UREA AND SELENIUM NUTRITION OF MARINE PHYTOPLANKTON:
A PHYSIOLOGICAL AND BIOCHEMICAL STUDY

by

NEIL MARTIN PRICE

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Department of Botany

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date March 24, 1987
ABSTRACT

Laboratory and field experiments measured urea uptake and assimilation with $^{14}\text{C}$- and $^{15}\text{N}$-urea and by disappearance of dissolved urea. A modified diacetyl monoxime method was developed, which accurately and precisely determined dissolved urea concentrations in seawater. In the Strait of Georgia, chlorophyll $a$ (chl $a$) specific uptake rates of ammonium ($\text{NH}_4^+$) and urea were greatest in stratified water; whereas, chl $a$ specific uptake rates of nitrate ($\text{NO}_3^-$) were greatest in frontal water. Ammonium and urea regeneration rates were calculated by a mass balance method and the rates were similar. Differences between measurements of particulate nitrogen, dissolved $\text{NH}_4^+$, $\text{NO}_3^-$ and urea, and $^{15}\text{N}$ uptake were used to explain the dominant N transformations in frontal and stratified seawater. Uptake rates measured by $^{14}\text{C}$-urea were ca. 1.4 times faster than those determined by $^{15}\text{N}$-urea in the Sargasso Sea. Turnover times of urea in the surface-mixed layer were ca. 12 h. Within some seawater samples, phytoplankton utilized urea at rates which approximated the maximum rates of utilization. In a nitrate-sufficient culture of *Thalassiosira pseudonana* (clone 3H) (Hustedt) Hasle and Heimdal, urea uptake rates measured by three methods disagreed; whereas, no discrepancies occurred in a nitrate-starved culture. $\text{NH}_4^+$ was released from cells after urea was taken up and was later reabsorbed. A model of urea uptake and assimilation by *T. pseudonana* is proposed.

An obligate selenium (Se) requirement for growth of *T.*
*psudonana* was demonstrated in axenic culture in artificial seawater. The addition of $10^{-9}$M SeO$_3^{2-}$ to culture medium was sufficient for good growth of this alga; SeO$_4^{2-}$ was only effective at concentrations greater than $10^{-7}$M. To elucidate the biochemical role of Se in *T. pseudonana*, cells were cultured in medium containing $10^{-9}$ M Na$_2^{75}$SeO$_3$. Two soluble polypeptides of 21 and 29 kD contained $^{75}$Se. Glutathione peroxidase was detected on non-denaturing polyacrylamide gels and $^{75}$Se co-migrated with the enzyme. It was concluded that Se is an essential element for growth of *T. pseudonana* due, in part, to the presence of the selenoenzyme glutathione peroxidase.
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Band (b) reacted with both peroxides ............... 262

Figure 45. (A) Glutathione peroxidase activity detected on a polyacrylamide gel using \( \text{H}_2\text{O}_2 \) as substrate. (B) Amount of \(^{75}\text{Se}\) in the gel (dpm·slice\(^{-1}\))................................. 264
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PART I: UREA

Introduction

Overview and objectives

The first part of this thesis examines urea uptake and assimilation by phytoplankton communities in coastal and open ocean environments and by phytoplankton grown in laboratory culture. Two methods for measuring dissolved urea concentrations in seawater were refined, and are compared in the first chapter. A modified diacetyl monoxime method gave the best results for urea analysis; this technique was used in subsequent chapters to determine urea uptake rates by phytoplankton, and to measure urea concentrations in seawater samples during field experiments. In Chapter 2, urea uptake rates by phytoplankton in the Strait of Georgia, in nitrate-replete and nitrate-deplete seawater, were measured and compared to rates of nitrate and ammonium uptake. Specific questions of this study addressed phytoplankton nitrogen preferences, the diel pattern of nitrogen uptake, and ammonium and urea regeneration. These results provided unique information regarding nitrogen uptake and its transformation in these two contrasting coastal environments. Experiments conducted in the Sargasso Sea determined in situ and maximum urea uptake rates, and evaluated $^{14}$C- and $^{15}$N-labelled urea uptake. These results are presented in Chapter 3. Discrepancies between the urea uptake rates measured by the two isotopes were evident. It is argued that phytoplankton in
these regions are adapted to the low ambient urea concentrations and, within some communities, utilize urea at rates which approximate the maximum rates of utilization. Laboratory experiments examined urea uptake and assimilation in an attempt to explain results obtained from experiments conducted in the Sargasso Sea. Chapter 4 presents the results of urea uptake by the marine diatom *Thalassiosira pseudonana* measured by three different methods during nitrate-sufficient growth and nitrate-starvation. On the basis of these results, a model for urea uptake and assimilation by *T. pseudonana* is proposed. Support for this model is found in the literature, and the model is consistent with other results reported in this thesis.

*Nitrogen limitation of phytoplankton productivity*

Substantial evidence has accumulated to suggest that nitrogen is frequently a growth-limiting factor for phytoplankton in many nearshore waters (Ryther and Dunstan 1971, Goldman *et al.* 1973, Thomas *et al.* 1974, Goldman 1976). The rate of supply of nitrogen to phytoplankton in the upper mixed layer of the ocean, by regenerative processes and by allochthonous inputs, regulates phytoplankton productivity. Nevertheless, numerous arguments have been advanced to suggest that phytoplankton in the open ocean are not nitrogen deficient (Thomas 1970, Morris *et al.* 1971, Goldman *et al.* 1979, DiTullio and Laws 1983, Laws *et al.* 1984, Kanda *et al.* 1985); by no means is this question resolved.

In the mixed layer of the ocean, in late spring and
summer, nitrogen concentrations are often near or below our limits of detection (limits of detection of nitrate $[\text{NO}_3^-]$, 50 ng at $\text{N} \cdot \text{L}^{-1}$, ammonium $[\text{NH}_4^+]$, 30 ng at $\text{N} \cdot \text{L}^{-1}$; urea, 50 ng at $\text{N} \cdot \text{L}^{-1}$) (McCarthy 1980, Raymont 1980, Garside 1985). During these periods, phytoplankton primarily utilize regenerated forms of nitrogen, such as ammonium and urea, for growth (Dugdale and Goering 1967, McCarthy et al. 1977, Glibert et al. 1982a, Cochlan 1986), and nitrate and nitrite are of less importance. Nitrogen uptake and regeneration are envisaged to be tightly coupled in these regions (Goldman 1984). Close coupling between supply and utilization of ammonium is evident in the results of a number of studies conducted in coastal and oceanic regions. These studies report that ammonium regeneration by zooplankton, and in particular those organisms less than 10 um in diameter, can supply most, if not all, of the phytoplankton ammonium requirements for growth (Harrison 1978, Caperon et al. 1979, Glibert 1982, Harrison et al. 1983, Cochlan 1986). Much less is known regarding urea regeneration and utilization by plankton communities.

Recent reports by Laws et al. (1985) and Wheeler and Kirchman (1986) question our current concepts of nitrogen cycling in marine systems. These investigators presented results that suggested that in the oligotrophic ocean, and in coastal waters, most of the ammonium is taken up by heterotrophic bacteria and not by phytoplankton. Since much significance is given to ammonium as a nitrogen source for phytoplankton growth, these results are profound. If
utilization by heterotrophic bacteria is the primary fate of ammonium, then other nitrogen compounds, previously overlooked or given less importance, must supply the phytoplankton nitrogen requirements for growth. In some marine environments, urea is a major form of nitrogen taken up by phytoplankton (Kaufman et al. 1983, Harrison et al. 1985).

Sources and sinks of urea in seawater

A significant source of urea in seawater is from excretion by invertebrates and fish (e.g. Corner and Newell 1967, McCarthy and Whitledge 1972, Whitledge 1981). As much as 50% of the total ammonium and urea excreted by a mixed macrozooplankton assemblage was urea (Eppley et al. 1973). McCarthy (1971) found that urea excretion rates of Calanus helgolandicus were roughly 30% of the NH$_4^+$ excretion rates. A similar proportion of the excreta from fish is made up of urea. Bacterial production of urea from dissolved organic matter in seawater has received less attention. Maita et al. (1973) argued that bacterial decomposition of arginine was the major source of urea in seawater. They failed to address the significance of this pathway in situ, and instead they only demonstrated the potential for urea production from arginine. In freshwater lakes, Satoh (1980) and Satoh et al. (1980) reported that decomposition of phytoplankton by bacteria was a major source of urea. The extent and importance of this process in the ocean is unknown, but decomposition of zooplankton by marine bacteria does produce urea (Mitamura and Saijo 1980b). Nearshore waters may receive urea via
terrestrial drainage (Remsen et al. 1974), and atmospheric precipitation has also been identified as a urea source (Timperley et al. 1985), which presumably contributes some urea to seawater.

Size fractionation studies have been conducted to quantify the relative importance of different types of phytoplankton and the role of bacteria in urea utilization, in seawater. The initial results of Remsen et al. (1972) implicated phytoplankton as the most important component of the plankton responsible for urea decomposition in coastal waters and associated estuaries of Georgia, U.S.A. Additional investigations by Mitamura and Saijo (1975) and Webb and Haas (1976) confirmed these results in different ocean environments, and Billen (1984) also concluded that phytoplankton were primarily responsible for utilizing urea in seawater. These studies were based, for the most part, on urea utilization by coastal plankton. Remsen et al. (1974) found that in coastal waters off Georgia, 86% of the urea uptake could be attributed to organisms greater than 10 um in diameter; whereas, only 49% of the urea uptake was by plankton of this size in the North Atlantic. These results cannot be used as evidence to support a greater role of heterotrophic bacteria in urea uptake in the open ocean because phytoplankton would certainly be a large component of the less-than-10 um size fraction. Recently, Wheeler and Kirchman (1986) found negligible uptake of urea by Gulf Stream and coastal organisms less than 1 um in diameter. It is
concluded, from the results of these studies, that phytoplankton are responsible for most, if not all, of the urea uptake in the ocean.

Utilization of urea as a nitrogen substrate for growth of phytoplankton in the ocean has received renewed interest since the initial studies of McCarthy, Remsen and coworkers (McCarthy, 1970, 1972a, Remsen 1971, Carpenter et al. 1972a, McCarthy and Eppley 1972, McCarthy and Kamykowski 1972, Remsen et al. 1972, 1974). This is a result, at least in part, of our growing realization of the importance of dissolved organic nitrogen in phytoplankton nutrition (e.g. Fisher and Cowdell 1982). Current estimates indicate that urea constitutes a significant fraction (20 to 50%) of the total nitrogen utilized by phytoplankton in coastal (McCarthy 1972a, Harvey and Caperon 1976, McCarthy et al. 1977, Kaufman et al. 1983, Kristiansen 1983, Harrison et al. 1985) and oceanic (Eppley et al. 1973, 1977) environments. Furthermore, rapid turnover times of urea (Herbland 1976, Savidge and Hutley 1977) in seawater emphasizes the strategic role of urea in the marine nitrogen cycle.

**Phytoplankton growth on urea**

Numerous reports confirmed that urea is an excellent source of nitrogen for growth of many phytoplankton (Guillard 1963, McCarthy 1971, Antia et al. 1975, 1977, Turner 1979, Neilson and Larsson 1980, Fisher and Cowdell 1982). However, in these studies, there were some species that were unable to grow or that showed poor growth with urea. Antia et al.
(1977) were able to culture some of these species by increasing the urea concentration in the medium. Most recently, Oliveira and Antia (1984, 1986b) demonstrated that by supplementing the culture medium with Ni\(^{2+}\), some algae, previously unable to utilize urea, grew well with urea as the sole nitrogen source. These results extend the list of phytoplankton known to be able to use urea as a nitrogen source for growth. The early research of Harvey (1940) documented preferential utilization of urea by particular phytoplankton species. He showed that the phytoplankton community that developed in seawater samples enriched with urea differed from the community in ammonium-enriched samples. The implication of his results was that some species are better able to sequester and utilize urea than others.

*Laboratory studies of urea uptake*

Uptake of urea by marine and freshwater phytoplankton has been the subject of a number of investigations. These studies examined the kinetics of urea uptake by phytoplankton grown in laboratory batch culture using a variety of isotopic tracers including \(^{15}\)N-urea (McCarthy 1972b, Collos and Slawyk 1979, Horrigan and McCarthy 1981, 1982), \(^{14}\)C-urea (Carpenter *et al.* 1972a, b, Williams and Hodson 1977, Kirk and Kirk 1978a, b, Bekheet and Syrett 1979, Rees and Syrett 1979a, Horrigan and McCarthy 1981, 1982, Syrett *et al.* 1986), \(^{35}\)S-thiourea (Syrett and Bekheet 1977, Rees and Syrett 1979b), and by chemical measurements of dissolved urea concentration (Healey 1977).
The general results from this work can be summarized as follows: Urea uptake is a constitutive property of phytoplankton growing on nitrate as the sole nitrogen source, but an induction period is required before ammonium-grown cells take up urea. Phytoplankton possess a urea uptake system that follows saturation kinetics. Phytoplankton are able to accumulate urea intracellularly at a rate, and to a concentration, that suggests that uptake is mediated by an active transport process. Although the energy requirements for urea transport are not well documented, uptake is stimulated by light and inhibited by the presence of proton ionophores. In total, these and other experiments have established that ATP is necessary for urea transport by microalgae. Following a period of nitrogen deprivation, phytoplankton take up urea at rates which are in excess of those required to meet their nitrogen demands during balanced growth.

Urea metabolism

The degradation of urea occurs by one of two enzymes, urease (EC 3.5.1.5) or ATP:urea amidolyase. The ATP:urea amidolyase enzyme complex consists of two distinct enzyme activities, urea carboxylase (EC 6.3.4.6) and an allophanate hydrolase (EC 3.5.1.13); together they catalyze the degradation of urea to CO₂ and NH₃ (Whitney and Cooper 1972). Urease and ATP:urea amidolyase are mutually exclusive in algae, and ATP:urea amidolyase appears confined to a small group of chlorophyll b containing microalgae (Syrett and Al
Houty 1984). Urease of some higher plants is a metalloenzyme containing nickel as an integral component (Dixon et al. 1975a). Evidence provided by Syrett (1981), Rees and Bekheet (1982) and Oliveira and Antia (1984, 1986a) indicates that this is probably true for microalgae as well.

Depending on the algal species, and the duration of the uptake experiment, once urea is transported through the cell membrane it may remain intact or it may be degraded to CO$_2$ and 2NH$_3$. Phaeodactylum tricornutum concentrates urea intracellularly, and during 10 min uptake experiments 80% of the $^{14}$C retained by the cells remains as urea (Rees and Syrett, 1979a). In other algae, such as Chlorella fusca var. vacuolata, Skeletonema costatum and Thalassiosira pseudonana, 50-98% of the C from urea is released from the cells during 5 min incubations (Bekheet and Syrett 1979, Horrigan and McCarthy 1981).

Ellner and Steers (1955) showed that urea carbon was incorporated into cellular constituents in Chlorella pyrenoidosa and Scenedesmus basilensis, and much of the activity of $^{14}$C was present in guanine. Webster et al. (1955) confirmed that urea carbon can be utilized as a carbon source. Their results showed, as did those of Allison et al. (1954), that a similar pattern of labelling of amino acids occurred in plants supplied with bicarbonate (H$^{14}$CO$_3^-$) and NH$_4^+$ as with $^{14}$C-urea. These results suggested that urea carbon is equivalent to HCO$_3^-$.

Nevertheless, differences in the types of amino acids synthesized by bean leaves exposed to $^{14}$C-urea
compared with $\text{H}^{14}\text{CO}_3^-$ and ammonium, as well as greater levels of $^{14}\text{C}$ activity in certain amino acids in the urea-spiked plants, provided evidence that urea carbon is not necessarily assimilated by photosynthetic pathways. Since the specific activity of $^{14}\text{C}$ from urea was less than in the plants supplied with $\text{H}^{14}\text{CO}_3^-$, a much greater proportion of C from urea was fixed into amino acids than was C from $\text{H}^{14}\text{CO}_3^-$. Webb and Haas (1976) and Mitamura and Saijo (1975) concluded that light-driven photosynthetic carboxylating reactions were not the sole mechanism for $^{14}\text{C}$-urea incorporation by natural communities of phytoplankton. In white spruce seedlings, Durzan (1973) reported that carbamyl compounds (carbamyl aspartate and citrulline) were formed more readily from $^{14}\text{C}$-urea than from $\text{H}^{14}\text{CO}_3^-$. In his proposed scheme, urea was preferentially incorporated into carbamyl phosphate, the precursor of other carbamyl compounds. Some of the urea-N was also incorporated into organic nitrogen without first being degraded to $\text{NH}_3$; independent evidence exists which indicates that urea-N may be assimilated into amino acids prior to its conversion to $\text{NH}_3$ (Kitoh and Hori 1977). Metabolism of $\text{NH}_3$, produced by urea degradation, into amino acids is by nitrogen assimilatory pathways which operate when $\text{NH}_4^+$ is supplied to the cells (see Turpin and Harrison 1978, Syrett 1981).

**Test organism**

In three chapters of this thesis the unicellular centric marine diatom *Thalassiosira pseudonana* (clone 3H) (Hustedt) Hasle and Heimdal was used to investigate physiological and
biochemical aspects of phytoplankton nutrition. This clone was isolated from Moriches Bay, Long Island in 1958 by R.R.L. Guillard. In 1970, it was obtained from N.J. Antia and maintained in the Northeast Pacific Culture Collection at the University of British Columbia. A complete morphological description of this alga was published by Hasle and Heimdal (1970) and Hasle (1976). *Thalassiosira pseudonana* has been the choice of many investigators as an experimental organism for studies examining nutrient uptake (Goldman and McCarthy 1978, Dortch *et al.* 1982, Parslow *et al.* 1984a, b, 1985) trace metal requirements and interactions (Murphy *et al.* 1981, Sunda and Huntsman 1983), and temperature and salinity effects (Guillard and Ryther 1962, Brand *et al.* 1981). *Thalassiosira pseudonana* grows rapidly, has been extensively studied, and is easily maintained in axenic culture. These criteria make it an ideal organism in which to examine physiological and biochemical processes.
CHAPTER 1. A COMPARISON OF METHODS FOR THE MEASUREMENT OF DISSOLVED UREA CONCENTRATIONS IN SEAWATER

Background

The concentration of dissolved urea in oceanic environments is generally less than 0.50 uM (Eppley et al. 1977, Mitamura and Saijo 1980a, McCarthy 1980); whereas, in coastal and estuarine environments, concentrations tend to be higher and more variable (Remsen et al. 1974, McCarthy 1980a). Variability in urea concentration is also observed in vertical profiles of marine waters (Remsen 1971, McCarthy 1972) and can be attributed to patchy distribution of urea-regenerating organisms. In fact, McCarthy and Kamykowski (1972) reported temporal variation in urea concentrations which was associated with a shark infestation.

Measurement of dissolved urea concentrations reveals little regarding the role of urea in nitrogen cycling or phytoplankton nutrition. However, accurate and precise measurements are required to determine uptake and excretion rates of urea and to provide an indication of the nitrogen status of phytoplankton communities. Throughout this thesis, the units uM (umol·l$^{-1}$) and ug at N·l$^{-1}$ are used interchangeably. The reader is reminded that 1 uM urea is equivalent to 2 ug at urea-N·l$^{-1}$ because urea contains two nitrogen atoms per molecule. For compounds containing a single nitrogen atom, such as ammonium (NH$_4^+$), nitrate and nitrite, values expressed as uM or ug at N·l$^{-1}$ are identical.

Currently, two techniques for measuring the concentration
of dissolved urea in seawater are in use. The urease method (McCarthy 1970) involves enzymatic hydrolysis of urea, by urease, to carbon dioxide and ammonia. Subsequently, the liberated ammonia is assayed by the procedure of Solorzano (1969), as outlined in McCarthy (1970), and the concentration of urea nitrogen is determined. The diacetyl monoxime method is a colourimetric analysis which directly determines the concentration of dissolved urea. The chemistry of this reaction has been examined (Butler et al. 1981). The diacetyl monoxime method was first adapted for use in seawater by Newell et al. (1967) and has since been modified by DeManche et al. (1973), Whitledge et al. (1981), Aminot and Kerouel (1982) and Koroleff (1983).

This chapter evaluates and compares the diacetyl monoxime and urease methods for measuring the concentration of dissolved urea in artificial seawater, phytoplankton culture filtrate and both natural and urea-spiked seawater samples from coastal and oceanic environments.

Materials and Methods

Urease method

Urea concentration was determined by the urease method of McCarthy (1970). Worthington URC and Sigma Type III urease were used as enzyme sources as suggested by McCarthy (1970) and McCarthy and Kamykowski (1972), respectively. The activity of Worthington URC urease was 72 units (U) mg-solid$^{-1}$ and for Sigma Type III urease it was 40 U mg-solid$^{-1}$. I
determined urease activities independently of the manufacturers' specifications, by measuring rates of $^{14}\text{CO}_2$ production from $^{14}\text{C}$-urea in phosphate buffer, pH 7.6, at 25°C. Incubations were conducted in acid-washed 25 ml Erlenmeyer flasks sealed with rubber septa. Small glass vials, containing filter paper impregnated with 50 ul phenethylamine to trap enzymatically released $^{14}\text{CO}_2$, were suspended inside the flasks. The reaction was terminated after 5 min by the addition of 0.5 ml 6N HCl, and recovery was 100%. Similar procedures were employed to examine the effect of urease enzyme concentration on $^{14}\text{C}$-urea hydrolysis in seawater.

With the exception of experiments designed to examine the effects of temperature and incubation duration on urea hydrolysis, urease-seawater samples were heated in a temperature-regulated water bath at 50°C for 20 min.

Ammonium concentration was measured manually as described by McCarthy (1970) and by automated analysis on a Technicon Autoanalyzer® following the procedure of Slawyk and MacIsaac (1972). These two methods gave identical results for $\text{NH}_4^+$ determinations in solutions of deionized distilled water (DDW), DDW containing 3% NaCl (w/v) (referred to hereafter as 3% NaCl), artificial seawater (ESAW; Harrison et al. 1980) and natural seawater. Both $\text{NH}_4^+$ techniques were employed for urea analysis using the urease method. They gave identical results, and consequently both $\text{NH}_4^+$ analyses have been used in this study.

Standard solutions of urea spanned the range of values encountered in the analyses. Unless stated otherwise,
standards were prepared in 3% NaCl. For comparison I have calculated a factor, F, defined by McCarthy (1970) as:

$$F = \frac{\text{concentration of urea standard}}{(\text{absorbance of standard-absorbance of blank})}$$

for different concentrations of urea. The background NH$_4^+$ concentrations in the Worthington URC and Sigma Type III urease preparations, when added to seawater in concentrations suggested by McCarthy (1970) and McCarthy and Kamykowski (1972), were 0.17 and 0.25 uM, respectively.

The precision (± 1 SD) of replicate samples containing 0.50 uM urea, prepared in DDW, was 0.01 uM urea (n=5).

Diacetyl monoxime method

Urea concentration was measured using the diacetyl monoxime method of Rahmatullah and Boyde (1980). This method was modified and automated for a Technicon Autoanalyzer® by Whitledge et al. (1981). Further modifications to this procedure were made, and for clarification I have included these details (Fig. 1). The compositions of the reagents are as follows – Reagent 1: 5 ml Brij-35 (Technicon) in 500 ml DDW; Reagent 2: 300 ml H$_2$SO$_4$ and 100 ml H$_3$PO$_4$ in 600 ml DDW, add 50.0 mg FeCl$_3$·6H$_2$O when the solution has cooled; Reagent 3: 5.0 g diacetyl monoxime and 50.0 mg thiosemicarbazide in 250 ml DDW. To determine the identity of potentially interfering substances, 29 organic and inorganic nitrogen compounds were tested. Concentrated solutions of each
Fig. 1. Schematic diagram of Autoanalyzer® system for urea analysis by the diacetyl monoxime method. Composition of reagents is given in the "Materials and Methods - Diacetyl monoxime method". The debubbler line pulled 0.60 ml·min⁻¹ to prevent any large air bubbles from entering the system. A boiling water bath gave a more constant incubation temperature and helped eliminate the oscillating baseline that was seen when less constant and lower temperatures were used.
compound were prepared in urea-free DDW, and samples were analyzed for urea. In solutions where urea was found to be present, subsamples were treated with urease and then reanalyzed by the diacetyl monoxime method. This was done to determine whether the compound mimicked urea and reacted with the colour reagents, or if the solution contained contaminating urea which was introduced with the compound being tested.

Solutions used to set the baseline on the autoanalyzer had the identical composition (minus urea) to the samples being analyzed. However, for natural seawater samples urea-free NaCl or ESAW solutions were used for the baseline, and urea standards were prepared in these same solutions.

As suggested by Aminot and Kerouel (1982), a Chelex 100 (Bio-Rad Laboratories, Richmond, California, USA) column was positioned in the sample line to remove potentially interfering metals; however, in the natural seawater samples I examined, there were no beneficial effects of the column and it was not used for subsequent analysis. In a separate experiment, dissolved amino acids were removed from seawater samples by ligand-exchange chromatography (Siegal and Degens 1966), and the seawater samples were reanalyzed for urea.

The precision (± 1 SD) of replicate samples of 0.50 μM urea prepared in DDW, was 0.01 μM urea (n=5) and the lower limit of detection was 0.025 μM urea.

Experimental methods

Urea concentrations in solutions of DDW, 3% NaCl, ESAW
and phytoplankton culture filtrate were measured simultaneously by the diacetyl monoxime and urease methods. Phytoplankton culture filtrate (referred to hereafter as "culture filtrate") was obtained by growing *Thalassiosira pseudonana* (clone 3H) in ESAW. The cells were harvested in early stationary phase by filtration through combusted Whatman GF/F filters (460°C for 4 h), and the culture filtrate was collected in an acid-rinsed sterile side-arm flask.

Natural seawater samples were collected from the Strait of Georgia, British Columbia and English Bay, British Columbia (49°18'N; 123°12'W) in August, 1984. Samples were filtered through combusted Whatman GF/F filters and stored frozen at -20°C in polypropylene bottles. There was no effect of freezing samples of filtered seawater or samples spiked with known concentrations of urea for periods up to one year (Price, unpublished). Seawater collected at 10 m from Ocean Station P, central North Pacific (49°55'N; 144°40'W) was screened through 5 um Nitex® netting and stored in 20-litre polyethylene carboys in a dark, cold-room. The seawater was later refiltered through combusted Whatman GF/F filters in the laboratory. To determine the accuracy with which urea was measured, internal standards were run in the natural seawater samples.

**Results**

*Standard curves*

Urea solutions prepared in DDW, 3% NaCl, ESAW and culture
filtrate were analyzed by the diacetyl monoxime (Fig. 2A) and urease (Fig. 2B) methods. Using the diacetyl monoxime method, calibration curves were indistinguishable and linear \( r^2 = 1.00 \) over a range of urea concentrations prepared in DDW, 3% NaCl, ESAW and culture filtrate. By contrast, the slopes of the regression lines using the urease method were significantly different \( p < 0.001 \). These urea standard curves were also linear \( r^2 = 1.00 \) for urea solutions prepared in DDW, 3% NaCl, ESAW and culture filtrate. An average factor, \( F \), calculated for concentrations of dissolved urea prepared in DDW or 3% NaCl, was 6.23. In ESAW and culture filtrate, the factor \( F \) was 12.4 and 24.6, respectively. These discrepancies were not the result of errors in the \( \text{NH}_4^+ \) analysis. It was shown in subsequent experiments that ammonium standard curves prepared in DDW, ESAW and culture filtrate were identical with urea standard curves prepared in DDW or 3% NaCl. Therefore, the urease method accurately measured dissolved urea concentrations only in DDW or 3% NaCl. A 2.50 \( \mu \text{M} \) urea standard prepared in culture filtrate was analyzed by the diacetyl monoxime method, and a concentration of 2.52 ± 0.01 \( \mu \text{M} \) \( n=3 \) urea was measured. The same standard was also analyzed by the urease method; the urea concentration was determined to be 1.07 ± 0.02 \( \mu \text{M} \) \( n=3 \). Small aliquots of the urease-treated standard were reanalyzed by the diacetyl monoxime method. It was found that urea was present in these samples \( 1.55 ± 0.05 \mu \text{M}; \ n=3 \), indicating that the urease had not completely hydrolyzed the urea to
Fig. 2. Standard curves for urea determined by (A) automated diacetyl monoxime method and (B) urease method, with urea standards in deionized distilled water or 3% NaCl (▲), in artificial seawater (●) and in culture filtrate (○).
ammonium.

**pH effects**

The high pH of the culture filtrate (pH 9.2) could explain the incomplete hydrolysis of urea in the samples analyzed by the urease method. The effect of pH on hydrolysis of urea in ESAW and culture filtrate, using the urease method, is shown in Figure 3. The concentration of urease added to these samples was twice the concentration recommended by McCarthy (1970). Hydrolysis of urea proceeded most completely at pH 7.2 in both solutions; however, there was less urea hydrolysis in culture filtrate compared with ESAW. There was no effect of sample pH on the diacetyl monoxime method.

**Heating time**

Time-course measurements of urea concentration analyzed by the urease method, as a function of heating time, are shown in Figure 4. Complete hydrolysis of a 1.00 μM urea standard prepared in 3% NaCl occurred after 5 min of heating. Only 85% of the urea added to the natural seawater samples was hydrolyzed after 45 min heating.

**Enzyme concentration**

To determine the effect of enzyme strength on urea hydrolysis, the activity of the enzyme solution was initially determined (Table I). Results demonstrated that the activities of the Worthington URC enzyme preparations were less than the manufacturer's specified activities and all
Fig. 3. Hydrolysis of urea in artificial seawater (dashed line) and culture filtrate (continuous line) by urease, as a function of seawater pH. Urease concentration was twice that recommended by McCarthy (1970). Total amount of urea was determined by the diacetyl monoxime method, and concentrations determined by the urease method are expressed as a percentage of this value. Error bars indicate the range of duplicates.
Fig. 4. Urea hydrolyzed as a function of heating time of the urease-seawater samples at 50°C. A 1.00 uM urea standard was prepared in 3% NaCl (●) and in seawater samples collected from the Strait of Georgia (O). Samples had cooled to room temperature before the NH₄⁺ measurements were made. Concentration of urea in each water sample was determined by the diacetyl monoxime method, and values are reported as a percentage of this value.
Table I

Measured urease activity of enzyme stock solutions compared to theoretical activity determined by manufacturer.

<table>
<thead>
<tr>
<th>Enzyme Type</th>
<th>Date prepared</th>
<th>Date measured</th>
<th>Measured activity (U·ml⁻¹)</th>
<th>Theoretical activity (U·ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Worthington URC</td>
<td>15.II.1984</td>
<td>21.XI.1984</td>
<td>500</td>
<td>720</td>
</tr>
<tr>
<td>Worthington URC</td>
<td>8.VII.1984</td>
<td>21.XI.1984</td>
<td>480</td>
<td>720</td>
</tr>
<tr>
<td>Sigma Type III</td>
<td>20.I.1983</td>
<td>21.XI.1984</td>
<td>840</td>
<td>950</td>
</tr>
<tr>
<td>Sigma Type III</td>
<td>26.XI.1984</td>
<td>27.XI.1984</td>
<td>1860</td>
<td>---²</td>
</tr>
</tbody>
</table>

¹ One unit (U) of urease releases 1 umol NH₃·min⁻¹; see "Materials and Methods" and "Discussion" for assay conditions.
² Not available.
three batches were similar. The enzyme preparations were stable for periods up to 9 months. In the case of the Sigma product, urease activity was similar to the theoretical activity for up to 22 mo. Using a range of urease concentrations (Sigma Type III), hydrolysis of a single urea standard solution was measured (Fig. 5). Incomplete hydrolysis of the urea standard was evident at all enzyme activities. Upon cooling the samples, it was evident from the NH$_4^+$ measurements that urea was still being hydrolyzed; in no case was all of the urea-NH$_4^+$ measured. At higher urease concentrations (1.9 to 2.3 U·ml$^{-1}$ seawater), it appeared as if little urea was being hydrolyzed when the samples cooled. In seawater samples containing lower enzyme concentrations (less than 1.4 U·ml$^{-1}$ seawater), urea continued to be hydrolyzed during cooling (Fig. 5).

**Specificity of diacetyl monoxime method**

To examine the specificity of the diacetyl monoxime method, 29 organic and inorganic nitrogen compounds of varying concentration were analyzed (Table II). Citrulline was the only compound which gave significant interference. Three amino acids gave positive reactions with the diacetyl monoxime reagents; however, after treating these solutions with urease, urea could no longer be detected. Apparently, these samples had been contaminated with urea, and there was no evidence that the amino acids were interfering with the technique.
Fig. 5. Urea hydrolyzed in urease-treated seawater samples, as a function of final enzyme activity in the urease seawater samples. Samples were collected from Ocean Station P and spiked with 1.00 μM urea. Incubations were conducted at 50°C for 20 min, and samples were cooled for 0 (■), 32 (○), and 76 min (●). Amount of urea hydrolyzed was expressed as a percentage of the concentration determined by the diacetyl monoxime method.
Table II

Organic and inorganic compounds tested for interference with the diacetyl monoxime method. Solutions were prepared in deionized distilled water.

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>Concentration tested (µM)</th>
<th>Measured Urea conc. (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Ammonium</td>
<td>500.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Arginine</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Asparaginie</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Aspartate</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Citrulline</td>
<td>10.0</td>
<td>11.7 (10.2)</td>
</tr>
<tr>
<td>Creatine</td>
<td>1000.0</td>
<td>0.64</td>
</tr>
<tr>
<td>Cysteine</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Glutamine</td>
<td>10.0</td>
<td>0.18 (&lt;0.025)</td>
</tr>
<tr>
<td>Glutamate</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Glutathione</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Glycine</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Histidine</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>10.0</td>
<td>0.15 (&lt;0.025)</td>
</tr>
<tr>
<td>Lysine</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Methyonine</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Nitrate</td>
<td>5500.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Nitrite</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Ornithine</td>
<td>1000.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>10.0</td>
<td>0.07 (&lt;0.025)</td>
</tr>
<tr>
<td>Potassium cyanate</td>
<td>1000.0</td>
<td>0.68</td>
</tr>
<tr>
<td>Serine</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Tris(hydroxymethyl)amino-methane</td>
<td>8000.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Threonine</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>10.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Uracil</td>
<td>100.0</td>
<td>0.47</td>
</tr>
<tr>
<td>Uric acid</td>
<td>100.0</td>
<td>0.50</td>
</tr>
</tbody>
</table>

1 Concentration of urea measured after treating sample with urease.
Natural seawater samples

Ammonium analysis of samples of Ocean Station P seawater (pH 8.32), spiked with various urease concentrations, indicated that some NH$_4^+$ was lost from the samples during heating. There was also evidence that the higher urease concentrations inhibited the NH$_4^+$ measurement (Table III). A urease concentration of 3 U·ml$^{-1}$ seawater did not alter the seawater pH and only slightly affected the pH of the reaction mixture in the manual NH$_4^+$ analysis. There was no effect on the pH of the reaction mixture in the automated procedure. To avoid any confounding effects from the NH$_4^+$ determination step of the urease assay, $^{14}$C-urea hydrolysis by urease was measured. 3% NaCl and Ocean Station P seawater were spiked with 0.5 uM $^{14}$C-urea, and the amount of $^{14}$CO$_2$ released by urease hydrolysis was determined. The results (Fig. 6) indicated that in 3% NaCl urea hydrolysis by urease was complete at enzyme concentrations greater than 1.0 U·ml$^{-1}$; consistent with the previous result. In the Ocean Station P sample, urea hydrolysis increased with increasing urease concentration and was almost complete at the highest enzyme concentration (3.0 U·ml$^{-1}$ seawater).

