

C. Fan · P.M. Glibert · J. Alexander · M.W. Lomas

Characterization of urease activity in three marine phytoplankton species, *Aureococcus anophagefferens*, *Prorocentrum minimum*, and *Thalassiosira weissflogii*

Received: 12 December 2001 / Accepted: 16 December 2002 / Published online: 15 February 2003
© Springer-Verlag 2003

Abstract The availability of different forms of nitrogen in coastal and estuarine waters may be important in determining the abundance and productivity of different phytoplankton species. Although urea has been shown to contribute as much as 50% of the nitrogen for phytoplankton nutrition, relatively little is known of the activity and expression of urease in phytoplankton. Using an in vitro enzyme assay, urease activities were examined in laboratory cultures of three species: *Aureococcus anophagefferens* Hargraves et Sieburth, *Prorocentrum minimum* (Pavillard) Schiller, and *Thalassiosira weissflogii* (Grunow) Fryxell et Hasle. Cultures of *P. minimum* and *T. weissflogii* were grown on three nitrogen sources (NO_3^- , NH_4^+ , and urea), while *A. anophagefferens* was grown only on NO_3^- and urea. Urease was found to be constitutive in all cultures, but activity varied with growth rate and assay temperature for the different cultures. For *A. anophagefferens*, urease activity varied positively with growth rate regardless of the N source, while for *P. minimum*, urease activity varied positively with growth rate only for cultures grown on urea and NH_4^+ . In contrast, for *T. weissflogii*, activity did not vary with growth rate for any of the N sources. For all species, urease activity increased with assay temperature, but with different apparent temperature optima. For *A. anophagefferens*, in vitro activity increased from near 0–30°C, and remained stable to 50°C, while for *P. minimum*, increased in vitro activity was noted from near 0–20°C, but constant activity was

observed between 20°C and 50°C. For *T. weissflogii*, while activity also increased from 0°C to 20°C, subsequent decreases were noted when temperature was elevated above 20°C. Urease activity had a half-saturation constant of 120–165 $\mu\text{g atom N l}^{-1}$ in all three species. On both an hourly and daily basis, urease activity in *A. anophagefferens* exceeded nitrogen demand for growth. In *P. minimum*, urease activity on an hourly basis matched the nitrogen demand, but was less than the demand on a daily basis. For *T. weissflogii*, urease activity was always less than the nitrogen demand. These patterns in urease activity in three different species demonstrate that while apparently constitutive, the regulation of activity was substantially different in the diatom. These differences in the physiological regulation of urease activity, as well as other enzymes, may play a role in their ecological success in different environments.

Introduction

Under most growth conditions, phytoplankton tend to prefer reduced forms of nitrogen (NH_4^+ and urea) over oxidized forms (NO_3^- and NO_2^-), even when oxidized forms are available in higher concentrations. The contribution of NH_4^+ to phytoplankton growth and nutrition has been extensively studied, while the use of urea has been less well studied. Although urea generally constitutes only a small fraction of the total dissolved organic nitrogen pool in coastal and estuarine waters, it has been shown to contribute >50% of the total nitrogen used by phytoplankton in some estuarine and coastal environments (McCarthy 1972, 1977; Harvey and Caperon 1976; Furnas 1983; Kaufman et al. 1983; Harrison et al. 1985; Glibert et al. 1991). In addition, urea can serve as the sole source of nitrogen for many phytoplankton species in the field and in the laboratory (Thomas 1968; Carpenter et al. 1972; Antia and Landymore 1975; Bekheet and Syrett 1977; McCarthy

Communicated by J.P. Grassle, New Brunswick

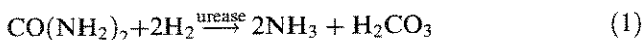
C. Fan (✉) · P.M. Glibert · J. Alexander · M.W. Lomas
Horn Point Laboratory, University of Maryland Center
for Environmental Science, P.O. Box 775, Cambridge,
MD 21613, USA

E-mail: cfan@hpl.umces.edu
Fax: +1-410-2218490

Present address: M.W. Lomas
Bermuda Biological Research Station, 17 Biological Lane,
GE01, Ferry Reach, St. Georges, Bermuda

1980; Oliveira and Antia 1986; Glibert 1998). The availability of different forms of nitrogen and their relative rates of utilization are considered to be important factors contributing to the success and productivity of different phytoplankton species at different times. For example, preference for urea uptake over nitrate has been demonstrated for the "brown tide" organism, *Aureococcus anophagefferens*, and urea also stimulates growth of this species (Berg et al. 1997; Lomas et al. 2001). Most previous research on urea metabolism and associated urea enzyme activity has been focused on chlorophytes and diatoms (Antia et al. 1991). In the present study, a comparison of urease activity under a range of laboratory growth and assay conditions was undertaken among a diatom (*Thalassiosira weissflogii*), a pelagophyte (*A. anophagefferens*), and a dinoflagellate (*Prorocentrum minimum*).

Urea may be taken up by phytoplankton by passive diffusion, but its metabolism is an enzymatic process (Antia et al. 1991). Following its uptake, urea is metabolized to NH_4^+ and H_2CO_3 via one of two pathways in marine algae: the urease pathway or the ATP:urea amidolyase pathway. The ATP:urea amidolyase pathway has been demonstrated only in several classes of Chlorophyceae. This enzymatic reaction is believed to be a two-step process involving the ATP-dependent catalysis of urea to allophanate by urea carboxylase, followed by the allophanate hydroxylase-mediated breakdown to NH_3 and H_2CO_3 (Leftley and Syrett 1973; Bekheer and Syrett 1977). The predominant pathway of urea hydrolysis in other algal classes involves a urease (Oliveira and Antia 1986), which is a nickel-dependent, metal-protein catalyzing the hydrolysis of urea according to the following equation:



The resulting NH_3 is then assimilated primarily by the glutamine synthase–glutamate synthase pathway. Interestingly, the production of H_2CO_3 from the hydrolysis of urea can also contribute carbon for cell metabolism (Antia et al. 1977).

Current knowledge about urease activity and expression in marine phytoplankton is limited. The objectives of the present study were to assess the characteristics of urease activity in three bloom-forming phytoplankton species under varying nitrogen sources, temperatures, rates of cell growth, and times of day.

Materials and methods

Culture sources and growth conditions

Three common bloom-forming phytoplankton species were chosen for this study: the pelagophyte *Aureococcus anophagefferens* Hargraves et Sieburth, the dinoflagellate *Prorocentrum minimum* (Pavillard) Schiller, and the diatom *Thalassiosira weissflogii* (Grunow) Fryxell et Hasle. Cultures of *A. anophagefferens* and *T. weissflogii* were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP clone B9TW

and CCMP clone 1708, respectively). The *P. minimum* culture was isolated from a Chesapeake Bay water sample by A. Lewitus and subsequently maintained in the Horn Point Laboratory culture collection.

From 1999 to 2000, cultures were grown under semi-batch conditions, and experiments were performed during the exponential phases of growth. For growth rate comparisons, some experiments were also conducted in early and late exponential phases. All cultures were grown on artificial seawater media (400 mM NaCl, 20 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mM CaCl_2 , 1.7 mM KBr, 10 mM KCl, 20 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and *f/2* vitamin mix and trace metals), with *f/2* NO_3^- , NH_4^+ , or urea as the nitrogen source, depending on the particular experiment being conducted. For *A. anophagefferens*, cultures failed to grow on NH_4^+ , consistent with previous observations of batch culture growth of this organism (Cosper et al. 1989). Thus, *A. anophagefferens* was grown only on NO_3^- and urea, while *P. minimum* and *T. weissflogii* were grown on NO_3^- , NH_4^+ , and urea. In all cases, the artificial seawater was autoclaved for 2 h, and the nitrogen substrates were added by sterile filtration to limit contamination by bacteria. Cultures of *A. anophagefferens* were also supplemented with H_2SeO_3 at a final concentration of 10 nM. All cultures were grown in 2-l bottles at 20°C, at 100 μM photons $\text{m}^{-2} \text{s}^{-1}$ light intensity, under a 12 h light:12 h dark cycle.

For the *P. minimum* and *T. weissflogii* cultures, cell counts were made using a Coulter multisizer. Specific growth rates (μ) were calculated from cell counts using the formula:

$$\mu = \ln(C_1/C_0)/(t_1 - t_0) \quad (2)$$

where C_1 and C_0 represent the cell counts at time 1 (t_1) and time 0 (t_0). For *A. anophagefferens*, the size of the cells precludes use of the multisizer for cell counts. Therefore, cell fluorescence was used and calibrated against cell number in an independent experiment. A conversion factor was obtained based on the regression of the fluorescence measurement (Turner Designs model 10 fluorometer) and cell counts (flow cytometer, Becton Dickinson; $r^2=0.99$, $n=8$). Cellular nitrogen content of all cultures was obtained by filtering 50-ml aliquots of culture onto pre-combusted 25-mm GF/F filters and analyzing with a Control Equipment CHN analyzer.

Cultures used were unialgal, but not axenic. There has been only limited success in growing *A. anophagefferens* axenically (e.g. Berg et al. 2002). Protocols were used throughout all phases of the experiments to minimize bacterial contamination. Bacterial contributions to the enzyme assays cannot, however, be discounted. Thus, in an attempt to estimate bacterial contribution to the enzyme assays, a comparison using two sets of filters was undertaken using *P. minimum*. Bacterial contribution to the overall urease activity of *P. minimum* was estimated by modifying the cell-harvesting method to either exclude or include proportionately more bacteria. The assay method utilized for this experiment requires the use of glass fiber filters when harvesting cells. Glass fiber filters break apart during the tissue-grinding phase of the assay, and actually aid in rupturing cellular membranes. To estimate the potential contribution of bacterial urease activity to the total activity in the culture, urease activities of *P. minimum* from cultures grown on urea and NH_4^+ sources at exponential growth phase, and filtered onto Whatman GF/C filters (nominal pore size=1.3 μm) and GF/F filters (nominal pore size=0.7 μm) were compared. At the same time, the bacterial counts from whole culture water (NH_4^+ -grown culture) and from the filtrates from the GF/F and GF/C filtrations were determined by flow cytometry.

Internal urea pools and cell volume estimations

Internal urea concentrations for these three species were determined for the cultures grown on urea at mid-exponential phase. Triplicate samples, 25–50 ml in volume, were filtered onto precombusted GF/F filters at low vacuum and rinsed with copious amounts of artificial seawater. The filters, along with 5 ml of boiling deionized water, were placed in scintillation vials that had been acid-washed and baked

(450°C for 1 h). They were capped using a liner-less polyethylene cap, shaken vigorously, and then frozen at -20°C. At a subsequent time, but within 30 days, the extracts were thawed and analyzed for urea concentration (Parsons et al. 1984).

Microscopic examination showed *T. weissflogii* was cylindrical and *P. minimum* was roughly spherical. Cell volumes were determined using the equations for a cylinder and sphere, with diameters determined from the multisizer. Cell volumes of *A. anophagefferens* were also calculated as a spherical shape, with diameters determined from the flow cytometer.

Enzyme extraction and assay

There are several techniques for measuring urease activity based on one of the products of the hydrolysis of urea. The indophenol colorimetric method measures the NH_4^+ released by enzymatic hydrolysis (Mobley and Hausinger 1989; Peers et al. 2000) and has been shown to be very sensitive for detailed kinetic analyses. In the present study, the urea hydrolysis method of Peers et al. (2000) was used with some modifications. First, the use of bovine serum albumin (BSA) to stabilize the enzyme, as recommended by Peers et al. (2000), was eliminated, in order to avoid the high background contamination of NH_4^+ associated with BSA. To test for loss of stability of the enzyme in the absence of BSA, replicate urease samples ($n=6$) of *P. minimum* cultures grown on urea at mid-exponential phase were collected, and run with and without the addition of BSA. Those experiments (data not shown) indicated that there was no loss of urease stability as a consequence of the removal of BSA. Second, the Peers' method recommends inactivation of the enzyme by one of two methods: heat (100°C water bath, 60 s), or addition of HCl, followed by subsequent neutralization with NaOH. However, the indophenol method of NH_4^+ determination is pH sensitive. To avoid possible problems associated with pH shifts, the addition of HCl to inactivate the enzyme was not used. Furthermore, preliminary results suggested incomplete inactivation of the enzyme following a 100°C, 60 s water bath. To compensate for this incomplete inactivation of urease activity after heat treatment, the method was modified to include the addition of the indophenol NH_4^+ reagents to the t_0 samples immediately following the addition of substrate. The NH_4^+ assay results from this modification were treated as a blank in the calculation of NH_4^+ production from urea hydrolysis.

In a preliminary experiment, the pH optimum for conducting the urease assay was determined for *A. anophagefferens* and *P. minimum* (data not shown). Duplicate samples were collected from cultures ($n=6$) grown on urea at mid-exponential growth, then the rates of urease activity were measured at three different pH levels (pH 6.0, 7.9, and 10.0) that bracketed the pH used by Peers et al. (2000). The optimum for both species was determined to be pH 7.9.

Thus, the detailed protocol for enzyme extraction was as follows. Filters containing the cultured material were transferred to a Teflon pestle tissue homogenizer tube (Thomas Scientific) on ice. To this was added 1 ml extraction buffer (50 mM HEPES, pH 7.9; 150 mM phosphate buffer, pH 7.9; 0.3% w/v PVP; 0.1% v/v Triton 100x; 5 mM EDTA), and the sample was homogenized. The contents were transferred to a clean 1.5 ml microcentrifuge tube. The supernatant was then clarified by centrifugation at 15,000 rpm for ~5 min. Of the clarified supernatant, 400 μl was then transferred to a disposable sterile polystyrene test tube, along with 700 μl cold assay buffer solution (50 mM HEPES; 150 mM phosphate buffer, pH 7.9), and 800 μl deionized H_2O .