Seawater samples collected from the Strait of Georgia were analyzed by the diacetyl monoxime and urease methods (Table IV). Standards were prepared in 3% NaCl. The null hypothesis, i.e., the concentration of urea determined by the urease method equals the concentration of urea determined by the diacetyl monoxime method, was rejected (p < 0.05,
Table III

An Ocean Station P seawater sample was spiked with 2.00 uM ammonium and divided into two portions. To one portion, urease was added to give a final concentration of 2.3 U·ml⁻¹; the other portion served as a control. The samples were heated at 50°C for 20 min and the concentration of ammonium was measured immediately (0 min) and after the samples had cooled (55 min). Incubations were conducted in 125 ml Erlenmeyer flasks covered with aluminum foil.

<table>
<thead>
<tr>
<th>Urease conc. (U·ml⁻¹)</th>
<th>Ammonium conc. (uM)</th>
<th>0 min</th>
<th>55 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td></td>
<td>1.91</td>
<td>1.84</td>
</tr>
<tr>
<td>2.3</td>
<td></td>
<td>1.58</td>
<td>1.55</td>
</tr>
</tbody>
</table>
Fig. 6. Hydrolysis of 0.50 uM $^{14}$C-urea in 3% NaCl (●) and Ocean Station P seawater (O), as a function of enzyme concentration. Samples were 50 ml and were heated for 20 min at 50°C. The reaction was terminated once the samples had cooled to room temperature. The amount of urea hydrolyzed is expressed as a percentage of the $^{14}$C-urea added, which was determined by liquid scintillation counting.
Table IV

Concentration (uM) of dissolved urea in seawater samples collected from the Strait of Georgia, as determined by the diacetyl monoxime and urease methods. Each value represents a single measurement.

<table>
<thead>
<tr>
<th>Station</th>
<th>Location</th>
<th>Depth (m)</th>
<th>Diacetyl monoxime method</th>
<th>Urease method</th>
</tr>
</thead>
<tbody>
<tr>
<td>T8</td>
<td>49°48.6'N; 124°50.7'W</td>
<td>0</td>
<td>0.28</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1.01</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.5</td>
<td>0.46</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5</td>
<td>0.57</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.5</td>
<td>0.52</td>
<td>0.33</td>
</tr>
<tr>
<td>T10</td>
<td>49°55.6'N; 125°02.3'W</td>
<td>0</td>
<td>0.33</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>0.16</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.55</td>
<td>0.29</td>
</tr>
<tr>
<td>A530b</td>
<td>49°53.0'N; 125°05.9'W</td>
<td>0</td>
<td>0.42</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.43</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5</td>
<td>0.30</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>0.34</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>0.21</td>
<td>0.43</td>
</tr>
<tr>
<td>T14</td>
<td>49°53.4'N; 125°05.7'W</td>
<td>0</td>
<td>0.19</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>0.40</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>0.43</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>0.17</td>
<td>0.12</td>
</tr>
<tr>
<td>T183b</td>
<td>49°46.7'N; 124°53.5'W</td>
<td>2.5</td>
<td>0.25</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.5</td>
<td>0.19</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>0.26</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>0.30</td>
<td>0.16</td>
</tr>
<tr>
<td>T81b</td>
<td>49°48.6'N; 124°50.7'W</td>
<td>0</td>
<td>0.37</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.37</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>0.15</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>0.08</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Wilcoxon-matched-pair-signed-rank test). The diacetyl monoxime method measured significantly higher concentrations of urea in these natural seawater samples.

Measurement of urea in natural seawater samples, spiked with a known concentration of urea, demonstrated that sampling location and time of collection were important variables affecting urea determination by the urease method (Fig. 7). With the exception of one seawater sample from English Bay, urea hydrolysis was incomplete in the samples; moreover, it was evident that as the samples cooled urea was still being hydrolyzed by the urease. Analysis of the same natural seawater samples by the diacetyl monoxime method demonstrated that urea was accurately measured in these samples regardless of seawater type.

Ocean Station P seawater was spiked with varying concentrations of urea, and the concentrations were determined by the diacetyl monoxime and the urease methods (Fig. 8). Single determinations by the diacetyl monoxime method were in excellent agreement with the amount of urea added; whereas, the urease method underestimated the urea concentrations.

Discussion

pH effects

The pH dependence of enzymes is well known, and for urease the pH optimum appears to be a function of the buffer system (Lynn 1967). Fishbein (1969a) reported the pH optimum of jack bean urease to be 7.0; however, broad maxima have been
Fig. 7. Time course of hydrolysis of a 1.00 uM urea standard prepared in natural seawater collected from English Bay, Vancouver, British Columbia (△), and Strait of Georgia (Station T3; 49°50.4'N; 125°00.5'W) at 15:47 (○) and 22:30 hrs (●) and from Ocean Station P (□). Urease-seawater samples were heated for 20 min at 50°C, and time on the abscissa refers to time following heating, when samples were cooling at room temperature. Concentration of urea in each water sample was determined using the diacetyl monoxime method, and values measured by the urease method are reported as a percentage of this value. Error bars indicate the range of duplicates.
Fig. 8. Comparison between the diacetyl monoxime and urease methods for measuring urea in a natural seawater sample (Ocean Station P) spiked with known concentrations of urea. Urea concentrations were measured using the diacetyl monoxime method (□) and the urease method (●, O). The heated urease-seawater samples were allowed to cool at room temperature for 37 (O) and 104 (●) min.
reported (Fishbein et al. 1965), and the existence of urease isozymes may be one explanation for these results. In ESAW, maximum urease activity occurred at pH 7.2 and, although this was the lowest pH tested, an examination of lower pH values was not warranted since they would be much less than the normal pH of seawater.

The pH of seawater is largely determined by the bicarbonate/borate concentration. In the open ocean, the pH of surface water is between 8.1 and 8.3 (Sverdrup et al. 1942). While in coastal regions, pH values are more variable as a result of freshwater influences and greater primary productivity; they are typically between 7.5 and 8.5. Recent measurements by Zirino et al. (1983) revealed pH values as high as 8.91 in the Peruvian upwelling zone, and Tully and Dodimead (1957) recorded pH values of 8.8 in surface water of the Strait of Georgia. Tully and Dodimead (1957) also noted spatial variation in pH, and they attributed these observations to patchy biological activity and complex mixing patterns in the strait.

The results of this study firmly establish the importance of pH in the hydrolysis of urea in artificial seawater solutions. The observations imply that pH may be an important parameter influencing urea hydrolysis in natural seawater samples. Strickland and Parsons (1972, p. 91-95) pointed out the potential problem of high pH in freshwater, and suggested that the pH should be adjusted if necessary. I concur with this suggestion; however, in order to avoid inhibition of the indophenol blue reaction used to determine NH$_4^+$, special care
should be taken in adjusting the pH since this reaction is highly pH-dependent (Harwood and Huyser 1970). Tris® buffer interferes with the phenol-hypochlorite method and is not suitable for buffering (McCarthy 1970). To my knowledge, there are no published reports of the effects of Good's buffers (Good et al. 1966) on the phenol-hypochlorite method, but MOPS [3-(N-morpholino) propanesulfonic acid] causes no interference at the 5 mM level (C.A. Suttle, personal communication).

Urea hydrolysis

Urea was incompletely hydrolyzed in ESAW, and the amount of urea hydrolyzed was not consistent between samples (see Figs. 2 and 3). The concentration of urease added to the samples was different between these experiments; better results were obtained with a higher enzyme concentration. This discrepancy can also be attributed to differences in sample pH and rates of cooling between the samples, important factors I was unaware of initially. It has also been reported that the specific activity of a concentrated urease solution, diluted with the same solvent, increases with time after dilution (Peterson et al. 1948). This was not controlled during the experiments. This observation suggests that the amount of urea hydrolyzed may depend upon the time the working urease preparation (sensu McCarthy 1970) has been prepared, and when it is added to the seawater sample. Urease is sensitive to heavy metals (Hughes et al. 1969, Olson and
Christensen 1982) and is also inhibited by Na\textsuperscript{+} and K\textsuperscript{+} (Fasman and Niemann 1951) at concentrations similar to those found in seawater. The latter observation could explain the incomplete hydrolysis of urea in ESAW, except that urea hydrolysis proceeds quickly in 3% NaCl solutions. An examination of the individual reagent-grade salts of artificial seawater demonstrated that urea hydrolysis proceeds most slowly and incompletely in solutions of MgCl\textsubscript{2} and CaCl\textsubscript{2} (Price, unpublished results). Urease inhibition by Ca\textsuperscript{2+} has been previously reported (Olson and Christensen 1982).

Using the urease method, hydrolysis of a 1.00 uM urea standard, prepared in natural seawater, was incomplete following 20 min at 50°C. These data are in disagreement with McCarthy (1970) who reported complete hydrolysis of 7.5 uM urea under the same assay conditions; the composition of his sample solution was not reported. The results from this study indicate that only urea standards prepared in DDW or 3% NaCl are completely hydrolyzed under these conditions.

The efficiency of recovery of internal standards of urea from natural seawater was dependent upon seawater type. This observation invalidates the previous assumption that there is a constant recovery of urea from seawater samples. To correct for this, it is advised that internal standards be run with each sample. If internal standards are properly used, it may be possible to determine accurately dissolved urea concentrations in seawater with the urease method. The results from this study have also shown that there is a time-dependence of the urease method, which is a result of the
continued hydrolysis of urea in urease-treated samples during cooling. In my experimental protocol, samples were allowed to cool as suggested by McCarthy (1970). Depending on air currents and other factors, samples that cooled passively reached room temperature ca. 30 min after their removal from the heating bath.

The independent estimates of urease activity are in close agreement with the manufacturers' specifications and are evidence of an active enzyme preparation. The discrepancies, particularly with the Worthington URC product, are most likely a result of different enzyme assay conditions. Worthington determines urease activity in a glutamate dehydrogenase-coupled system in phosphate buffer, pH 7.6, at 25°C; whereas, the enzyme assay I used measured $^{14}$CO$_2$ release from $^{14}$C-urea. McCarthy and Kamykowski (1972) have also reported receiving two batches of the Worthington product which yielded less activity and a higher blank than in the initial publication of McCarthy (1970). Following this, they suggested using Sigma Type III urease as the enzyme source for the urease method.

Both types of urease enzyme preparations were unsatisfactory for obtaining complete hydrolysis of urea in seawater. However, complete recovery of urea in one seawater sample collected from English Bay was demonstrated, and identical results were obtained by the diacetyl monoxime technique. In four seawater samples from the Strait of Georgia, urea concentrations were similar when determined by both techniques (Table IV: Station T8, 0 and 7.5 m; Station
T10, 1.5 m; Station T81b, 4 m), and in three other samples the urease method yielded higher concentrations of urea than the diacetyl monoxime method (Table IV: Station A530b, 20 m; Station T183b, 9 m; Station T81b, 13 m). Reasons for this latter observation are not clear, but hydrolysis of dissolved organic nitrogen compounds in the seawater by urease may be one explanation (see below "Specificity of methods").

Specificity of methods

Although the diacetyl monoxime method is not specific for urea, there was no evidence of interference from citrulline in seven coastal seawater samples (Station T8, 3.5, 7.5, and 18 m; Station A530b, 0 and 2 m; Station T14, 1.5 and 7 m). These data support a similar conclusion made by Aminot and Keroeul (1982) who analyzed 20 seawater samples from the English Channel. Urease, long considered to be an absolutely specific enzyme for urea, is now known to catalyze hydrolysis of other compounds producing $\text{NH}_4^+$ as an end product (Fishbein et al. 1965, Fishbein 1969b, Sundaram and Laidler 1970).

Of the many urease inhibitors (see for example Olson and Christensen 1982), hydroxamic acids are extremely potent (Kobashi et al. 1962, Gale and Atkins 1969, Dixon et al. 1975b). A number of organisms are known to produce hydroxamic acids, including cyanobacteria (Estep et al. 1975, Armstrong and Van Baalen 1979) and eukaryotic phytoplankton (Trick et al. 1983). However, the presence or absence of these compounds as dissolved constituents in seawater is unclear. In phytoplankton culture filtrate, measured urea
concentrations were less than in ESAW even after corrections were made for pH (Fig. 3). A possible explanation is that extracellular metabolites produced by the phytoplankton (or bacterioplankton) may have inhibited the urease. A number of additive factors are most likely responsible for the low activity of urease in seawater, ultimately resulting in the incomplete hydrolysis of urea during the urease assay.

*Analysis of natural seawater*

Increasing the urease concentration did not result in complete recovery of urea added to natural seawater samples. Analysis of $^{14}\text{CO}_2$ evolved from $^{14}\text{C}$-urea demonstrated that urea hydrolysis proceeds more completely at the higher urease concentrations than is indicated by the $\text{NH}_4^+$ measurement. Nonetheless, by using greater enzyme concentrations I failed to measure all of the urea which had been added to the sample. These data suggest that the high urease enzyme concentrations are responsible for affecting the $\text{NH}_4^+$ determination step in the urease assay. Direct measurement of $\text{NH}_4^+$ in the presence of high levels of urease confirmed this, although I found the results were variable. Loss of $\text{NH}_4^+$ during heating was a minor factor contributing to the lower urea measurements by the urease assay. Using an empirically derived stability constant of the equilibrium, $\text{NH}_3 + \text{H}^+ = \text{NH}_4^+$ (Johansson and Wedborg 1980), it was determined that ca. 40% of total $\text{NH}_3$ and $\text{NH}_4^+$ in a 50°C-heated seawater sample, pH 8.3, will be in the $\text{NH}_3$ form. This $\text{NH}_3$ gas, dissolved in the seawater, represents
a significant portion of the total NH$_3$ and NH$_4^+$. The NH$_3$ has the potential to diffuse out of solution, thereby decreasing the NH$_3$ and NH$_4^+$ concentration in the water. The use of screw-capped tubes with little air space above the seawater would help diminish loss of NH$_3$. Finally, it has been noted by several investigators that urease activity is dependent upon enzyme concentration. In dilute solutions, the specific activity per unit weight of enzyme is greater than in more concentrated solutions (Hofstee 1948, Peterson et al. 1948, Ambrose et al. 1950).

Modifications to the urease method of McCarthy (1970) have been published (McCarthy and Kamykowski 1972, Strickland and Parsons 1972, Parsons et al. 1984b). These procedures have with other changes, increased the amount of enzyme added to the sample. Unfortunately, there has been no emphasis on using a specified activity of urease for the hydrolysis of urea. Enzymes purchased from Worthington and Sigma may vary in activity by as much as two-fold between batches. For this reason, the activity of dialyzed urease may vary similarly. Inattention to this fact may result in enzyme solutions of extremely low activity, and consequently, urea hydrolysis in the seawater samples will be very much reduced.

When urease was added at concentrations suggested by McCarthy (1970) (ca. 0.7 U·ml$^{-1}$) it contributed 0.17 to 0.25 uM NH$_4^+$ to the seawater samples. These concentrations for the NH$_4^+$ blank are slightly greater than those obtained by McCarthy (1970). If higher concentrations of urease are added to the samples, for example 2.5 U·ml$^{-1}$, the amount of
background $\text{NH}_4^+$ will increase proportionately to 0.6 to 0.9 $\mu$M $\text{NH}_4^+$. Uncertainty in the urea measurement will also increase, because this $\text{NH}_4^+$ concentration, which may be many times greater than the actual urea concentration, must be subtracted to calculate the urea concentration.

A number of researchers have supported the use of either the diacetyl monoxime (Carpenter et al. 1972a, Nakas and Litchfield 1977) or urease (McCarthy 1970, Strickland and Parsons 1972, Parsons et al. 1984) methods for measuring urea concentrations; however, their conclusions were not based on published data. Kristiansen (1983) found no significant difference between the diacetyl monoxime method of Newell et al. (1967) and the urease method of McCarthy (1970), but he only tested seawater from two stations within Oslofjord, and he did not run internal standards. These data demonstrate that, in general, the urease method measures lower concentrations of dissolved urea, in a variety of seawater samples, than the diacetyl monoxime method.

In conclusion, the determination of urea concentrations in seawater by the diacetyl monoxime procedure is accurate and reproducible. This method is recommended in preference to the urease method. The diacetyl monoxime method is suitable for urea measurements in artificial and natural seawater samples. It is only subject to interference by citrulline, an organic nitrogen compound that appears to be absent from the coastal seawater samples I examined. The method described is an automated procedure, although a manual application of this
method is published (Rahmatullah and Boyde 1980).

The urease method requires a more complex blanking correction, and the results from this investigation suggest that internal standards must be run with each sample. Other factors are also important for measuring urea by the urease technique and they must be rigorously controlled. Increasing the heating time or the concentration of urease improves the results. These data demonstrate that a constant recovery of urea cannot be assumed with the urease technique, and that seawater type and pH are important factors affecting the activity of urease in this assay.

Summary

A comparison between the diacetyl monoxime and urease methods for measuring dissolved concentrations of urea in seawater was conducted in artificial seawater, phytoplankton culture filtrate and both natural and urea-spiked field samples from coastal and oceanic environments during 1984. The urease technique underestimated urea concentrations in unbuffered phytoplankton culture filtrate. Incomplete hydrolysis of urea in these samples was a result of the inhibition of the urease enzyme. Factors responsible for inhibiting urease included pH, seawater ions, and possibly extracellular metabolites produced in unialgal cultures. Seawater type and time of sample collection were important variables affecting urea measurement by the urease method, and recovery of internal standards ranged from 40 to 100%. Increasing the heating time of the urease assay, or the
concentration of urease added to the seawater samples, increased the amount of urea determined by the urease method. However, measured values were still less than the concentration of the urea internal standards. The diacetyl monoxime method was suitable for urea determinations in all the seawater samples examined; it was easily automated, and the results were accurate and reproducible. This modified technique is recommended for measuring dissolved concentrations of urea in seawater.
CHAPTER 2. TIME COURSE OF UPTAKE OF INORGANIC AND ORGANIC NITROGEN BY PHYTOPLANKTON IN THE STRAIT OF GEORGIA: COMPARISON OF FRONTAL AND STRATIFIED COMMUNITIES

Background

Shallow sea fronts are areas of high primary productivity (Pingree et al. 1975, Parsons et al. 1981, 1983, Holligan et al. 1984) located at the boundary between mixed and stratified water. These regions are characterized by having high phytoplankton biomass in surface water, with measurable concentrations of dissolved nitrate, and a shallow pycnocline which extends to the surface at the frontal boundary (e.g. Simpson and Pingree 1978). In the Strait of Georgia, a coastal basin off the west coast of Canada, several tidally-induced frontal regions have been described (Parsons et al. 1981).

The nitrogen dynamics of frontal regions has received little attention. Floodgate et al. (1981) measured urea decomposition rates in frontal waters of the Irish Sea. These rates were greater than rates in mixed and stratified water, and were concomitant with higher dissolved urea concentrations. High rates of carbon and nitrate uptake have been observed in the proximity of a front by Parsons et al. (1984a). Holligan et al. (1984) calculated that ammonium excretion by zooplankton could account for greater than 50% of the potential phytoplankton requirements on the stratified side of a front, but neither uptake nor regeneration were measured. It is apparent that additional information is required in order to describe and understand the nitrogen
cycling between dissolved and particulate components in these areas.

A surface transect normal to a frontal boundary progresses from a region of high nitrate concentration on the mixed side to nitrogen-depleted water on the stratified side. This transect represents a gradient of nitrogen availability and hence phytoplankton nutritional states. Most of the nitrogen demands of phytoplankton in nitrogen-impoverished water are supplied by ammonium and urea from regenerative processes; whereas, in nitrogen-rich areas nitrogen compounds appear to be utilized at rates proportional to their availability (e.g. Dugdale and Goering 1967, McCarthy et al. 1977). In the nitrogen-rich regions, nitrate supplies most of the phytoplankton nitrogen requirements by virtue of its abundance.

Experiments using laboratory cultures of phytoplankton have demonstrated that the preconditioning nitrogen substrate affects the response of phytoplankton to the additions of different forms of nitrogen (Horrigan and McCarthy 1981, 1982, Dortch and Conway 1984). In nitrogen-starved phytoplankton, the ability to take up nitrate may be lost and must be induced (Dortch et al. 1982, review by Collos 1983, Parslow et al. 1984b). These observations suggest that phytoplankton communities in frontal and stratified water may differ in their response to perturbations of nitrogen by their preference for, and uptake rates of, different nitrogen substrates.
Previous nitrogen uptake experiments have involved single end-point measurements of accumulated $^{15}$N-labelled substrates in particulate matter over long time intervals (Goldman et al. 1981, review by Harrison 1983a). In theory, these experiments provide important information concerning daily rates of nitrogen utilization, as they invariably take into account diel patterns of uptake (providing they are of 24 h duration). The existence of uptake periodicity has been reported for cyclostat cultures of Skeletonema costatum (Eppley et al. 1971b) and Chaetoceros sp. (Malone et al. 1975), and natural phytoplankton communities (e.g. Goering et al. 1964, Eppley et al. 1970, 1971a, McCarthy and Eppley 1972, MacIsaac 1978, Fisher et al. 1982).

The experiments presented in this chapter were designed to examine the time course of nitrogen uptake by phytoplankton from nitrate-deplete stratified water and nitrate-replete frontal water, over a 24 h cycle. The response of the phytoplankton to additions of ammonium, nitrate, and urea was measured in these two regions. From measurements of nitrogen uptake rate using $^{15}$N isotope incorporation, and changes in the concentration of dissolved nitrogen over time, regeneration rates of ammonium and urea by intact plankton communities were calculated. Measured particulate nitrogen concentrations were compared with theoretical values calculated from the changes in the concentrations of dissolved inorganic nitrogen and urea. These results are discussed within the current concepts of nitrogen cycling in marine planktonic ecosystems.
Materials and Methods

Sample collection

Three 24 h time course experiments were conducted in the Strait of Georgia, B.C., Canada aboard the C.S.S. 'Vector' (July 1984); station locations for Time Courses 1, 2 and 3 are shown in Figure 9, and a description of the sample stations is given in Table V. At approximately 0900 h, water samples were collected from a depth corresponding to 50% of the sea-surface irradiance using 5-litre PVC Niskin bottles and transferred into a 20-litre Nalgene carboy.

Chemical, physical and biological analyses

Subsamples for nutrient analyses were filtered through combusted (460°C for 4 h) Whatman GF/F filters using an acid-washed syringe and a 25 mm Millipore Swinex® filter holder. Samples were gently filtered into acid-washed polyethylene bottles and were either analyzed immediately for dissolved inorganic nitrogen (DIN) as ammonium ($NH_4^+$) and nitrate ($NO_3^- + NO_2^-$), and urea concentrations or filtered samples were stored frozen (-20°C), and analyzed within 24 h. $NH_4^+$ and $NO_3^- + NO_2^-$ were measured with a Technicon Autoanalyzer® II following the procedures outlined in Slawyk and MacIsaac (1972) and Wood et al. (1967), respectively. Urea was determined by the diacetyl monoxime thiosemicarbazide technique described in Chapter 1. Duplicate samples for chlorophyll a (chl a) (coefficient of variation, CV, = 4.4 ±
Fig. 9. Station locations for Time Course 1 (T3, frontal station), Time Course 2 (A5, frontal station) and Time Course 3 (T4, stratified station).
Table V

Location of stations and time of sampling.

<table>
<thead>
<tr>
<th>Station and Location</th>
<th>Description</th>
<th>Date</th>
<th>Sample time</th>
<th>Sample depth (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3 49°50.42'N;</td>
<td>Frontal</td>
<td>24.VII.1984</td>
<td>0730</td>
<td>2</td>
</tr>
<tr>
<td>125°00.54'W</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A5 49°53.02'N;</td>
<td>Frontal</td>
<td>28.VII.1984</td>
<td>1000</td>
<td>3</td>
</tr>
<tr>
<td>125°05.48'W</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4 49°55.30'N;</td>
<td>Stratified</td>
<td>29.VII.1984</td>
<td>0800</td>
<td>3</td>
</tr>
<tr>
<td>124°55.30'W</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Data pairs) were collected on Whatman GF/F filters and stored frozen in a desiccator. Chl a was extracted in 90% acetone and analyzed by in vitro fluorometry (Strickland and Parsons 1972) using a Turner Designs model 10 fluorometer. Particulate organic carbon and nitrogen (POC and PON) (CV = 5.2 ± 4.8% and 3.8 ± 4.1%; 7 data pairs), collected on combusted Whatman GF/F filters, were stored similarly and analyzed later with a Perkin Elmer model 240 elemental analyzer. Vertical profiles of temperature and salinity were obtained from continuous profiles, run prior to bottle sampling, using an Interocian CTD system. In vivo chl a fluorescence was measured simultaneously with a Turner model 111 fluorometer. Incident solar irradiance (P.A.R.) was monitored continuously with a Lambda Instruments Li-185 light meter equipped with a Li-190S Surface Quantum Sensor. Phytoplankton species samples (250 ml) were preserved in Lugol's solution and 10 ml subsamples were settled and counted on an inverted microscope; 100 ml subsamples were examined for microzooplankton.

**Nitrogen uptake**

Within 1 h of collection, water was transferred into 500 ml Wheaton glass bottles (clear: light bottles, or darkened with black tape: dark bottles) with teflon-lined caps and saturating additions of either $^{15}$NH$_4$Cl, Na$^{15}$NO$_3$ or CO($^{15}$NH$_2$)$_2$ (all 99 at % $^{15}$N) were added. In Time course 1, 2 ug at N·l$^{-1}$ of $^{15}$NO$_3^-$ or CO($^{15}$NH$_2$)$_2$ was added and in Time Courses 2 and 3, 6 ug at N·l$^{-1}$ of $^{15}$NH$_4^+$, $^{15}$NO$_3^-$ or CO($^{15}$NH$_2$)$_2$ was added. The
precision (± 1 SD) of the nutrient determinations for the time-zero samples was ± 0.09 ug-at N·1⁻¹ (n=5) for NH₄⁺, ± 0.07 ug-at N·1⁻¹ (n=5) for NO₃⁻ and ± 0.02 ug at N·1⁻¹ (n=4) for urea. Light and dark bottle uptake rates of each nitrogen substrate were measured over the time course, and all sample bottles were mixed hourly. Time-zero samples for dissolved nitrogen were withdrawn immediately and analyzed for NH₄⁺, NO₃⁻ and urea concentrations in all bottles. Incubations were conducted under natural light in clear Plexiglas® deck incubators, cooled with surface seawater and covered with neutral density screening to simulate the irradiance at the 50% light depth. At 3 h intervals, particulate matter from duplicate samples was collected by filtration (vacuum less than 125 mm Hg) onto combusted Whatman GF/F filters and stored frozen in a desiccator. Samples for dissolved nitrogen concentrations were taken concurrently, and samples for chl a, and POC and PON were taken every 6 h. It is important to note that samples were taken from randomly selected incubation bottles and chl a and POC and PON samples were taken from bottles distinct from those analyzed for ¹⁵N atom % excess in particulate matter and dissolved nitrogen concentrations.

Calculations

Nitrogen in the particulate samples was converted to N₂ (g) by the micro-Dumas dry combustion technique as described by LaRoche (1983). Samples were analyzed for ¹⁵N enrichment with a JASCO model N-150 emission spectrometer (Fiedler and
Proksch 1975). Nitrogen uptake rates were calculated according to the equations of Dugdale and Goering (1967) and are presented as nitrogen-specific (h⁻¹) and absolute (ug at N·l⁻¹·h⁻¹) rates. The ratio of nitrogen uptake in the dark bottle (continuous darkness) to that in the light bottle (exposed to the natural light cycle) (V_D:V_L) is also reported. Ammonium and urea regeneration rates (d) have been determined using the approach of Fisher et al. (1981). These rates were calculated using the Blackburn-Caperon equation (Blackburn 1979, Caperon et al. 1979)

\[ d = \frac{P}{t} + i \]

where \( P \) = change in concentration of NH₄⁺ or urea (ug at N·l⁻¹) over time interval t (h), and \( i \) = nitrogen uptake rate (ug at N·l⁻¹·h⁻¹) calculated from ¹⁵N accumulation in the particulate matter (Dugdale and Goering 1967). Disappearance uptake rates (V_d) were calculated from the change in concentration of dissolved nitrogen per unit time (ΔP/t) and, like the nitrogen-specific and absolute ¹⁵N uptake rates (V_i), are reported for the time intervals over which they have been calculated.

Results

Station profiles

The vertical profiles of temperature, relative in vivo chl a fluorescence, and NO₃⁻ concentration for the frontal water stations (Time Course 1 [T3]; and Time Course 2 [A5])
showed almost identical trends with depth; thus only the synoptic profile of Time Course 2 is presented (Fig. 10A). Throughout the Results and Discussion, reference to frontal water pertains to Time Course 2 unless specified otherwise. The results of Time Course 1 are discussed only briefly because of the paucity of data. The diagnostic features of the frontal water were the shallow thermocline and high fluorescence at the depth of the nitracline (3 to 7 m). Time Course 3 was conducted in stratified water at Station T4, and the depth profile (Fig. 10B) demonstrated a subsurface fluorescence maximum (ca. 10 m) which was overlain by nitrate-depleted mixed water. A summary of the initial biomass data and environmental conditions for each station is given in Table VI.

**Plankton community composition**

The species composition of the phytoplankton community in the frontal and stratified water was very different (Table VII). In the frontal water, chain-forming diatoms of the genus *Chaetoceros* formed aggregates, less than or equal to 1 mm, which contained some pennate diatoms belonging to *Navicula* spp. and *Nitzschia* spp. Because of the size of the diatom flocs, the water samples were not screened through Nitex® netting in order to minimize macrozooplankton predation during incubations. In spite of the absence of these aggregates in stratified water, to remain consistent, these water samples were not passed through Nitex® netting. Small flagellates less than 5 um were the most common phytoplankton in the
Fig. 10. Depth profiles of temperature, *in vivo* fluorescence, and NO$_3^-$ concentration. (A) Frontal station A5, Time Course 2. (B) Stratified station T4, Time Course 3.
Table VI

Initial environmental conditions of seawater collected for time course experiments.

<table>
<thead>
<tr>
<th>Station</th>
<th>Dissolved nutrient concentration</th>
<th>Chl a</th>
<th>PON</th>
<th>POC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(ug·l⁻¹) (ug at N·l⁻¹) (ug at C·l⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>NO₃⁻</td>
<td>urea</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(ug at N·l⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>--</td>
<td>2.99</td>
<td>0.18</td>
<td>6.55</td>
</tr>
<tr>
<td>2</td>
<td>0.27</td>
<td>4.55</td>
<td>0.60</td>
<td>2.12</td>
</tr>
<tr>
<td>3</td>
<td>0.19</td>
<td>&lt;0.05</td>
<td>0.33</td>
<td>0.39</td>
</tr>
</tbody>
</table>
Table VII

Phytoplankton community composition in frontal and stratified water.

<table>
<thead>
<tr>
<th>Station</th>
<th>Phytoplankton (10^6 cells·l⁻¹)</th>
<th>Diatoms</th>
<th>Dinoflagellates</th>
<th>Flagellates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal A5</td>
<td>2.3</td>
<td>0.023</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Stratified T4</td>
<td>0.43</td>
<td>0.049</td>
<td>1.6</td>
<td></td>
</tr>
</tbody>
</table>
stratified water. *Chaetoceros* spp., *C. socialis* and *Skeletonema costatum* were the most abundant of the diatoms, whereas dinoflagellates were almost exclusively *Gymnodinium* spp. Water samples were not originally taken for zooplankton species enumeration. The abundance of these organisms, as seen in the phytoplankton samples, suggested that they could have been important grazers and nitrogen remineralizers. As a first approximation, the numbers and types of these organisms in samples were determined (Table VIII).

15N uptake and nitrogen disappearance

During the time course experiments, changes in the concentration of dissolved NH_4^+, NO_3^- and urea, and the incorporation of 15N-labelled nitrogen into the particulate matter were measured. Both approaches yield different information concerning nitrogen utilization by the phytoplankton. Changes in dissolved nitrogen concentration represent net community flux of that nutrient and encompass regenerative and uptake processes. By contrast, 15N-isotope accumulation gives a measure of the gross uptake by the phytoplankton providing there is no recycling of 15N, and 15N enrichment remains constant. Results from Time Course 2 (frontal water) and Time Course 3 (stratified water) experiments are shown in Figures 11 and 12, respectively. Data from Time Course 2 demonstrate multiple nitrogen substrate utilization by phytoplankton; specifically for NH_4^+, NO_3^- and urea (Fig. 11C, E) and NO_3^- and urea (Fig. 11G). By virtue of the high ambient NO_3^- concentration in the frontal
Table VIII

Zooplankton community structure in frontal and stratified water.

<table>
<thead>
<tr>
<th>Station</th>
<th>Tintinnids</th>
<th>Calanoid copepods</th>
<th>Ciliates excl. tintinnids</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal A5</td>
<td>470</td>
<td>50</td>
<td>730</td>
<td>280</td>
</tr>
<tr>
<td>Stratified T4</td>
<td>180</td>
<td>60</td>
<td>140</td>
<td>300</td>
</tr>
</tbody>
</table>

1 50 to 60% were *Noctiluca* sp., the remainder were *Oikopleura* sp. and unidentified zooplankton.
Fig. 11. Time course measurements at frontal station (A5), Time Course 2. (A) Daily incident irradiance during experiment. (B, D, F) $^{15}$N atom % excess in particulate matter for light and dark bottle incubations following addition of 6 ug at N·l$^{-1}$ of (B) NH$_4^+$, (D) NO$_3^-$ and (F) urea (error bars represent the range of duplicates). (C, E, G) Corresponding measurements of dissolved NH$_4^+$ (●), NO$_3^-$ (○) and urea (Δ) in (C) NH$_4^+$, (E) NO$_3^-$, and (G) urea-spiked samples. Dashed line indicates no measurements of dissolved urea at 3 and 6 h.

Fig. 12. As Fig. 12 except at stratified station (T4), Time Course 3.
waters, disappearance uptake rates of $\text{NO}_3^-$ in the $\text{NH}_4^+$ and urea-spiked samples were determined. Disappearance uptake rates for nitrate were similar in the presence ($V_{0-6h} = 0.521 \ \mu\text{g at N} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$) and absence ($V_{0-9h} = 0.567 \ \mu\text{g at N} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$) of urea but were reduced in the $\text{NH}_4^+$-spiked samples ($V_{0-9h} = 0.267 \ \mu\text{g at N} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$). The $^{15}\text{N}$-urea atom % accumulation rate was constant over the first 15 h, but prior to the end of the dark period it increased and remained constant until the end of the incubation (Fig. 11F). The increase in urea uptake rate coincided with the depletion of external $\text{NO}_3^-$; moreover, the change in urea concentration was minimal over the first 6 h, when $\text{NO}_3^-$ concentrations were high (4.55 to 1.4 $\mu$g at N·l⁻¹) and $\text{NO}_3^-$ was being taken up (Fig. 11G). $^{15}\text{N}$-$\text{NO}_3^-$ and $^{15}\text{N}$-$\text{NH}_4^+$ incorporation was not constant during the incubation, but substrate exhaustion did not occur until the 21 to 24 h time interval.