Control tubes at time 0 (t_0) were placed in a 100°C water bath for 60 s. Samples from t_{final} (t_f) were transferred to a 20°C incubation water bath. Then, 300 μl of 5 mM urea (equivalent to 10 mg atom N l^{-1}) stock was added to all tubes to initiate incubation. Assay reagents for NH_4^+ detection (Parsons et al. 1984) were added immediately to t_0 controls to determine initial NH_4^+ background levels. Following a 1 h incubation, t_f samples were transferred to a 100°C water bath for 60 s to inactivate enzymatic activity, and then NH_4^+ detection assay reagents were added. Samples were allowed to develop color for 2.5 h, and were read in a spectrophotometer at 640 nm.

Experimental manipulations

Separate cultures were grown under semi-continuous batch conditions to characterize the in vitro urease activity of these species as a function of nitrogen substrate and growth rate, assay temperature, substrate (urea) concentration, and time of day. For all experiments, comparisons of in vitro urease activity are based on measurements made from the mid-exponential growth phase; however, in the examination of the relationship between urease activity and culture growth rate, urease activity from cultures grown from early exponential to early stationary phase were included to increase the range of growth rate variation. For assays at different temperatures, the cultures for these three species were grown on urea, samples were harvested during mid-exponential phase, and crude enzyme was extracted, dispensed into test tubes, and exposed to temperatures ranging from 0°C to 50°C for 1 h, when enzyme activity was determined. However, cells were not acclimated to the temperature prior to the enzyme assay. For the substrate concentration experiment, the cultures were grown on urea as the nitrogen source. Samples were harvested during mid-exponential growth, and crude enzyme extracts were prepared and enriched with urea in concentrations ranging from 0 to 3 mg atom N l^{-1} . Lastly, to determine variability in urease activity with time of day, cultures of *A. anophagefferens* and *P. minimum* were grown on both NO_3^- and urea to mid-exponential growth, and at eight intervals during a day/night period, aliquots were withdrawn and processed as above.

Data analysis

All of the statistical comparisons were done using the Student's *t*-test, with $\alpha=0.05$. The urease enzyme kinetics parameters were iteratively curve-fit, using the Michaelis-Menten equation (Berges et al. 1994).

Results

Bacterial effects

Using filters of differing porosity, a variable bacterial contribution to enzyme activity should be measurable if bacteria were contributing significantly to the measured rates. The larger pore size of the GF/C filters should allow a larger fraction of the bacterial assemblage to pass through the filter and be excluded in the assay of enzyme activity. Although some bacteria would be expected to pass through a GF/F filter, it has previously been shown that ~50% of bacteria are retained (Kirchman 1990; Glibert et al. 1995; Bronk et al. 1998). In these experiments, GF/F filters retained ~80% of the total bacteria, while GF/C filters retained a significantly lower (~50%) proportion of total bacteria ($P<0.05$, Fig. 1A). However, no statistically significant difference ($P>0.05$) in the activities of the GF/C and the GF/F filtered samples was found for the cultures grown on either NH_4^+ or urea (Fig. 1B, C; $n=6$), suggesting that the bacterial contribution to the overall urease activity was likely to be minimal in this study. Measured bacterial abundance levels in the cultures of *Prorocentrum minimum* were about 10% of those determined in natural estuarine waters of the Chesapeake Bay, from which *P. minimum* was originally isolated (Fig. 1A).

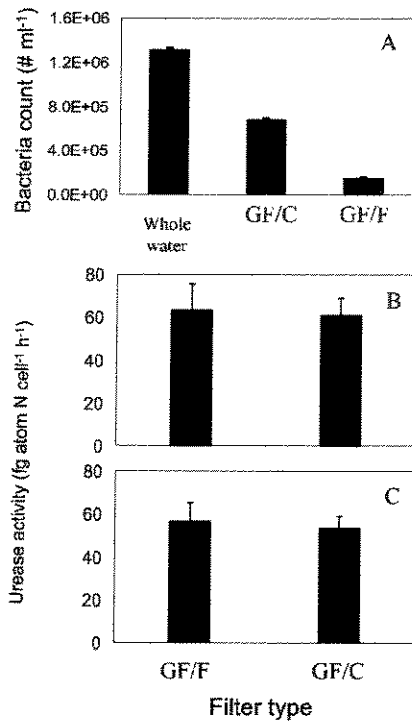


Fig. 1A–C *Prorocentrum minimum*. **A** Comparison of bacterial counts for cultures grown on NH_4^+ and filtered using GF/C and GF/F filters in relation to cell count from a natural bloom of *P. minimum* in the river from which this species was originally isolated. **B** Comparison of urease activity of cells grown on NH_4^+ , collected on GF/F filters versus GF/C filters. **C** Same comparison for cells grown on urea. Error bars represent SE of three measurements

Urease activity as a function of nitrogen growth source

Urease activity was observed for all three species regardless of the nitrogen source or growth rate. For *Aureococcus anophagefferens*, there were no significant differences in urease activity between the cultures that were grown on NO_3^- and those that were grown on urea. For *P. minimum*, urease activity also showed no significant difference among the cultures grown on three different nitrogen sources (Table 1). Furthermore, the

growth rates of the *A. anophagefferens* and *P. minimum* cultures grown on urea were only slightly higher than those of the cultures grown on NO_3^- (Table 1).

In contrast, the urease activity of *Thalassiosira weissflogii* grown on NH_4^+ and urea was significantly ($P < 0.05$, $n = 6$) higher than that of the same species grown on NO_3^- (Table 1). The growth rate for *T. weissflogii* cultures grown on urea ($\mu = 0.77$, $\text{SE} = 0.13$) was significantly lower than that measured for comparable cultures grown on NO_3^- and NH_4^+ ($P < 0.05$, $n = 6$).

Urease activity as a function of growth rate

For *A. anophagefferens*, urease activity per cell showed a significant positive relationship with growth rates, for the cultures grown both on urea ($P < 0.05$, $n = 7$) and on NO_3^- ($P < 0.05$, $n = 7$; Fig. 2). For *P. minimum*, this positive trend for urease activity with growth rate was only observed for cultures grown on urea and NH_4^+ ($P < 0.05$, $n = 6$; Fig. 2); there was no significant relationship between urease activity and growth rate for cultures grown on NO_3^- ($P > 0.05$, $n = 6$). For *T. weissflogii*, overall urease activity did not vary with culture growth rate, but higher activities were observed exclusively when the cells were grown on NH_4^+ and urea, while lower activities were observed when the cells were grown on NO_3^- ($P < 0.05$, $n = 6$; Fig. 2). For *A. anophagefferens* and *P. minimum*, higher urease activity was also observed during the exponential phase of the culture, when growth rates were higher than in early exponential and early stationary phases. However, this relationship was not found for *T. weissflogii* urease activity (Fig. 2D–F).

Urease activity as a function of substrate concentration

The kinetics of urease as a function of substrate (urea) concentration for these three phytoplankton species followed the general kinetics of enzyme activity and was described by a Michaelis–Menten kinetics model (Fig. 3; Table 2). The values of K_m for urease for the

Table 1 Summary of the urease activities and growth rates ($\pm \text{SE}$) of three phytoplankton species grown on NO_3^- , NH_4^+ , and urea media. Data represent mean ($\pm \text{SE}$) values for the number of experiments shown. All measurements were conducted during mid-exponential growth phase

Species	Culture medium	<i>n</i>	Urease activity (fg atom N cell ⁻¹ h ⁻¹)	Culture growth rate (day ⁻¹)
<i>Aureococcus anophagefferens</i>	NO_3^-	17	6.54 \pm 2.07	0.54 \pm 0.13
	Urea	17	6.03 \pm 1.53	0.66 \pm 0.24
<i>Prorocentrum minimum</i>	NO_3^-	32	61.75 \pm 10.74	0.47 \pm 0.17
	Urea	32	64.96 \pm 12.35	0.58 \pm 0.12
	NH_4^+	6	48.47 \pm 22.02	0.56 \pm 0.13
<i>Thalassiosira weissflogii</i>	NO_3^-	6	19.80 \pm 5.67	1.63 \pm 0.08
	Urea	6	45.07 \pm 13.68	0.77 \pm 0.13
	NH_4^+	6	44.71 \pm 9.23	1.39 \pm 0.23

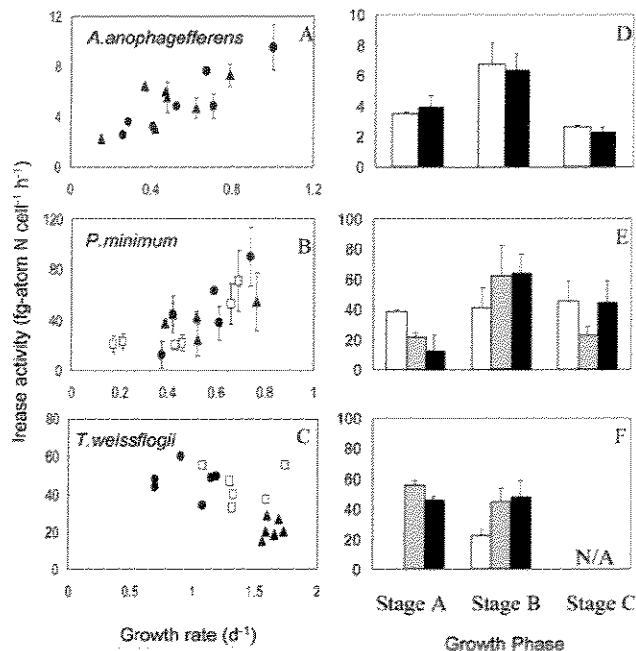


Fig. 2 Urease activity as a function of growth rate for *Aureococcus anophagefferens* (A), *Prorocentrum minimum* (B), and *Thalassiosira weissflogii* (C) grown on urea (circles), NH_4^+ (squares) and NO_3^- (triangles). For *A. anophagefferens*, each plotted point, with error bar (\pm SE), represents three measurements derived from individual batch cultures. For *P. minimum* grown on NH_4^+ , error bars represent six measurements from replicated cultures. Panels D–F show data replotted as a function of growth phase (stage A early exponential phase; stage B exponential phase; stage C early stationary phase). Open bars represent cultures grown on NO_3^- , grey bars represent cultures grown on NH_4^+ , and black bars represent cultures grown on urea. Data for *T. weissflogii* at early stationary phase are not available (N/A)

three species were in the same range (120–165 $\mu\text{g atom N l}^{-1}$; Table 2). However, as these species vary in size and in vacuolar content, the calculated ρ_{max} varied considerably between species. Values of ρ_{max} also were dependent on the unit by which this parameter was normalized. On a cellular basis, the ρ_{max} ranged from a low of $< 5 \text{ fg atom N cell}^{-1} \text{ h}^{-1}$ in *A. anophagefferens* to a high of $> 100 \text{ fg atom N cell}^{-1} \text{ h}^{-1}$ in *P. minimum* (Table 2). However, *A. anophagefferens* had the highest urease activity per cell volume, $0.77 \text{ fg atom N } \mu\text{m}^{-3} \text{ h}^{-1}$, roughly sixfold higher than that for *P. minimum*, and more than an order of magnitude higher than that for *T. weissflogii* (Table 2). As diatoms such as *T. weissflogii* have large vacuoles, the use of cell volume for normalization may underestimate biomass-specific activity in these cells. Furthermore, the α (ρ_{max}/K_m) value for urease activity, which is an index of urease affinity, showed no difference among these three species (Table 2).

The activity of urease and the kinetic ranges reported are based on nitrogen-sufficient cells. The intracellular concentrations of urea were highest for *A. anophagefferens* and lowest for *T. weissflogii* (Table 2), and these

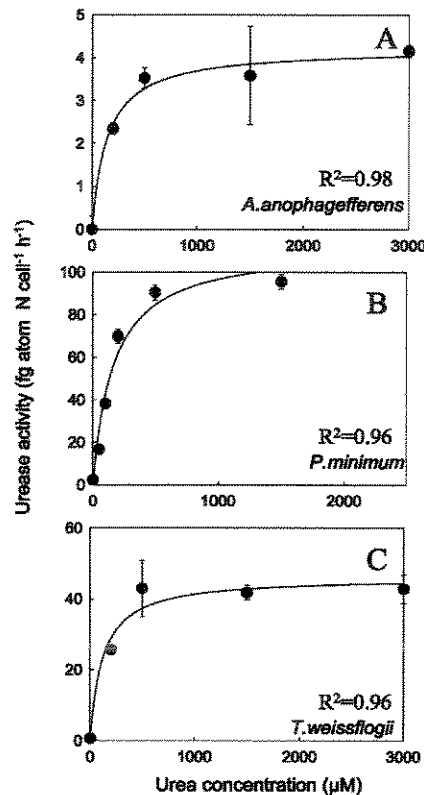


Fig. 3 Urease activity as a function of urea concentration for *Aureococcus anophagefferens* (A), *Prorocentrum minimum* (B), and *Thalassiosira weissflogii* (C) grown on urea. Each plotted point, with error bar (\pm SE), represents three measurements derived from individual batch cultures

intracellular concentrations were all higher than the K_m of urease for these studied species.

Urease activity as a function of temperature

The in vitro activity of urease varied with temperature, but the extent of this variation differed among species (Fig. 4). For all species tested, there was an increase in enzyme activity with temperature from 0°C to 20°C . However, for *A. anophagefferens*, at temperatures of 20 – 50°C , the activity of urease increased further, while, for the other two species, there appeared to be either a leveling off of activity, as in the case of *P. minimum*, or an actual decline, as in the case of *T. weissflogii*.