Time Course 1 was conducted in phytoplankton-rich water and $\text{NO}_3^-$ depletion occurred in less than 3 h. Nitrogen-specific uptake rate of $\text{NO}_3^-$ ($V_{0-3h} = 0.070 \ \text{h}^{-1}$) was the highest of any nitrogen substrate measured in all time course experiments. The disappearance uptake rate over the same time interval ($V_{0-3h}$) was 1.35 $\mu$g at N·l⁻¹·h⁻¹, and using a time averaged particulate nitrogen, calculated from the amount of $\text{NO}_3^-$ taken up and the initial measured particulate nitrogen, the nitrogen-specific uptake rate ($V_{0-3h} = 0.081 \ \text{h}^{-1}$) was in fair agreement with the rate determined by $^{15}\text{N}$ uptake. As a consequence of substrate exhaustion, both techniques yielded rates which were underestimates. Simultaneous uptake of $\text{NO}_3^-$
and urea was evident in the urea-spiked samples. The maximum disappearance rate of urea \( (V_{3-8h} = 0.314 \text{ ug at N·l}^{-1}·	ext{h}^{-1}) \) was less than the \( \text{NO}_3^- \) rate \( (V_{0-6h} = 0.441 \text{ ug at N·l}^{-1}·	ext{h}^{-1}) \).

The pattern of \( ^{15} \text{N} \) uptake by the phytoplankton in the stratified water was similar in the \( \text{NH}_4^+ \), \( \text{NO}_3^- \) and urea-spiked samples (Fig. 12B, D, F). Uptake was linear over the first 9 to 12 h and was subsequently reduced during the dark period and increased again in the early morning. Substrate depletion did not occur in these experiments, and utilization of nitrogen was minimal in the \( \text{NH}_4^+ \), \( \text{NO}_3^- \) and urea-spiked samples (23, 18 and 10%, respectively).

**Uptake periodicity**

Clear indications of urea regeneration, and to a lesser extent \( \text{NH}_4^+ \) regeneration, were evident from increases in their concentrations over the time course in all 3 experiments (Fig. 12C, E, G). Furthermore, the pattern of \( \text{NH}_4^+ \) and urea production in the samples suggested that there was a periodicity in uptake and/or regeneration processes. Similar results were seen in Time Course 2 (Fig. 11C, E, G) particularly for urea production over the 15 to 21 h time interval. Results from both frontal and stratified time course experiments demonstrate that simultaneous utilization of two or more nitrogen substrates occurs even when the concentration of one of the nitrogen substrates (\( \text{NH}_4^+ \), \( \text{NO}_3^- \), or urea) is in excess of the other(s). This suggests that such a phenomenon may naturally occur in these communities. The pattern of \( ^{15} \text{N} \)-labelled \( \text{NH}_4^+ \), \( \text{NO}_3^- \) and urea uptake rates
suggests the existence of a diel periodicity in nitrogen uptake, in both frontal and stratified water (Fig. 13). The decrease in uptake of $\text{NH}_4^+$ and $\text{NO}_3^-$ from 21 to 24 h in Time Course 2 was due to substrate exhaustion (see Fig. 11C, E). In the frontal community, uptake rates of $\text{NO}_3^-$ were greatest throughout the time course, and there was significant dark uptake of $\text{NO}_3^-$. In comparison, $\text{NH}_4^+$ uptake rates were highest in the stratified community, and $\text{NO}_3^-$ and urea uptake rates were similar but of a lesser magnitude. In both time courses there was a tendency for nitrogen uptake rates to increase prior to the onset of the light period; this was most evident in the urea-spiked samples.

**Chl a specific uptake rates**

Uptake rates normalized per unit chl a showed that $\text{NH}_4^+$ and urea uptake were on average 2 and 2.4 times higher in the stratified water; whereas, $\text{NO}_3^-$ uptake rates were on average 1.6 times higher in frontal water (Table IX). Chl a specific uptake rates for each nutrient, when compared between stations, were most similar over the dark period (12 to 18 h), while the greatest disparity was found over the first 6 h.

**Comparison of rates**

The $^{15}$N uptake rate, disappearance uptake rate and the rate of change of the PON, calculated from the difference between measured values, are presented in Figure 14. All rates were calculated over 6, 12, 18, and 24 h time intervals,
Fig. 13. Nitrogen-specific uptake rates of NH$_4^+$ (●), NO$_3^-$ (○) and urea (△) in (A) frontal and (B) stratified water. Rates determined for 3 or 6 h intervals; each point indicates a rate calculated over the time interval between it and the next point on the curve. Shaded area on the abscissa delimits the dark period.
Table IX

Chlorophyll a specific uptake rates of NH$_4^+$, NO$_3^-$ and urea in frontal (A5) and stratified (T4) water. The dark period occurs during the 12 to 18 h time interval.

<table>
<thead>
<tr>
<th>Nitrogen substrate</th>
<th>Time interval (h)</th>
<th>Chl a specific N-uptake rate [ug at N (ug chla)$^{-1}$.h$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frontal stn</td>
<td>Stratified stn</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 6</td>
<td>0.091</td>
<td>0.261</td>
</tr>
<tr>
<td>6 - 12</td>
<td>0.060</td>
<td>0.133</td>
</tr>
<tr>
<td>12 - 18</td>
<td>0.025</td>
<td>0.030</td>
</tr>
<tr>
<td>18 - 24</td>
<td>0.028</td>
<td>0.047</td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 6</td>
<td>0.162</td>
<td>0.098</td>
</tr>
<tr>
<td>6 - 12</td>
<td>0.075</td>
<td>0.082</td>
</tr>
<tr>
<td>12 - 18</td>
<td>0.042</td>
<td>0.019</td>
</tr>
<tr>
<td>18 - 24</td>
<td>0.068</td>
<td>0.039</td>
</tr>
<tr>
<td>Urea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 6</td>
<td>0.040</td>
<td>0.127</td>
</tr>
<tr>
<td>6 - 12</td>
<td>0.028</td>
<td>0.125</td>
</tr>
<tr>
<td>12 - 18</td>
<td>0.026</td>
<td>0.019</td>
</tr>
<tr>
<td>18 - 24</td>
<td>0.050</td>
<td>0.053</td>
</tr>
</tbody>
</table>
Fig. 14. Nitrogen uptake rates determined by $^{15}$N atom % excess accumulation in the particulates (●), change in dissolved nitrogen concentration (○) and by change in the particulate nitrogen concentration (▲) over 6, 12, 18 and 24 h time intervals. (A) NH$_4^+$, (B) NO$_3^-$, and (C) urea-spiked samples in frontal water and (D) NH$_4^+$, (E) NO$_3^-$ and (F) urea-spiked samples in stratified water.
and this approach was taken, rather than calculating the rates over successive 6 h intervals, to minimize fluctuations due to sample variability. Comparison of data from the frontal station indicates that in the NH$_4^+$ and urea-spiked samples, the rate of change of the particulate nitrogen is greater than the accumulation of $^{15}$N or the disappearance of either nutrient (Fig. 14A, C). In the nitrate-spiked samples (Fig. 14B), the rate of nitrate uptake as determined by the disappearance of NO$_3^-$, the incorporation of $^{15}$N-NO$_3^-$ and the change in PON are similar. A general feature of the data from the stratified station is the more rapid change in PON than the $^{15}$N uptake or disappearance uptake rates (Fig. 14D, E, F). Furthermore, the disappearance rates of NH$_4^+$ and urea are consistently less than the $^{15}$N uptake rates.

**Dark/Light uptake**

The ratio of dark to light $^{15}$N uptake rate ($V_D:V_L$) for NH$_4^+$, NO$_3^-$ and urea is given in Table X. At both station, NH$_4^+$ dark uptake rates were a significant fraction of the light uptake rates throughout the entire time course. The $V_D:V_L$ for NH$_4^+$ in frontal water was constant (38%) and was less than the ratio in stratified water (52 to 102%). Initial dark uptake rates of urea were 60 to 81% of the light rates in all three time course experiments. Dark urea uptake in the stratified water was always a measurable fraction of the light rate and appeared less light dependent than at the frontal stations. The light dependency of NO$_3^-$ uptake was more similar to that of urea than ammonium in both stratified and
Table X

Ratio of dark to light uptake rates \((V_D:V_L)\) of \(\text{NH}_4^+\), \(\text{NO}_3^-\) and urea for frontal and stratified water.

<table>
<thead>
<tr>
<th>Station</th>
<th>Time interval (h)</th>
<th>(\text{NH}_4^+) ((V_D:V_L))</th>
<th>(\text{NO}_3^-) ((V_D:V_L))</th>
<th>Urea ((V_D:V_L))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal T3</td>
<td>0 - 6</td>
<td>--</td>
<td>--</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>6 - 11</td>
<td>--</td>
<td>--</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>11 - 18</td>
<td>--</td>
<td>--</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>18 - 24</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Frontal A5</td>
<td>0 - 6</td>
<td>0.37</td>
<td>0.08</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>6 - 12</td>
<td>0.39</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12 - 18</td>
<td>0.37</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>18 - 24</td>
<td>0.39</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stratified T4</td>
<td>0 - 9</td>
<td>0.58</td>
<td>0.18</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>9 - 18</td>
<td>1.02</td>
<td>0.60</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>18 - 24</td>
<td>0.52</td>
<td>0</td>
<td>0.06</td>
</tr>
</tbody>
</table>
frontal water.

Regeneration rates

The regeneration rates of NH$_4^+$ and urea in the frontal water were similar (Table XI). Note that the change in substrate concentration was much greater than the $^{15}$N uptake rate over the 18 to 24 and 6 to 12 h time periods for NH$_4^+$ and urea, respectively. As a result, negative rates of regeneration have been calculated. A consistent pattern was seen for the calculated values of NH$_4^+$ and urea regeneration rates in stratified water. The disappearance rates of both nutrients surpassed the $^{15}$N uptake rates in the 12 to 18 and 18 and 24 h time intervals. Urea regeneration rates were approximately 5 and 2 times greater than the corresponding NH$_4^+$ regeneration rates for the first two 6 h intervals.

Mass balance

Changes in the PON over 6, 12, 18 and 24 h time intervals for each set of nitrogen-spiked samples were calculated (Table XII). By way of comparison, the change in the total DIN and urea ($\Delta P_T$) over the same time interval and the amount of nitrogen accumulated in particulate matter ($\Sigma V_T^i$), as determined by $^{15}$N atom percent excess data, are reported. The results indicate that the frontal and stratified communities were very different. The change in $P_T$ in the frontal water samples consistently overestimated the change in the PON for all 3 nutrients. However, the opposite was true in the stratified water samples where the change in PON was always
Table XI

Regeneration rates of $\text{NH}_4^+$ and urea in frontal and stratified water.

<table>
<thead>
<tr>
<th>Station</th>
<th>Nitrogen addition</th>
<th>Time (h)</th>
<th>Change in concentration of added $\text{N}^{15}$</th>
<th>Uptake Regeneration rate</th>
<th>Regeneration rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(ug at N·1$^{-1}$·h$^{-1}$)</td>
</tr>
<tr>
<td>Frontal</td>
<td>$\text{NH}_4^+$</td>
<td>0 - 6</td>
<td>.177</td>
<td>.224</td>
<td>.047</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 - 12</td>
<td>.206</td>
<td>.232</td>
<td>.026</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 - 18</td>
<td>.086</td>
<td>.141</td>
<td>.055*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 - 24</td>
<td>.420</td>
<td>.174</td>
<td>-.246</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>0 - 6</td>
<td>.040</td>
<td>.094</td>
<td>.054*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 - 12</td>
<td>.398</td>
<td>.094</td>
<td>-.305</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 - 18</td>
<td>.116</td>
<td>.132</td>
<td>.016</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 - 24</td>
<td>.287</td>
<td>.308</td>
<td>.021</td>
</tr>
<tr>
<td>Stratified</td>
<td>$\text{NH}_4^+$</td>
<td>0 - 6</td>
<td>.085</td>
<td>.103</td>
<td>.018</td>
</tr>
<tr>
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<td>6 - 12</td>
<td>.052</td>
<td>.073</td>
<td>.021*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 - 18</td>
<td>.039</td>
<td>.025</td>
<td>-.014*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 - 24</td>
<td>.074</td>
<td>.053</td>
<td>-.021</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>0 - 6</td>
<td>-.044</td>
<td>.050</td>
<td>.094</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 - 12</td>
<td>.023</td>
<td>.066</td>
<td>.043</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 - 18</td>
<td>.026</td>
<td>.014</td>
<td>-.012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 - 24</td>
<td>.088</td>
<td>.050</td>
<td>-.033</td>
</tr>
</tbody>
</table>

1 Equivalent to $V_d$; negative value indicates an increase in substrate concentration over the incubation period.

2 Regenerative fluxes were calculated using a mass balance; see "Materials and Methods".

* Indicates that disappearance of dissolved nutrient was greater than uptake rates calculated from $\text{N}^{15}$. 
Table XII

Changes over time in measured DIN and urea concentration ($\Delta P_T$), particulate nitrogen ($\Delta PON$), and amount of $^{15}$N-nitrogen accumulated in the particulate matter ($\Xi V^i_T$) in frontal and stratified water.

<table>
<thead>
<tr>
<th>Station</th>
<th>Nitrogen addition</th>
<th>Time (h)</th>
<th>$\Delta P_T$ (ug at N·l$^{-1}$)</th>
<th>$\Delta PON$ (ug at N·l$^{-1}$)</th>
<th>$\Xi V^i_T$ (ug at N·l$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal</td>
<td>NH$_4^+$</td>
<td>0 - 6</td>
<td>3.51</td>
<td>1.94</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 12</td>
<td>5.79</td>
<td>4.86</td>
<td>2.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 18</td>
<td>5.59</td>
<td>7.24</td>
<td>3.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 24</td>
<td>10.10</td>
<td>8.19</td>
<td>4.67</td>
</tr>
<tr>
<td>A5</td>
<td>NO$_3^-$</td>
<td>0 - 6</td>
<td>3.41</td>
<td>2.66</td>
<td>2.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 12</td>
<td>5.36</td>
<td>4.99</td>
<td>4.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 18</td>
<td>7.74</td>
<td>5.58</td>
<td>5.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 24</td>
<td>10.58</td>
<td>9.18</td>
<td>7.82</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>0 - 6</td>
<td>3.21</td>
<td>2.97</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 12</td>
<td>6.56</td>
<td>3.65</td>
<td>1.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 18</td>
<td>7.70</td>
<td>5.13</td>
<td>1.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 24</td>
<td>9.62</td>
<td>7.60</td>
<td>3.79</td>
</tr>
<tr>
<td>Stratified</td>
<td>NH$_4^+$</td>
<td>0 - 6</td>
<td>-0.01</td>
<td>0.98</td>
<td>0.62</td>
</tr>
<tr>
<td>T4</td>
<td></td>
<td>0 - 12</td>
<td>0.58</td>
<td>1.01</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 18</td>
<td>0.66</td>
<td>1.42</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 24</td>
<td>1.51</td>
<td>1.47</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td>NO$_3^-$</td>
<td>0 - 6</td>
<td>-1.08</td>
<td>0.21</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 12</td>
<td>0.01</td>
<td>1.28</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 18</td>
<td>0.16</td>
<td>1.55</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 24</td>
<td>0.65</td>
<td>1.66</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>0 - 6</td>
<td>-0.24</td>
<td>0.57</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 12</td>
<td>-0.08</td>
<td>1.15</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 18</td>
<td>0.08</td>
<td>1.19</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - 24</td>
<td>0.59</td>
<td>1.60</td>
<td>1.08</td>
</tr>
</tbody>
</table>
greater than \( \Delta P_T \). Summation of the \(^{15}\text{N} \) accumulation in the particulate matter over time indicates that this nitrogen contribution cannot account for the change in the particulate nitrogen except in the nitrate-spiked time course in frontal water and the \( \text{NH}_4^+ \)-spiked time course in stratified water.

**Discussion**

*Experimental considerations*

In these experiments, saturating additions of each nitrogen compound (\( \text{NH}_4^+ \), \( \text{NO}_3^- \) and urea) were required to ensure that substrate exhaustion did not occur during the time course. This approach was chosen, rather than collecting water samples at various times and determining *in situ* rates of nitrogen uptake, in order to eliminate potential complicating factors such as diel migration of phytoplankton (Blasco 1978), surface water advection and problems associated with adding tracer amounts of \(^{15}\text{N} \)-substrate (Goldman *et al.* 1981, Glibert *et al.* 1982b). Consequently, these rates of nitrogen uptake are potential rates (with the exception of \( \text{NO}_3^- \) uptake in frontal stations), as they will only be realized under conditions where the nitrogen substrate concentration is elevated to a level sufficient to saturate the uptake system. Empirical observations, such as deep water injection (Walsh *et al.* 1977), soliton enrichment (Holligan *et al.* 1985), diel migratory behavior (Cullen and Horrigan 1981), phytoplankton sinking (Bienfang *et al.* 1982) and patchy excretion (Lehman and Scavia 1982) plus theoretical
considerations (McCarthy and Goldman 1979, Parslow et al. 1985) lend credence to this approach. More importantly, this approach has enabled me to derive additional information concerning the physiological state of, and the nitrogen cycling within, the plankton community of these two types of coastal ecosystems.

**Simultaneous uptake of nitrogen compounds**

Simultaneous utilization of NH$_4^+$ and NO$_3^-$ is well documented (Collos and Lewin 1974, Eppley and Renger 1974, Bienfang 1975, Conover 1975, Caperon and Ziemann 1976, Conway 1977, Maestrini et al. 1982), and the results of this study not only demonstrate dual nitrogen substrate utilization but that NH$_4^+$, NO$_3^-$ and urea may be taken up concurrently. As pointed out by Collos (unpubl.), multiple nitrogen substrate utilization will result in a reduction of the nitrogen-specific uptake rate of the $^{15}$N-labelled compound compared to the nitrogen-specific uptake rate determined when only the $^{15}$N-labelled compound is being taken up. I calculated the absolute uptake rates using the final PON, determined at the end of an incubation, which gives an accurate measure of the uptake rate of the $^{15}$N-labelled nutrient into the phytoplankton and avoids potential artifacts caused by the incorporation of non-$^{15}$N-labelled nitrogen. Recently, Maestrini et al. (1982) demonstrated that microalgae of oyster ponds took up NH$_4^+$ and NO$_3^-$ at the same rate once the NH$_4^+$ concentration had decreased to ca. 7 ug at N·l$^{-1}$. The results from the frontal community demonstrated the similarity of NH$_4^+$...
and NO₃⁻ disappearance uptake rates, in the NH₄⁺-spiked samples. However, the NO₃⁻ disappearance uptake rate was reduced by 50% in the NH₄⁺-spiked samples by comparison to the nitrate-spiked samples. Similar NH₄⁺ suppression of NO₃⁻ uptake has been reported for both laboratory (e.g. Conway 1977, Cresswell and Syrett 1979) and natural phytoplankton assemblages (e.g. Blasco and Conway 1982). NO₃⁻ and urea uptake interactions from two time course experiments in frontal water are contradictory. In Time Course 1, there was a 70% reduction in the NO₃⁻ disappearance uptake rate in the presence of urea, but the NO₃⁻ disappearance uptake rate was unaffected or slightly enhanced in the presence of urea in Time Course 2. The reasons for this discrepancy are not apparent, nonetheless, variation in phytoplankton community structure, relative growth rates and internal nitrogen status may be important differences between the two stations. These variables have been identified as affecting uptake interactions among nitrogen compounds (Dortch and Conway 1984). In Time Course 2, the apparent slow disappearance uptake rate of urea, over the first 6 h, may be explained by regeneration of urea over this period. Alternatively, McCarthy and Eppley (1972) have reported NO₃⁻ inhibition of urea uptake in natural seawater samples. Irrespective of the concentration of NH₄⁺, NO₃⁻ or urea (less than equal to 6 ug at N·l⁻¹), phytoplankton in both the frontal and stratified water are capable of utilizing low concentrations of regenerated nitrogen (NH₄⁺ and urea).
Variations in nitrogen uptake rate

In these experiments, sampling intervals were long relative to phytoplankton rapid uptake responses seen in the laboratory (Conway et al. 1976, Parslow et al. 1984a, b) and the field (e.g. Glibert and Goldman 1981); therefore, short term variations in uptake rate were not evident. Enhanced uptake of NH$_4^+$ and urea by NO$_3^-$ sufficient phytoplankton has been reported (Horrigan and McCarthy 1981, 1982, Parslow et al. 1984b). In the light of the slower long-term rates of NH$_4^+$ and urea uptake in the frontal station, relative to NO$_3^-$, it appears unlikely that such a process occurred on time scales shorter than these measurements. On long time scales, changes in uptake rate due to diel periodicity were evident in the results. Bottle confinement effects have been shown to lead to serious underestimates of rate processes (Venrick et al. 1977), but the constant rates of chl a and POC and PON synthesis indicate no such artifacts in these experiments. Olson and Chisholm (1983) have shown that cell division patterns of nitrogen-limited phytoplankton cultures may be entrained by NH$_4^+$ pulses. Although samples were spiked with saturating additions of each nitrogen compound, there is evidence from an earlier cruise in the Strait of Georgia (August 1983) of uptake periodicity at ambient concentrations of dissolved nitrogen (Parslow et al. unpubl.). Uptake periodicity was also evident in nitrogen-sufficient frontal water.
Effects of light/dark regime on nitrogen uptake

The constancy of $V_D:V_L$ for $\text{NH}_4^+$ in frontal water, when $\text{NH}_4^+$ uptake rates of phytoplankton exposed to the natural light/dark cycle were periodic, suggests that $\text{NH}_4^+$ uptake is circadian; in absence of the light/dark cycle the rhythm is free running (see Chisholm 1981). This conclusion is supported by Goering et al. (1964) who found rhythmic variation in both $\text{NH}_4^+$ and $\text{NO}_3^-$ uptake by natural communities under continuous light. The results for $\text{NO}_3^-$ and urea demonstrate the dependency of uptake on light, and in this respect both nutrients are comparable. The light dependence of uptake of both nutrients is well established (MacIsaac and Dugdale 1972, Mitamura and Saijo 1975, 1980a, Harvey and Caperon 1976, Webb and Haas 1976, Nelson and Conway 1979). Other reports have shown that nitrogen-deprived phytoplankton have higher dark uptake rates of nitrogen than nitrogen-replete phytoplankton (e.g. Syrett 1962, Eppley and Coatsworth 1968, Malone et al. 1975, Rees and Syrett 1979b). In this study, dark nitrogen uptake rates normalized to chl $a$ were highest in the nitrogen-depleted stratified water, in agreement with these observations. Dark uptake rates were also a greater proportion of the light rates for $\text{NH}_4^+$, $\text{NO}_3^-$ and urea in stratified water. The higher chl $a$ specific uptake rates of $\text{NH}_4^+$ and urea in stratified water and of $\text{NO}_3^-$ in frontal water are consistent with the way we envisage nitrogen supply to these areas. Regenerated nitrogen ($\text{NH}_4^+$ and urea) has been shown to supply most of the phytoplankton nitrogen
demand in nitrogen-depleted waters and as the concentration of ambient $\text{NO}_3^-$ increases so does the relative importance of $\text{NO}_3^-$ for the phytoplankton nitrogen ration (e.g. McCarthy et al. 1977, Harrison 1980, Glibert et al. 1982a, Cochlan 1986).

$\text{NH}_4^+$ and urea regeneration

These estimates of $\text{NH}_4^+$ regeneration rates are in agreement with previously published rates for coastal waters (0.01 to 0.31 ug at N·l$^{-1}$·h$^{-1}$) (Caperon et al. 1979, Cochlan 1982, Glibert 1982, Glibert et al. 1982b, Paasche and Kristiansen 1982, LaRoche 1983). The method used to calculate regeneration rates is inferior by comparison to the isotope dilution method (Blackburn 1979, Caperon et al. 1979). Unlike experiments employing trace additions of $^{15}$N, the concentration of regenerated nitrogen was small relative to the added $^{15}$N substrates, and thus the $^{15}$N enrichment factor remained constant over the incubation. Consequently, there is little error associated with the uptake measurement. The rates of urea regeneration are of similar magnitude to the $\text{NH}_4^+$ regeneration rates, and the rates are comparable in both communities. These experiments have enabled me to quantify urea regeneration by an intact plankton community. These results are an improvement over previous attempts which have quantified urea production by species, or size fractionated assemblages, of zooplankton. The patterns of $\text{NH}_4^+$ and urea production over the time course experiments indicate a periodicity which is not a result of reduced uptake rates. This corroborates data of Caperon et al. (1979) and Glibert
(1982) who reported higher rates of NH$_4^+$ regeneration at night and early morning. Additionally, Collos and Lewin (1974) and Hattori (1982) have shown diel variations in dissolved NH$_4^+$ concentration in coastal waters. Unlike the dissolved nitrogen concentration measurements, the calculated regeneration rates do not show the same periodicity, since the time scales over which they were calculated are much greater than these physiological processes.

Regeneration of nitrogen has long been recognized as a possible artifact in determining $^{15}$N uptake rates (Dugdale and Goering 1967), and recently it has been shown that these rates may be underestimated by a factor of ca. 2 when a constant $^{15}$N atom % enrichment is assumed (Glibert et al. 1982b). I have not corrected these uptake rates for isotope dilution, but as I will now show, regeneration of NH$_4^+$ and urea in these experiments, has little effect on uptake rates calculated with the initial enrichment; however, such a process may dramatically affect the disappearance uptake rates.

Initial additions of nitrogen were 6.0 ug at N·l$^{-1}$, correcting for background and purity of the substrate this represents 5.92 ug at $^{15}$N l$^{-1}$. When no regeneration occurs and $^{15}$N is conserved;

$$P_t = P_o - V^i(t)$$

and

$$P_o - P_t = V^i \frac{t}{t}$$
where $P_o$ and $P_t$ = initial and final concentrations of dissolved nitrogen; $V^i = ^{15}N$ uptake rate; $t = time$; and disappearance uptake rate equals $^{15}N$ uptake rate. It is obvious that large additions of $^{15}N$ to samples cause the isotope enrichment factor ($R$) to be relatively insensitive to additions of regenerated nitrogen. For example, if $R$ changes from 0.9394 to 0.8500 and I assume that the pulse of regenerated $^{14}N$ (0.66 ug at N·l$^{-1}$) is added immediately after time zero, disappearance uptake rates will be underestimated by 62%, while $^{15}N$ uptake rates will decrease by only 10%. Therefore, regenerative processes are of lesser consequence to $^{15}N$-uptake rate calculations when the concentration of $^{15}N$ is large; if the total concentration of dissolved nitrogen becomes low, for example toward the end of a time-course experiment, regeneration of $^{14}N$ will have a greater effect on $^{15}N$ uptake rate calculations.

With this line of reasoning and as discussed earlier, I have interpreted discrepancies between the disappearance uptake rate and $^{15}N$ uptake rate as indicative of regeneration. In the $NH^+_4$ and urea-spiked samples from stratified water, regeneration is evident; however, the disappearance uptake rates are in closer agreement with the $^{15}N$ uptake rates at the end of the time course compared with the beginning (Fig. 14). In frontal water, the discrepancies between disappearance uptake rates of $NH^+_4$ and $^{15}NH^+_4$ uptake rates may be adequately explained by regeneration. The exception is the final 6 h period where changes in dissolved concentrations exceed $^{15}N$ incorporation rates. The urea disappearance rates are greater
than $^{15}$N incorporation rates after the first 6 h, and the very fast disappearance rates from 6 to 12 h make the 0 to 18 and 0 to 24 rates high as well.

Particulate nitrogen balance

In a two-compartment system consisting of DIN + urea and PON, regardless of the flux rates between the two pools, changes in the concentration of one component should be reflected by corresponding changes in the other. Using this approach, nitrogen will be conserved providing the system is closed. Additionally, by including $\text{NH}_4^+$, $\text{NO}_3^-$ and urea as part of the dissolved nitrogen pool I am able to account for circumstances when regenerated nitrogen differs from the assimilated form. The corollary of this is that the regenerated nitrogen is in the form of $\text{NH}_4^+$ and/or urea. In summary, this relation may be expressed as:

$$\text{PON}_\alpha + \text{P}_{\text{To}} = \text{PON}_\tau + \text{P}_{\text{Tt}}$$

and

$$\Delta\text{PON} = \Delta\text{P}_{\text{T}}$$

where $\text{PON}_\alpha$ and $\text{PON}_\tau$ = initial and final particulate nitrogen concentrations; $\text{P}_{\text{To}}$ and $\text{P}_{\text{Tt}}$ = initial and final DIN + urea concentrations; $\Delta\text{PON} = \text{PON}_\tau - \text{PON}_\alpha$; $\Delta\text{P}_{\text{T}} = \text{P}_{\text{To}} - \text{P}_{\text{Tt}}$.

Deviations from this model are instructive since they provide information concerning nitrogen cycling and its transformation in aquatic systems. From the results of this study, it is apparent that additions and losses of nitrogen are occurring
and that this trend is consistent within the frontal and stratified communities.

The discrepancies between $\Delta \text{APON}$ and $\Delta \text{P}_T$ (Table XII) in frontal water indicate that nitrogen is being lost from the system. Given the precision and accuracy for determining the concentration of the different dissolved nitrogen fractions (see "Materials and Methods"), I argue that nitrogen losses are occurring from the PON compartment. Changes in PON for the $^{15}$N-nitrate-spiked samples from frontal water, as predicted by $^{15}$N uptake ($\Sigma V_i^N$) are not significantly different from the measured values ($\Delta \text{APON}$) (paired t-test, $p > 0.05$). Therefore, the incorporation of $^{15}$N into particulate matter accounts for the increase in PON. The discrepancies between $\Delta \text{APON}$ and $\Sigma V_i^N$ in the $\text{NH}_4^+$ and urea-spiked samples are, in part, a consequence of the simultaneous uptake of unlabelled $\text{NO}_3^-$ and its contribution to PON. Further statistical analysis of the data from the $^{15}$N-nitrate-spiked samples showed that the hypotheses $\Delta \text{P}_T = \Sigma V_i^N$ and $\Delta \text{P}_T = \Delta \text{APON}$ must be rejected ($p < 0.05$ and $p < 0.05$). Therefore, because $\Delta \text{P}_T > \Delta \text{APON}$ and $\Sigma V_i^N$, I conclude that the lost PON is labelled with $^{15}$N and that it has the same isotopic composition as the dissolved $\text{NO}_3^-$. Furthermore, these results suggest that nitrogen losses from the PON pool can be most easily explained by excretion or grazing losses to a dissolved organic pool (DON). The alternative explanations, that PON or DON is lost directly via methodological artifacts, are untenable for the following reasons. The effective retention size of a Whatman GF/F filter (0.7 um) is sufficient to have caught all of the
large chain-forming diatoms which dominated the frontal water. Phytoplankton samples collected on 0.2 um Nuclepore filters and examined with a Zeiss epifluorescence microscope showed that there were no chlorophyll-containing organisms less than 2 um and that bacterioplankton were ca. 1 um. Secondly, the low filtration pressure differential (less than 125 mm Hg) would have minimized cell lysis on the filter, and in the stratified water, dominated by soft-bodied flagellates, loss of nitrogen was not seen. Feeding zooplankton have been shown to contribute to the dissolved organic carbon pool by loss of phytoplankton cell contents during handling and feeding (Lampert 1978). The high zooplankton biomass in the frontal and stratified stations suggests that such processes may have contributed to the loss of PON, as DON, during the incubations. It is not clear why similar losses were not seen in the stratified station. Excretion of DON by phytoplankton has been reported (Newell et al. 1972, Mague et al. 1980). The interpretation of these results is consistent with the analysis by Laws (1984) that losses of $^{15}$N seen in these data and from previously published results could be attributed to losses of DO$^{15}$N, at least for experiments lasting 6 h.

In stratified water, the differences between $\Delta$PON and $\Delta$P$_T$ are exactly opposite to the results from the frontal station. Anomalously high PON values indicate the phytoplankton must be utilizing additional nitrogen sources other than those accounted for in the initial mass balance. These compounds are most probably DON as the nitrogen fixing
microorganisms were absent from the samples. In the $^{15}\text{NH}_4^+$-spiked samples, $\Sigma V_T$ is in good agreement with $\Delta\text{PON}$ suggesting that no additional nitrogen was required to account for the increase in PON; also, at the end of the experiment, $\Delta P_T$ is the same as $\Delta\text{PON}$. This is certainly not true for the $^{15}\text{NO}_3^-$ and $^{15}\text{N}$-urea-spiked samples but reason(s) for this difference are not apparent. Since $\text{NH}_4^+$ inhibits uptake of $\text{NO}_3^-$ and urea, it may also inhibit DON utilization by phytoplankton.

Characterization of seawater DON remains an enigma and current estimates suggest that free and combined amino acids and humic acids can account for only 50% of the DON pool (Sharp 1983). Wheeler et al. (1974) and Geesey and Morita (1979) have shown that these types of compounds can be utilized by marine phytoplankton and bacteria, respectively. Similarly, Hollibaugh (1978) has reported degradation of several amino acids in natural seawater samples incubated in the dark. Support for in situ DON utilization is scarce, but indirect evidence from depth profiles in the Indian Ocean (Fraga 1966) and work by Armstrong et al. (1966) showed sea-surface depletion of DON relative to deep samples. More significantly, Fisher and Cowdell (1982) reported that eight diatom clones were able to utilize at least some natural DON.

A schematic representation of simplified nitrogen cycles in frontal and stratified water is presented in Figure 15. It is impossible to distinguish between direct utilization of DON or indirect utilization via regenerated $\text{NH}_4^+$ and/or urea in stratified water.
Fig. 15. Schematic diagram of nitrogen cycling in the euphotic zone of frontal and stratified water. Arrows indicate major pathways of nitrogen transformation between the various pools; other pathways are excluded since they were not found to be dominant in these experiments. The dissolved organic nitrogen pool includes amino acids, proteins and other nitrogen containing macromolecules. Excretion may involve active and passive processes.
Frontal Water

Stratified Water
Summary

In both frontal and stratified water of the Strait of Georgia, changes in dissolved nitrogen concentrations provided evidence for the simultaneous uptake of ammonium, nitrate and urea by a summer phytoplankton community. Chlorophyll $a$ specific uptake rates of ammonium and urea were ca. 2 and 2.4 times greater in stratified water than in frontal water; whereas, chlorophyll $a$ specific nitrate uptake rates were ca. 1.6 times greater in frontal water. Ammonium and urea regeneration rates, calculated using a mass balance approach, were similar in frontal water, but urea regeneration rates were 2 to 5 times greater in the stratified water during the first 12 h of the experiment. Increases in particulate nitrogen could not be accounted for by corresponding decreases in total concentration of dissolved inorganic nitrogen and urea, or by $^{15}$N accumulation in the particulates. In frontal water, the change in total dissolved inorganic nitrogen and urea consistently overestimated the change in particulate nitrogen, while in stratified water, the change in total dissolved inorganic nitrogen and urea consistently underestimated the change in particulate nitrogen. These data suggest that the plankton community in frontal water was losing nitrogen in the form of dissolved organic nitrogen. By contrast, the plankton community in stratified water took up nitrogen compounds which were not measured as part of the total dissolved inorganic and urea nitrogen, but were most likely dissolved organic nitrogen compounds. These results
stress the importance of determining uptake rates of all three nitrogen substrates (NH$_4^+$, NO$_3^-$ and urea) using $^{15}$N isotopes and by simultaneously measuring the change in concentration of these compounds throughout the incubation period.
CHAPTER 3. UREA UPTAKE BY SARGASSO SEA PHYTOPLANKTON:
SATURATED AND IN SITU UPTAKE RATES

Background

Results of recent studies have contradicted our conventional view of phytoplankton nitrogen nutrition, in which the majority of the phytoplankton nitrogen requirements are met by ammonium ($\text{NH}_4^+$). In fact, these studies showed that urea is quantitatively as important as, if not more important than, $\text{NH}_4^+$ for phytoplankton nutrition in certain ocean environments (Kaufman et al. 1983, Harrison et al. 1985).