Urease activity as a function of time of day

Variations in urease activity with time of day were determined for two of the three species. For *A. anophagefferens*, urease activity was higher during the day and lower at night (Fig. 5). For *P. minimum*, urease activity was more variable, both with respect to light and dark conditions and nitrogen growth source. For the

Table 2 Urease kinetics parameters (\pm SE) for the three study species. The half saturation constant (K_m) is expressed in micrograms atomic nitrogen per liter, maximum urease activity (ρ_{max}) is expressed both on a per cell and per cell volume basis, and affinity index α is based on volumetric comparisons. Intracellular urea

Species	K_m ($\mu\text{g atom N l}^{-1}$)	ρ_{max} (fg atom N cell $^{-1}$ h $^{-1}$)	ρ_{max} (fg atom N μm^{-3} h $^{-1}$)	α (h $^{-1}$)	Intracellular urea concentration (mg atom N l $^{-1}$)
<i>Aureococcus anophagefferens</i>	144 \pm 39.4	4.22 \pm 0.22	0.76 \pm 0.04	0.68	4.65 \pm 0.31
<i>Prorocentrum minimum</i>	165 \pm 45.7	112.48 \pm 9.94	0.12 \pm 0.01	0.71	2.71 \pm 0.21
<i>Thalassiosira weissflogii</i>	120 \pm 53.8	46.26 \pm 3.6	0.01 \pm 0.001	0.64	0.42 \pm 0.08

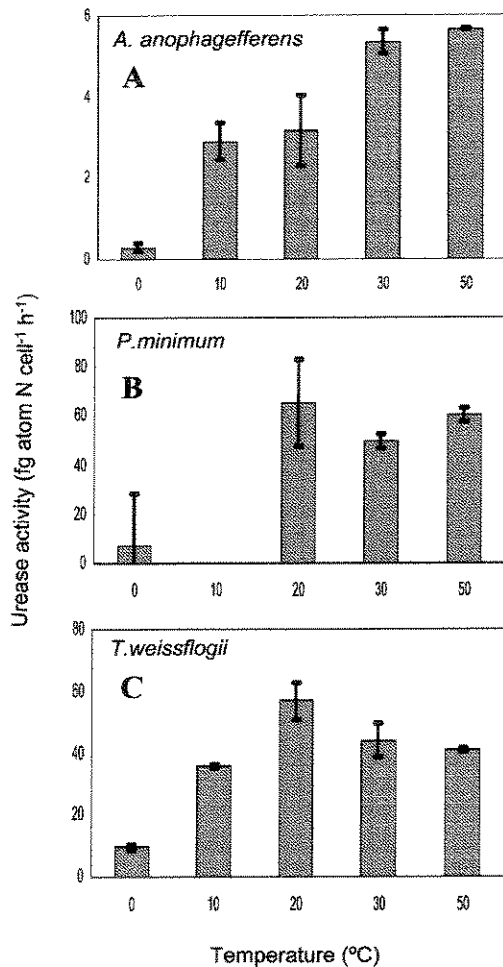


Fig. 4 In vitro urease activity as a function of assay temperature for *Aureococcus anophagefferens* (A), *Prorocentrum minimum* (B), and *Thalassiosira weissflogii* (C) grown on urea. Each bar, with error bar (\pm SE), represents three measurements derived from individual batch cultures

urea-grown *P. minimum* culture, urease activity was not significantly different between day and night ($P > 0.05$, $n = 12$), while for the NO_3^- -grown culture, the highest urease activity was expressed near noon, ~ 4 h after lights came on, and the lowest activity was expressed ~ 6 h after the lights went off (Fig. 5).

concentrations (\pm SE) for three phytoplankton species are also indicated. All cultures were grown on urea as the growth N source and measurements were determined at mid-exponential growth phase

Discussion

The role of urea in aquatic nitrogen cycling is receiving increasing attention as it is now recognized as a dynamic component that contributes significantly to phytoplankton nutrition, and potentially to species dynamics (Berg et al. 1997; Glibert and Terlizzi 1999). However, there are very few studies that have focused on the regulation of metabolism of urea relative to the wealth of studies on the metabolism of inorganic nitrogen. For aquatic phytoplankton, central to the study of urea metabolism is an understanding of the dynamics of urease. A decade ago, virtually all that was known about urea uptake and metabolism came from studies based on chlorophytes and diatoms (Antia et al. 1991); this remains largely true today.

The present study focused on several bloom-forming algae from different taxonomic groups. *Aureococcus anophagefferens* and *Prorocentrum minimum*, are commonly observed in the spring and summer in mid-Atlantic coastal and estuarine waters, and their abundance has previously been shown to correlate with urea concentrations in the water column and with high rates of urea uptake (Berg et al. 1997; Glibert et al. 2001). Since *A. anophagefferens* and *P. minimum* have been associated with increased urea availability in the water column, nitrogen-sufficient conditions were used in this study.

Species differences in urease activity

One of the most significant findings of this study was that urease activity was expressed in all three phytoplankton species regardless of the nitrogen source. Urea uptake has been previously shown to be a constitutive property of many phytoplankton species growing on nitrate or urea (e.g. Singh 1990) for two freshwater cyanobacteria (*Anacystis nidulans* and *Nostoc muscorum*). However, uptake of urea and urease expression in cells growing on NH_4^+ were previously thought to require an induction period (Antia et al. 1991). For *Thalassiosira weissflogii* and *P. minimum* in our study, this was not the case. Inasmuch as *A. anophagefferens* did not grow on NH_4^+ , the relationship between NH_4^+ availability and urease activity is yet to be determined. This study

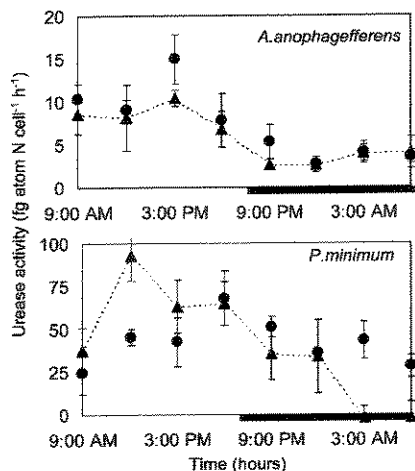


Fig. 5 Urease activity as a function of time of day for *Aureococcus anophagefferens* (upper panel) and *Prorocentrum minimum* (lower panel) grown on NO_3^- (triangles) and urea (circles). Each plotted point, with error bar (\pm SE), represents three measurements derived from individual batch cultures. Black bar represents 12 h of darkness

indicates that these organisms may remain poised to assimilate urea when it becomes available, even if they have been primarily growing on an alternate nitrogen source. Moreover, for *A. anophagefferens* and *P. minimum* grown on urea, urease activity was positively correlated with growth rate, suggesting that urease is regulated by growth within the cells (Fig. 2).

Urease activity in the diatom *T. weissflogii*, however, differed from activity in the other species, both as a function of growth rate and of nitrogen source, indicating that its regulation within the cell may be different from that of the other two species. A similar variation in urease activity as a function of nitrogen source was also observed for the diatom *Cyclotella cryptica* (Oliveira and Antia 1986). In that study, rates of urease activity for cells grown on NO_3^- were about 75% those of urea-grown cells, and the addition of urea induced urease activity. In the present study, urease activity in *T. weissflogii* grown on NO_3^- was <50% of that in NH_4^+ and urea-grown cells. Previously, however, Peers et al. (2000) found, for the same diatom, that urease expression did not differ with nitrogen source in nitrogen-replete conditions. Peers et al. (2000) also found that urease activity in the diatom *T. pseudonana* was low in NH_4^+ -grown cultures and was induced by the additions of NO_3^- and urea.