Urea uptake by phytoplankton in the oligotrophic ocean has been poorly studied. Rapid urea turnover times of less than one day were measured by Herbland (1976) in the tropical South Atlantic Ocean. Nevertheless, since $^{14}$C-urea was used in these experiments, the contribution of urea-N to phytoplankton growth was not addressed. Without exception, in other oligotrophic environments, turnover times of urea are on the order of several days to months (Remsen et al. 1974, Eppley et al. 1973, 1977, Mitamura and Saijo 1980a, Kanda et al. 1986). These results imply that urea is not an important nitrogen compound in these regions or that the organisms which utilize urea are growing slowly.

This study examines urea uptake by phytoplankton communities in the western North Atlantic Ocean. Saturating concentrations of $^{14}$C- and $^{15}$N-urea were added to seawater samples to determine maximum urea uptake rates and to compare
the uptake of both isotopes. Rates of nitrate and ammonium uptake were measured concurrently. The ratio of maximum urea uptake rate/in situ urea uptake rate was used to evaluate physiological differences between phytoplankton communities in these waters.

**Materials and Methods**

*Sample collection*

Experiments were performed on board the R/V Cape Hatteras during August 1985 (Cruise: Corsair 10) at one station in continental slope water, S1 (36°30'N; 74°35'W) and two stations in the Sargasso Sea, S2 (33°12'N; 66°28'W) and S3 (33°54'N; 70°17'W) (Fig. 16).

Water samples were collected with 5-litre Glo-Flo bottles from depths corresponding to 100, 55, 30, 10, 3 and 1% of the sea-surface irradiance. A light extinction coefficient of 0.09 m⁻¹ and 0.04 m⁻¹, measured on a previous cruise (R. Rivkin, pers. comm.), was used to determine these depths in continental slope water and the Sargasso Sea, respectively. Samples were collected from S1 at 11:30, S2 at 09:30 and S3 at 11:20 h. Water from each depth was dispensed into 20-litre Nalgene® carboys covered with black plastic to shade the samples collected from low light.

*Physical, chemical and biological analyses*

Vertical profiles of temperature and salinity were obtained coincidently with the bottle casts with a Neil Brown
Fig. 16. Location of stations in the western North Atlantic Ocean. S1 is in continental slope water, S2 and S3 are in the Sargasso Sea.
CTD attached to the rosette sampler. Samples for nutrient analysis were filtered through combusted (460°C for 4h) Whatman GF/F filters using an acid-washed syringe and a 25 mm Millipore Swinex® filter holder into acid-washed polypropylene sample bottles and stored frozen (-20°C). Ammonium (NH$_4^+$) concentration was determined in fresh samples taken from a separate bottle cast at the same station using the manual method of Solorzano (1969). The frozen samples were analyzed for NH$_4^+$ and nitrate plus nitrite (NO$_3^-$ + NO$_2^-$) with a Technicon Autoanalyzer® II following the procedure of Slawyk and MacIsaac (1972) and Wood et al. (1967), respectively. Urea was determined by the diacetyl monoxime thiosemicarbazide technique described in Chapter 1. Chlorophyll a (chl a) samples (1.0 litre) were filtered onto Whatman GF/F filters and stored frozen in a desiccator. Samples were extracted in 90% acetone and analyzed by in vitro fluorometry (Strickland and Parsons 1972) using a Turner Designs model 111 fluorometer. Particulate organic carbon and nitrogen (POC and PON) samples (1.0 litre), collected on combusted Whatman GF/F filters, were stored similarly and analyzed with a Carlo Erba Elemental Analyzer model 1106.

**Saturated uptake experiments**

Experiments were conducted using $^{15}$N-labelled NH$_4^+$ and NO$_3^-$ and both $^{15}$N- and $^{14}$C-labelled urea. All nitrogen uptake experiments were initiated within 1 h of sampling. Incubations were conducted on deck in clear Plexiglas® incubators, cooled with surface seawater. Where necessary,
incubation bottles were covered with neutral density screening to simulate the *in situ* light regime at each sample depth.

\[ ^{15}N \text{-labelled substrates} \]

Saturating additions of \(^{15}N\)-labelled \(NH_4^+\), \(NO_3^-\) or urea (all 99 at \(^{15}N\)) were added to separate 1200 ml Wheaton glass bottles, and measurements were duplicated for all N substrates at each depth. \(NH_4^+\) and urea uptake rates were determined at six depths, but \(NO_3^-\) uptake was only measured at the 55 and 3\% sea-surface light depths. Water was not prescreened through Nitex\(^R\) netting because of the abundance of *Trichodesmium* colonies at 2 of the 3 stations. In water collected from the 55\% sea-surface light depth, incorporation of \(^{15}N\)-urea was measured at 6 h intervals over the 24 h incubation to test for constant uptake at each station. Experiments were terminated by filtration (vacuum less than 100 mm Hg) onto combusted Whatman GF/F filters, and the samples were stored frozen in a desiccator. Nitrogen in the particulate matter was converted to \(N_2\) (g) by the micro-Dumas dry combustion technique as described by LaRoche (1983), and then analyzed for \(^{15}N\) enrichment with a JASCO model N-150 emission spectrometer (Fiedler and Proksch 1975). \(^{15}N\) uptake rates were calculated according to the equations of Dugdale and Goering (1967) except that initial PON measurements were used in place of final PON. Correction for changes in the initial PON due to uptake of added N during the incubation were made as described by Collos (1984).
Concurrent with the saturating $^{15}$N uptake experiments, $^{14}$C-urea (Amersham) was added at saturating (10 μg at N·l$^{-1}$; 0.072 GBq·mmol$^{-1}$) and trace (5 ng at N·l$^{-1}$; 2.04 GBq·mmol$^{-1}$) concentrations. A subsample (100 ml) from one of the duplicate 1200 ml Wheaton glass incubation bottles was taken after 6 h of incubation, and samples were taken again from both bottles after 24 h. Uptake of $^{14}$C-urea was determined by measuring $^{14}$CO$_2$ released into the seawater, as a result of $^{14}$C-urea hydrolysis, and by measuring the quantity of $^{14}$C in the particulate matter retained by a Whatman GF/F filter. The particulate matter was collected by gentle filtration, and the filters were rinsed with filtered (0.45 um Millipore filter) seawater. To correct for abiotic degradation of urea, formalin-killed (1 ml, 20% formalin) controls were run with each set of samples. Immediately following the addition of $^{14}$C-urea, samples were withdrawn and filtered to determine the amount of filter adsorption. To determine the amount of $^{14}$CO$_2$ in the filtrate, small glass vials containing filter paper impregnated with 50 ul phenethylamine were suspended inside 250 ml Erlenmeyer flasks containing 100 ml of the sample. The flasks were sealed with rubber septa, and 0.5 ml 6N HCl was added to the filtrate. After 24 h, the filters were removed from the flasks and placed in scintillation vials containing 10 ml Aquasol II. Recovery of $^{14}$CO$_2$ was determined to be 62.5%. Uptake of urea was calculated by adding the amount of $^{14}$CO$_2$, which was produced during the incubation and the amount
of $^{14}$C retained by the filter minus the controls. These uptake rates are actually urea hydrolysis rates and represent a measurement based on $^{14}$C and not nitrogen. To convert these rates to nitrogen uptake rates, I assumed that for each $^{14}$C appearing in CO$_2$, or in the particulate fraction, 2 nitrogen atoms were taken up (see Discussion).

In situ urea uptake rates

Trace additions of $^{14}$C-urea were added to 1200 ml water samples collected from the 55 and 3% sea-surface light depths. Duplicate 5 ml samples taken from each bottle at the start of the experiment were added to scintillation vials containing 10 ml of fluor. At 2, 4, and 6 h, duplicate 100 ml samples were filtered through Whatman GF/F filters, and the filters were rinsed with 15 ml filtered seawater. Precaution was taken to ensure that the filters did not become dry prior to rinsing. The filtrate (25 ml) was dispensed into 50 ml Erlenmeyer flasks. To remove $^{14}$CO$_2$ produced from $^{14}$C-urea hydrolysis, the filtrate was acidified by adding 0.5 ml 6H HCl and allowed to degas in a vacuum desiccator under reduced pressure for 12 h. This procedure removed all $^{14}$CO$_2$ from the samples.

The data were fitted to an exponential equation to determine the rate coefficient. The reciprocal of this coefficient is the turnover time of dissolved urea measured in hours. Trace $^{14}$C-urea uptake rates were determined from the product of the turnover time and the ambient dissolved urea concentration. All $^{14}$C samples were counted by Packard Tri-
Carb 460c liquid scintillation counter, and the standard deviation of each count was less than 2%.

Results

Environmental parameters

Initial biomass data for the three stations are presented in Table XIII. POC:PON was similar in continental slope water and the Sargasso Sea; however, PON/chl a measurements indicated that there was considerably more detrital N in the Sargasso Sea samples. This was particularly evident in the deep samples, where the PON/chl a was 8 times greater than particulate matter in slope water collected from a similar light regime. Size fractionated measurements indicated that ca. 90% of the chl a was in the less than 3 um size fraction (R. Rivkin, unpublished results).

Dissolved NO$_3^-$ + NO$_2^-$ were detectable throughout the surface-mixed layer at Stations 1 and 2, but were undetectable at Station 3 except at 115 m (Fig. 17). These analyses did not differentiate between NO$_3^-$ and NO$_2^-$; since NO$_2^-$ is generally in lower abundance, I have referred to these measurements as NO$_3^-$ concentrations. NH$_4^+$ concentration was determined in freshly collected samples from an earlier cast at the same station. These values were often detectable; with the exception of Station 3, they were lower than the NH$_4^+$ concentrations measured in the frozen samples. Data are reported for the frozen samples. Urea concentration was undetectable in the surface and at 15 m at Stations 2 and 3.
### Table XIII

Biomass data from water samples collected for nitrogen uptake experiments.

<table>
<thead>
<tr>
<th>Station</th>
<th>Date</th>
<th>Mixed layer depth (m)</th>
<th>Sample depth (m)</th>
<th>Chl a (ug·l⁻¹)</th>
<th>PON (ug at N·l⁻¹)</th>
<th>POC:PON (atoms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4/VIII/85</td>
<td>18</td>
<td>0</td>
<td>0.12</td>
<td>0.75</td>
<td>11.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6.6</td>
<td>0.13</td>
<td>0.75</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>13</td>
<td>0.19</td>
<td>0.82</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>26</td>
<td>1.65</td>
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<td>7.1</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>38</td>
<td>0.33</td>
<td>0.64</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>51</td>
<td>0.13</td>
<td>0.42</td>
<td>9.9</td>
</tr>
<tr>
<td>2</td>
<td>8/VIII/85</td>
<td>25</td>
<td>0</td>
<td>0.028</td>
<td>0.33</td>
<td>11.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15</td>
<td>0.030</td>
<td>0.44</td>
<td>10.5</td>
</tr>
<tr>
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<td></td>
<td>30</td>
<td>0.044</td>
<td>0.48</td>
<td>10.2</td>
</tr>
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<td></td>
<td>58</td>
<td>0.15</td>
<td>0.48</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>88</td>
<td>0.14</td>
<td>0.35</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>115</td>
<td>0.057</td>
<td>0.32</td>
<td>11.5</td>
</tr>
<tr>
<td>3</td>
<td>13/VIII/85</td>
<td>28</td>
<td>0</td>
<td>0.037</td>
<td>0.29</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15</td>
<td>0.034</td>
<td>0.42</td>
<td>9.9</td>
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<td>30</td>
<td>0.042</td>
<td>0.44</td>
<td>9.2</td>
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<td></td>
<td>58</td>
<td>0.071</td>
<td>0.47</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>88</td>
<td>0.13</td>
<td>0.36</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>115</td>
<td>0.047</td>
<td>0.31</td>
<td>13.0</td>
</tr>
</tbody>
</table>
Fig. 17. Dissolved $\text{NH}_4^+$, $\text{NO}_3^-$ + $\text{NO}_2^-$ and urea concentration (ug at N·l$^{-1}$) measured in filtered frozen samples collected from (A) Station 1 (continental slope water), (B) Station 2 (Sargasso Sea), and (C) Station 3 (Sargasso Sea).
Time course measurements of $^{15}$N-urea uptake by plankton collected from the 50% sea-surface light depth demonstrated that incorporation of $^{15}$N was constant over the 24 h incubation (Fig. 18). Similar time course experiments were not run for NH$_4^+$ or NO$_3^-$.

Saturated $^{15}$N uptake rates were higher in slope water than in the Sargasso Sea, because phytoplankton biomass (measured as chl a) was an order of magnitude greater (Fig. 19A). At all depths in slope water, NH$_4^+$ uptake rates were 2-3 times greater than urea uptake rates and 3-3.5 times greater than NO$_3^-$ uptake rates. Nitrogen-specific uptake rates of NH$_4^+$ ranged from 0.15 to 0.70 d$^{-1}$. NO$_3^-$ uptake rate ($0.031 \pm 0.012$ ug at N$\cdot$l$^{-1}$$\cdot$d$^{-1}$) in the 38 m water sample was representative of the in situ uptake rate, since ambient NO$_3^-$ concentrations were high (5.3 ug at N$\cdot$l$^{-1}$). At Station 2, urea uptake rates were less than NH$_4^+$ uptake rates (Fig. 19B). Urea uptake rates were the same at the three depths within the surface-mixed layer; however, in the upper portion of the chlorophyll maximum, the urea uptake rate decreased. In the surface waters of Station 3, little uptake of NH$_4^+$ was evident, but NH$_4^+$ uptake increased with depth (Fig. 19C). The saturated urea uptake rates were 5 times greater than the NH$_4^+$ uptake rates, and NO$_3^-$ uptake rates were also higher. Below the pycnocline, NH$_4^+$ uptake rates increased and were greater than both NO$_3^-$ and urea uptake rates.
Fig. 18. Time course of incorporation of $^{15}\text{N}$-urea into particulate matter in water samples collected from 6.6 m at Station 1 (●), and 15 m at Stations 2 (■) and 3 (○). Incubation times were measured from the starting time of the incubation. The dark period during the time course at Stations 1 and 3 occurred between 8.5 and 18 h and between 10.5 and 20 h at Station 2.
$^{15}\text{N} - \text{UPTAKE}$ (atom % excess)

INCUBATION TIME (h)

$S_1$, $S_2$, $S_3$
Fig. 19. Daily nitrogen uptake rates determined with saturating additions of $^{15}$N-labelled NH$_4^+$, NO$_3^-$ and urea by plankton in seawater samples collected at (A) Station 1, (B) Station 2 and (C) Station 3. Error bars are the range of duplicate samples.
\[ ^{14}\text{C-urea uptake} \]

Incorporation of \[^{14}\text{C}\] from \[^{14}\text{C}\text{-urea}\], into the particulate matter was negligible in all water samples except in the chlorophyll maximum (ca. 10% surface light depth). At Station 1, \[^{14}\text{C}\] retained by the filter accounted for 63\% of the total urea taken up after 6 h. There was no further incorporation of \[^{14}\text{C}\] during the last 18 h, but some urea continued to be taken up. \[^{14}\text{C}\] incorporation into the particulate matter over the 24 h incubation amounted to 3 and 4\% at Stations 2 and 3, respectively; no measurable retention of \[^{14}\text{C}\] occurred during the first 6 h. All of the \[^{14}\text{C}\] in \[^{14}\text{C}\text{-urea}\] was released into the seawater as \[^{14}\text{CO}_2\].

The \[^{14}\text{C}\text{-urea uptake rates}\] were greater than \[^{15}\text{N}\text{-urea uptake rates}\], determined over the same incubation period, at the three stations (Table XIV). There were no apparent trends with depth or between stations. \[^{14}\text{C}\text{-urea uptake rates}\] were on average 1.4 times greater than \[^{15}\text{N}\text{-urea uptake rates}\], but at two depths \[^{15}\text{N}\text{-urea uptake rates}\] exceeded \[^{14}\text{C}\text{-urea uptake rates}\].

By contrast to \[^{15}\text{N}\text{-urea uptake}\], \[^{14}\text{C}\text{-urea uptake}\] was not constant over the 24 h incubation period. On an hourly basis, the \[^{14}\text{C}\text{-urea uptake rate}\] during the first 6 h greatly exceeded the rate measured over the 24 h incubation, in extreme cases by as much as 25 times (Table XV). In seven of the twelve samples from the Sargasso Sea, less \[^{14}\text{C}\text{-urea uptake}\] was measured during the 24 h incubation than over the first 6 h. Since only the \[^{14}\text{CO}_2\] in the seawater and the \[^{14}\text{C}\] retained by
Table XIV

Comparison of $^{15}$N-urea and $^{14}$C-urea uptake rates determined over 24 h incubations. Rates of $^{14}$C-urea uptake were converted to equivalent nitrogen uptake rates as described in the text. Values in brackets are daily $^{15}$N-urea uptake rates (ug at N·l$^{-1}$·d$^{-1}$).

<table>
<thead>
<tr>
<th>Light Depth (% of surface irradiance)</th>
<th>$^{15}$N Uptake Rate</th>
<th>$^{14}$C Uptake Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S1</td>
<td>S2</td>
</tr>
<tr>
<td>100</td>
<td>0.54 (0.22)</td>
<td>0.62 (0.038)</td>
</tr>
<tr>
<td>55</td>
<td>0.68 (0.15)</td>
<td>0.78 (0.041)</td>
</tr>
<tr>
<td>30</td>
<td>1.30 (0.24)</td>
<td>0.32 (0.046)</td>
</tr>
<tr>
<td>10</td>
<td>0.76 (0.62)</td>
<td>0.06 (0.006)</td>
</tr>
<tr>
<td>3</td>
<td>0.86 (0.041)</td>
<td>0.84 (0.036)</td>
</tr>
<tr>
<td>1</td>
<td>0.48 (0.022)</td>
<td>0.60 (0.024)</td>
</tr>
</tbody>
</table>
Table XV

Ratio of $^{14}$C-urea uptake rates (ug at urea-C·l$^{-1}$·h$^{-1}$) determined during 6 and 24 h incubations at three stations (S1, S2, and S3) in water samples collected from 6 depths. Values in brackets are the $^{14}$C-urea uptake rates during 6 h incubations (ug at urea-C·l$^{-1}$·h$^{-1}$).

<table>
<thead>
<tr>
<th>Light Depth (% of surface irradiance)</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.32 (0.0055)</td>
<td>25.0 (0.065)</td>
<td>4.68 (0.018)</td>
</tr>
<tr>
<td>55</td>
<td>1.39 (0.013)</td>
<td>2.49 (0.0054)</td>
<td>4.54 (0.013)</td>
</tr>
<tr>
<td>30</td>
<td>1.64 (0.013)</td>
<td>16.3 (0.090)</td>
<td>4.78 (0.012)</td>
</tr>
<tr>
<td>10</td>
<td>1.06 (0.036)</td>
<td>1.76 (0.0075)</td>
<td>3.90 (0.012)</td>
</tr>
<tr>
<td>3</td>
<td>1.69 (0.0034)</td>
<td>13.0 (0.023)</td>
<td>2.81 (0.0031)</td>
</tr>
<tr>
<td>1</td>
<td>1.28 (0.0024)</td>
<td>11.9 (0.020)</td>
<td>2.92 (0.0035)</td>
</tr>
</tbody>
</table>
the particulate matter was accounted for during these uptake experiments, it is possible that some $^{14}$C was lost to a compartment which was not measured by these methods. At Station 1, the rates calculated from the 6 and 24 h incubations were in fair agreement, and the 6 h incubation rate was on average 1.2 times faster. In the surface water sample, there was an initial lag in urea uptake. At Station 2, the hourly uptake rates during the 6 and 24 h incubations were most dissimilar. In neither of these stations were any depth related trends apparent. This contrasts the results from Station 3 where the greatest disparity between the rates measured during the 6 and 24 h incubations occurred in the surface-mixed layer. Below 28 m, the 6 and 24 h $^{14}$C-urea uptake rates were in better agreement.

\textit{In situ urea uptake rates}

The disappearance of $^{14}$C-urea from seawater samples spiked with 5 nM $^{14}$C-urea is shown in Figure 20. The slopes of these curves, calculated from an exponential regression, equal the turnover rates of urea.

The average turnover time of urea in the surface-mixed layer of the Sargasso Sea was 13 h; this was slightly faster than the turnover time in slope water (Table XVI). At the 3% surface light depth, the turnover times of urea were slower than in surface samples. \textit{In situ} urea uptake rates, referred to here as urea trace uptakes or $V_{\text{trace}}$, could not be accurately computed in instances where the ambient urea concentration was below detection. In these cases, I assumed
Fig. 20. The amount of $^{14}$C-urea remaining in seawater during 6 h time course experiments. Water was collected from the 55 (O) and 3% (●) sea-surface light depths at (A) Station 1 (continental slope water), (B) Station 2 (Sargasso Sea), and (C) Station 3 (Sargasso Sea). Particulate matter was removed from samples by filtration, and the filtrate was acidified to remove $^{14}$CO$_2$. The remaining activity was measured. Error bars are the range of duplicate subsamples from a single sample bottle.
Table XVI

Turnover times of dissolved urea concentration in surface-mixed and deep water at Stations 1, 2 and 3. The correlation coefficient for each exponential equation is given. Dissolved urea concentrations are the mean of duplicates (± 1 SD).

<table>
<thead>
<tr>
<th>Station</th>
<th>Depth (m)</th>
<th>Hydrolysis rate (d⁻¹)</th>
<th>Turnover time (h)</th>
<th>r²</th>
<th>Urea concentration (ug at N·l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.6</td>
<td>1.67</td>
<td>21.1</td>
<td>0.953</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>0.66</td>
<td>58.5</td>
<td>0.834</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>1.86</td>
<td>12.6</td>
<td>0.923</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>88</td>
<td>0.61</td>
<td>43.6</td>
<td>0.992</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>1.81</td>
<td>13.3</td>
<td>0.957</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>88</td>
<td>0.70</td>
<td>34.3</td>
<td>0.924</td>
<td>0.14 ± 0.07</td>
</tr>
</tbody>
</table>
that the urea concentration was equal to the limit of detection: 50 ng at N·l⁻¹. As a result, these estimates of Vtrace are an upper limit. A comparison of urea uptake rates determined using saturating and trace additions of substrate is given in Table XVII. Saturating uptake rates of urea were in excess of in situ uptake rates at all samples with the exception of the 88 m sample from Station 3. In this sample, one of the dissolved urea samples was twice as high as the other, consequently the mean value may be too high. This results in an overestimate of the actual uptake rate.

Discussion

Environmental parameters

In oligotrophic waters, concentrations of dissolved NO₃⁻ + NO₂⁻ measured by colourimetric techniques are usually below our limits of detection (Garside 1985). This was only true in this study at Station 3. Although the samples were filtered and stored frozen before analysis, MacDonald and McLaughlin (1982) found this procedure had no effect on the storage of NO₃⁻ samples. Cochlan (1982) measured an average NO₃⁻ + NO₂⁻ concentration of 0.15 ug at N·l⁻¹ in the mixed layer at a single station in the northern Sargasso Sea. On a separate cruise during July 1986, analysis of freshly collected water samples from the Sargasso Sea indicated the presence of low, but detectable concentrations of NO₃⁻ + NO₂⁻ (Price, unpublished results). The estimates of the contribution of detritus to POC and PON measurements in this study are only
Table XVII

Uptake rates of $^{14}$C-urea determined during 6 h incubations in seawater samples collected from the 50 and 3% surface light depths at three stations (S1, S2, and S3), and spiked with trace and saturating concentrations. Rates are expressed in terms of urea-N uptake.

<table>
<thead>
<tr>
<th>Station</th>
<th>Depth (m)</th>
<th>$V_{\text{trace}}$</th>
<th>$V_{\text{sat}}$</th>
<th>$V_{\text{sat}}/V_{\text{trace}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(ug at N·l$^{-1}$·h$^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.6</td>
<td>0.0085</td>
<td>0.0127</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>0.0021</td>
<td>0.00335</td>
<td>1.6</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>0.0039</td>
<td>0.00545</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>88</td>
<td>0.0032</td>
<td>0.0233</td>
<td>7.3</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>0.0038</td>
<td>0.0130</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>88</td>
<td>0.0041*</td>
<td>0.00312</td>
<td>0.8*</td>
</tr>
</tbody>
</table>

* One of the duplicate measurements of ambient dissolved urea concentration was 2 times the concentration of the other; consequently, the mean value that was used in the calculation may be too high. This would result in an overestimate of the actual uptake rate, and an underestimate of the $V_{\text{sat}}/V_{\text{trace}}$ ratio.
relative. The PON/chl \(a\) values reported here are higher than those reported by McCarthy and Nevins (1986) in a warm-core ring and Glibert and McCarthy (1984) in the Caribbean and the Sargasso Sea. This indicates that there was a greater detrital contamination of the samples collected in this study. There was no relationship between PON or POC and chl \(a\) at either of the Sargasso Sea stations. Other investigators have found good relationships between POC and chl \(a\) and are able to estimate the proportion of detritus in their samples from a linear regression of these variables. In the oligotrophic North Pacific, the proportion of living POC, as estimated from cell volumes and numbers and from ATP measurements (Sharp et al. 1980, Winn and Karl 1984), was ca. 30% of the total POC. Laws et al. (1985) determined from ATP measurements that 42% of the total particulate C was living in oligotrophic Hawaiian waters. In these studies, the PON/chl \(a\) in the surface waters (less than 30 m) were less than the samples I collected within the mixed layer. From these data, I conservatively estimate detrital contamination of POC and PON to be greater than 50% of the total POC and PON present in the Sargasso Sea stations.

*Saturated* \(\textsuperscript{15}N\) uptake rates

Linear incorporation of \(\textsuperscript{15}N\) over long incubation times (24 h) has been reported for: NO\(_3^-\) and NH\(_4^+\), in coastal waters of the Scotian Shelf (Cochlan 1986) and in a warm-core ring (McCarthy and Nevins 1986); NH\(_4^+\), in Vineyard Sound (Goldman et al. 1981), and NO\(_3^-\), NH\(_4^+\) and urea in the eastern Canadian Arctic (Harrison 1983b). However, there are generally
pronounced diel patterns in nitrogen uptake, with rates being greatest during the daylight hours (Collos and Slawyk 1976, MacIsaac 1978, Olson 1980). Although the $^{15}$N-urea uptake rates were linear over the time course, it is not known whether NH$_4^+$ and NO$_3^-$ uptake rates were also linear because time course experiments were not run. Since uptake of NO$_3^-$ is known to be more light dependent than the uptake of either NH$_4^+$ or urea, it seems unlikely that $^{15}$NO$_3^-$ incorporation was constant over the time course.

The measured rates of NO$_3^-$ uptake at the 3% sea-surface light depth at Stations 1 and 2 are *in situ* uptake rates because ambient NO$_3^-$ concentrations were already saturating. NH$_4^+$ and urea uptake rates were greater than NO$_3^-$ uptake rates. It is possible that this is a result of reduced NO$_3^-$ uptake in the dark, although Horrigan and McCarthy (1981) found that nitrate-sufficient cultures of phytoplankton had enhanced uptake rates for NH$_4^+$ and urea.

Saturated uptake rates of NH$_4^+$, NO$_3^-$ and urea demonstrate the potential capacity for nitrogen uptake by the plankton communities. These results indicate the spatial variability in nitrogen uptake by phytoplankton assemblages at different depths and between stations. Generally, NH$_4^+$ uptake rates exceeded urea uptake rates; however, the differences depended upon the depth from which the samples were collected. The vertical distribution of phytoplankton in ocean water provides evidence for variation in the community composition with depth. In the Sargasso Sea, maximum cell densities of
chroococcoid cyanobacteria occur in surface waters (less than 40 m), and the eukaryotic phytoplankton are found in greatest numbers below this depth (Murphy and Haugen 1985). Most of these picoplankton have not been tested for their ability to use urea under laboratory conditions. The utilization of urea by the plankton community represents an integrated response of the individual species of phytoplankton and bacterioplankton in the sample. Selective utilization of certain types of nitrogen compounds by different sizes of plankton was shown in size-fractionated nitrogen uptake experiments in the Benguela upwelling and the Antarctic Ocean (Probyn 1985, Probyn and Painting 1985). Although the results presented here do not allow the same resolution as obtained by Probyn, they demonstrate that plankton assemblages in the different water samples possess the capability to utilize different nitrogen sources to varying degrees.

Plankton collected at Station 2 from the chlorophyll maximum had the lowest urea uptake rates measured by $^{15}\text{N}$ compared with plankton collected from other depths. $\text{NH}_4^+$ uptake rates were maximum at this depth. Results from the surface waters of Station 3 are unique: urea uptake rates were much greater than $\text{NH}_4^+$ uptake rates. This appears related to a low capacity for $\text{NH}_4^+$ uptake, since urea uptake rates were similar to rates seen at Station 2; $\text{NO}_3^-$ uptake rates were also greater than $\text{NH}_4^+$ uptake rates. This response by the phytoplankton has not been previously reported, and is surprising in light of the almost universal preference of phytoplankton for $\text{NH}_4^+$. Not only does the preconditioning
nitrogen source effect the ability of phytoplankton to utilize other nitrogen sources (Horrigan and McCarthy 1981, 1982), but growth rate (Dortch and Conway, 1984) and light regime (Bates 1976) may also be important factors.

**Urea uptake: $^{14}$C and $^{15}$N tracers**

The discrepancy between $^{14}$C-urea uptake rates during 6 and 24 h incubations contrasts the linear uptake of $^{15}$N-urea at the three stations. These results provide new information regarding urea cycling in seawater and its uptake and assimilation by planktonic organisms. Measured over short time intervals (on the order of minutes), the initial uptake rates of $^{14}$C- and $^{15}$N-urea represent influx of urea. As incubation times increase the transported substrate is metabolized, and the rate of metabolism may regulate the uptake rate. A comparison of the utilization of $^{14}$C- and $^{15}$N-labelled urea is confounded by the fact that urea is degraded rapidly by the plankton and the radioactive ($^{14}$C) and stable ($^{15}$N) isotope labels are metabolized by different pathways. The length of the incubation time required to measure $^{15}$N incorporation in the oligotrophic ocean is of sufficient duration that the transported substrate is metabolized. Wheeler *et al.* (1982) demonstrated that greater than 80% of the $^{15}$NH$_4^+$ taken up by Chesapeake phytoplankton was incorporated into protein within 15 minutes. Even if rate processes are slower in open ocean water compared to coastal regions, $^{15}$N uptake may also be subject to regulation by
feedback mechanisms associated with enzymatic nitrogen assimilation. $^{14}$C-urea uptake rates are determined from rates of urea hydrolysis, which certainly cannot be confused with a transmembrane flux.

The observation of negligible retention of $^{14}$C by the particulate matter is in agreement with Herbland (1976), but contrasts with the results of Mitamura and Saijo (1975). This may be explained by the much greater phytoplankton biomass in the coastal water samples of Mitamura and Saijo (1975). The only instance where incorporation of $^{14}$C into the particulate fraction was significant was in a water sample which contained one to two orders of magnitude more chl $a$.

The fate of urea-N is unclear from the $^{14}$C-urea tracer uptake studies; however, from these results and those of Harrison et al. 1985, it appears that only 50-80% of the nitrogen from urea is incorporated into particulate matter retained by GF/F filters. Results from this study also demonstrate that there are temporal differences in the fate of the $^{14}$C and $^{15}$N tracers. Initial rates of $^{14}$C-urea hydrolysis indicate that urea is rapidly hydrolyzed to CO$_2$ and NH$_3$. The observation that initial rates of urea hydrolysis were greatest at Station 2 compared with the other 2 stations may be a result of the earlier starting time of these incubations. The only plausible explanation to account for the loss of $^{14}$C during the 24 h incubations is its loss to a fraction not measured by these methods, such as DOC.

The initial rapid hydrolysis of urea, indicated by the liberation of $^{14}$CO$_2$ and the slower rate of urea-N uptake,
might be anticipated if urease were an extracellular enzyme. However, our current knowledge suggests that urease is an intracellular enzyme in eukaryotic microalgae (Leftley and Syrett 1973), cyanobacteria (Berns et al. 1966), and heterotrophic bacteria (McLean et al. 1985). Consequently, hydrolysis rates of urea, when measured over the time intervals such as used in these experiments, occur on time scales similar to those for urea uptake, since urea must be taken into the cells before it is degraded. These rates may be considered gross urea uptake rates. To account for the lower urea-N uptake rates, I postulate that either the N from urea is lost as DON, incorporated into particulate matter not retained by GF/F filters, or that it diffuses out of the cells as NH$_3$ prior to being assimilated into amino acids and proteins. During nitrogen uptake experiments, loss of $^{15}$N has been attributed to DO$^{15}$N excretion or loss from the particulate matter (Laws 1984, Price et al. 1985), although direct evidence is lacking. Both Li et al. (1983) and Cuhel et al. (1983) demonstrated that GF/F filters retain the small photosynthetic picoplankton. Laboratory evidence for NH$_4^+$ release was reported by Uchida (1976). He observed that *Prorocentrum minimum* excreted NH$_4^+$ when grown in urea-enriched culture medium. Rees (1979) also reported NH$_4^+$ release by urea-grown *Phaeodactylum tricornutum*. These results contrast the findings of Horrigan and McCarthy (1981). They observed that uptake rates of $^{14}$C- and $^{15}$N-urea were identical in two diatom species. Since their measurements were made over much
shorter time intervals than the studies of Uchida and Rees, it is possible that NH$_4^+$ loss from the cells may not have been evident in their results.

I propose that, when phytoplankton are exposed to elevated urea concentrations, urea is rapidly hydrolyzed by urea degrading enzymes. The CO$_2$ from urea diffuses out of the cells, and although some NH$_4^+$ is incorporated into macromolecules, some NH$_4^+$ diffuses out of the cells as NH$_3$.

**Urea turnover times**

The turnover time of urea in seawater depends upon the ambient dissolved urea concentration, phytoplankton biomass and nutritional state, and water temperature. Previous investigators found that urea turnover times in oligotrophic ocean environments were generally slow (Table XVIII); however, the results of Herbland (1976) are an exception. He reported rapid turnover times (ca. 1.2 d) in tropical South Atlantic waters. In instances where turnover times were not directly reported by the investigator, I calculated rates from their data. The results presented in this study are the most rapid turnover times of urea measured in an oligotrophic oceanic gyre; these rates are as fast or faster than turnover times of urea in coastal waters. The turnover time of urea, determined by a variety of techniques in several coastal waters, varies between 5-10 d (Carpenter *et al.* 1972, Remsen *et al.* 1974, Mitamura and Saijo 1975, 1980a, Savidge and Hutley 1977, Harrison *et al.* 1985). In two exceptional studies, Kristiansen (1983) and Turley (1985) measured urea
turnover times on the order of a few hours in Oslofjord and at a front in the western Irish Sea, respectively. Some of the difference between the Sargasso Sea data and data collected by other investigators arises because of the method used for measuring urea turnover and uptake. The following example serves to demonstrate this point. Urea turnover times, calculated from data given in Eppley et al. (1977), averaged 52 d. Turnover times were calculated from the ambient dissolved urea concentrations and in situ urea uptake rates measured with $^{15}$N-urea. Using data collected on the same cruises of Eppley et al., Sharp et al. (1980) reported an average urea turnover time of 22 d. They used urea uptake measurements determined over a range of substrate concentrations, and calculated turnover times from linearized plots of the Michaelis-Menten equation. This method, first used by Parsons and Strickland (1962) for measuring glucose turnover in seawater, requires no knowledge of the ambient dissolved urea concentration. Eppley et al. (1977) recognized that the concentrations of urea they reported were in error; this could be responsible for the differences between the two methods for calculating turnover times.

Many of the early studies measuring urea uptake by phytoplankton, which ultimately led to our recognition of the importance of urea for phytoplankton nutrition in the ocean, were derived from long incubations with substrate additions similar to ambient levels. These uptake rates may be biased. The limitations of the $^{15}$N method are now widely known, and
### Table XVIII

Urea turnover times in oligotrophic ocean waters.

<table>
<thead>
<tr>
<th>Region</th>
<th>Sample depth (m)</th>
<th>$T(°C)$</th>
<th>Chl $a$ (ug·l$^{-1}$)</th>
<th>[Urea] (ug at N·l$^{-1}$)</th>
<th>Turnover time (d)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>N Atlantic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.62</td>
<td>98</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.28</td>
<td>59.2</td>
<td>1</td>
</tr>
<tr>
<td>Tropical</td>
<td>&lt;40</td>
<td>28.4</td>
<td>0.4</td>
<td>-</td>
<td>1.44</td>
<td>2</td>
</tr>
<tr>
<td>S Atlantic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;15</td>
<td>27</td>
<td>0.1</td>
<td>-</td>
<td>0.71</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>&lt;10</td>
<td>25</td>
<td>0.1</td>
<td>-</td>
<td>1.91</td>
<td>2</td>
</tr>
<tr>
<td>Central</td>
<td>&lt;85</td>
<td>21</td>
<td>0.07</td>
<td>0.28</td>
<td>52 a</td>
<td>3</td>
</tr>
<tr>
<td>N Pacific</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;30</td>
<td>-</td>
<td>-</td>
<td>0.37</td>
<td>140</td>
<td>4</td>
</tr>
<tr>
<td>Subarctic</td>
<td>&lt;30</td>
<td>-</td>
<td>-</td>
<td>0.23</td>
<td>90</td>
<td>4</td>
</tr>
<tr>
<td>Pacific</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sargasso</td>
<td>15</td>
<td>25.5</td>
<td>0.32</td>
<td>0.09</td>
<td>0.54</td>
<td>5</td>
</tr>
<tr>
<td>Sea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N Atlantic</td>
<td>6.6</td>
<td>26</td>
<td>0.13</td>
<td>0.15</td>
<td>0.88</td>
<td>5</td>
</tr>
<tr>
<td>Slope</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Sharp *et al.* 1980 calculated an average turnover time of 22 d using data collected by Eppley, but not reported in Eppley *et al.* 1977.
- Remsen *et al.* 1974
- Herbland 1976
- Eppley *et al.* 1977
- Mitamura and Saijo 1980a
- This study
are recently reviewed by Dugdale and Wilkerson (1986). The use of $^{14}$C-urea in these experiments allowed accurate measurements of in situ urea turnover rates. These rates were measured during short incubations and without significantly perturbing the plankton community by the addition of the tracer. Herbland (1976) used a similar methodology to that reported here. Current methodology is insufficient to determine urea regeneration rates in a manner similar to that used for NH$_4^+$. As was discussed earlier, there are many sources of urea in seawater; there is a significant potential for urea regeneration in seawater samples. In Chapter 2, a mass balance method was used to quantify urea regeneration by plankton in a coastal environment. The results showed that urea regeneration rates were similar in magnitude to NH$_4^+$ regeneration rates. By comparison to other nitrogen compounds, the rapid urea turnover times in the Sargasso Sea are similar. Glibert and McCarthy (1984) measured NH$_4^+$ turnover times in the Sargasso Sea of ca. 11 h. These rates were calculated from in situ uptake rate data and dissolved ammonium concentrations. When concentrations were undetectable, they were set equal to 30 nM: the limit of detection. There was no significant difference between the turnover times of ammonium in the surface-mixed layer, or at the 5% sea-surface light depth, but the median value for the NH$_4^+$ turnover time in surface water was less. Although most of the phytoplankton biomass was below the thermocline, I found faster turnover times of urea in surface waters, in agreement with results of Herbland (1976). These rapid
turnover times of urea indicate that urea regeneration rates are of similar magnitude to NH$_4^+$ regeneration rates. Therefore, calculation of *in situ* urea uptake rates, using $^{14}$C- or $^{15}$N-urea, must take this into consideration. Failure to do this will result in an underestimate of the *in situ* urea uptake rate.

**Urea uptake: saturating/trace rates**

Harvey and Caperon (1976) compared rates of urea uptake by phytoplankton populations in Kaneohe Bay with saturating (8 ug at N·l$^{-1}$) and trace (0.8 ug at N·l$^{-1}$) additions. They found that saturated uptake rates were on average 1.25 times greater than *in situ* urea uptake rates, but statistical analysis of their data indicated that the uptake rates were not significantly different. This result implied that urea uptake rates were saturated at ambient *in situ* concentrations. Kristiansen (1983) measured urea uptake rates in Oslofjord following 1 and 10 ug at N·l$^{-1}$ additions, although he did not compare the two rates. Glibert and McCarthy (1984) suggested comparing these measurements, using NH$_4^+$ as the nitrogen source, as a means of quantifying the nitrogen status of phytoplankton communities. My results are too few to show any apparent patterns. Trace and saturating urea uptake rates were in fairly good agreement in continental slope water, with saturating rates only 1.5 times the *in situ* rates. The dissolved urea concentrations were 0.12 and 0.18 ug at N·l$^{-1}$ in surface and deep water, respectively. Laboratory studies
on a limited number of phytoplankton species show that these concentrations are near the $K_s$ for uptake of urea, so the small discrepancy seen between the two rates is expected. In the Sargasso Sea $V_{saturated}/V_{trace}$ is more variable, the greatest disparity between the rates occurred in water collected from 88 m at Station 2. McCarthy and Nevins (1986) found a similar variability in $V_{saturated}/V_{trace}$ for $NH_4^+$ uptake in their warm-core rings study. In the deep water sample from Station 3, $V_{trace}$ was similar to $V_{saturated}$, however, $V_{trace}$ may be overestimated because one of the duplicate urea samples was two times the concentration of the other. In instances where urea concentrations were below the limit of detection, I arbitrarily chose the limit of detection as the ambient concentration.

In conclusion, the similarity between $V_{saturated}$ and $V_{trace}$ demonstrates that in the Sargasso Sea there is both horizontal and vertical variability in the urea-N status of the plankton communities. In slope water, *in situ* urea uptake rates were in close agreement with saturated uptake rates. The rapid turnover times of urea in the Sargasso Sea and the low ambient concentrations suggests that urea is supplied by regenerative processes at rates which are close to the rates of utilization. In some phytoplankton communities in the Sargasso and in continental slope water, urea is utilized at rates which approximate the maximum rates of utilization, implying that the phytoplankton are able to effectively sequester nanomolar concentrations of urea from seawater.
Summary

Uptake rates, determined with saturating additions (10 ug at N·l⁻¹) of ¹⁵N-labelled ammonium, nitrate and urea, were measured at two stations in the western Sargasso Sea and at one station over the continental slope off Cape Hatteras in August 1985. Daily rates of nitrogen uptake were determined during 24 h incubations in water samples collected within the euphotic zone. Urea uptake, as measured by ¹⁵N isotopes, was constant over the incubation at all stations. Throughout the euphotic zone in slope water, NH₄⁺ uptake rates were 2-3 times greater than urea uptake rates and 3-3.5 times greater than NO₃⁻ uptake rates. In the surface waters of one Sargasso Sea station, urea uptake rates were 5 times greater than NH₄⁺ uptake rates, but below the 30 m, uptake rates of NH₄⁺ were greater than urea uptake rates. At the other Sargasso station, NH₄⁺ uptake rates were generally greater than urea uptake rates.

Urea uptake rates measured by ¹⁴C- and ¹⁵N-urea were not equivalent: rates measured by ¹⁴C-urea were on average 1.4 faster than those determined with ¹⁵N-urea.

In situ urea turnover times were determined by trace additions of ¹⁴C-urea. In the surface-mixed layer of the Sargasso Sea, urea turnover times were ca. 12 h, and they were ca. 2 d in water samples collected from the base of the euphotic zone. The ratio of saturated uptake rate/in situ uptake rate was near unity in 4 of the 6 samples. These results provide evidence that urea uptake rates in situ are near the maximum potential uptake rates, suggesting that the phytoplankton in these regions have a high
affinity for dissolved urea.
CHAPTER 4. FATE OF UREA-C AND N DURING UREA UPTAKE BY THE
COASTAL MARINE DIATOM _THALASSOSIRA PSEUDONANA_

Background


In the results of the last chapter, there were discrepancies between uptake rates of urea calculated with \(^{14}\text{C} \)-urea and \(^{15}\text{N} \)-urea and differences in the time course of uptake of both labelled substrates. \(^{15}\text{N} \)-urea incorporation by phytoplankton was constant over the 24 h incubation period, while \(^{14}\text{C} \)-urea uptake was most rapid over the first 6 h of the
incubation. During 24 h incubations, some $^{14}$C was lost to a compartment not measured in the analyses. In contrast to these results, Mitamura and Saijo (1986) found no difference between $^{14}$C- and $^{15}$N-urea uptake rates in Lake Biwa. Discrepancies between $^{14}$C-urea uptake and disappearance of dissolved urea from seawater were evident in the data of Harvey and Caperon (1976). This might be expected if urea regeneration is an important process, and the difference between the two measurements should favour higher uptake rates determined with $^{14}$C-urea. This was not the case in Kaneohe Bay, as Harvey and Caperon (1976) reported that rates of urea disappearance were generally greater than $^{14}$C-urea uptake rates.

In laboratory experiments with unialgal cultures of *Thalassiosira pseudonana* and *Skeletonema costatum*, Horrigan and McCarthy (1981) found no difference in urea uptake rates determined by $^{14}$C- and $^{15}$N-urea. However, there was evidence in their results to suggest that the two isotopes were not measuring the same process. Ammonium completely inhibited $^{15}$N-urea incorporation by *T. pseudonana*; whereas, in a separate experiment $^{14}$C-urea uptake rate was only reduced by 60% in the presence of 10 ug at NH$_4^+$-N·L$^{-1}$.

In this study, uptake of urea by axenic cultures of *Thalassiosira pseudonana* was measured by $^{14}$C-urea, $^{15}$N-urea and by chemical analysis of dissolved urea. Urea uptake rates were determined in nitrate-sufficient and nitrate-starved batch cultures, following the addition of 10 ug at urea-N·L$^{-1}$. 
Materials and Methods

Culturing procedure

An axenic culture of *Thalassiosira pseudonana* clone 3H, obtained from the Northeast Pacific Culture Collection, Dept. Oceanography, U.B.C., was established with antibiotic treatment using a procedure similar to that outlined by Droop (1967). The absence of bacteria was verified by epifluorescence microscopy (Hobbie et al. 1977), and by the use of sterility test medium (Provasoli et al. 1957). Batch cultures of *T. pseudonana* were grown in modified artificial seawater medium (ESAW) of Harrison et al. (1980), described in detail in Chapter 5, with NO$_3^-$ (50 ug at N·l$^{-1}$) as the nitrogen source. Cultures were grown in a 2.5 litre polycarbonate Fernbach flask or 250 ml Erlenmeyer flasks under continuous illumination at an irradiance of 120 uE·m$^{-2}$·s$^{-1}$ and 18°C. Cultures were continuously stirred by teflon-coated magnetic stir bars.

Cell growth was monitored by *in vivo* chlorophyll $a$ fluorescence measured on a Turner Designs model 10 fluorometer, and by cell counts using a Coulter Counter® model TA II. Dissolved NO$_3^-$ + NO$_2^-$ and NH$_4^+$ were measured with a Technicon Autoanalyzer® II using the methods of Wood et al. (1967) and Slawyk and MacIsaac (1972), respectively. Dissolved urea concentration was analyzed by the modified diacetyl monoxime method described in Chapter 1. Particulate nitrogen (PON) samples were collected on combusted Whatman GF/F filters, oven dried at 60°C and measured on a Carlo Erba
Elemental Analyzer.

**Urea purity**

The purity of the urea stock solutions used during uptake studies was verified by analyzing them for ammonium and other potential contaminants. Crystalline $^{14}$C-urea purchased from Amersham (specific activity 55 uCi·umol$^{-1}$; $2.04$ GBq·mmol$^{-1}$) was reported by the manufacturer to be 99% pure. A stock solution was prepared in sterile deionized distilled water, and stored frozen at $-30^\circ$C. This solution was analyzed for NH$_4^+$ to determine if any urea had decomposed during storage. Results showed that in a $10$ ug at N·l$^{-1}$ solution of $^{14}$C-urea, NH$_4^+$ was undetectable. Urease ($1860$ units·ml$^{-1}$) was added to $10$ ml samples of $10$ ug at N·l$^{-1}$ $^{14}$C-urea stock in a final concentration of $9.3$ units·ml$^{-1}$. After incubation at room temperature for $1$ h, the samples were acidified and allowed to degas overnight. All but $0.16\%$ of the initial $^{14}$C activity was lost from solution, indicating there was negligible contamination by $^{14}$C-containing compounds other than urea.

The $^{15}$N-urea purchased from Kor Isotopes was 99% $^{15}$N. A stock solution was prepared in a manner similar to that described for $^{14}$C-urea; no NH$_4^+$ was detected in a $10$ ug at N·l$^{-1}$ solution.

Analytical reagent grade urea (ACS approved), purchased from Fisher Chemical company, was used in the preparation of the "cold" urea stock solution. Its purity was confirmed by chemical analysis, and no contaminating NH$_4^+$ was detected in a
10 ug at urea-N·l⁻¹ solution.

Urea uptake

Urea uptake experiments were performed with *T. pseudonana* during nitrate-sufficient growth and after 24 h of nitrate-starvation. Aliquots of phytoplankton culture (100 ml) were aseptically added to sterile 250 ml polycarbonate Erlenmeyer flasks. Uptake rates were determined in duplicate with ¹⁵N-urea, ¹⁴C-urea, and by measuring the change in urea concentration in the medium by chemical analysis. All uptake experiments were initiated within 45 min of each other, following the addition of a saturating concentration (10 ug at N·l⁻¹) of urea. Urea uptake rates were measured during 2 h incubations for the nitrate-sufficient cultures and 1 h for the nitrate-starved cultures. Average uptake rates are reported ± 1 SD.

Rate measurements

*Change in dissolved urea concentration*

At designated time intervals (1, 5, 15, 30, 60 and 120 min), samples were withdrawn and filtered through Swinex® filter holders containing combusted (4 h at 460°C) Whatman GF/F filters. Previously acid-washed sample cups were rinsed once with sample. The filtered samples were immediately analyzed for NH₄⁺, NO₃⁻ + NO₂⁻ and urea.

The rate of decrease of dissolved urea concentration was calculated from the slope of a linear regression through the
data points. This rate is the disappearance uptake rate or net urea uptake rate expressed as ug at N·1⁻¹·h⁻¹.

$^{15}$N-urea uptake

Samples for $^{15}$N-urea uptake (10 ml) were collected by filtration onto combusted Whatman GF/F filters and rinsed with 10 ml filtered ESAW (0.2 um Nuclepore filter). Filtration pressures were always less than 100 mm Hg. Nitrogen in the particulate samples was converted to N₂ (g) by the micro-Dumas dry combustion technique, as described by LaRoche (1983), and then analyzed for $^{15}$N enrichment with a JASCO model N-150 emission spectrometer (Fiedler and Proksch 1975).

Uptake rates were calculated according to the equations of Dugdale and Goering (1967) and are presented as absolute uptake rates (ug at N·1⁻¹·h⁻¹) determined using the final PON. The difference in the initial and final $^{15}$N atom percent excess (ape) in successive samples was divided by the length of the time interval, and uptake rates (V) were calculated as:

$$V = \frac{ape_f - ape_i}{(t_f - t_i)} \frac{(PON_f)}{R}$$

where PON is the particulate nitrogen, t is the time, subscripts i and f designate initial and final measurements, respectively, and R is the enrichment factor. These rates are plotted against the average incubation time. The PON at the end of each time interval was calculated from an exponential regression equation through PON values measured at the start, middle and end of the experiment. A linear regression was
used for the nitrate-starved cultures.

$^{14}$C-urea uptake

Subsamples of culture (10 ml) were filtered through combusted Whatman GF/F filters, and the filters rinsed before running dry with 10 ml filtered ESAW. Filtration pressures were always less than 100 mm Hg. The filters were added to scintillation vials containing 10 ml of Aquasol II. The filtrate was retained, and the $^{14}$CO$_2$ recovered as described in Chapter 1. The amount of $^{14}$C in the phytoplankton samples, and the amount released as $^{14}$CO$_2$ was determined by liquid scintillation counting on an Isocap/300 (Searle Analytical Inc.). Samples were counted until the standard deviation of each count was 2%.

$^{14}$C-urea uptake rates were calculated over successive time intervals in a manner similar to the $^{15}$N-urea uptake calculations. The total amount of urea taken up was calculated by summing the amount of $^{14}$C retained on the filters and the amount of $^{14}$CO$_2$ released by the cells. Recovery of known quantities of $^{14}$C-HCO$_3^-$ from seawater samples was 91.5% (n=4), and uptake rates were corrected accordingly. Rates are expressed as nmol·cell$^{-1}$·min$^{-1}$, but they are also given in absolute rates for comparison with other data.
Results

Culture conditions

Growth of *Thalassiosira pseudonana*, and concentration of dissolved NO$_3^-$ in the medium are given in Figure 21. The exponential growth rate was 1.63 d$^{-1}$, and the initial biomass parameters for each experiment are summarized in Table XIX.

Nitrate-sufficient culture

Urea disappearance rate from the culture medium averaged 1.88 ± 0.08 ug at N·l$^{-1}$·h$^{-1}$, and was constant. Nitrate concentration measured in the same cultures indicated that NO$_3^-$ uptake was not constant over the 2 h incubation (Fig. 22). During the first 15 min, no NO$_3^-$ uptake was detected. Between the 15-60 min interval, NO$_3^-$ uptake rate averaged 1.14 ± 0.08 ug at N·l$^{-1}$·h$^{-1}$, but over the final hour of the incubation this rate decreased to 0.29 ± 0.14 ug at N·l$^{-1}$·h$^{-1}$. From these chemical measurements, an average total nitrogen uptake rate (i.e. NO$_3^-$ and urea) was 2.78 ug at N·l$^{-1}$·h$^{-1}$. This nitrogen uptake rate exceeded the growth requirements of *T. pseudonana* during this stage of the growth cycle. Nitrogen demand, calculated from the growth rate (1.63 d$^{-1}$) and an exponential average of the particulate nitrogen over the incubation (28.7 ug at N·l$^{-1}$), equalled 1.95 ug at N·l$^{-1}$·h$^{-1}$.

Uptake of $^{14}$C-urea varied over the incubation period (Fig. 23A). The apparent decrease in cellular uptake rate of urea, which was evident during the 5-15 min time interval, was confirmed in a separate repeat experiment using shorter
Fig. 21. Growth of *T. pseudonana* measured by *in vivo* chlorophyll *a* fluorescence (O), and the concentration of dissolved NO$_3^-$ in the culture medium (●) measured over time. The arrows labelled 1 and 2 indicate the times at which urea uptake experiments were initiated for the nitrate-sufficient and nitrate-starved culture, respectively.
**Table XIX**

Summary of culture conditions at the beginning of each experiment.

<table>
<thead>
<tr>
<th>Culture Description</th>
<th>[NO$_3^-$] (ug at N·l$^{-1}$)</th>
<th>PON$^1$ (ug at N·l$^{-1}$)</th>
<th>Cell Numbers ($10^8·l^{-1}$)</th>
<th>$Q_N^2$ (fmol N·cell$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate-sufficient</td>
<td>22.0</td>
<td>26.8</td>
<td>3.14</td>
<td>85.4</td>
</tr>
<tr>
<td>Nitrate-starved</td>
<td>&lt; 0.05</td>
<td>42.6</td>
<td>8.3</td>
<td>51.3</td>
</tr>
</tbody>
</table>

$^1$ Particulate nitrogen  

$^2$ Cell nitrogen quota
sampling times (Fig. 23B). A high initial uptake rate occurred during the 0.5-2 min time interval and was primarily the result of incorporation of the $^{14}$C label into the cells (Fig. 23C). The amount of $^{14}$CO$_2$ released by the cells over this time was only 37% of the total urea taken up (Fig. 23D). Hydrolysis of urea, and subsequent release of $^{14}$CO$_2$ became a greater fraction of the total urea taken up as the incubation progressed.

For comparison, cellular uptake rates were converted to rates of urea disappearance from the culture medium. In this calculation, I assumed that for each $^{14}$C transported across the cell membrane and retained by the cell or released as $^{14}$CO$_2$ two nitrogen atoms are transported into the cell. The average $^{14}$C-urea uptake rate was 1.03 ug at N·l$^{-1}$·h$^{-1}$.

The incorporation of $^{15}$N-urea into the phytoplankton was not constant over the incubation (Fig. 24A). The rate of increase of cellular $^{15}$N decreased markedly after 30 min, and the initial rate of $^{15}$N-urea incorporation was less than the maximum $^{15}$N uptake rate. During the 30-60 and 60-120 min intervals, the rates of urea-N uptake were less than 30% of the maximum $^{15}$N-urea uptake rate (Fig. 24B). The total amount of urea-N retained by the phytoplankton during the incubation was 0.59 ± 0.05 ug at N·l$^{-1}$. This yields an average $^{15}$N-urea uptake rate of 0.29 ± 0.03 ug at N·l$^{-1}$·h$^{-1}$.

Over the incubation period, cellular nitrogen, as measured by elemental analysis, increased at an average rate of 1.5 ± 0.1 ug at N·l$^{-1}$·h$^{-1}$. This increase was identical with the decrease in NO$_3^-$ concentration measured in the
Fig. 22. Dissolved urea (●) and nitrate (O) concentration in duplicate samples (solid and dashed lines) of a nitrate-sufficient culture of *T. pseudonana* spiked with 10 ug at urea-N·l\(^{-1}\). Urea disappearance was constant over the incubation; the regression coefficients for the two lines were: \( r^2 = 0.996 \) (dashed line) and \( r^2 = 0.998 \) (solid line).
Fig. 23. (A) $^{14}$C-urea uptake rate measured in duplicate samples (●,○) of a nitrate-sufficient culture of *T. pseudonana*. (B) $^{14}$C-urea uptake by nitrate-sufficient *T. pseudonana* determined in a separate experiment. A single uptake determination was made on two replicate cultures (●,○), and sampling times were of shorter duration during the first 5 min of the experiment. (C) $^{14}$C accumulation in *T. pseudonana*. (D) $^{14}$CO$_2$ released into the medium. All rates were calculated between successive sampling points and are plotted against the average incubation time.
Fig. 24. $^{15}N$-urea uptake rate measured in duplicate samples (●, ○) of a nitrate-sufficient culture of *T. pseudonana*. (A) $^{15}N$ accumulation expressed as $^{15}N$ atom percent excess in the cells determined over time. (B) $^{15}N$-urea uptake rate during each incubation period plotted against the average incubation time.
unperturbed culture during the same time period (1.5 ug at N·l⁻¹·h⁻¹).

**Nitrate-starved culture**

An uptake rate of 5.5 ± 0.1 ug at N·l⁻¹·h⁻¹ was determined by chemical analysis of dissolved urea, and this rate was constant over the incubation (Fig. 25). Many of the samples were lost due to an instrument malfunction, nevertheless, uptake rates of the replicate cultures were comparable.

Uptake of $^{14}$C-urea was constant over the incubation, although the shortest time interval was only 1-5 min (Fig. 26). These cellular rates were similar to those found for nitrate-sufficient cells. The urea uptake rate was determined to be 5.3 ± 0.04 ug at N·l⁻¹·h⁻¹, and was in excellent agreement with the rate calculated by chemical analysis of urea disappearance from the medium.

Incorporation of $^{15}$N-urea into *T. pseudonana* is shown in Figure 27A. Elevated $^{15}$N-urea uptake rates occurred during the 5-15 min time interval. Following this, the $^{15}$N-urea uptake rate decreased to the initial rate, although it appeared that the uptake rate increased during the final incubation periods (Fig. 27B). An average $^{15}$N-urea uptake rate was 5.18 ± 0.04 ug at N·l⁻¹·h⁻¹ and was in good agreement with the rates measured by the other methods.

From the direct measurement of the cellular PON, at the beginning and end of the experiment, a nitrogen uptake rate of 5.3 ± 0.05 ug at N·l⁻¹·h⁻¹ was calculated.
Fig. 25. Dissolved urea concentration measured in duplicate (●, ○) samples of a nitrate-starved culture of *T. pseudonana* spiked with 10 ug at urea-N·l⁻¹.
Fig. 26. $^{14}$C-urea uptake rate measured in duplicate samples (●, ○) of a nitrate-starved culture of *T. pseudonana*. 
14C-UREA UPTAKE RATE (nmol·cell⁻¹·min⁻¹) x 10⁻⁸

TIME (min.)

0 20 40 60

0 10 20 30 40 50

∞ 4 2 0

163
Fig. 27. $^{15}$N-urea uptake rate measured in duplicate samples (●, ○) of a nitrate-starved culture of T. pseudonana. (A) $^{15}$N accumulation in the cells over time. (B) $^{15}$N-urea uptake rate during each incubation period plotted against the average incubation time.
Concentration of dissolved NH$_4^+$ increased in the culture medium after the addition of urea to the nitrate-sufficient and nitrate-starved cultures (Fig. 28). Then the NH$_4^+$ concentration decreased during the remainder of the experiment. All results for the nitrate-sufficient and nitrate-starved cultures are summarized in Table XX.

Discussion

Interpretation of measurements

Urea uptake rates measured by $^{15}$N-urea, $^{14}$C-urea and by chemical analysis of dissolved urea differ depending on the nitrogen status of the culture, and the times over which they were determined. Throughout this discussion, I will refer to urea uptake rate measured by urea disappearance from the culture medium as the net urea uptake rate. The change in dissolved urea concentration over time provides an unambiguous measure of net urea uptake by phytoplankton, since it is the difference between the influx and efflux rates. With $^{14}$C- and $^{15}$N-labelled urea, assumptions must be made regarding the fate of the label once it is inside the cell. Failure to account for a portion of the label will result in an underestimate of the uptake rate and may obscure the true physiological process. This very problem was pointed out by Stephens and North (1971) in their study of $^{14}$C-amino acid uptake by marine phytoplankton. They found that not only are amino acids metabolized after they are taken up by phytoplankton, but a portion of the amino acid carbon skeleton is released to the
Fig. 28. Dissolved ammonium concentration in (A) nitrate-sufficient, and (B) nitrate-starved cultures of *T. pseudonana*, following the addition of 10 ug at urea-N·l⁻¹. The ammonium concentration in the cultures prior to the addition of urea is indicated by the arrows.
Table XX

Summary of urea uptakes rates measured by $^{14}$C- and $^{15}$N-labelled urea and by disappearance in nitrate-sufficient and nitrate-starved cultures. Nitrate uptake rate measured in the nitrate-sufficient culture is also given, as are the measured rates of change of the particulate nitrogen over the incubation.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Parameter Measured</th>
<th>Rate Measured$^1$ (ug at N·l$^{-1}$·h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate-sufficient</td>
<td>Urea Disappearance</td>
<td>1.88 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Nitrate Disappearance</td>
<td>0.90 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>$^{14}$C-urea Uptake</td>
<td>1.03 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>$^{15}$N-urea Uptake</td>
<td>0.29 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>Particulate Nitrogen</td>
<td>1.50 ± 0.10</td>
</tr>
<tr>
<td>Nitrate-starved</td>
<td>Urea Disappearance</td>
<td>5.5  ± 0.1</td>
</tr>
<tr>
<td></td>
<td>$^{14}$C-urea Uptake</td>
<td>5.3 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>$^{15}$N-urea Uptake</td>
<td>5.2 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Particulate Nitrogen</td>
<td>5.3 ± 0.03</td>
</tr>
</tbody>
</table>

$^1$ All rates are averaged over the incubation period and are reported on an hourly basis ± 1 SD.
medium as CO₂ and acid-stable products.

**Methodological considerations**

The discrepancy between methods for measuring urea uptake rates are only evident in the nitrate-sufficient culture. These results will be explained later by dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) excretion. Other investigators have reported loss of ¹⁴C-labelled DOC from phytoplankton, in filtered samples which were rinsed with seawater after the filters have dried (Goldman and Dennett 1985). But in general, losses are not seen when the rinse solution is added before the filters go dry, and little or no loss is seen when using glass fibre filters. I am confident that the filtration technique used in this study prevented loss of either ¹⁴C- or ¹⁵N-labelled metabolites from *T. pseudonana*. At all times during these experiments, filtrations were done using vacuum pressures less than 100 mm of Hg, and the rinse solution was added prior to the filter drying. Additional evidence refuting the loss of labelled metabolites during rinsing is provided in the data from the nitrate-starved cultures pulsed with urea. Goldman and Dennett (1985) showed that N-limited phytoplankton pulsed with NH₄⁺ at the start of a ¹⁴C uptake experiment lost significantly greater DO¹⁴C than phytoplankton which did not receive NH₄⁺. If any losses were to occur as an artifact of filtration during experiments in this study, I expect they would have been most obvious in the nitrate-starved cultures. But in the results from the nitrate-starved cultures all
methods for measuring urea uptake were in excellent agreement, and the increase in PON was exactly balanced by the uptake of urea-nitrogen.

*Nitrate-sufficient cultures*

In the nitrate-sufficient cultures, the disappearance rate of urea, and the simultaneous uptake of $\text{NO}_3^-$, resulted in a total nitrogen flux into the cells of 2.78 μg at N·1⁻¹·h⁻¹. However, by contrast to the urea disappearance rate, it was evident that $^{15}$N-urea incorporation was not constant and only represented 15% of the net urea uptake rate. Since the change in PON over the incubation period could be accounted for by $\text{NO}_3^-$ uptake and $^{15}$N-urea incorporation (Table 2), these data indicate that most of the urea nitrogen was lost from the cells to a nitrogen pool not measured by the methods used in this study.

The reduced rates of $^{15}$N-urea uptake are not due to dilution of $^{15}$N-urea by coincident $^{14}$NO$_3^-$ uptake. As it was pointed out in Chapter 2, and by Dugdale and Wilkerson (1986), simultaneous utilization of $^{14}$N-labelled nitrogen substrates and a $^{15}$N-labelled compound will result in a decrease in the N-specific uptake rate of the $^{15}$N-labelled compound. In this study, the $^{15}$N-urea uptake rates were calculated using the PON at the end of the incubation thereby avoiding this problem. Since the released $\text{NH}_3/\text{NH}_4^+$ was largely reassimilated by the cells, I conclude that most of the urea-N is lost from the nitrate-sufficient cells as $\text{DO}^{15}$N. There is ample evidence in
the literature to support this theory.

Release of DON

The release of dissolved free amino acids (DFAA) by phytoplankton during nitrate-sufficient growth has been unequivocally demonstrated in axenic culture by HPLC analysis (Admiraal et al. 1986). Although DFAA excretion is species specific, and related to the stage of the growth cycle, in the extreme case of Coscinodiscus granii as much as 3% of assimilated \( \text{NO}_3^- \) is excreted as amino acids. Failure to account for 85% of the urea-nitrogen taken up by the \( T. \) pseudonana suggests that this nitrogen must be lost from the cells as DO\(^{15}\)N. Observations by other investigators support this interpretation of the results. Furthermore, estimates of PON, calculated from the amount of \( \text{NO}_3^- \) taken up by \( T. \) pseudonana during exponential growth, overestimated the actual PON determined by elemental analysis. This is consistent with Admiraal's observations that phytoplankton release DON which is not reutilized. It is well known, that in axenic culture, phytoplankton are capable of utilizing many forms of DON; but some amino acids may not be utilized (Guillard 1963, Wheeler et al. 1974, Antia et al. 1975). Admiraal et al. (1986) showed that some phytoplankton release DFAA which they are unable to reassimilate, at least while \( \text{NO}_3^- \) is present. They did not examine nitrogen-starved cultures, but extracellular organic phosphates, which are produced during exponential growth of some coastal diatoms, are incompletely reabsorbed during phosphorus starvation (Admiraal and Werner 1983).
*Pattern of urea uptake*

The pattern of $^{14}$C-urea uptake observed in the first experiment was confirmed and clarified in a separate experiment using shorter sampling times. The time course of urea uptake involves an initial rapid uptake period followed by a shutdown and a return to an intermediate rate, which is maintained throughout the remainder of the experiment. The initial rate of $^{14}$C-urea uptake, determined during the 0.5-2 min time interval from the data presented in Figure 23B, averages $7.9 \pm 1.9 \cdot 10^{-8}$ nmol·cell$^{-1}$·min$^{-1}$. Although the average $^{14}$C-urea uptake rate (over the 2 h incubation) was only 55% of the net urea uptake rate ($5.0 \pm 0.3 \cdot 10^{-8}$ nmol·cell$^{-1}$·min$^{-1}$), the initial $^{14}$C urea uptake rate was actually greater. The initial $^{14}$C-urea uptake rate is the most accurate measurement of the urea influx rate. As uptake proceeds, efflux of urea becomes a more significant fraction of the influx, and eventually $^{14}$C-urea uptake rate should equal the net urea uptake rate. The discrepancy between net urea uptake rate and the $^{14}$C-urea uptake rate can only be reconciled by proposing that some of the $^{14}$C is lost to a compartment not accounted for in these analyses. This compartment may be DOC. Since urea degradation occurs very rapidly in *T. pseudonana*, as evidenced by the rapid appearance of $^{14}$CO$_2$, initial uptake measurements will be least affected by metabolic processes. In fact, it was observed that the initial $^{14}$C-urea uptake rate was predominantly a result of the
accumulation of $^{14}$C-urea by *T. pseudonana*. This probably represents the filling of an intracellular urea pool. The lack of the rapid initial $^{14}$C-urea uptake in Figure 23A and in the nitrate-starved cultures is solely a consequence of the long sampling times relative to the time over which this process is occurring. Long times between sample points will tend to smooth out this transient, pool-filling phase, and only the constant long term uptake rate will be evident.

*Urea pools and efflux*

The concentration of urea that accumulated intracellularly was estimated during the 0.5-2 min time interval as follows: Using the $^{14}$C-urea data, $2.4 \times 10^{-8}$ nmol $^{14}$C·cell$^{-1}$ was retained by the phytoplankton during this period, and the average cell volume was 43 femtolitres·cell$^{-1}$. If 80% of the label is present as $^{14}$C-urea, similar to that which is found in *Phaeodactylum tricornutum* (Rees and Syrett 1979a), the average internal dissolved urea concentration is 0.45 mM. I suggest that the decrease in urea uptake rate and intracellular $^{14}$C observed during the 2-5 min interval is due, in part, to urea efflux. Using a urea permeability coefficient of $5 \times 10^{-6}$ cm·s$^{-1}$ (Raven 1980), the urea efflux rate is $8.5 \times 10^{-8}$ nmol·cell$^{-1}$.min$^{-1}$. Providing the initial influx rate (*ca.* $8 \times 10^{-8}$ nmol·cell$^{-1}$.min$^{-1}$) remains constant throughout the experiment, it is obvious from a comparison of the influx and efflux rates that net urea uptake should equal zero. Since net uptake is measurable, urea efflux must decrease subsequently. This proposed reduction in urea efflux
can be explained as a result of a reduction of the intracellular urea concentration by urea degradation and assimilation. Evidence for this is provided by the observed increase in $^{14}\text{CO}_2$ evolution from the cells (Fig. 23D).