The urease of all species tested had a similar affinity for urea, when grown on urea as the growth N source, as shown by comparable K_m and α values (Table 2; Fig. 3). Variability observed in the maximal velocity (ρ_{max}) of the enzyme reaction is a function of cell size and cell volume. Cell size and volume are also related to the internal concentration of nutrient within these cells. In this study, cultures were grown under nitrogen-replete conditions, so the intracellular urea concentrations reflected

near-maximal values. In all cases, the internal concentrations were higher than the K_m value of the enzyme. The diatom *T. weissflogii* has previously been shown not to accumulate urea in high concentration in internal pools, even when retaining the ability to utilize urea for growth (Conover 1975; Oliveira and Antia 1986; Antia et al. 1991), and our results for internal urea concentrations in *T. weissflogii* also showed a significantly lower concentration than for the other species (Table 2).

Ambient urea concentrations in estuarine and coastal waters are normally <1 μM , although significantly higher concentrations, up to 10 μM , have been reported for the southern German Bight and Chesapeake Bay (Turley 1986; Glibert et al. 2001). However, these ambient urea concentrations are still very low compared to urease K_m values measured in the present study. Thus, the extent to which phytoplankton can maintain either a high intracellular urea concentration under low ambient urea concentration or a high uptake rate of urea into the cells will determine the importance of urea in phytoplankton nutrition. Based on rates of urea uptake in a previous study of a natural *P. minimum* bloom (Fan et al., unpublished data, 0.98–21.22 fg atom N cell⁻¹ h⁻¹) and measurements of cell volume from the present study, *P. minimum* would have the ability to build an internal urea pool of 2–40 mg atom N l⁻¹ in 1 h in an ambient concentration of 0.5 μg atom N l⁻¹, if assimilation and release were negligible. If assimilation were occurring, higher uptake rates would be needed to saturate the activity of urease. However, the mechanism by which these phytoplankton take up urea and maintain a high internal pool of urea is still unclear.

Internal pools of urea may also be a product of another metabolic pathway, in addition to transport across the cell membrane. This pathway, the urea cycle, converts surplus nucleic acid bases (purines and pyrimidines) to simpler amino acids and urea. This cycle is also thought to be regulated by the nitrogen content of the phytoplankton cells (Antia and Landymore 1975). Although energy-requiring, this pathway is a means for cells to conserve nutrients. Urea produced by this pathway is then available for assimilation within the cell, the same as urea acquired from the external medium. Regulation of the urea cycle within these species, or the extent to which this process may have contributed to internal urea pools in these experiments, is not understood at this time.

Differences in urease activity among the study species were also observed in the enzymatic responses to changes in temperature and to day–night cycles. Although the temperatures tested in this study encompassed a wider range than would normally be encountered in the field for these species, they do give an indication of the general responses to temperature shifts from ambient growth conditions. For *P. minimum* and *T. weissflogii*, highest activities were observed at 20°C. For *A. anophagefferens*, activity continued to increase up to 50°C, with a near doubling of activity as temperatures were elevated well above 20°C (Fig. 4). Many enzymes

start to denature at temperatures near 50°C. On the other hand, one of the common methodologies for assaying urea concentrations involves the enzymatic degradation of urea to NH_4^+ (McCarthy 1972). This assay is carried out at 50°C, and even 60°C under some modified protocols, to optimize the reaction. Jahns et al. (1995) also reported that urease from two cyanobacteria was heat stable.

Differences in urease activity were also noted with time of day for the two non-diatom species, and were also a function of the nitrogen source. For urea-grown cells, *A. anophagefferens* had a clear day–night difference in activity, while *P. minimum* maintained similar activity throughout the day. For NO_3^- -grown cells, however, both species had generally lower urease activity levels in the dark than in the light (Fig. 5).

Urea activity and nitrogen demand

If urease activity is indeed expressed under virtually all growth conditions examined, it is interesting to calculate the contribution of urea to the cellular nitrogen demand. Peers et al. (2000) first reported that urease activity in two marine diatoms could account for the cell nitrogen assimilation rate in a urea medium. In our study, urease activity and daily nitrogen demand can be calculated on an hourly basis for all three species grown on urea, and for *A. anophagefferens* and *P. minimum* comparisons of urease activity and nitrogen demand can also be made on a daily basis.

To calculate the hourly nitrogen demand, the particulate nitrogen per cell content for each species was multiplied by the mean daily growth rate and then divided by 24. For *A. anophagefferens*, the urease activity based on daylight-measured rates exceeded the nitrogen demand for cell growth nearly fourfold, while for *P. minimum*, the urease activity nearly balanced the nitrogen demand for cell growth (Fig. 6). On the other hand, for the diatom *T. weissflogii*, urease activity was not sufficient to account for the cellular nitrogen demand (Fig. 6).

Daily rates may differ from hourly rates due to diel patterns in activity. When a daily rate was estimated, based only on the number of hours during which urease activity was expressed, the rates for *A. anophagefferens* and *P. minimum* were lower than the estimated hourly rates. For *A. anophagefferens*, however, even on a daily basis, the activity of the enzyme still exceeded the nitrogen demand of the cells. The daily urease activity for *A. anophagefferens* was $0.16 \text{ pg atom N cell}^{-1} \text{ day}^{-1}$, fourfold higher than the daily demand of $0.04 \text{ pg atom N cell}^{-1} \text{ day}^{-1}$. For this species, the only species for which urease activity appeared to exceed both the hourly and the daily nitrogen demand, there are important implications for maintaining such high activity. The result could be a flux of NH_4^+ from the cells, due to the high rate of urea hydrolysis and an excess of NH_4^+ that would not be assimilated by the cells (Price and Harrison 1988).

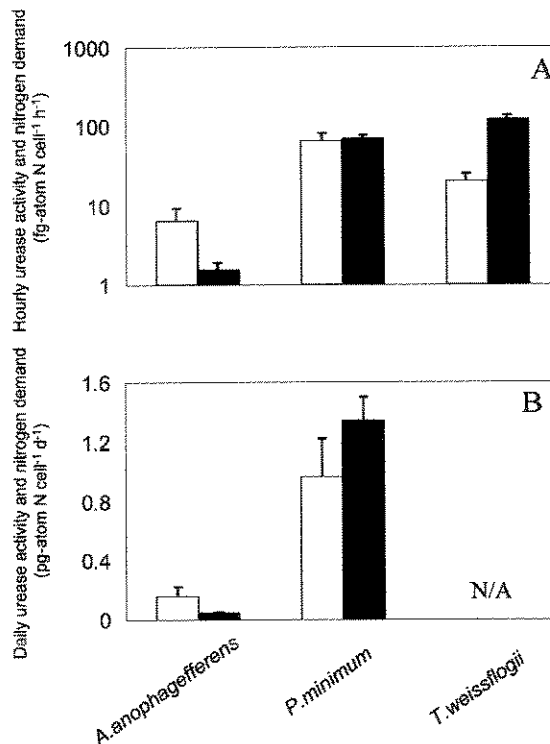


Fig. 6 **A** Comparison of urease activity (Open bars) versus nitrogen demand (solid bars) calculated on an hourly basis for *Aureococcus anophagefferens*, *Prorocentrum minimum*, and *Thalassiosira weissflogii* grown with urea as nitrogen substrate. **B** Same comparison calculated on a daily basis for *A. anophagefferens* and *P. minimum*. Each bar, with error bar (\pm SE), represents three measurements derived from individual batch cultures

For *P. minimum*, urease activity, which on an hourly basis under daylight conditions nearly balanced the nitrogen demand, was lower on a daily basis ($0.96 \text{ pg atom N cell}^{-1} \text{ day}^{-1}$) than the daily nitrogen demand ($1.34 \text{ pg atom N cell}^{-1} \text{ day}^{-1}$). The daily rate for *T. weissflogii* cannot be calculated from the data presented, but, were there a diel change in activity, it would only exaggerate the discrepancy observed on an hourly basis.