Following the initial transient in urea uptake, net uptake is reduced to 60% of urea influx rate (0.5-2 min). This results in a continuous efflux rate of $3 \cdot 10^{-8}$ nmol·cell$^{-1}$·min$^{-1}$. The intracellular concentration required to support this rate, when the external urea concentration equals 10 ug at N·l$^{-1}$, is 0.16 mM. A comparison of this average cell concentration with the Km for urease in *Phaeodactylum tricornutum* (0.46 mM) (Syrett and Leftley 1976) suggests that, all things being equal, to account for the rapid metabolism of urea, urea must be compartmentalized in *T. pseudonana*. Dagestad *et al.* (1981) postulated that urea is stored in the chloroplast of *Chlamydomonas*, but not metabolized there.

**Nitrate-starved culture**

Urea uptake rates measured by $^{14}$C- and $^{15}$N-labelled urea and by chemical analysis of dissolved urea concentration were in excellent agreement over the duration of the experiment in the nitrate-starved culture. However, there were transient patterns in the uptake data which were consistent with the results from the nitrate-sufficient culture.

During the 5-15 min time interval, the rate of $^{15}$N-urea uptake (7.7 ± 0.7 ug at N·l$^{-1}$·h$^{-1}$) was ca. 2 times greater than the initial and subsequent rate. This rate was also in
excess of the net uptake rate measured by disappearance (5.5 ± 0.1 ug at N·l⁻¹·h⁻¹). The initial rate of ¹⁵N-urea uptake, calculated over the first 5 min was 3.7 ug at N·l⁻¹·h⁻¹, and was significantly less than the net uptake rate. This difference can be accounted for by the release of ¹⁵NH₃/¹⁵NH₄⁺ into the medium by the phytoplankton (see Fig. 28). ¹⁵N incorporation rate over the 5-15 min time interval is greater than net urea uptake, and the ¹⁴C-urea uptake rate. Therefore, released ¹⁵N must be taken back up over this time interval. During this time interval approximately 0.7 ug at ¹⁵N·l⁻¹ must be taken up at the same time as ¹⁵N-urea to account for the increased ¹⁵N incorporation rate.

Results from McCarthy (1972b) are entirely consistent with these observations and the interpretation of the results in this study. He reported that the long term (3.5-7 h) uptake rate of ¹⁵N-urea by Cyclotella nana (3H) (sic) (renamed Thalassiosira pseudonana) was 1.8 times greater than the rate measured over 10 min. Although the precise physiological state of the cultures in McCarthy's experiments is unknown, urea uptake experiments were started as soon as nitrogen (NO₂⁻) could no longer be detected in the medium. This would suggest that the cells were in early stages of nitrogen-starvation (see also Parslow et al. 1984b).

The total amount of NH₃ released into the medium is too great to be accounted for solely on the basis of urea-nitrogen excretion. Although precautions were taken to ensure that the sample containers were NH₄⁺-free, contamination may have occurred. This may explain why such high concentrations of
NH₄⁺ were initially measured. An alternative explanation, which has not received consideration, is that during uptake of urea intracellular ¹⁴NH₃/¹⁴NH₄⁺ is released as well as ¹⁵NH₃/¹⁵NH₄⁺ from urea. If urea uptake is coupled to cotransport of H⁺, as shown in Chlorella fusca (Rees and Syrett 1984), or Na⁺ as suggested for Phaeodactylum tricornutum (Rees et al. 1980), then perhaps NH₄⁺ may serve as a counter ion to maintain charge balance during urea uptake in T. pseudonana. In higher plants, it is observed that efflux of Ca²⁺ is a non-specific response of low salt plants to monovalent cation uptake (Siddiqi and Glass 1984). It is argued that these fluxes are directed towards maintaining the charge balance within the cells. In nitrate-sufficient T. pseudonana, there is a large intracellular NH₄⁺ pool. Dortch et al. (1984) found that the intracellular concentration of NH₄⁺ in nitrate-sufficient T. pseudonana was 0.3% of the total PON. For the cultures in this study, this amounts to 0.80 μg at NH₄⁺.l⁻¹ or 6 mM on a cell volume basis. Although Dortch et al. (1984) reported that following 4 d of NO₃⁻ starvation NH₄⁺ pools were below detection, no data were given for shorter periods of NO₃⁻ starvation.

Ammonia efflux

Direct measurement of NH₃ efflux from algal cells is lacking, although on theoretical grounds, a finite leakage is unavoidable (Raven 1980). Circumstantial evidence indicates that NH₃ loss from cells is common. Castorph and Kleiner
(1984) found that the ammonium transport mutant of *Klebsiella pneumoniae* lacks the ability to accumulate NH\(_4^+\) intracellularly, but constantly loses NH\(_3\) by diffusion. They propose that efflux is the norm, and that active transport acts to circumvent this loss. Pulse chase experiments by Wheeler (1980), Wheeler and Hellebust (1981) and Balch (1986) showed that methylamine efflux, and by analogy NH\(_3\) efflux, occurs in marine phytoplankton.

The appearance of NH\(_4^+\) in the medium following the addition of urea, and its reassimilation, is entirely consistent with the rapid efflux of NH\(_3\) produced during urea metabolism in *T. pseudonana*. This response is seen in nitrate-sufficient and nitrate-starved phytoplankton. Confirmation of these observations and the proposal of NH\(_3\) efflux during urea uptake by *T. pseudonana* is found in exaggerated form in the results of Uchida (1976). He observed that *Prorocentrum micans*, previously deprived of nitrogen, excreted NH\(_3\) into the medium after the addition of 210 ug at urea-N·l\(^{-1}\). As much as 40 ug at NH\(_4^+\)-N·l\(^{-1}\) accumulated in the medium; it was then reassimilated by the cells: Little urea was taken up during this time. Once the external NH\(_4^+\) concentration decreased below 7 ug at N·l\(^{-1}\), urea uptake increased, and NH\(_4^+\) was excreted back into the medium and then reabsorbed.

The transient increase in \(^{15}\)N urea uptake by *T. pseudonana* can only be explained on the basis of simultaneous uptake of additional \(^{15}\)N compounds with urea. I suggest that the cells reabsorb excreted \(^{15}\)NH\(_4^+\) produced from urea
A model for urea uptake by *Thalassiosira pseudonana*

On the basis of these results, I propose a model of urea uptake in the diatom *Thalassiosira pseudonana*. The major events associated with urea transport, and the assimilation of urea-C and urea-N are depicted in Figure 29. Regardless of the nitrogen status of the cells, the initial stages of urea uptake involve a rapid influx and the accumulation of an intracellular urea pool. Urea uptake is reduced after this initial surge, and this apparent shutdown can be explained as a result of efflux of urea. A decrease in the intracellular urea concentration, as a result of metabolic degradation, reduces the urea pool size, and consequently the efflux rate. Resumption of urea uptake, albeit at a lower rate than seen initially, is evidence for this. This intracellular urea pool is rapidly metabolized by a urea-degrading enzyme to CO$_2$ and 2NH$_3$. It seems likely that this enzyme is urease, since it is reported in other diatoms (Leftley and Syrett 1973, Oliveira and Antia 1986a).

During nitrogen-sufficiency, when cells are growing at rates which are not limited by abiotic factors, $^{14}$C-labelled organic products derived from urea-C are excreted into the medium (see earlier Discussion). The net loss of $^{14}$C is approximately 45% of the total urea-C transported into the cells. Under nitrate-sufficiency and nitrate-starvation, there is an efflux of NH$_3$/NH$_4^+$ derived from urea, and the
Fig. 29. Diagrammatic representation of urea uptake, and assimilation of urea-C and urea-N by *Thalassiosira pseudonana*. Stippled region represents cell membrane. The arrows passing through open circles in the membrane represent transport by membrane porters; and arrows passing directly through the membrane represent diffusion. GS/GOGAT is Glutamine synthetase/glutamate oxoketoglutarate amino transferase. Losses of \((CH_2O)_n\) and \(R-NH_2\) are shown to occur by diffusion and active or mediated transport processes; the actual mechanism is not known. Consult the text for clarification of rates and processes.
\[ \text{CO(NH}_2\text{)}_2 \rightarrow \text{CO}_2 \rightarrow \text{CO(NH}_2\text{)}_2 \]

\[ \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{NH}_3 \rightarrow \text{NH}_4^+ \rightarrow \text{GS/GOAT} \rightarrow R-\text{NH}_2 \rightarrow \text{PROTEIN} \]

\[ \rightarrow (\text{CH}_2\text{O})_n \rightarrow \text{CARBOHYDRATE} \]

\[ \rightarrow (\text{CH}_2\text{O})_n \]

\[ \rightarrow \text{CARBOXYLASE} \]

\[ \rightarrow \text{NH}_3 + \text{H}^+ \rightarrow \text{NH}_4^+ \]
initial rate of $^{15}$N-urea incorporation is low. Within 5-15 min, this effluxed $\text{NH}_4^+$ is coincidently taken back up by the phytoplankton, with urea. The cell membrane becomes less permeable to $\text{NH}_4^+$ and influx and efflux of $\text{NH}_4^+$ are balanced. A further complication arises in N-sufficient cultures where a large fraction of the urea-N is released from the cells in the form of organic nitrogen. This DON is not reassimilated during the short term. There was no evidence of DON release by nitrate-starved phytoplankton.

Nitrate-replete T. pseudonana did not have enhanced uptake rates for urea, contrary to the results of Horrigan and McCarthy (1981, 1982). Nitrogen-specific uptake rate was a variable and averaged 0.26 d$^{-1}$ under nitrogen-sufficiency, but increased to 3.2 d$^{-1}$ in the nitrate-starved cultures. This was a consequence of a reduction in the nitrogen cell quota and the retention of all the urea-nitrogen by the nitrate-starved cells. From a comparison of net urea uptake rates, determined from chemical measurements, it is apparent that in T. pseudonana there is no increase in urea uptake rate upon nitrate starvation. Similar results have been found in Chlorella pyrenoidosa (Bekheet and Syrett 1979); however, Syrett et al. (1986) reported increased urea uptake rates upon increasing nitrogen starvation in Phaeodactylum tricornutum.

Implications for urea uptake measurements in nature

There are advantages in using of $^{14}$C- and $^{15}$N-labelled urea for measuring urea uptake by phytoplankton. The most obvious advantage is the greater sensitivity of both
techniques compared to chemical analysis of dissolved urea concentrations. This is particularly true in nature because cell densities are usually too low to allow accurate measurements of urea disappearance to be made; since urea concentrations are low in the ocean, isotopes are the only alternative. As stressed earlier, urea disappearance from the culture medium represents the net uptake of urea, since efflux of urea also occurs. These results demonstrate that $^{14}$C- and $^{15}$N-urea do not necessarily measure the same process, and $^{14}$C-urea uptake cannot be equated with simultaneous incorporation of urea-N. There is little doubt that $^{15}$N-urea incorporation by phytoplankton represents the overall contribution of urea-N to phytoplankton nitrogen requirements. What these results have shown, though, is that under nitrate-sufficiency this contribution can be very much less than the total urea-nitrogen taken up by the cells.

If a similar mechanism of urea uptake is present in other phytoplankton, the outcome of the results of urea uptake measurements conducted in regions of differing nitrogen status may differ. Under conditions of nitrate-sufficiency, urea uptake rates measured by $^{14}$C- and $^{15}$N-urea should be most divergent, while the converse will be true in nitrate-starved phytoplankton. A portion of the urea-N is released by T. pseudonana as NH$_3$/NH$_4^+$ and later reabsorbed. The discrepancies between measurements of $^{14}$C- and $^{15}$N-urea uptake in field experiments, reported in Chapter 3, provide evidence that some urea-N is lost from phytoplankton and is not
reabsorbed. A portion of this urea-N may be NH$_4^+$

The ability of algae to sequester this NH$_4^+$ will depend upon the ambient concentration of NH$_4^+$, and its rate of recycling. When the external NH$_4^+$ concentration is great, the amount of urea-derived $^{15}\text{NH}_4^+$ that is reassimilated will be small because of its dilution by the ambient NH$_4^+$ pool. Experiments designed to determine the effects of NH$_4^+$ on urea uptake must also take this into consideration. The true effect of ammonium on urea uptake can be determined by urea disappearance measurements.

These results provide circumstantial evidence for DON$^{15}$N loss from nitrate-sufficient phytoplankton spiked with $^{15}$N-urea. Direct measurement of DON production is now required to substantiate this claim. It is possible that the failure to account for all of the $^{15}$N during field experiments (see for example Laws 1984, Price et al. 1985) may be due to DON$^{15}$N loss. Other processes may also contribute to this loss, such as nitrification and denitrification, but their significance is undetermined.

Summary

Urea uptake rates were measured in nitrate-sufficient and nitrate-starved batch cultures of *Thalassiosira pseudonana* (clone 3H) by $^{15}$N-urea, $^{14}$C-urea, and by measuring the disappearance of dissolved urea from the medium.

In nitrate-sufficient cultures, urea uptake was determined by adding 10 ug at urea-N·L$^{-1}$, and uptake was measured during a 2 h incubation. Initial urea influx rate
measured by $^{14}$C-urea was greater than the net urea uptake rate measured by disappearance of urea from the medium during the first 2 min. But the long term average $^{14}$C-urea uptake rate was only 60% of the net uptake rate. Nitrate uptake continued in the presence of 10 ug at urea-N·l$^{-1}$ at a reduced rate. Only 15% of the urea-N was retained by the phytoplankton, and the increase in the particulate nitrogen was accounted for by the total NO$_3^-$ and urea-N uptake.

In 24 h nitrate-starved cultures, the average urea uptake rates measured by all methods were in excellent agreement with the increase in the particulate nitrogen during the incubation. $^{15}$N-urea uptake rates were not constant, and maximum rates were measured 5-15 min after the addition of urea. Uptake of $^{14}$C-urea and the net uptake rate were constant during the incubation.

A model of urea uptake and assimilation by *Thalassiosira pseudonana* that involves urea-N efflux as NH$_3$ and its rapid reabsorption is proposed. These results explain earlier observations in the literature and have implications for urea-N utilization and cycling by phytoplankton in nature.
PART II: SELENIUM

INTRODUCTION

Overview and objectives

In the second part of this thesis, the selenium (Se) nutrition of the coastal marine diatom, *Thalassiosira pseudonana*, was investigated. This alga was used during the initial stages of this thesis for urea uptake experiments. *Thalassiosira pseudonana* grew well in the artificial seawater medium routinely used in our laboratory to culture successfully other species. Suddenly, for no apparent reason, it became increasingly difficult to culture *T. pseudonana* in this medium; finally it stopped growing. The research described in the forthcoming chapters was initiated to elucidate the missing factor(s), which prevented growth of this alga. As the research progressed, it became apparent that Se was an essential element for growth of *T. pseudonana*, and that it was absent from the culture medium. Results presented in Chapter 5 document the essential growth requirement of *T. pseudonana* for selenium. The growth promoting properties of two forms of inorganic Se present in seawater, selenate and selenite, were examined. Observations of morphological features diagnostic of selenium-deficient cells were reported.

The specific nutritional role of selenium in algae, and in plants in general, is not understood. Collaborative research with G.J. Doucette (Doucette *et al.* 1987), which
examined ultrastructural alterations in Se-deficient *T. pseudonana*, provided a working hypothesis to explain the selenium requirement in *T. pseudonana*. By testing this hypothesis, I sought to explain the biochemical basis for the obligate selenium requirement in this alga. These results are presented in Chapter 6. An axenic culture of *T. pseudonana* was grown in the presence of $\text{Na}_2^{75}\text{SeO}_3$; the distribution of selenium among the biochemical constituents, measured by solvent extraction procedures, was determined. Gel electrophoresis was used to identify specific selenoproteins, and cell extracts were assayed for the selenoenzyme, glutathione peroxidase. Since the plant biology of selenium is so poorly understood and comparatively little work has been done in this field, a detailed account of our current knowledge of Se biology is provided.

**Historical perspective**

Biological selenium (Se) research began in the 1930's when poisoning among livestock was connected to high Se content of foodstuffs (Robinson 1933, Byers 1935). Franke (1934) demonstrated experimentally that grain grown in certain types of soil was toxic to animals, and these results led to the discovery that Se derived from plants was the causative agent of alkali disease in livestock. In their surveys of plants and soils of Wyoming, Beath and co-workers (Beath *et al.* 1934, 1935) observed that certain genera were restricted to soils containing high levels of Se. Some of these so-called selenium-indicator, or accumulator plants, species of
Astragalus, accumulated up to 10,000 ppm Se from soil containing less than 10 ppm Se. Selenium toxicity was of overwhelming concern at this time because of its impact on agriculture, and its potential as a public health hazard. Perhaps it was for these reasons, that the reports by Trelease and Trelease (1938a, b), which stated that some indicator plants required Se for growth, went largely unnoticed. Even now the Se requirements of plants remain a poorly studied aspect of Se nutrition.

The beneficial effects of Se in animals were realized in the late 1950's. Schwarz and Foltz (1957) reported that Se was an essential trace element, which was completely effective in preventing liver necrosis in rats grown on Se-deficient diet. Simultaneously, Patterson et al. (1957) and Schwarz et al. (1957) published results attributing exudative diathesis in vitamin E deficient chicks to Se deficiency. Thereafter, a number of researchers discovered that some previously unexplained illness in livestock were a result of the lack of Se in the diet (Muth et al. 1958, Proctor et al. 1958): Suddenly Se had gained a reputable status.

Selenium requirements in plants

Unlike the unequivocal results demonstrating Se essentiality in animals, much controversy, albeit based on little study, surrounds the role of Se in plants. A considerable period of time elapsed between the publications of Trelease and Trelease (1938a, b) and other research
investigating the growth promoting properties of Se in plants. Rosenfeld and Beath (1964) found the dry weight of *Astragalus racemosus* grown in the presence of selenite or selenate was twice that of the control plants which were not supplied with Se. But Broyer *et al.* (1966) failed to observe any stimulatory effects of Se added to the non-accumulators, *Medicago sativa* and *Trifolium subteraneum*. Even though control plants in the study of Broyer *et al.* (1966) were grown in Se-free culture (actually 50 ng Se was present in the nutrient solutions of each plant) they each contained 1.5 ug Se when the experiments were terminated. Measurements of Se accumulated in the carbon filter used to purify the greenhouse air confirmed the presence of atmospheric Se; this led Broyer *et al.* (1966) to postulate that plants were able to derive Se from the air. The lack of methodology available to grow plants in a Se-free environment appears to be one of the major shortcomings of this and similar research. In all studies conducted to date, the fact that the control plants were able to grow in the absence of Se enrichments confounds the interpretation of the results and questions the validity of conclusions that Se is an essential element for plants. True essentiality can only be demonstrated if in the absence of an element, plant growth is abnormal, the plants life cycle can not be completed, or if an essential metabolite contains the element as an integral component (Salisbury and Ross 1978). Clearly, the results for higher plants fall short of satisfying these criteria. To make matters worse, Broyer *et al.* (1972a, b) reported that the results of Trelease and
Trelease (1938a, b) could be explained as an artifact caused by phosphate toxicity in the control plants. Broyer et al. (1972a) argued that the additions of Se as SeO$_3^{2-}$ or SeO$_4^{2-}$ ameliorated the toxic effects of phosphate and thereby created the impression that Se stimulated growth. Nevertheless, the addition of 5 ug at Se·l$^{-1}$, two orders of magnitude less than the phosphate concentration, increased the dry weight of the plants by two-fold and reduced the total plant phosphorus concentration by one-third. This is a pronounced effect for such a relatively small addition of Se. Ziebus and Shrift (1971) found no evidence for Se essentiality in callus tissue of Astragalus species. Finally, Anderson and Scarf (1983) documented a stimulatory effect of Na$_2$SeO$_3$ on the growth of Trifolium repens and Neptunia amphexicaulis, but in light of the evidence put forth by Broyer et al. (1972b) regarding ionic interactions of SeO$_3^{2-}$, one cannot rule out this criticism of their work.

Although research with higher plants has left many unanswered questions, studies on algae have given more conclusive results. In two phytoplankton species, the obligate growth requirements for Se are well established (Lindstrom and Rodhe, 1978, Wehr and Brown 1985), and in other species stimulatory effects of Se on growth are reported. This thesis examines the selenium nutrition of a marine alga, but I will forgo further discussion of this aspect of Se biology until Chapter 5.
Chemical interactions of selenium

Chemical interactions of Se with other ions in the growth medium, and at the membrane level, may act to alter the availability of either ion to the plant. Through this mechanism the beneficial effects of Se may not be manifested in the utilization of selenium per se, but rather selenium may alter the availability of the other ion(s) to the plant. The data of Broyer et al. (1972b) provide one example. They found that the amount of Mn in plant tissue varies according to the amount of Se supplied in the culture medium. Since SeO$_3^{2-}$ is known to adsorb to Fe oxyhydroxides (Harrison and Berkheiser 1982), its adsorption to Mn oxides may reduce the availability of Mn to the plant and result in low Mn levels in plant tissue. Similarly, if chemical interactions of Se with a toxic element reduces the toxicity of this element to the organism in question, then the beneficial effects of Se may incorrectly be viewed as those attributed to essential elements. The results of Gotsis (1982) demonstrate that Se/Hg and Se/Cu antagonisms result in an elimination of the inhibitory effects seen when these elements are supplied individually. Whether this interaction occurs in solution or intracellularly is not apparent, although high Se levels in the tissues of animals protects against the toxic effects of heavy metals (Diplock 1976). By contrast to these indirect effects of Se, a number of specific Se-containing macromolecules are known in a diverse group of organisms.
Specific selenium-containing macromolecules

Prior to the discovery that Se was an essential element for animals, Pinsent (1954) provided the first example of a specific catalytic role for Se. She demonstrated that trace amounts of Se, in addition to Mo and Fe, were necessary for expression of active formate dehydrogenase activity in *Escherichia coli*. But it wasn't until 23 years later that Se was shown to be an integral component of this enzyme in *E. coli* (Enoch and Lester 1975). Since then, Se-dependent formate dehydrogenases have been described in several anaerobic bacteria (Jones *et al.* 1979). Other selenoproteins in bacteria are well characterized. The best known is glycine reductase of *Clostridium stricklandii* (Turner and Stadtman 1973). Nicotinic acid hydroxylase of *Clostridium barkeri* (Imhoff and Andresen 1979) and xanthine dehydrogenases from *Clostridium acidiurici* and *C. cylindrosorum* show enhanced activity when selenite is added to the medium (Wagner and Andresen 1979). In *C. barkeri*, Dilworth (1980) found that $^{75}$Se and nicotinic acid hydroxylase activity co-migrate during electrophoresis. Finally, thiolase isolated from *Clostridium kluyveri* also appears to be a selenoprotein (Hartmanis 1980).

Not only are selenoproteins common in bacteria, specific selenonucleosides of tRNA's from *Clostridium stricklandii* (Chen and Stadtman 1980), *Methanococcus vannielii* (Ching *et al.* 1984), *Escherichia coli* (Wittwer 1983) and cultured mouse leukemia cells (Ching 1984) have been identified. In *Clostridium stricklandii*, one Se-containing tRNA is the major
glutamate-accepting species among all the tRNA's, and its aminoacylation activity is dependent upon the presence of Se (Ching and Stadtman 1982).

The number of selenoproteins identified in mammals has increased dramatically since the demonstration that glutathione peroxidase is a selenoenzyme (Rotruck et al. 1972, 1973, Flohe et al. 1973). Currently, as many as 11 unique selenoproteins have been detected in mice mammary epithelial cells (Danielson and Medina 1986), and including different isoelectric forms detected by 2-D SDS-PAGE, a total of 25 polypeptides contain Se stably associated with the protein in the form of selenocysteine. The specific enzymatic or biochemical functions of these proteins is unknown.

Selenometabolites in plants

No specific Se-containing compounds have been isolated from higher plants, but the accumulation of selenometabolites has been well examined in the Se-indicator species. Nearly 80% of Se in *Astragalus* is hot-water extractable and is present in low-molecular weight compounds, which are for the most part Se analogs of the S-containing amino acids (Horn and Jones 1940, Trelease et al. 1960) In non-Se-indicator plants, such as *Atriplex* sp. and *Machaeranthera ramosa*, Se is accumulated in the form of $\text{SeO}_4^{2-}$ (Rosenfeld and Beath 1964); whereas, Peterson and Butler (1962) found in *Lolium perenne*, *Triticum vulgare*, *Trifolium repens* and *T. pratense* that greater than 60% of cellular Se was incorporated into protein.

Recent evidence of the enzyme glutathione peroxidase in
cultured plant cells (Drotar et al. 1985) and earlier reports of this enzyme in spinach chloroplasts (Flohe and Menzel 1971) and its possible existence in plant tissue (Neubert et al. 1962) have provided a new line of evidence to address the question of Se essentiality in plants. Although these results conflict with observations of Smith and Shrift (1979), it is apparent that a glutathione peroxidase does exist in some plants; whether it is a Se-dependent enzyme remains to be resolved.

**Glutathione peroxidase**

Oxygen radicals and related products are detoxified intracellulary by a number of enzymes including superoxide dismutase, catalase, and glutathione peroxidase. These radicals are able to initiate free radical chain reactions leading to organic and lipid hydroperoxide formation (Halliwell 1974, Tappel 1977, Burton and Ingold 1986). Production of radicals in cells may occur by a number of pathways: Superoxide, for example, is formed during oxidation of several reduced compounds, such as ferredoxin and hemoproteins; by oxidative enzymes, including xanthine oxidase; and by chloroplasts and mitochondria (Fridovich, 1984). Polyunsaturated fatty acids, components of cell membranes, are particularly susceptible to oxidation by peroxyradicals. Since cell membranes are essential for compartmentalizing biochemical processes and regulating the flow of materials into and out of the cell, destruction of
membrane integrity ultimately leads to cell death.

Glutathione peroxidase (GSH-Px) is an enzyme which catalyzes the reduction of a variety of hydroperoxides (Flohe et al. 1979), but reduced glutathione (GSH) is the only reductant with which it is active (Flohe 1976). This enzyme plays an important protective role in the cell removing injurious hydroperoxides. Two major types of GSH-Px have been found. In mammalian and avian tissues, GSH-Px (EC 1.11.1.9) is a selenoenzyme which is active with hydrogen peroxide and a variety of organic peroxides. A selenium-independent enzyme showing GSH-Px activity was isolated from rat tissue (Lawrence and Burk 1976), but similarities between this enzyme and glutathione-S-transferases (EC 2.5.1.18) indicate that they are probably the same enzyme (Prohaska 1980). The glutathione-S-transferases can be differentiated from true GSH-Px's by their inability to reduce $\text{H}_2\text{O}_2$ and their lack of Se. The molecular weight of glutathione peroxidase (EC 1.11.1.9) from a variety of sources ranges from 76,000 to 96,000 daltons (Stadtman 1980a). The enzyme is a tetramer composed of identical subunits (19,000 to 23,000 daltons) each containing one selenocysteinyI residue. In the literature, enzymes which produce oxidized glutathione in the presence of organic hydroperoxides are often erroneously referred to as GSH-Px, when they may be GSH-S-transferases. In this thesis, GSH-Px refers specifically to the selenoenzyme GSH-Px (EC 1.11.1.9), but enzymes showing "GSH-Px activity" may or may not be true GSH-Px; they may also be GSH-S-transferases.
CHAPTER 5. SELENIUM: AN ESSENTIAL ELEMENT FOR GROWTH OF THE COASTAL MARINE DIATOM THALASSIOSIRA PSEUDONANA

Background

The nutritional importance of selenium (Se) for algal growth is becoming increasingly apparent. Pioneering work by Pintner and Provasoli (1968) documented the stimulatory effects of Se on the growth of three marine Chrysochromulina spp. Since then, two other studies have shown, using axenic cultures, that Peridinium cinctum fa. westii (Lindstrom and Rodhe 1978) and Chrysochromulina breviturrita (Wehr and Brown 1985) have an absolute growth requirement for Se. Other investigators have demonstrated that Se additions to natural and artificial seawater media stimulates the growth of axenic phytoplankton (Wheeler et al. 1982), axenic macroalgae (Fries 1982), and xenic phytoplankton (v.Stoch 1980, Lindstrom 1983, 1985, Keller et al. 1984) cultures. In these reports, the algae were able to grow without Se enrichments, although the background concentrations of Se in the growth media were not known. To date, representatives from six classes of algae have been shown to have a requirement for Se which is essential for, or which markedly stimulates, growth.

The concentration of Se required to support growth of algae depends upon the chemical form of the element, and the requirement is species specific. In seawater, dissolved Se is present as selenite (SeO_3^{2-}, Se IV), selenate (SeO_4^{2-}, Se VI) and organic Se. In general, it appears that selenite is the more biologically active form of inorganic Se; however, the
growth promoting properties of organic Se compounds have received much less study. The importance of selenite in phytoplankton nutrition is further supported by the finding of Wrench and Measures (1982). They observed that decreases in Se IV, but not Se VI, concentration were inversely correlated with increases in phytoplankton biomass and particulate Se in a coastal seawater environment. Selenomethionine and selenocystine may be utilized by some phytoplankton, and at least three species grow as well on these organic forms of Se as on Na$_2$SeO$_3$ (Lindstrom 1983, Wehr and Brown 1985).

*Chrysochromulina breviturrita* has also been shown to utilize dimethyl selenide (DMSe) as a Se source (Wehr and Brown 1985). This ability may be relevant to Se cycling in aquatic systems, as DMSe and other volatile Se compounds can be produced by microbial assemblages in lake sediments (Chau *et al.*, 1976).

Current attention to Se toxicity in organisms from marine and freshwater environments is warranted. Increases in anthropogenic inputs of Se, from acid rain and agricultural run-off, to aquatic systems is well documented (Gissel-Nielsen and Gissel-Nielsen 1973, Andren *et al.* 1975, Furr *et al.* 1977, Parekh and Husain 1981) and publicized (Marshall 1985, 1986). However, little information is available on the requirements of Se by marine phytoplankton or on the effects of elevated concentrations of this element on algal growth.

The present study documents the essential requirement of Se for growth of the coastal marine diatom *Thalassiosira pseudonana* (clone 3H). *T. pseudonana* could not be maintained in artificial seawater medium without the addition of
nanomolar quantities of Se. Se-limitation and Se-starvation in *T. pseudonana* resulted in a reduction in cell growth rate and subsequent cessation of cell division and a pronounced increase in cell size.

**Materials and Methods**

**Algal culture**

*Thalassiosira pseudonana* (clone 3H) was obtained from the Northeast Pacific Culture Collection (N.E.P.C.C. #58), Department of Oceanography, University of British Columbia, where it was maintained on nutrient enriched natural seawater. Axenic cultures of *T. pseudonana* were established by repeated transfers on agar medium (1% agar, w/v), and the absence of bacteria was verified by acridine orange epifluorescent microscopy (Hobbie *et al.* 1977). Stained samples were viewed under a Zeiss model D-7802 epifluorescent microscope. Axenic cultures were used in initial experiments (Figs. 31 and 32) to confirm that the stimulatory effects of Se occurred in the absence of bacteria. Thereafter, unialgal cultures were used, but precautions were taken to minimize bacterial contamination. Selenium was an essential element for growth of *Thalassiosira pseudonana* in xenic and axenic culture.

**Culture medium and flasks**

Cultures of *T. pseudonana* were grown in filter-sterilized (0.22 um Millipore filters) nutrient enriched artificial seawater based on ESAW (Harrison *et al.* 1980). Axenic
cultures were grown in autoclaved ESAW with filter-sterilized vitamins and NaHCO$_3$ added after the medium had cooled. Reagent grade chemicals were used throughout, and nutrient enrichment solutions were prepared in deionized distilled water (DDW). In some experiments, the seawater salts of ESAW were purified of cationic transition metals by the use of Chelex 100, following the procedure of Morel et al. (1979). Modifications to ESAW included replacing FeNH$_4$(SO$_4$)$_2$·6H$_2$O by an equimolar concentration of FeCl$_3$·6H$_2$O, and by adding all the Fe to a Na$_2$EDTA solution to give an EDTA:Fe molar ratio of 1.6. The remaining Na$_2$EDTA added to ESAW was included with the trace metal stock solution, and 0.0126 g·l$^{-1}$ Na$_2$MoO$_4$ and 0.0059 g·l$^{-1}$ NiCl$_2$·6H$_2$O were also added. Na$_2$glycerophosphate was replaced with an equimolar concentration of Na$_2$HPO$_4$. Na$_2$SiO$_3$·9H$_2$O was prepared and added as described by Suttle et al. (1986). Se was added to ESAW, when required, as aqueous solutions of Na$_2$SeO$_3$ or Na$_2$SeO$_4$, and these solutions were freshly prepared for each experiment.

Glassware and polycarbonate flasks were used for culturing algae and storing ESAW. They were soaked overnight in 10% (v/v) HCl and were autoclaved before use. Alternatively, glassware were soaked in 1M HNO$_3$ and rinsed thoroughly in DDW, following 24 h of soaking in DDW. Pyrex® and Kimax® 50-ml screw-capped tubes with teflon liners were used for culturing T. pseudonana, and they were treated similarly except they were filled with fresh DDW and autoclaved twice prior to use. The tubes were then
reautoclaved without DDW and used immediately.

Culture conditions and growth measurements

Cultures were continuously illuminated from two sides by Vita-Lite® UHO and Sylvania® VHO daylight fluorescent tubes. The light was filtered through a 3 mm thick sheet of blue Plexiglas® (No. 2069, Rohm and Hass), and the irradiance, measured at the surface of the culture vessels, was 120 uE·m⁻²·s⁻¹. Growth temperature was maintained at 18°C in a temperature regulated water bath. All experiments were conducted in batch cultures. In one experiment, a subsample from a continuous culture was used as an inoculum source for the batch cultures.

Cell growth was monitored by in vivo chlorophyll a fluorescence measured by a Turner Designs model 10 fluorometer and by cell counts on a Coulter Counter® model TA II. Average cell volumes were computed from the cell distribution in the various channels of the Coulter Counter®, using a 70 um aperture sample tube. The Coulter Counter® was calibrated with microspheres of 5.07 um in diameter. In experiments designed to examine the effects of Na₂SeO₃ and Na₂SeO₄ concentration on the growth rate of T. pseudonana, cells were preconditioned in the medium for at least 10 cell doublings.

Water mounts of T. pseudonana were prepared for light microscopy and were examined with a Zeiss Photomicroscope II light microscope using Nomarski interference optics and brightfield illumination.
Results

Selenium requirement and recovery

*Thalassiosira pseudonana* was inoculated from nutrient enriched natural seawater (ESNW) into ESAW containing no added Se (-Se) or ESAW enriched with $10^{-9} \text{ M Na}_2\text{SeO}_3$ (+Se) (Fig. 30). The cultures were serially diluted to maintain the cells in exponential growth. The inoculum was diluted 1000-fold with fresh medium to minimize any carry over of dissolved Se from the ESNW. The initial cell concentration in each culture was ca. 3400 cells·ml$^{-1}$. Exponential growth rates of *T. pseudonana* were identical for the -Se and +Se cultures during the first transfer. However, the cells in the -Se ESAW had a reduced growth rate and fluorescence yield by comparison to the +Se culture for the second transfer. When *T. pseudonana* was transferred for the third time into -Se ESAW the cells failed to grow. The +Se culture maintained the same growth rate over the three transfers. Cell counts of *T. pseudonana* verified that fluorescence was accurately representing growth of this alga and these have been included, for comparison, with the fluorescence data (Fig. 30).