Comparisons between urease and nitrate reductase activity

In a recent study (Lomas and Glibert 2000), two of the same test species, *T. weissflogii* and *P. minimum* were compared in similar batch experiments focused on NO_3^- reductase activity. The differences between species, and between enzyme activities of NO_3^- reductase and urease, can be compared (Table 3). For *T. weissflogii*, whereas the internal urea concentrations were relatively low and the extent to which urea contributed to the nitrogen demand was also lowest of the species studied, for NO_3^- reductase a vastly different pattern was observed. In that case, very large internal NO_3^- pools were measured, and the uptake of NO_3^- was nearly twice as high as the

Table 3 *Thalassiosira weissflogii*, *Prorocentrum minimum*. Comparison of biochemical parameters for NO_3^- reductase (NR) and urease for N-sufficient, semi-continuous batch cultures. Results for NR from Lomas and Glibert (2000)

	<i>T. weissflogii</i>		<i>P. minimum</i>	
	NO_3^- concentration or NR activity	Urea concentration or urease activity	NO_3^- concentration or NR activity	Urea concentration or urease activity
Internal nutrient concentration (mg atom N l^{-1})	40.1	0.42	Not detected	2.71
Enzyme K_m (mg atom N l^{-1})	0.05	0.12	1.16	0.17
Cell-specific activity (fg atom $\text{N cell}^{-1} \text{ h}^{-1}$)	91	20	31	65
Ratio of cell-specific activity to demand (hourly basis)	1.9	0.17	0.3	0.97

nitrogen demand (Table 3). Furthermore, the K_m for NO_3^- reductase activity was quite low, 0.05 mg atom N l^{-1} , indicating very high affinity for this substrate, while that for urease was higher, 0.12 mg atom N l^{-1} . The enhanced capacity of *T. weissflogii* to take up and reduce NO_3^- over that needed to balance the nitrogen demands of the cells may be independent of nutritional requirements. This capacity has been hypothesized to compensate short-term energy imbalances in the cell, which may occur when cells are exposed to environmental conditions such as rapid changes in light or temperature (Lomas and Glibert 1999, 2000).

For *P. minimum*, the affinity for NO_3^- , as measured by the K_m for NO_3^- reductase was much lower than that for urea (Lomas and Glibert 1999). The K_m for NO_3^- reductase was about seven-fold higher than that for urease for this species (Table 3). Internal NO_3^- concentrations were not detectable (Lomas and Glibert 1999), whereas large internal urea concentrations were measured in the present study (e.g. Table 2). Furthermore, when activity was compared to nitrogen demand for *P. minimum*, there was rough equality with urease activity on an hourly basis, but insufficient NO_3^- reductase activity to meet the nitrogen demand (Table 3).

Ecological implications

There is considerable evidence that different species of phytoplankton tend to dominate under environmental regimes differing in their nutritional quality (Paerl 1988; Smayda 1990, 1997; Lomas and Glibert 1999). One measure of nutritional quality is the type of nitrogen available. Of the three phytoplankton species in this study, blooms of the non-diatom species *A. anophagefferens* and *P. minimum* tend to be correlated with low NO_3^- and higher NH_4^+ and/or DON supply (Glibert et al. 2001; Lomas et al. 2001). In fact, in tributaries of Chesapeake Bay, high urea concentrations were found to precede large blooms of *P. minimum* in spring (Glibert et al. 2001). Also, comparisons of uptake kinetics suggest that these species prefer to take up NH_4^+ and urea over NO_3^- (Berg et al. 1997; Fan, unpublished data). Similarly, high concentrations of other dinoflagellates have been shown to be associated with either elevated concentrations of urea or elevated rates of

uptake of this nitrogen source (Glibert and Terlizzi 1999; Kudela and Cochlan 2000). By contrast, diatom blooms are more frequently associated with conditions of higher NO_3^- supply, as they have physiological adaptations that permit them to exploit NO_3^- (Takahashi and Fukazawa 1982; Lomas and Glibert 1999, 2000). Enzyme regulation, including urease and NO_3^- reductase, is central to the physiological regulation of nitrogen uptake by phytoplankton. The differences in enzyme activities reported here are consistent with diatoms having greater affinity for NO_3^- uptake and reduction, while the dinoflagellate and pelagophyte studied here have higher affinity for urea. Ecologically, there are many other factors that contribute to the high abundance of one species over another, including turbulence in the water column (e.g. Margalef 1978) and the rate of grazing. As we gain a further understanding of the factors regulating these enzymes in different species, we will gain a better understanding of their competitive ability in different environments.

Acknowledgements We thank the Analytical Services Laboratories of the Horn Point Laboratory for technical assistance. Additional assistance was provided by E. Haramoto. This study was supported by the U.S. ECOHAB Program, sponsored by the National Oceanic and Atmospheric Administration, U.S. Environmental Protection Agency, National Science Foundation, National Aeronautics and Space Administration, and Office of Naval Research. This is contribution number 3628 from the University of Maryland Center for Environmental Science, and number 64 from the U.S. ECOHAB Program.

References

- Antia NJ, Landymore AF (1975) The non-biological oxidative degradation of dissolved xanthopterin and 2,4,6-trihydroxypteridine by the pH or salt content of seawater. *Mar Chem* 3:347–363
- Antia NJ, Berland BR, Bonin DJ, Maestrini SY (1977) Effects of urea concentration in supporting growth of certain marine microplanktonic algae. *Phycologia* 16:105–111
- Antia NJ, Harrison PJ, Oliveira L (1991) The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology, and ecology. *Phycologia* 30:1–89
- Bekheet IA, Syrett PJ (1977) Urea-degrading enzymes in algae. *Br Phycol J* 12:137–143
- Berg GM, Glibert PG, Lomas ML, Burford M (1997) Organic nitrogen uptake and growth by the chrysophyte *Aureococcus anophagefferens* during a brown tide event. *Mar Biol* 129:377–387