Further evidence for a Se requirement by *T. pseudonana* is presented in Figure 31. The addition of $10^{-10} \text{ M Na}_2\text{SeO}_3$ to a Se-deplete stationary phase culture caused a resumption in growth of the cells. Cell counts and microscopic observation confirmed that the increase in *in vivo* fluorescence was attributed to an increase in cell numbers and was not just an increase in chlorophyll per cell or a change in the
Fig. 30. Growth of *T. pseudonana* over three successive transfers in artificial seawater (ESAW) in the absence of Se (O) and in ESAW supplemented with $10^{-9}$ M $Na_2SeO_3$ (●). The growth curves for the first, second and third transfers begin at 0, 114 and 212 h, respectively. When the cells were in late exponential growth, a portion of the culture was transferred into fresh medium. Growth of the culture was then followed by monitoring *in vivo* chlorophyll *a* fluorescence (solid lines). The arrows indicate when the cultures were transferred. During the third transfer, cell numbers (dashed line) were also determined in the Se-replete culture.
Fig. 31. Early stationary phase *T. pseudonana* was transferred from Se-deplete ESAW into two culture tubes containing the same medium. At 120 h (indicated by the arrow) one tube was spiked with $10^{-10}$ M Na$_2$SeO$_3$ (●), and the other tube served as the control (O).
photosynthetic efficiency.

**Morphological changes**

An ammonium (NH$_4^+$)-limited chemostat culture of *T. pseudonana* was grown at a dilution rate of 1.0 d$^{-1}$. The medium reservoir contained ESAW without added Se, and the culture started to wash out. At this time, aliquots of the chemostat culture were transferred to tubes enriched with 25 uM NH$_4^+$, with and without 10$^{-9}$ M Na$_2$SeO$_3$. Significantly greater growth rates were seen in the +Se cultures (mean value ± 1 SD) (1.49 ± 0.08 d$^{-1}$, n=4) compared to the -Se cultures (1.03 ± 0.07 d$^{-1}$, n=4). The Coulter Counter® cell size distribution indicated that the average cell volume of the -Se cultures increased two-fold over the duration of the experiment; the cells growing in ESAW +Se decreased in volume (Fig. 32). Microscopic examination verified that changes in cell volume were not attributed to cell clumping or to the formation of cell chains (see Discussion).

Cells of *T. pseudonana* starved for Se were greatly elongated by comparison to cells growing in Se-replete ESAW (Fig. 33a, c). Cell length along the pervalvar axis was as great as 60 um in the -Se grown cultures; experimental cultures grown in the presence of 10$^{-9}$ M Na$_2$SeO$_3$ contained cells ca. 4-5 um in length (Fig. 33a). There was no difference in the valve diameter between the -Se and +Se grown cells (Fig. 33b, c). Other morphological changes seen in some Se-starved cells included bent and twisted cells and cells with cytoplasmic protrusions from the girdle region. To
Fig. 32. Average cell volume of *T. pseudonana*, following transfer of cells from an ammonium-limited chemostat culture growing on Se-deplete ESAW into Se-deplete (O) or Se-replete (●) ESAW. The initial cell volume was determined in duplicate, and the range of the values was less than the width of the symbol. The experiment was done in triplicate, but only the final cell volumes of the other two cultures are given.
Fig. 33. Photomicrographs of vegetative cells of *T. pseudonana* grown in ESAW enriched with $10^{-9}$ M Na$_2$SeO$_3$; (a) girdle view and (b) valve view and in Se-deplete ESAW; (c) girdle view. Scale bar = 5 um.
eliminate the possibility of trace metal toxicity in the cultures, excess chelator was added to the medium. The addition of 100 μM Na₂EDTA to ESAW -Se failed to alleviate the growth inhibition seen in the -Se cultures of *T. pseudonana*. Moreover, these cells underwent the same morphological changes seen in the Se-deplete cultures containing normal ESAW concentrations of EDTA (20 μM). Similar results were obtained with *T. pseudonana* grown in Se-deplete chelexed ESAW. Also, increasing the concentration of trace metals added to ESAW by 5 times yielded results which were identical to those obtained by growing cells in ESAW -Se with normal trace metal concentrations. Eleven trace elements were tested for their ability to support growth of *T. pseudonana* in the absence of Se (Table XXI). The results demonstrated that Se was the only element which stimulated growth and that Na₂SeO₃, but not Na₂SeO₄, was effective at a concentration of 10⁻⁹ M.

*Growth on selenite and selenate*

Various concentrations of Na₂SeO₃ were tested to determine the lower limit of requirement and the upper limit of tolerance for this compound (Fig. 34). Excellent growth was obtained with Na₂SeO₃ concentrations of 10⁻⁹ to 10⁻⁶ M. An increased lag period was seen with 10⁻¹⁰ M Na₂SeO₃; however, the exponential growth rate was the same as that obtained with 10⁻⁹ to 10⁻⁶ M. *Thalassiosira pseudonana* grew at a much reduced growth rate in ESAW enriched with 10⁻¹¹ M Na₂SeO₃ and the lag period was ca. 175 h. There was considerably more variation between the duplicate cultures
Table XXI

Trace elements tested for their ability to support growth of *T. pseudonana* in Se-deplete artificial seawater (ESAW). The form of the element added is given, and $10^{-9}$ M was added in each case. Each treatment was performed in duplicate. No growth (-), good growth (+).

<table>
<thead>
<tr>
<th>Element</th>
<th>Growth Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al- $\text{Al}_2(\text{SO}_4)_3$</td>
<td>-</td>
</tr>
<tr>
<td>Ba- $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$</td>
<td>-</td>
</tr>
<tr>
<td>Cr- $\text{K}_2\text{CrO}_4$</td>
<td>-</td>
</tr>
<tr>
<td>Cu- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$</td>
<td>-</td>
</tr>
<tr>
<td>Li- $\text{LiCl}$</td>
<td>-</td>
</tr>
<tr>
<td>Ni- $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$</td>
<td>-</td>
</tr>
<tr>
<td>Rb- $\text{RbCl}$</td>
<td>-</td>
</tr>
<tr>
<td>S- $\text{Na}_2\text{SO}_3$</td>
<td>-</td>
</tr>
<tr>
<td>Se- $\text{Na}_2\text{SeO}_3$</td>
<td>+</td>
</tr>
<tr>
<td>Se- $\text{Na}_2\text{SeO}_4$</td>
<td>-</td>
</tr>
<tr>
<td>Te- $\text{K}_2\text{TeO}_3$</td>
<td>-</td>
</tr>
<tr>
<td>V- $\text{NH}_4\text{VO}_3$</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 34. Growth of *T. pseudonana* as a function of Na$_2$SeO$_3$ concentration. An aliquot of cells from an early stationary phase culture growing in Se-deplete ESAW was added to ESAW enriched with; (A) $10^{-7}$, $10^{-8}$, $10^{-9}$ (O), $10^{-10}$ (●), (B) $10^{-11}$ (●) or $10^{-15}$ M (O) Na$_2$SeO$_3$. Error bars indicate the range of duplicates and where absent the range was less than the width of the symbol.
growing on $10^{-11}$ M Na$_2$SeO$_3$ than there was at the higher concentrations of Na$_2$SeO$_3$. *Thalassiosira pseudonana* failed to grow when the Na$_2$SeO$_3$ concentration was $10^{-15}$ M. Cell growth resumed following a lag time of 400 h, in ESAW containing $10^{-12}$ M Na$_2$SeO$_3$ (Fig. 35).

Selenium was supplied as selenate in the form of Na$_2$SeO$_4$ and the growth rate of *T. pseudonana* was measured as a function of Na$_2$SeO$_4$ concentration (Fig. 36). *Thalassiosira pseudonana* failed to grow in ESAW supplemented with $10^{-9}$ and $10^{-8}$ M Na$_2$SeO$_4$. The maximum growth rate of $1.45 \text{ d}^{-1}$, observed in cultures growing with $10^{-4}$ M Na$_2$SeO$_4$, was less than the maximum growth rate seen in cultures growing on Na$_2$SeO$_3$. Concentrations of $10^{-3}$ and $10^{-2}$ M Na$_2$SeO$_4$ were inhibitory to growth. As a comparison, growth rate of *T. pseudonana* over a range of Na$_2$SeO$_3$ concentrations is also included (Fig. 36). *Thalassiosira pseudonana* grew well at $10^{-3}$ and $10^{-2}$ M Na$_2$SeO$_3$, and the growth rates were only slightly less for cells exposed to $10^{-3}$ M compared with the maximum growth rate of 1.7 d$^{-1}$. A crystalline precipitate formed on the bottom of the culture tubes containing ESAW enriched with $10^{-2}$ Na$_2$SeO$_3$. While the composition of this material is unknown, it may have been a Ca$^{2+}$ and/or Mg$^{2+}$ salt of SeO$_3^{2-}$. If this is true then the concentration of dissolved SeO$_3^{2-}$ in these tubes was less than $10^{-2}$ M. To test for possible Se-phosphate interactions, the growth rate of *T. pseudonana* was measured in the Se-deplete and Se-replete ($10^{-9}$ M Na$_2$SeO$_3$) ESAW containing three different dissolved phosphorus (Na$_2$HPO$_4$) concentrations. *Thalassiosira pseudonana* failed to grow at any of the PO$_4^{3-}$
Fig. 35. Duplicate cultures tubes enriched with $10^{-12}$ M Na$_2$SeO$_3$ were inoculated with Se-depleted T. pseudonana. Growth was monitored to examine long term effects of exposure to low concentrations of Se.
Fig. 36. Maximum exponential growth rate as a function of Na₂SeO₃ (●) and Na₂SeO₄ (O) concentration. Growth rates are the mean of four replicates and error bars indicate ±1 SD.
concentrations (100, 21 and 0.5 μM) in Se-deplete ESAW. In ESAW enriched with $10^{-9}$ M Na$_2$SeO$_3$, *T. pseudonana* grew at the maximum growth rate of 1.7 d$^{-1}$ at all PO$_4^{3-}$ concentrations. In ESAW, phosphate is added at 20 μM and this was used in all other experiments.

Discussion

*Selemium-limitation and recovery*

In ESAW unenriched with Se, *T. pseudonana* was able to undergo ca. 9 cell divisions prior to Se starvation and the cessation of growth (Fig. 30). This growth should represent a reduction of intracellular Se concentration to the minimum cell quota and the time required for cells to express Se limitation is a function of their Se preconditioning. The initial inoculum for these experiments had been maintained on natural seawater, collected from English Bay, Vancouver, which was enriched with ES nutrient solutions (Harrison *et al.* 1980) or the inocula were from cultures growing on ESAW supplemented with $10^{-9}$ M Na$_2$SeO$_3$. The background concentration of Se in ESAW is unknown, however, the growth data (Fig. 33) indicate that it must be less than $10^{-11}$ M.

At times during the experiments, I encountered problems with Se contamination of the Se-deplete ESAW. This appeared to result, at least in part, from an inability to remove Se from glassware used for culturing the algae. A more rigorous procedure was adopted where glassware were soaked in 1 N HCl for 24 h and were then autoclaved twice containing fresh DDW.
This was a successful means of cleaning glassware. I also tested HNO₃, a strong oxidizing acid, in an effort to convert the Se present in the glassware to the more soluble species of SeO₄⁻², but this proved no more effective than HCl. Autoclaved Se-deplete ESAW gave erratic results, and I noticed that *T. pseudonana* was often able to grow in this medium without Se enrichments. I suspect that the autoclave was contaminating the Se-deplete ESAW with sufficient Se to allow the alga to grow. In filter-sterilized ESAW, *T. pseudonana* was never able to grow without Se enrichments.

Se additions, in the form of Na₂SeO₃, have been observed to stimulate the growth of *Peridinium cinctum f*a. *westii* at concentrations as low as 2.5 × 10⁻¹³ M (Lindstrom and Rodhe 1978). Other algal species require considerably higher concentrations of Na₂SeO₃, such as *Platymonas* spp. which grew better than control cultures only when concentrations greater than 1.3 × 10⁻⁶ M were added to the medium (Wheeler *et al.* 1982). *Thalassiosira pseudonana* grew at its maximum growth rate (1.7 d⁻¹) in ESAW containing 10⁻¹⁰ M Na₂SeO₃, although there was a slightly increased lag period relative to those cells exposed to higher concentrations of Na₂SeO₃. When 10⁻¹⁰ M Na₂SeO₃ was added to a Se starved culture (Fig. 32) there was a greater lag period than that observed with the preconditioned cells, and the growth rate was reduced to 0.4 d⁻¹. This further emphasizes the importance of the cells' preconditioning to Se. It also demonstrates that cell growth can resume once the limiting nutrient (Se) is resupplied to the cells. The use of fluorescence is too insensitive to give
an accurate measure of the recovery time of the cells from Se-starvation. Roughly 100 h elapsed before there was any evidence of cell growth. This recovery time is an upper limit, and in the future the use of $^{14}$CO$_3^-$ may be one method to increase resolution.

The morphological changes seen in the Se-limited and starved *T. pseudonana* were not unlike those seen in phytoplankton subjected to high concentrations of toxic metals or silica deficiency (Thomas *et al.* 1980, Jahnke and Baumann 1983). However, the same growth inhibition and abnormal cells of *T. pseudonana* were observed in Se-deplete chelexed ESAW and Se-deplete ESAW supplemented with 100 uM Na$_2$EDTA. Only in ESAW containing Se could cells grow. It was demonstrated that other trace elements, perhaps present as contaminants in the Se stock solutions or made more available by the addition of Se, could not alleviate the growth requirement for Se. In these experiments, there was no evidence for PO$_4^{3-}$ toxicity in *T. pseudonana* grown in Se-replete ESAW enriched with 20 and 0.5 uM PO$_4^{3-}$. In the absence of Se, phosphate has been observed to be toxic to some higher plants (Broyer *et al.* 1972b). The fact that *T. pseudonana* was unable to grow in medium lacking Se, regardless of the PO$_4^{3-}$ concentration, further indicates that growth inhibition was a result of Se deficiency and not PO$_4^{3-}$ toxicity.

Growth on selenite compared with selenate

Overall, Na$_2$SeO$_3$ supported better growth of *T. pseudonana*
than Na$_2$SeO$_4$. The minimum concentration of Na$_2$SeO$_4$ required for growth of *T. pseudonana* was 10,000 times greater than Na$_2$SeO$_3$. The smallest addition of Na$_2$SeO$_3$ which supported good growth of *T. pseudonana* was 10$^{-10}$ M. This concentration may be an underestimate because the background concentration of Se in ESAW is unknown. Moreover, it has been shown that SeO$_3^{2-}$ and SeO$_4^{2-}$ can adsorb to mineral surfaces such as FeOOH (Hingston et al. 1968, Harrison and Berkheiser 1982) which are present in seawater. The observations of Peterson and Butler (1962) confirm that this can occur; this may have the effect of reducing the bioavailability of Se. Uncertainty in the amount of biologically available Se suggests that future work necessitates the development of a chemically-defined culture medium where the activities of the various Se forms may be regulated and determined. Such an approach has already been taken by Morel et al. (1979) for cationic trace metal speciation in artificial seawater.

In some algae, Na$_2$SeO$_4$ is as effectively utilized as Na$_2$SeO$_3$ (Fries 1982, Lindstrom 1985, Wehr and Brown 1985) and when these Se compounds are supplied at the same concentration, algal growth rate and final biomass yield are identical. This was not observed for *T. pseudonana*, although 10$^{-5}$ M Na$_2$SeO$_4$ was not tested for growth (Na$_2$SeO$_3$ data not shown). Higher concentrations of Na$_2$SeO$_4$ were required to support growth of *T. pseudonana*. This is consistent with the results of Lindstrom (1983). He found that long adaptation times were required for algal growth on Na$_2$SeO$_4$, relative to Na$_2$SeO$_3$, but I saw no evidence for this. Experiments were not
designed to determine long term adaptation times of *T. pseudonana* exposed to low concentrations of SeO$_3^{2-}$. However, it was noticed for one experiment that growth resumed after 400 h of exposure to $10^{-12}$ M Na$_2$SeO$_3$. At this concentration, I did not test whether repeated subculturing would reduce the long lag period for growth, but when cells which had been adapted to $10^{-11}$ and $10^{-10}$ M Na$_2$SeO$_3$ for at least 10 cell divisions were transferred to new medium, the lag periods were still observed. This does not preclude the possibility of cellular adaptation over still longer periods of time, and that this can occur in cells grown in increasing concentrations of Na$_2$SeO$_4$ (Kumar 1964) indicates that adaptation to low concentrations may also occur.

Selenite has been shown to be more inhibitory to growth of cyanobacteria than equivalent concentrations of Na$_2$SeO$_4$ and organic Se (Kumar and Prakash 1971). Sielicki and Burnham (1973) have also found that concentrations of Na$_2$SeO$_3$ of $10^{-5}$ M and greater were toxic to *Phormidium luridum* var. *olivacea*. This alga reduced Na$_2$SeO$_3$ to elemental Se at high Na$_2$SeO$_3$ concentrations. *Thalassiosira pseudonana* is tolerant of Na$_2$SeO$_3$ concentrations greater than $10^{-3}$ M and there is only a slight but significant ($p < 0.05$) reduction in cell growth rate. Wehr and Brown (1985) showed a reduction in growth and yield of *Chrysochromulina breviturrita* when Na$_2$SeO$_3$ concentrations were greater than $10^{-4}$ M. In other organisms, Na$_2$SeO$_4$ is the more toxic form of inorganic Se (Brown and Smith 1979). This appears to be the case in *T. pseudonana*,
and \( \text{Na}_2\text{SeO}_4 \) completely inhibited cell growth when supplied at \( 10^{-3} \) and \( 10^{-2} \) M. Even when cells which had been maintained for greater than 20 generations in ESAW supplemented with \( 10^{-4} \) M \( \text{Na}_2\text{SeO}_4 \) were used as an inoculum for the higher Se concentrations, growth was inhibited. The toxic effects of Se are believed to arise as the result of the indiscriminant incorporation of Se into sulfur (S) containing macromolecules through the S assimilatory pathways (Stadtman 1979). It is not surprising, therefore, that \( \text{SeO}_4^{2-} \), which is chemically more similar to \( \text{SO}_4^{2-} \) than is \( \text{SeO}_3^{2-} \), is the more potent of these two Se forms. Shrift (1954) pointed out that \( \text{SO}_4^{2-} \) and \( \text{SeO}_4^{2-} \) compete for a common porter in \textit{Chlorella}, and Brown and Shrift (1980a) have also demonstrated this to be true in a bacterium. Additionally, it appears that uptake of \( \text{SeO}_3^{2-} \) is distinct from \( \text{SeO}_4^{2-} \) and \( \text{SO}_4^{2-} \) uptake in bacteria (Brown and Shrift 1980a, Hudman and Glenn, 1984, 1985). In \textit{T. pseudonana}, the growth rate data, in response to different concentrations of \( \text{Na}_2\text{SeO}_4 \) and \( \text{Na}_2\text{SeO}_3 \), demonstrate that the uptake and/or assimilation of these Se compounds is dissimilar. Nevertheless, the fact that both Se forms can support the growth of \textit{T. pseudonana} indicates that there are some similarities. The lack of \( \text{Na}_2\text{SeO}_3 \) toxicity suggests that \textit{T. pseudonana} is capable of regulating the influx or efflux of this ion or is able to detoxify it intracellularly. Further work is necessary to resolve the differences between the cellular metabolism of these Se compounds in algae.
Morphological changes

Cell volumes of *T. pseudonana* based on Coulter Counter® data are underestimates, since the orientation of the cells passing through the aperture will effect the measurement. Microscopic observations demonstrated that Se-depleted cells were up to 10 times the length of the Se-replete cells, and a similar increase would also be expected in cell volume. Roughly 90% of the cells in the Se-deplete ESAW became elongated, but this depended on the stage of the growth cycle. Early evidence of Se limitation was observed by an increase in the number of cells which failed to separate following cytokinesis. *Thalassiosira pseudonana* is a unicellular diatom when grown under nutrient sufficiency. As the growth rate of *T. pseudonana* became reduced in the ESAW -Se, cells of 2-4 times the size of the +Se cells were seen in chains or as solitary cells. Finally, the cells became more elongated and some of the cells became twisted and bent.

Microscopic observation of cells stained with acridine orange showed that the elongate cells from the -Se culture were, for the most part, uninucleate and no cells contained multiple nuclei. Although binucleate cells were observed they were no more frequent than in the +Se culture. Thus, Se starvation prevents nuclear division and cytokinesis; however, cells are still able to increase in size in spite of the blockage of these events.

Doucette *et al.* (1987) have described the morphological changes associated with Se deficiency in more detail. It is
interesting that previous research examining Se nutrition has reported increases in algal cell size following exposure of cells to high concentrations of Se (Shrift 1954), but no such changes were evident in T. pseudonana. By contrast, Lindstrom (1983) has reported an increase in the cell diameter of Peridinium cinctum starved for Se. Although his results were less dramatic than the results with T. pseudonana, it is tempting to speculate that Se may play a similar role in these dinoflagellates.

Ecological considerations

By comparison with natural seawater concentrations of Se, the minimum amount of Na$_2$SeO$_3$ required to support growth of T. pseudonana is very similar to the concentration measured in natural seawater. In surface seawater of the Pacific and Indian Oceans, the concentration of Na$_2$SeO$_3$ is ca. 5·10$^{-11}$ M and concentrations in the Atlantic are 30-40% lower (Measures et al. 1983). Comparable results have been obtained by Cutter and Bruland (1984), and many of their measurements were at or below the limit of detection (10$^{-11}$ mol·Kg$^{-1}$). It is possible that in nature, growth of T. pseudonana could be limited by this concentration of Na$_2$SeO$_3$. There is some independent confirmation of this proposal. Wehr and Brown (1985) demonstrated that Se can limit growth of Chrysochromulina breviturrita in situ, and Keller et al. (1984) have found that Se enrichments to Sargasso seawater are necessary for the maintenance of marine ultraplankton clones isolated from oligotrophic oceanic environments. Although the concentration
of Na$_2$SeO$_4$ in seawater is greater than Na$_2$SeO$_3$ (Measures et al. 1980, 1983, Cutter and Bruland 1984), it is present in too low a concentration to be utilized by T. pseudonana. In coastal seawater, the total Se concentration is greater than in oceanic water, but values are still very low and they are more variable (0.38-1.8·10$^{-9}$ M) (Schultz and Turekian 1965, Sugimura et al. 1976, Measures and Burton 1980, Cutter 1982, Takayanagi and Wong 1984). The concentration of total Se in oligotrophic oceanic surface seawater averages 5·10$^{-10}$ mol·Kg$^{-1}$, and 80% of this is dissolved organic selenide (Cutter and Bruland 1984); at least a portion of the selenide is believed to be in the form of amino acids and polypeptides. Phytoplankton are capable of utilizing some forms of organic Se and this large pool of organic Se in seawater may be directly important in phytoplankton nutrition. However, as a note of caution, it was reported that a variable portion of the total dissolved Se in lake water is unavailable for phytoplankton growth (Lindstrom 1980). The bioavailability of dissolved forms of Se in seawater has not been examined.

The role of selenium

The biochemical basis for the requirement of Se in algae remains unknown. Naturally occurring selenoproteins have been identified in bacteria (reviewed by Stadtman 1980a, b) and the essential requirement of Se in mammals and birds is attributed to the Se-containing enzyme glutathione peroxidase. In higher plants, demonstration of an obligate Se requirement remains
equivocal. In some higher plants, it is apparent that Se is an important cellular constituent. Further experimentation is required to establish whether Se is essential for growth of all higher plants.

Enhanced glutathione peroxidation by cell extracts of *Dunaliella tertiolecta* and *Porphyridium cruentum* was evident when these two algae were cultured in medium containing Na$_2$SeO$_3$ (Gennity *et al.* 1985). However, it was concluded that the selenoenzyme glutathione peroxidase was absent and that hydroperoxide dependent oxidation of glutathione was non-enzymatic in nature. In *Euglena gracilis*, it has been shown that there exists two types of glutathione peroxidases, one of which is a Se-independent form of the enzyme (Overbaugh and Fall 1982, 1985).

Thus far I am unable to ascribe a specific metabolic role for Se in *T. pseudonana*. It is evident from these data, and it is further corroborated by results of Doucette *et al.* (1987), that Se is an indispensable trace element for the growth of *T. pseudonana*.

The development of ultra-clean methods for the culture of algae, through the use of chemically defined media prepared with ultrapure water and chemicals, has increased our knowledge of the nutritional requirements of these important primary producers. It has also made culturing of some phytoplankton more difficult. Previously, in some instances we have relied on contaminating sources of known and unknown essential elements in artificial seawater to fulfill the growth requirements of many phytoplankton (e.g. Oliveira and
Antia 1984). However, with the use of ultra-clean artificial seawater it may now become increasingly difficult to culture successfully some of the more difficult-to-grow phytoplankton until we have elucidated other essential growth requirements of these organisms.

Summary

An obligate requirement for selenium is demonstrated in axenic culture of the coastal marine diatom *Thalassiosira pseudonana* (clone 3H) (Hustedt) Hasle and Heimdal grown in artificial seawater medium. Selenium deficiency was characterized by a reduction in growth rate and eventually by a cessation in cell division. The addition of $10^{-10}$ M Na$_2$SeO$_3$ to nutrient enriched artificial seawater resulted in excellent growth of *T. pseudonana*. Only a slight inhibition of growth occurred with Na$_2$SeO$_3$ concentrations of $10^{-3}$ and $10^{-2}$ M. By contrast, Na$_2$SeO$_4$ failed to support growth of *T. pseudonana* when supplied at concentrations less than $10^{-7}$ M, and the growth rate at this concentration was only one quarter of the maximum growth rate. The addition of $10^{-3}$ and $10^{-2}$ M Na$_2$SeO$_4$ to the culture medium was toxic and cell growth was completely inhibited. Eleven trace elements were tested for their ability to replace the selenium requirement by this alga and all were without effect.

In selenium-deficient and selenium-starved cultures of *T. pseudonana*, cell volume increased as much as 10-fold as a result of an increase in cell length (along the pervalvar
axis), but cell width was constant. It is concluded that selenium is an indispensable element for the growth of *T. pseudonana*; it should be included as a nutrient enrichment to artificial seawater medium when culturing this alga.
CHAPTER 6. SPECIFIC SELENIUM-CONTAINING POLYPEPTIDES IN THE MARINE DIATOM THALASSIOSIRA PSEUDONANA

Background

The biochemical basis for the obligate growth requirement for selenium (Se) in some marine and fresh water phytoplankton is unknown. Although a number of selenoproteins are characterized in animals and prokaryotic heterotrophs, no specific Se-containing macromolecules have been isolated from photosynthetic organisms. In the preceding chapter, it was demonstrated that growth of the marine diatom Thalassiosira pseudonana was dependent upon Se. This requirement was specific for Se; no other elements were able to substitute for this growth requirement.

To date, the only investigations of Se-containing macromolecules in algae have examined species which have not been shown to have an obligate Se requirement for growth. In two phytoplankton species, Tetraselmis tetratele and Dunaliella minuta, Wrench (1978) observed that ca. 55% of $^{75}$Se associated with the cells was protein bound. A fraction of this was present as hydrogen selenide, and the remaining Se was in the form of selenoamino acids. Gennity et al. (1984) found that Se was associated with lipid in Dunaliella primolaeca and Porphyridium cruentum, when these algae were cultured in the presence of high concentrations of selenite (0.13 mM). However, they found no evidence that Se was incorporated into the covalent structure of any lipids.

Ultrastructural alterations to the membranes of Se-
deficient *T. pseudonana* were similar to changes seen in Se-deficient mammalian tissue (Doucette *et al.* 1987). In mammals, Se is known to be required for the selenoenzyme glutathione peroxidase (Rotruck *et al.* 1972, 1973, Flohe *et al.* 1973), and Se-deficiency results in decreased levels of this enzyme (Chow and Tappel 1974). In the absence of Se, chloroplast thylakoids of *T. pseudonana* were disrupted and in some instances formed balloon-like structures. Similar observations were made in spinach chloroplasts, which were induced to swell in high light (Murakami and Nobel 1967). The results of Murakami and Nobel (1967) were attributed to lipid peroxidation and the accumulation of lipid peroxides in the membranes. The lipid peroxides caused changes in the integrity of the membranes and thereby altered their physiochemical properties (Heath and Packer 1965, Murakami 1968). Glutathione induces swelling of mitochondria, and lipid peroxides accumulate concurrently (Hunter *et al.* 1959, Lehninger and Schneider 1959). Neubert *et al.* (1962) showed that glutathione peroxidase reversed this swelling by preventing the accumulation of lipid peroxides. In both mitochondria and chloroplasts, it appears that glutathione peroxidase acts to detoxify injurious lipid peroxides, or their precursors, and maintains membrane integrity (Flohe and Zimmerman 1970). On the basis of these observations, I hypothesized that the selenoenzyme glutathione peroxidase occurs in *T. pseudonana*. Although some enzymes showing glutathione peroxidase activity have been detected in algae, there is no evidence to indicate whether or not they are Se-
dependent enzymes.

The purpose of this chapter is two-fold: 1) to describe Se uptake and the distribution of Se within the biochemical constituents of *T. pseudonana*, and 2) to assay cell-free extracts for glutathione peroxidase activity.

**Materials and Methods**

**Culture conditions**

*Thalassiosira pseudonana* was grown in modified artificial seawater medium (ESAW) of Harrison et al. (1980) as described in Chapter 5. An axenic inoculum of *T. pseudonana* was transferred into 8 litres of autoclaved ESAW supplemented with $10^{-8}$ M Na$_2^{75}$SeO$_3$ (Amersham; 5.72 mCi·mg$^{-1}$; 0.21 GBq·mg$^{-1}$). Cells were grown at 17°C under a continuous irradiance of 145 uE·m$^{-2}·s^{-1}$ supplied by Vita-Lite® fluorescent bulbs, and growth was monitored by measuring *in vivo* chlorophyll *a* fluorescence. A Spencer Bright-Line® hemacytometer was used to count cells. The culture was continuously stirred by a teflon-coated magnetic stir bar. Incorporation of $^{75}$Se into the phytoplankton was determined by filtering 5 ml subsamples of culture through Whatman GF/C filters. The filters were rinsed with 5 ml of filter-sterilized seawater (0.2 um Millipore filter), and the amount of radioactivity retained by the filters was measured.

**Measurement of $^{75}$Se**

Selenium-75, a radioactive isotope of selenium, undergoes
electron capture decay with a half-life of 119.8 d. During radioactive decay, energy is released from $^{75}$Se atoms in the form of electromagnetic (gamma rays and X-rays) and corpuscular (internal-conversion-electron transitions and Auger electrons) radiation (NCRP Report No. 58 1985). Gamma counting is the conventional method for measuring $^{75}$Se, however, the isotope may also be detected using liquid scintillation techniques. Like beta particles, Auger electrons cause liquid fluorescent materials to emit light which can be detected by sensitive photo-multiplier tubes present in liquid scintillation counters. Since the use of this procedure to measure $^{75}$Se is not reported in the literature, the preliminary experiments used to validate this methodology are reported here. $^{125}$I, which also decays by electron capture, can be counted equally well using gamma or liquid scintillation counting (Helman and Ting 1973); $^{51}$Cr can be counted more efficiently by liquid scintillation counting than by gamma counting (Sheppard and Marlow 1971, Helman and Ting 1973).

The energy distribution of the Auger electrons emitted by $^{75}$Se is shown in Figure 37. The total number of cpm measured by liquid scintillation counting, with Aquasol II as scintillation fluor, was linearly related to the amount of $^{75}$Se added to each sample (Fig. 38). A quench curve was constructed using acetone as the quenching agent (Fig. 39). In all cases, quenching by phytoplankton samples was negligible, and counting efficiency was approximately 93%.
Fig. 37. Energy spectrum of $^{75}$Se as measured by liquid scintillation counting with Aquasol II as fluor.
Fig. 38. Relationship between the amount of $^{75}$Se measured by liquid scintillation counting (cpm) and the amount of $^{75}$Se added to scintillation vials containing Aquasol II.
Fig. 39. Quench curve for $^{75}$Se determined by using the H-Number method of quench monitoring. Acetone was used as the quenching agent. The equation of the line is $y = -3.253x + 1.104; r^2 = 0.997$. 
Collection of cells

When the culture was in late exponential growth phase, the cells were harvested by filtration onto Whatman GF/C filters. Cells were removed from the filters with a spatula, placed in Cryovials® (Simport Plastics Ltd., Quebec), and immediately frozen in liquid nitrogen. Phytoplankton cells were lyophilized in the centre-well of a freeze-drier (Virtis Company Inc., N.Y.). The lyophilized cells were stored under vacuum in a desiccator at -30°C. A similar procedure is described by Antia and Kripps (1978) for the preparation and storage of algal powders. They found that when algal samples were treated in this manner, the enzymes remained active with no loss in activity for periods up to one year.

During exponential growth, duplicate 25 ml subsamples of culture were collected for analysis of $^{75}$Se in various biochemical constituents.

Biochemical analysis

Biochemical constituents, determined during exponential growth, were separated into lipids ($\text{CHCl}_3$ fraction), proteins (TCA-insoluble fraction), polysaccharides and nucleic acids (TCA-soluble fraction) and low molecular weight compounds ($\text{KCl-MeOH-H}_2\text{O}$ fraction) according to the procedure of Terry et al. (1983). Since this procedure is discussed later, the experimental protocol is outlined here. Duplicate subsamples of culture were collected by filtration and rinsed with 5 ml Se-free filter-sterilized ESAW. The filters were immediately
placed in test tubes containing 3 ml cold CHCl$_3$-MeOH (2:1; v/v) and mixed on a vortex generator until the filter was disrupted. The contents of the test tube were refiltered, and the test tube and material on the filter were rinsed three times with 3 ml of CHCl$_3$-MeOH. The CHCl$_3$-MeOH fractions were combined and extracted with 3 ml 0.88% KCl solution and then re-extracted with 3 ml MeOH-H$_2$O (1:1; v/v). The KCl and MeOH-H$_2$O fractions were combined in a liquid scintillation vial and evaporated to dryness at room temperature in a fume hood. The CHCl$_3$ fraction was added to a scintillation vial and was treated similarly. The filters containing the CHCl$_3$-MeOH residual matter were added to a test tube containing 3 ml 5% TCA and placed in a boiling water bath for 30 min. The contents were filtered, and the test tube and filter were rinsed twice with 2 ml of cold 5% TCA. The TCA soluble fractions were combined in a scintillation vial, and the remaining filter was placed in a separate scintillation vial. Samples were counted by liquid scintillation techniques, and the counts were corrected for quenching. Samples were counted until the standard deviation of each count was 2%.

Lyophilized cells were suspended in ice cold buffer containing 65 mM Tris-HCl (pH 6.8) (Ultra Pure, Schwarz/Mann) and sonicated on ice for 1 min with a Branson Sonifier$^\text{®}$ cell disrupter (model 200) at 60% output and 47% duty cycle. It was determined by microscopic observation that no cells remained intact following this treatment. The sample was centrifuged at 180,000 g (TI-50 rotor, 40,000 rpm) for 2 h at 4°C in a Beckman$^\text{®}$ L8-M Ultracentrifuge to pellet the membranes
and cell walls. The supernatant was decanted and kept on ice. The membrane pellet was resuspended in an equal volume of Tris-HCl buffer. Protein was measured in each fraction by the Lowry method (Lowry et al. 1951) following the procedure of Markwell et al. (1978). Protein was co-precipitated with 50 ul of 25 mg.ml⁻¹ soluble yeast RNA (Polachek and Cabib 1981), and the precipitate pelleted by centrifugation at 25,000 rpm for 30 min. The activity of ⁷⁵Se was measured in the membrane and soluble protein and in the supernatant of each fraction.

**Gel filtration**

Supernatant from the crude cell extract was passed through a Sephadex G-150 column (1.6 × 45 cm) equilibrated with Tris-KCl buffer (65 mM Tris-HCl, 0.1 M KCl (Bio-Rad); pH 6.8) in a cold room (4°C). Void volume was determined by Blue Dextran 2000 (Pharmacia), and standards purchased from Sigma were used to calibrate the column. The standard proteins and their molecular weights (daltons; D) were; cytochrome c, 12,400; carbonic anhydrase, 29,000; bovine serum albumin, 66,000; and alcohol dehydrogenase, 150,000. Fractions (1.5 ml) were collected with a Gilson micro-fractionator, and protein was detected spectrophotometrically (280 nm) on a Bausch and Lomb Spectronic 2000. Activity of ⁷⁵Se in the crude cell extracts was measured in 50 ul samples of each fraction. Molecular weight was estimated from calibration plots of log molecular weight versus eluted volume/void volume (Cooper, 1977).
Electrophoresis

Membrane and soluble proteins were separated on SDS-polyacrylamide gels using the procedure of Laemmli (1970). All electrophoretic separations were performed in a cold room (4°C) with prechilled buffer and gels. The sample buffer consisted of 65 mM Tris-HCl (pH 6.9), 5 mM dithiothreitol, 1% lauryl sulfate (SDS) (w/v), and 10% glycerol. The running buffer consisted of 0.19 M glycine, 0.025 M Tris, and 0.1% SDS (w/v). Proteins were dissociated by heating in the presence of the buffer at 70°C for 2-5 min. Proteins were run in the stacking gel with a current of 15 mA and separated in the resolving gel at 25 mA (Searle DC Power Supply). Gels were stained with 0.2% Coomassie Brilliant Blue R in 30% (v/v) methanol and 7% (v/v) acetic acid, and destained overnight in 20% methanol and 7% acetic acid. Standard proteins obtained from Sigma (SDS-7) were acetylated according to Lane (1978). The standard proteins and their molecular weights (D) were; bovine serum albumin, 66,000; ovalalbumin, 45,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; trypsin inhibitor, 20,100; and a-lactalbumin, 14,200. The electrophoretic front was determined by adding bromphenol blue to the samples and standards. Autoradiograms were run to detect the location of 75Se-labelled polypeptides. Molecular weights of the sample polypeptides were estimated from a calibration plot of log protein molecular weight versus Rf (distance of protein migration/distance of electrophoretic front) as described by
Glutathione peroxidase activity

The glutathione reductase-coupled assay described by Drotar et al. (1985) was used to measure glutathione peroxidase (GSH-Px) in *T. pseudonana*. Glutathione peroxidase activity was assayed in cell-free extracts in Tris-HCl buffer. The reaction was initiated by the addition of 0.09 mM H$_2$O$_2$ or 2 mM tert-butyl oxyhydroxide (tBOOH), and oxidation of NADPH$_2$ was measured spectrophotometrically (340 nm) over 5 min. The oxidation rates determined in controls lacking reduced glutathione (GSH), H$_2$O$_2$ or tBOOH (collectively ROOH), glutathione reductase (GSH-reductase) or cell extract were subtracted from the total oxidation rate to calculate GSH-Px activity (Smith and Shrift 1979).

Glutathione peroxidase activity was assayed on non-denaturing polyacrylamide gels (lacking SDS) by a method similar to that described by Beutler and West (1974). Soluble proteins in cell-free extracts were separated by electrophoresis on a 10% polyacrylamide resolving gel for 8 h with a current of 20 mA. The gel was sliced longitudinally, and filter paper strips soaked in the enzyme assay solution were placed on top of the gel slices. The GSH-Px assay solution consisted of 0.2 M KH$_2$PO$_4$/K$_2$HPO$_4$ buffer, pH 7.0; 10 mM GSH; 10 units.ml$^{-1}$ GSH-reductase; 1 mM NADPH$_2$ and 5 mM EDTA. H$_2$O$_2$ (0.7 mM) and tBOOH (2 mM) were used as substrates. Controls were run in the absence of GSH and ROOH. After 30
min at 25°C, the filter paper strips were removed, and the gel slices were examined on a short wavelength (250 nm) UV light table (Fotodyne) using transmitted UV light. Enzyme activity was evident in bands lacking NADPH$_2$ fluorescence. Photographs of the gels were taken with Polaroid type 57 film using an orange filter. The gel strips were sliced into 0.25 cm sections, and each section was counted to determine $^{75}$Se activity.

Results

Uptake of $^{75}$Se

Growth of *Thalassiosira pseudonana*, and incorporation of $^{75}$Se-selenite into the cells is reported in Figure 40. The rate of $^{75}$Se uptake was in fair agreement with the growth rate of the alga (1.27 and 1.56 d$^{-1}$, respectively), and $^{75}$Se uptake ceased once the cells stopped growing. Assuming that background levels of Se were negligible compared to the $^{75}$Se addition (see Chapter 5 "Discussion"), approximately 12 nmol Se was taken up by the cells. This corresponds to $1.6 \cdot 10^{-18}$ mol Se·cell$^{-1}$ or $9.8 \cdot 10^5$ atoms Se·cell$^{-1}$.

Biochemical distribution of $^{75}$Se

The majority of $^{75}$Se was present in the protein and the polysaccharide and nucleic acid fractions (Table XXII). There was negligible incorporation of Se into lipids (CHCl$_3$ fraction), and only 11% of the label was associated with the low molecular weight compounds (KCl-MeOH-H$_2$O fraction). $^{75}$Se
Fig. 40. Growth of *T. pseudonana* as measured by *in vivo* chlorophyll *a* fluorescence (●) over the duration of the experiment. The accumulation of $^{75}\text{Se}$ by cells (O) was measured in 5 ml portions of culture collected by filtration and rinsed with 5 ml of filter-sterilized ESAW. The arrow indicates when the culture was harvested.
\( ^{75}\text{Se} \text{ ACCUMULATION (dpm)} \)
Table XXII

Partitioning of $^{75}$Se between protein, polysaccharides and nucleic acids, lipids and low molecular weight compounds in exponentially growing *T. pseudonana*, as determined by solvent extraction techniques. Values are reported as dpm: 25 ml of culture was extracted. Each measurement is the mean of quadruplicate determinations ± 1 SD, and units are dpm · $10^3$.

<table>
<thead>
<tr>
<th>Protein &amp; Nucleic Acids (TCA-insoluble)</th>
<th>Polysaccharides (TCA-soluble)</th>
<th>Lipids (CHCl$_3$)</th>
<th>Low Molecular Wt Compounds (KCl-MeOH-H$_2$O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.3 ± 0.5</td>
<td>11.5 ± 0.7</td>
<td>0.59 ± 0.07</td>
<td>3.6 ± 0.09</td>
</tr>
</tbody>
</table>
was equally distributed between the soluble and membrane-bound proteins (Table XXIII). Over 90% of the total cellular $^{75}$Se was extracted from the cells in the TCA precipitate. This precipitated material includes protein and nucleic acids. Cellular protein comprised only 17% of the dry weight of the phytoplankton. This value is low because diatoms contain a siliceous cell wall which accounts for up to 35% of the dry weight (Parsons et al. 1961). There may also have been an undetermined contribution to the phytoplankton dry weight by sea salts, which were not removed during rinsing.

**Gel filtration and electrophoresis**

Gel filtration of the cell-free extract through Sephadex G-150 resolved three distinct peaks of $^{75}$Se activity (Fig. 41A). The highest molecular weight peak eluted in the void volume, and was associated with protein (Fig. 42B); the two peaks were not exactly coincident. A broad peak of $^{75}$Se activity eluted in fractions 28 to 40. The average molecular weight of this material was 87 kD. Although little protein was detected in these fractions, it was not surprising since the sensitivity of the method used to detect protein is low and very little protein was applied to the column (1.4 mg). The third peak of $^{75}$Se activity had an apparent molecular weight of 4.6 kD. Other cell-free extracts gave identical chromatograms. It was noted, however, that if the cell extract was frozen at $-30^\circ$C and rerun through the column, there was little $^{75}$Se associated with higher molecular weight material eluting from the column; as many as three separate
Table XXIII

Distribution of $^{75}\text{Se}$ between soluble and membrane proteins of *T. pseudonana*. Average concentrations (calculated from quadruplicate measurements) of protein are reported ± 1 SD.

<table>
<thead>
<tr>
<th>Protein Type</th>
<th>Protein (ng·mg dry wt cells$^{-1}$)</th>
<th>Selenium$^1$ (ng·mg protein$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane Protein</td>
<td>77 ± 2.8</td>
<td>13</td>
</tr>
<tr>
<td>Soluble Protein</td>
<td>95 ± 3.6</td>
<td>9.9</td>
</tr>
</tbody>
</table>

$^1$ calculated by assuming that $^{75}\text{Se}$ was the only source of Se.
Fig. 41. (A) Elution pattern of $^{75}\text{Se}$ from Sephadex G-150 column. Arrows indicate where the molecular weight standards eluted from the column: AD, alcohol dehydrogenase; BSA, bovine serum albumin; CA, carbonic anhydrase; CC, cytochrome c. Insert is a plot of the log molecular weight of standard proteins versus the volume of buffer required to elute the protein from the column expressed relative to the void volume measured using Dextran Blue 2000. Peak height was used to determine eluted volume ($V_e$) and void volume ($V_o$). The equation of the line is $y = -1.251x + 7.269$; $r^2 = 0.996$. (B) Elution pattern of protein from Sephadex G-150, as detected spectrophotometrically at 280 nm.
peaks of $^{75}$Se activity with molecular weights less than 5 kD, could be resolved.

The membrane and soluble proteins were separated by SDS-PAGE (Fig. 42A). After staining, the gel was exposed to X-ray film for 100 h (Fig. 42B). No Se-containing polypeptides were detected in the membrane fraction, but two soluble polypeptides of 29 and 21 kD were heavily labelled with $^{75}$Se. These $^{75}$Se-labelled polypeptides were not the most abundant of the soluble polypeptides.

Glutathione peroxidase activity

Glutathione peroxidase activity was detected in the cell-free extract using $\text{H}_2\text{O}_2$ and tBOOH as substrates (Table XXIV). Heat-denatured samples had no GSH-Px activity. The rates of NADPH$_2$ oxidation were similar for both substrates. Glutathione-S-transferase activity was also assayed using 1-chloro-2,4-dinitrobenzene as substrate (Habig et al. 1974), but it could not be detected.

Glutathione peroxidase activity was assayed on non-denaturing polyacrylamide gels. Two bands of enzyme activity were present using tBOOH as substrate (Fig. 43). Controls lacking GSH and tBOOH or both were run concurrently. In the absence of tBOOH, some defluorescence occurred at both bands, but it was of much less magnitude than the experimental treatments. To facilitate better separation between the two bands of enzyme activity, the cell extract was electrophoresed under identical conditions for 9 h. The higher molecular weight band showed less activity compared to the first gel and
Fig. 42. (A) Coomassie blue stained SDS-PAGE of soluble and membrane proteins from *T. pseudonana*. Standard proteins (for description see "Materials and Methods") were run in lane S; the number beside each standard is the molecular weight in kD. Different amounts of soluble and membrane protein extract were applied to the gel; lane 1 received 60 or 50 mg of soluble or membrane protein, respectively. Lanes 2 and 3 were loaded with twice the amount of protein in lane 1, and lane 4 contained four times the protein as lane 1. (B) Autoradiograms of gel exposed to X-ray film for 100 h. Two soluble proteins of 29 and 21 kD were labelled with $^{75}\text{Se}$.
Glutathione peroxidase activity measured in cell-free extracts of *T. pseudonana*. Enzyme activity is expressed as the rate of NADPH$_2$ oxidized and was normalized to protein. The reaction temperature was 25°C. Bracketed values are the number of different protein preparations assayed, and GSH-Px activity in each preparation was measured in duplicate; average values are reported ± 1 SD.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>GSH-Px Activity (nmol·min$^{-1}$·mg protein$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O$_2$</td>
<td>29.0 ± 1.0 (2)</td>
</tr>
<tr>
<td>tBOOH</td>
<td>36.0 (1)</td>
</tr>
</tbody>
</table>
Fig. 43. Glutathione peroxidase activity detected on a polyacrylamide gel following 6 h of electrophoresis. Two bands showing enzyme activity (NADPH₂ oxidation resulting in defluorescence) were evident on the gel. However, the extent of the reaction in both bands resulted in the separate bands becoming indistinct. Sample buffer, glycerol and bromphenol blue (bpb) were run as a control in lane 1, and cell-free extract in buffer with glycerol and bpb were run in lane 2.
reacted less strongly than the lighter band. Both \( \text{H}_2\text{O}_2 \) and tBOOH were used as substrates, and the appropriate controls were run for each. The higher molecular weight band was active with tBOOH, but no activity was detected with \( \text{H}_2\text{O}_2 \). The lower molecular weight band was active with both substrates (Fig. 44). A portion of the same gel was assayed for GSH-Px activity using \( \text{H}_2\text{O}_2 \) as substrate, and the gel was sliced in to 25 mm sections and counted for \( ^{75}\text{Se} \) activity. Glutathione peroxidase activity and \( ^{75}\text{Se} \) co-migrated on the gel (Fig. 45). There were also other peaks of \( ^{75}\text{Se} \) activity of lower molecular weight, and one small peak with a greater weight.

Discussion

Selenium uptake

*Thalassiosira pseudonana* accumulated selenite during exponential growth, and uptake stopped once cell division ceased. Sandholm *et al.* (1973) found that *Scenedesmus dimorphus* did not incorporate inorganic selenite to an appreciable extent during 1 h incubations. While this should not seem surprising, because of the short time interval over which the uptake rates were measured, it was in stark contrast to the uptake of selenomethionine. Their results showed that 60% of the \( ^{75}\text{Se} \)-selenomethionine added to the medium was taken up in 1 h. However, caution must be exercised in the interpretation of their results, since the phytoplankton cultures were not axenic. It is likely that the rapid uptake
Fig. 44. Glutathione peroxidase activity detected on polyacrylamide gels using tBOOH (lane 1) and $H_2O_2$ (lane 2) as a substrate, after electrophoresis for 9 h. The upper band (a) was not detected on the gel treated with assay solution containing $H_2O_2$. Band (b) reacted with both peroxides.
Fig. 45. (A) Glutathione peroxidase activity detected on a polyacrylamide gel using H₂O₂ as substrate. (B) Amount of ⁷⁵Se in the gel (dpm·slice⁻¹).
of this organic compound can also be attributed to bacterial utilization.

The cell quota for Se reported here invariably includes some extracellularly bound Se that was not removed from the cells during rinsing. Refinement of methods used to determine accurately Fe cell quotas have resulted in a reduction of recently measured Fe quotas by comparison to the historical values (Harrison and Morel 1986). This is a result of the removal of adsorbed metal oxides on the cell surface by the use of a strong reductant, such as ascorbate (Anderson and Morel 1982). Whether inorganic Se complexes, such as FeOOH/SeO$_3^{2-}$, adsorb to the phytoplankton cell surface is unknown. Also, the Se cell quota reported here may include a "luxury" component (sensu Droop 1975).

The minimum Se cell quota can be estimated from data presented in Chapter 5. When cells were transferred into Se-deplete ESAW they undergo ca. 9 cell divisions; at this time the minimum Se cell quota would be $3.5 \cdot 10^{-21}$ mol Se·cell$^{-1}$. It is interesting to note that this quota is very similar to the subsistence cell quota for vitamin B$_{12}$ reported by Droop (1975) in a similar-sized phytoplankton, *Monochrysis lutherii* (sic) (28-52 um$^3$·cell$^{-1}$) (Burmaster 1979). These calculations further emphasize why only the slightest contamination of Se in the medium will allow *T. pseudonana* to grow.

Cell quotas for other essential trace elements, such as Fe and Mn, have been measured in diatoms. The Mn cell quota of *T. pseudonana* and *T. weissflogii*, growing in nutrient sufficient medium is $1.4 \cdot 10^{-14}$ and $1.1 \cdot 10^{-14}$ mol Mn·cell$^{-1}$,
respectively (Sunda and Huntsman 1983, Harrison and Morel 1986). While the minimum Fe cell quota required to sustain maximum cell growth of \( T. \text{weissflogii} \) is \( 1 \times 10^{-13} \text{ mol Fe.cell}^{-1} \) (Harrison and Morel 1986). The Se cell quota of \( T. \text{pseudonana} \), determined during exponential growth, is 4-5 orders of magnitude less than these trace metals.

**Intracellular distribution of \( ^{75}\text{Se} \)**

Solvent extraction techniques have been used to quantify the biochemical constituents of plants, animals and microorganisms, and there are as many variations of these methods as there are investigators (for the phytoplankton literature see: Morris et al. 1974, Kochert 1978, Wrench 1978, Li et al. 1980, Smith and Morris 1980, Hitchcock 1983, Terry et al. 1983, Bottino et al. 1984, Dortch et al. 1984). From the work conducted with prokaryotes, it is apparent that the various solvent fractions contain a wide range of compounds, and that a single class of biochemicals (for example polysaccharides) may not be entirely extracted by one procedure (Sutherland and Wilkerson 1971). Only two studies have examined the efficacy of solvent extraction procedures for biochemical separation in marine phytoplankton (Morris et al. 1974, Hitchcock 1983).

In all of these methods, the isolation of lipids from biological samples involves \( \text{CHCl}_3-\text{MeOH} \) extraction: this method was first described by Polch et al. (1957). Re-extraction of the \( \text{CHCl}_3-\text{MeOH} \) fraction with KCl and MeOH removes polar
compounds, which include the free amino acids and other low molecular weight compounds such as carbohydrates. Lipids are retained in the CHCl₃ fraction. Although it is generally excepted that CHCl₃-MeOH extraction solubilizes all free amino acids, Wrench (1978) found this was not true during extraction of two green algae. Since his initial separation step is identical to what was used in this study, it is possible that not all of the free amino acids in *T. pseudonana* were extracted into the KCl-MeOH-H₂O fraction; some may also be contained in the TCA soluble fraction.

Whereas lipid extracts of *Dunaliella primolecta* and *Porphyridium cruentum* contained high levels of Se (Gennity *et al.* 1984), and were confined primarily to the carotenoids and xanthophylls, the results of this investigation found very little Se associated with lipid in *T. pseudonana*. Similar observations were made by Wrench (1978). He found that the lipids of *Tetraselmis tetrethelae* and *Dunaliella minuta* contained negligible quantities of Se when these algae were grown in the presence of nanomolar quantities of ⁷⁵Se. The results of Gennity *et al.* (1984) appear more of an artifact of the high Se concentration added to their culture medium (10 ppm Se as Na₂SeO₃). Since SeO₃²⁻ was shown to bind to lipids during extraction, their results provided no proof for the existence of Se-lipid associations *in vivo*. In fact, all extracted lipids containing Se were unsaturated, and gentle catalytic hydrogenation with PtO₂ decreased the Se content of the lipids by 95%. This result suggested that Se was associated with carbon double bonds (saturated lipids did not
contain Se), and there was no evidence for metabolic incorporation of Se.

Morris et al. (1974) verified that boiling TCA hydrolyzed polysaccharides in the CHCl₃-MeOH residual material, and this procedure gave similar results to the more conventional method of boiling sulfuric acid hydrolysis for 3 h (Casselton and Syrett 1962). Morris et al. (1974) made no mention of the stability of protein under these conditions. The treatment with hot TCA hydrolyzes nucleic acids as well as polysaccharides. Hitchcock (1983) criticized this method on the basis of results he obtained from experiments using bovine serum albumin (BSA) standards. He found that only 20% of the BSA was recovered in the TCA-insoluble fraction, but two other methods yielded 100% recovery of the BSA standards. In spite of this result, all three methods extracted the same amount of protein from two dinoflagellate species, implying that there may have been something unique about the BSA standard which was not reflected in the biological samples. It should be pointed out that the objective of the biochemical fractionation in this study was to determine the amount of ⁷⁵Se associated with the major groups of biochemicals in T. pseudonana. Nevertheless, although these methods may be suitable for isolating the organic molecules, they may disrupt any loose associations of these compounds with Se. Because of its reactivity, some extraction procedures, notably the hot TCA extraction, may eliminate Se from molecules in which it is normally a constituent.
Bottino et al. (1984) found most of the Se associated with *Dunaliella primolecta* and *Chlorella* sp. (73 and 98%, respectively) was extracted in the free amino acid and soluble carbohydrate fraction; whereas, 80% of the cellular Se was bound to lipids in *Porphyridium cruentum*. The major criticism of this work was that the levels of selenite added to the medium were unrealistically high \(10^{-2} \text{ g} \cdot \text{l}^{-1}\). It is not clear whether the results obtained by Bottino et al. (1984) are representative of the Se partitioning among the cellular constituents of these organisms under more natural conditions. Rather, these Se-containing compounds maybe the end products of metabolic pathways designed to detoxify high levels of Se, or the result of indiscriminate incorporation of Se into macromolecules, by processes which may or may not be enzymatically mediated.

Protein-bound Se represents 51% of the total particulate Se in *T. pseudonana*, and 36% of the Se was associated with polysaccharides and nucleic acids. During measurements of the soluble and membrane protein concentrations, soluble RNA was added to the samples to co-precipitate protein in the presence of TCA. The precipitate, containing protein and nucleic acids, and the supernatant from both fractions were analyzed for \(^{75}\text{Se}\) activity. Over 90% of the \(^{75}\text{Se}\) was present in the precipitate. Since only 51% of the total \(^{75}\text{Se}\) activity was associated with protein, as measured by solvent extraction methods, the additional \(^{75}\text{Se}\) in the protein/nucleic acid precipitate can only be due to Se bound to nucleic acids. This argues that, in the TCA soluble fraction, all of the \(^{75}\text{Se}\)
was associated with nucleic acids and none with polysaccharide. Selenonucleotides have recently been identified in bacteria (Stadtman 1980b). Nevertheless, if boiling TCA inadvertently removed some loosely bound $^{75}$Se from the protein, the $^{75}$Se found in the polysaccharide and nucleic acid fractions may be an artifact.

**Specific selenium-containing molecules**

Separation of soluble macromolecules by gel filtration showed that some $^{75}$Se was associated with high molecular weight material which eluted in the void volume. The exclusion limit of Sephadex G-150 is 300 kD, and this weight is approximately the lower limit of material eluted in the void volume. The two soluble polypeptides identified in the autoradiogram of the SDS-polyacrylamide gel had Se stably incorporated into their molecular structure. The molecular weight of these proteins, 21 and 29 kD, when compared to the weight of the $^{75}$Se-labelled material eluting from the column, suggested that they are polypeptide subunits. It was noticed that the $^{75}$Se in fractions 28-40 did not elute from the column in a symmetrical peak. There was some indication that two macromolecules containing $^{75}$Se with similar molecular weights were eluted from the column. In hindsight, the molecular weights of the polypeptide subunits provides evidence that this is true. Integral multiples of the subunit molecular weights equal the molecular weight of the material eluted from the column in fractions 28-40. These results provide
circumstantial evidence that *T. pseudonana* contains two Se-containing polypeptides which have a native molecular weight of approximately 87 kD. The simplest explanation is that one protein is a tetramer with a subunit molecular weight of 21 kD and the other is a trimer with a subunit molecular weight of 29 kD.

The nature of the chemical form of Se in these proteins was not addressed in this study. The fact that the $^{75}$Se remained bound to the proteins during electrophoresis suggests it is linked by a covalent bond. In selenoproteins of bacteria, and in mammalian and avian GSH-Px, the Se-containing moiety is selenocysteine; the Se analogue of the S-containing amino acid cysteine (Cone *et al.* 1976, Forstrom *et al.* 1978, Jones *et al.* 1979). Thiolase is a unique selenoprotein in which selenomethionine is the Se-containing moiety (Hartmanis and Stadtman 1982).

Although specific Se-containing proteins have not been identified in higher plants, chemical and enzymatic hydrolysis of crude protein extracts and their subsequent analysis have shown the presence of selenoamino acids in these organisms. Selenocysteine and its oxidized form selenocystine were identified in corn and in wheat grain (Smith 1949: as cited in Rosenfeld and Beath 1964) and selenocysteine, selenomethionine, and their oxidation products were present in proteins of clover and rye grass (Peterson and Butler 1962). Stadtman (1974) criticized the results of selenoamino acid composition of protein obtained from acid hydrolysis. She argued, on the basis of work conducted by Huber and Criddle
that selenocysteine is altered upon acid hydrolysis, and after 6 h all the amino acid is destroyed. From this observation, the results of the partitioning of $^{75}\text{Se}$ in protein of *T. pseudonana* may be underestimated as a consequence of the boiling TCA extraction. Additionally, since acid volatile Se was reported in protein extracts of *Tetraselmis tetraethela* (Wrench 1978), suggesting the presence of hydrogen selenide, protein bound Se in *T. pseudonana* may have been lost during this treatment. Recently, Ng and Anderson (1979) reported the in vitro synthesis of selenocysteine by cysteine synthases from a variety of plants. Brown and Shrift (1980b) used alkylating agents to stabilize the selenocysteinyl residues and were able to unequivocally demonstrate the presence of selenocysteine in proteins of pea and bean.

The lack of specific Se-labelled membrane protein(s), in spite of equivalent activity on a weight basis for both soluble and membrane proteins, is perplexing. A possible explanation may be that there was a non-specific incorporation of $^{75}\text{Se}$ into all the membrane proteins, and the gel was not exposed to the x-ray film for a sufficient length of time to detect these proteins. This first scenario seems unlikely. If all the proteins were equally labelled it might be anticipated that some of the more abundant proteins would have been detected in the autoradiogram. If the degree of incorporation of Se is a function of the amount of S-containing amino acids in a protein, perhaps this is too
simplistic a proposal. It is known that selenol groups are easily oxidized, and the oxidized products will undergo spontaneous elimination reactions producing elemental Se (Stadtman 1980a). The nature of the Se functionality in membrane proteins may favour this type of reaction. Alternatively, Schwarz and Sweeney (1964) reported that $\text{Na}^{75}\text{SeO}_3$ added to a cell extract will readily bind to protein. It is possible that the Se associated with the membrane proteins was loosely bound and was released when the proteins were solubilized and denatured prior to electrophoresis. Danielson and Medina (1986) offered this explanation to account for the lack of $^{75}\text{Se}$-labelled membrane polypeptides in their autoradiograms in spite of the high activity of $^{75}\text{Se}$ in the membrane protein extract.

**Glutathione peroxidase**

Recent attempts to demonstrate the presence of GSH-Px in *Dunaliella primolecta* and *Porphyridium cruentum* have failed (Gennity et al. 1985). But this enzyme has been detected in purified cell-free extracts of a number of microalgae, including *Euglena gracilis Z*(UTEX 753), *E. gracilis* var. *bacillaris* (UTEX 884) and (W3BUL), and *Astasia longa* (Overbaugh and Fall 1982, 1985). Overbaugh and Fall (1985) found that GSH-Px in *Euglena gracilis* was a Se-independent enzyme which was active with both $\text{H}_2\text{O}_2$ and organic hydroperoxides. This was the first report of a Se-independent GSH-Px which reduces $\text{H}_2\text{O}_2$. The molecular weight of this enzyme is much greater than previously described Se-dependent GSH-Px.
Glutathione peroxidase activity was evident in *T. pseudonana*, and activity was observed with H$_2$O$_2$ and tBOOH. Enzymatic activity was completely eliminated after heating the cell extract at 100°C for 5 min. This observation has relevance, since Gennity *et al.* (1985) concluded that oxidation of glutathione was non-enzymatic. They found that boiling the algal extract for 30 min did not eliminate GSH-Px activity.

The specific activity of GSH-Px in crude extracts of four planktonic euglenoids ranged from 16-100 nmol NADPH$_2$ oxidized·min$^{-1}$·mg protein$^{-1}$, with H$_2$O$_2$ as substrate (Overbaugh and Fall, 1982). Similar levels of this enzyme were measured in tissue-cultured plant cells (Drotar *et al.* 1985). In both reports, the activity of GSH-Px was generally less when organic hydroperoxides (cumene hydroperoxide and tBOOH) were used as substrates, but the measured activity with organic hydroperoxides compared with H$_2$O$_2$ was greater in corn and similar in *Lemna* and *E. gracilis* var. *bacillaris* (W3BUL). It is impossible to ascribe these rate measurements to a single enzyme since glutathione-S-transferases show GSH-Px activity when organic hydroperoxides are present. Rates determined with H$_2$O$_2$ represent the activity of the true GSH-Px enzyme. The activity of GSH-Px measured in *T. pseudonana* was slightly greater with tBOOH (36.0 nmol·min$^{-1}$·mg protein$^{-1}$) than H$_2$O$_2$ (29.0 nmol·min$^{-1}$·mg protein$^{-1}$). Since glutathione-S-transferase could not be detected in *T. pseudonana*, both rates may reflect GSH-Px activity. The levels of GSH-Px in *T. pseudonana* are
very similar to published values for other plants and protists.

Glutathione peroxidase activity on polyacrylamide gels

Two enzymes exhibiting GSH-Px activity with tBOOH were separated and assayed on non-denaturing polyacrylamide gels. The higher molecular weight protein did not use $H_2O_2$ as a substrate and did not show true characteristics of GSH-Px (EC 1.11.1.9). This protein appeared more labile than the lower molecular weight GSH-Px, and its activity was reduced following electrophoresis for 9 h. The lower molecular weight enzyme demonstrated characteristics of a true GSH-Px (EC 1.11.1.9); it was active with $H_2O_2$ and tBOOH and contained Se.

Selenium nutrition

The chemical similarities between Se and S are well known, and many enzymes which normally catalyze reactions of sulfur compounds function equally well with the corresponding Se analogs (Stadtman 1979). The toxic effects of Se are believed to be manifested in organisms as a consequence of the indiscriminant incorporation of Se into essential proteins and macromolecules in place of sulfur (S). Fortunately, this only occurs when organism are exposed to very high concentrations of Se relative to S. On the other hand, very specific pathways for the synthesis of selenoenzymes function in the presence of orders of magnitude greater S concentrations. In the ocean, planktonic organisms are bathed in a medium containing mM concentrations of S and pM concentrations of Se.
Not only must highly specific processes incorporate Se into necessary macromolecules, equally specific membrane porters must function to overcome potential competitive interactions with similar anions such as $\text{SO}_3^{2-}$, $\text{SO}_4^{2-}$ and $\text{NO}_2^-$. The ionic composition of the culture medium used in this study is representative of natural concentrations of Se and S. I argue that any selenometabolites produced by *T. pseudonana*, under the growth conditions of these experiments, are normal cellular constituents, and are not a consequence of non-specific Se incorporation.

**Summary**

These results are the first to provide evidence of specific selenium-containing polypeptides in a photosynthetic organism. Glutathione peroxidase activity was measured in cell-free extracts by a glutathione-reductase coupled assay and on non-denaturing polyacrylamide gels. Two enzymes showing GSH-Px activity were present on polyacrylamide gels. One enzyme was active with $\text{H}_2\text{O}_2$ and tBOOH, consistent with known Se-dependent glutathione peroxidases. The other enzyme was only active with tBOOH. Co-migration of the GSH-Px that was active with $\text{H}_2\text{O}_2$ and $^{75}\text{Se}$ supports the enzymatic evidence that *Thalassiosira pseudonana* contains a Se-dependent glutathione peroxidase. The subunit molecular weight of $^{75}\text{Se}$-labelled polypeptides agrees well with the weight of previously characterized GSH-Px from other sources. In addition, the weight of $^{75}\text{Se}$-containing macromolecules, as
measured by gel filtration, is consistent with the proposal that this glutathione peroxidase is a tetramer. Earlier observations of ultrastructural and morphological changes associated with Se-depleted cells support the conclusion that the obligate requirement for Se in *Thalassiosira pseudonana* is due, in part, to the presence of the selenoenzyme glutathione peroxidase.
GENERAL CONCLUSIONS

This thesis examined two aspects of marine phytoplankton physiology and nutrition: urea and selenium. The specific findings of this research are summarized below.

1. A modified diacetyl monoxime method for urea analysis in seawater gave superior results by comparison to the urease method. The urease method was plagued by a number of problems, which were a result of the inhibition of the urease enzyme used in the assay. The diacetyl monoxime method for urea analysis is recommended for measuring urea concentrations in seawater.

2. Results of experiments conducted in the Strait of Georgia, in nitrate-rich frontal water and nitrogen-depleted stratified water, were used to examine nitrogen cycling between the dissolved and particulate components in these two different coastal regions. This was the first study of nitrogen cycling in this area. I argued that in frontal water plankton were losing nitrogen in the form of dissolved organic nitrogen, and in stratified water phytoplankton assimilated DON concurrently with inorganic nitrogen and urea.

3. In both coastal and oceanic regions, maximum uptake rates of urea and ammonium by phytoplankton were similar. Nitrate uptake rates by nitrate-sufficient phytoplankton communities in the Strait of Georgia were faster than maximum uptake rates of ammonium and urea.

4. In situ urea regeneration rates were determined by intact
plankton communities. In coastal seawater, in both frontal and stratified communities, urea regeneration rates were comparable to ammonium regeneration rates.

5. Uptake and regeneration rates of regenerated forms of nitrogen are believed to be tightly coupled, but data only exist for ammonium. This thesis has provided supporting evidence for coupling between urea uptake and regeneration based on the following observations: Dissolved urea concentrations were low in the Sargasso Sea; the turnover time of the ambient pool of urea was 12 h, and phytoplankton were able to utilize these low concentrations of urea at rates, which were comparable to the maximum possible rates of utilization. These data represent the fastest turnover times of urea in any oligotrophic ocean region hitherto examined.

6. Discrepancies between $^{14}$C-urea and $^{15}$N-urea uptake were evident in data collected from the Sargasso Sea. Uptake rates determined by both isotopes were not equivalent. $^{14}$C-urea uptake rates were 1.4 times greater than rates measured by $^{15}$N-urea. It was proposed that urea-N is lost from phytoplankton as ammonia/ammonium, which is not taken back up, at least on the short term. Support for ammonia/ammonium excretion during urea uptake by phytoplankton was found in an axenic culture of a marine diatom.

7. A model of urea uptake and assimilation by the coastal marine diatom *Thalassiosira pseudonana* (clone 3H) was
proposed. One salient feature of this model includes urea-N excretion as ammonia/ammonium and its rapid reabsorption. During nitrate-sufficient growth, _T. pseudonana_ retained only 15% of the total urea-N taken up by the cells, suggesting that nitrogen is excreted as dissolved organic nitrogen. Losses of urea-C were also observed, but they were less than those of urea-N. Nitrate-starved cells took up urea and during assimilation retained all of the urea-N; the three methods used to measure urea uptake, \(^{14}\)C-urea, \(^{15}\)N-urea and the disappearance of dissolved urea were in excellent agreement during a 1 h urea uptake experiment with nitrate-starved cells.

8. Selenium is an essential element for growth of _Thalassiosira pseudonana_ (clone 3H). Of the two inorganic forms of Se examined, SeO\(_3^{2-}\) was the most readily utilized for growth; SeO\(_4^{2-}\) was only effective in supporting growth when concentrations were greater than \(10^{-7}\)M, four orders of magnitude greater than SeO\(_3^{2-}\). No other element was able to substitute for the Se requirement.

9. For the first time, the results in this thesis provide evidence of specific Se-containing polypeptides in a photosynthetic organism. Two soluble polypeptides of 21 and 29 kD contained Se. Glutathione peroxidase is a selenoenzyme in _T. pseudonana_, which reduces H\(_2\)O\(_2\) and tertiary-butyl hydroperoxide. This observation explains, at least in part, the reason for the obligate Se
requirement in this alga.
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