- Berg GM, Repeta DJ, Laroche J (2002) Dissolved organic nitrogen hydrolysis rates in axenic cultures of *Aureococcus anophagefferens* (Pelagophyceae): comparison with heterotrophic bacteria. *Appl Environ Microbiol* 68:400–404
- Berges JA, Montagnes DJS, Hurd CL, Harrison PJ (1994) Fitting ecological and physiological data to rectangular hyperbolae: a comparison of methods using Monte Carlo simulations. *Mar Ecol Prog Ser* 114:175–183
- Bronk DA, Glibert PM, Malone TC, Banahan S, Sahlsten E (1998) Inorganic and organic nitrogen cycling in Chesapeake Bay: autotrophic versus heterotrophic processes and relationships to carbon flux. *Aquat Microb Ecol* 15:177–189
- Carpenter EJ, Remsen CC, Watson SW (1972) Utilization of urea by some marine phytoplankters. *Limnol Oceanogr* 17:265–269
- Conover SAM (1975) Partitioning of nitrogen and carbon in cultures of the marine diatom *Thalassiosira fluviatilis* supplied with nitrate, ammonium, or urea. *Mar Biol* 32:231–246
- Cosper EM, Dennison WC, Milligan A, Carpenter EJ, Lee C, Holzapfel J, Milanese L (1989) An evaluation of the environmental factors important to initiating and sustaining "brown tide" bloom. In: Cosper EM, Carpenter EJ, Bricelj WM (eds) *Novel phytoplankton blooms: causes and impacts of recurrent brown tide and other unusual blooms*. Springer, Berlin Heidelberg New York, pp 391–340
- Furnas MJ (1983) Nitrogen dynamics in lower Narragansett Bay, Rhode Island. 1. Uptake by size-fractionated phytoplankton populations. *J Plankton Res* 5:657–676
- Glibert PM (1998) Interactions of top-down and bottom-up control in planktonic nitrogen cycling. *Hydrobiologia* 363:1–12
- Glibert PM, Terlizzi DE (1999) Co-occurrence of elevated urea levels and dinoflagellate blooms in temperate estuarine aquaculture ponds. *Appl Environ Microbiol* 65:5594–5596
- Glibert PM, Garside C, Fuhrman JA, Roman MR (1991) Time-dependent coupling of inorganic and organic nitrogen uptake and regeneration in the plume of the Chesapeake Bay estuary and its regulation by large heterotrophs. *Limnol Oceanogr* 36:895–909
- Glibert PM, Conley DJ, Fisher TR, Harding LW, Malone TC (1995) Dynamics of the 1990 winter/spring bloom in Chesapeake Bay. *Mar Ecol Prog Ser* 122:27–43
- Glibert PM, Magnien R, Lomas MW, Alexander J, Fan C, Haramoto E, Trace M, Kana TM (2001) Harmful algal blooms in the Chesapeake and coastal bays of Maryland, USA: comparison of 1997, 1998, and 1999 events. *Estuaries* 24:875–883
- Harrison WG, Head EJH, Conover RJ, Longhurst AR, Sameoto DD (1985) The distribution and metabolism of urea in the eastern Canadian Arctic. *Deep-Sea Res* 32:23–42
- Harvey WA, Caperon J (1976) The rate of utilization of urea, ammonium, and nitrate by natural populations of marine phytoplankton in a eutrophic environment. *Pac Sci* 30:329–340
- Jahns T, Schafer U, Kaltwasser H (1995) Heat-stable ureases from two filamentous cyanobacteria. *Microbiology* 141:737–741
- Kaufman ZG, Lively JS, Carpenter EJ (1983) Uptake of nitrogenous nutrients by phytoplankton in a barrier island estuary: Great South Bay, New York. *Estuar Coast Shelf Sci* 17:483–493
- Kirchman DL (1990) Limitation of bacterial growth by dissolved organic matter in the subarctic Pacific. *Mar Ecol Prog Ser* 62:47–54
- Kudela RM, Cochlan WP (2000) Nitrogen and carbon uptake kinetics and the influence of irradiance for a red tide bloom off southern California. *Aquat Microb Ecol* 21:31–47
- Leftley JW, Syrett PJ (1973) Urease and ATP:Urea amidolyase activity in unicellular algae. *J Gen Microbiol* 77:109–115
- Lomas MW, Glibert PM (1999) Temperature regulation of nitrate uptake: a novel hypothesis about nitrate uptake and reduction in cool-water diatoms. *Limnol Oceanogr* 44:556–572
- Lomas MW, Glibert PM (2000) Comparisons of nitrate uptake, storage, and reduction in marine diatoms and flagellates. *J Phycol* 36:903–913
- Lomas MW, Glibert PM, Clougherty DA, Huber DE, Jones J, Alexander J, Haramoto E (2001) Elevated organic nutrient ratios associated with brown tide blooms of *Aureococcus anophagefferens* (Pelagophyceae). *J Plankton Res* 23:1339–1344
- Margalef R (1978) Life-forms of phytoplankton as survival alternatives in an unstable environment. *Oceanol Acta* 1:493–509
- McCarthy JJ (1972) The uptake of urea by natural populations of marine phytoplankton. *Limnol Oceanogr* 17:738–748
- McCarthy JJ (1977) Nitrogenous nutrition of the plankton in the Chesapeake Bay. 1. Nutrient availability and phytoplankton preferences. *Limnol Oceanogr* 22:996–1011
- McCarthy JJ (1980) Nitrogen and phytoplankton ecology. In: Morris I (ed) *The physiological ecology of phytoplankton*. Blackwell, Oxford, pp 191–233
- Mobley HLT, Hausinger RP (1989) Microbial urease: significance, regulation, and molecular characterization. *Microbiol Rev* 53:85–108
- Oliveira L, Antia NJ (1986) Some observations on the urea-degrading enzyme of the diatom *Cyclotella cryptica* and the role of nickel in its production. *J Plankton Res* 8:235–242
- Paerl HW (1988) Nuisance phytoplankton blooms in coastal, estuarine, and inland waters. *Limnol Oceanogr* 33:823–847
- Parsons TR, Maita Y, Lalli CM (1984) *A manual of chemical and biological methods for seawater analysis*. Pergamon, New York
- Peers SG, Milligan AJ, Harrison PJ (2000) Assay optimization and regulation of urease activity in two marine diatoms. *J Phycol* 36:523–528
- Price NM, Harrison PJ (1988) Uptake of urea C and N by the coastal marine diatom *Thalassiosira pseudonana*. *Limnol Oceanogr* 33:528–537
- Singh S (1990) Regulation of urease activity in the cyanobacterium *Anabaena doliolum*. *FEMS Microbiol Lett* 67:79–84
- Smayda TJ (1990) Novel and nuisance phytoplankton blooms in the sea: evidence for a global epidemic. In: Graneli E, Sundstroem B, Edler L, Anderson DM (eds) *Proceedings of the 4th international conference on toxic marine phytoplankton*. Toxic marine phytoplankton. Elsevier, New York, pp 29–40
- Smayda TJ (1997) Harmful algal blooms: their ecophysiology and general relevance to phytoplankton blooms in the sea. *Limnol Oceanogr* 42:1137–1153
- Takahashi M, Fukazawa N (1982) A mechanism of "red-tide" formation. 2. Effect of selective nutrient stimulation on the growth of different phytoplankton species in natural water. *Mar Biol* 70:267–273
- Thomas WH (1968) Nutrient requirements and utilization: algae. In: Altman PL, Dittmer DS (eds) *Metabolism*. Federation of American Societies for Experimental Biology, Bethesda, Md., pp 210–228
- Turley CM (1986) Urea uptake by phytoplankton at different fronts and associated stratified and mixed waters on the European shelf. *Br Phycol J* 21:225–238