



Characterization of the affinity for nitrogen, uptake kinetics, and environmental relationships for *Prorocentrum minimum* in natural blooms and laboratory cultures

Chunlei Fan^{a,*}, Patricia M. Glibert^a, JoAnn M. Burkholder^b

^a Horn Point Laboratory, Center for Environmental Science, University of Maryland, P.O. Box 775, Cambridge, MD 21613, USA

^b Center for Applied Aquatic Ecology, North Carolina State University, 620 Hutton Street Suite 104, Raleigh, NC 27606, USA

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Abstract

During the late spring and early summer of 1998, an extensive bloom of the dinoflagellate *Prorocentrum minimum* (>93% of phytoplankton cell density) developed in several tributaries of the Chesapeake Bay, USA. In January 1999, a bloom of mixed dinoflagellates (*Heterocapsa rotundata*, *H. triquetra* and *P. minimum*, with *P. minimum* forming 21% of total phytoplankton cells and 39% of the total biovolume) developed in the mesohaline Neuse Estuary, North Carolina, USA. During these blooms, experiments were carried out to characterize the nitrogen uptake kinetics of these assemblages with ¹⁵N isotopic techniques. Four nitrogenous substrates (NO₃⁻, NH₄⁺, urea, and a mixed amino acids substrate) were used to determine uptake rate and substrate preference. Rates of nitrogen uptake were also measured in *P. minimum* cultures grown on varying growth nitrogen substrates. The calculated kinetic parameters determined for the *P. minimum*-dominated field assemblages and the cultures indicated a preference for NH₄⁺. NH₄⁺ was also the primary nitrogen source supporting the blooms. In addition, a high affinity for urea was also found, and urea contributed significantly to the Neuse Estuary bloom. Furthermore, results showed that the regulation of uptake for each of the substrates was different: strong positive relationships between affinity and temperature were found for NH₄⁺ and amino acids, while a negative response was found for NO₃⁻, and very little response to temperature was noted for urea. These differences suggest that a diversity of nitrogen uptake mechanisms may aid the development and maintenance of *P. minimum* blooms.

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1. Introduction

Prorocentrum minimum (Pavillard) Schiller is a common bloom-forming, photosynthetic dinoflagellate in temperate and subtropical areas. Large blooms have been reported along the East Coast of the USA

(Pierce and Turner, 1994; West et al., 1996; Stoecker et al., 1997), the north Pacific (Zou et al., 1985; Kondo et al., 1990; Qi et al., 1993), the northeastern Atlantic (Yallop, 2001), the Mediterranean Sea (Grzebyk and Berland, 1996), and the coasts of Australia (Cannon, 1990). During recent years, numerous blooms of *P. minimum* have been documented in areas affected by freshwater inputs and/or anthropogenic nutrient inputs (Smayda, 1990; Mallin, 1994; Moncheva et al.,

* Corresponding author.

E-mail address: cfan@hpl.umces.edu (C. Fan).

1995; Burns et al., 2000). It has been suggested that increased nutrient loading to these near-shore waters, together with changes in the nutrient composition, may contribute to blooms of this dinoflagellate species (Paerl, 1988; Glasgow and Burkholder, 2000; Glibert et al., 2001).

There has been considerable research on *P. minimum* in the field and in the laboratory, and this species is relatively easy to grow in culture (Granéli and Persson, 1982; Granéli and Moreira, 1990; Berland and Grzebyk, 1991; Lomas and Glibert, 2000). *P. minimum* has a broad range of tolerance to salinity, light, and temperature (Grzebyk and Berland, 1996). *P. minimum* can survive and grow under low light and/or nutrient conditions (Tyler and Seliger, 1981; Harding, 1988; Harding and Coats, 1988 [note that *Prorocentrum mariae-lebouriae* described in the latter two references has been renamed as *P. minimum*; Tomas, 1997]). Under natural nutrient-limited conditions *P. minimum* can maintain a nightly migration into the pycnocline where more nutrients are available (Tyler and Seliger, 1981). Stoecker et al. (1997) also showed that *P. minimum* can supplement its nitrogen and carbon demand through mixotrophic grazing on cryptophytes under nutrient stressed conditions.

Although *P. minimum* has been extensively studied in both estuaries and laboratory cultures, relatively little is known about its nutrient requirements and preferences leading to large blooms, and the maintenance of these blooms. Extensive *P. minimum* blooms formed in several tributaries of Chesapeake Bay during late spring 1998, and also in a mixed-species assemblage in the Neuse Estuary of the Albemarle–Pamlico Estuarine System during winter 1999. The two estuarine systems are the largest and second largest, respectively, on the US mainland (Epperley and Ross, 1986). These blooms afforded the opportunity to examine the nitrogen nutrition of *P. minimum*, alone and with other abundant species under variable field conditions. These data represent the first study in which rates of utilization of NO_3^- , NH_4^+ , urea, and dissolved free amino acids by *P. minimum* were simultaneously examined. To gain additional insights with regard to nitrogen nutrition of *P. minimum*, rates of uptake of the same substrates were determined on laboratory-grown cultures of *P. minimum*.

2. Materials and methods

2.1. Choptank River sampling and environmental parameters

The Choptank River is a major tributary on the eastern shore of Chesapeake Bay. An extensive *P. minimum* bloom occurred in this river during mid-to late spring of 1998. This bloom lasted more than 4 weeks, and was sampled from 7 to 29 May 1998, from the dock of Horn Point Laboratory, located on the Choptank River (Fig. 1). During the bloom, water temperature and salinity were measured using a YSI 85 hand-held meter (YSI Inc.) and in situ irradiance was measured with a Biospherical Instruments QSL-100 light meter. At 3–4-day intervals, ambient nutrient levels were characterized and intensive experiments were carried out to assess rates of nitrogen uptake. The sampling time was ca. 1300 h for all experiments. Water samples were taken from the surface using an acid-cleaned bucket, and stored in acid-cleaned 20 l carboys. The carboys were transferred to the laboratory immediately. Ambient nutrient and chlorophyll samples were filtered through pre-combusted (500 °C for 2 h) Whatman GF/F filters (0.7 μm nominal porosity) and frozen immediately for storage until further analysis. The filters were retained for analysis of particulate nitrogen (PN), particulate carbon (PC) and chlorophyll *a*.

Ambient inorganic nitrogen (NO_3^- , NO_2^- , and NH_4^+) concentrations were measured using a Technicon AutoAnalyzer, and urea samples were analyzed manually using a spectrophotometer with the method of Parsons et al. (1984). Dissolved free amino acids (DFAAs) were analyzed with the method of Lindroth and Mopper (1979). Particulate nitrogen (PN) and particulate carbon (PC) were measured on a Control Equipment Elemental Analyzer using acetanilide as a standard. Samples for chlorophyll *a* analysis were ground in 90% acetone on ice and concentrations determined fluorometrically (Parsons et al., 1984) on a Turner Designs Model 10 fluorometer calibrated against an HPLC-measured chlorophyll *a* standard. Cell counts were measured using a Coulter Counter (Coulter Multisizer II) in glutaraldehyde (1% final) preserved water samples. Samples were also examined microscopically to verify the size range of *P. minimum*, to calibrate the

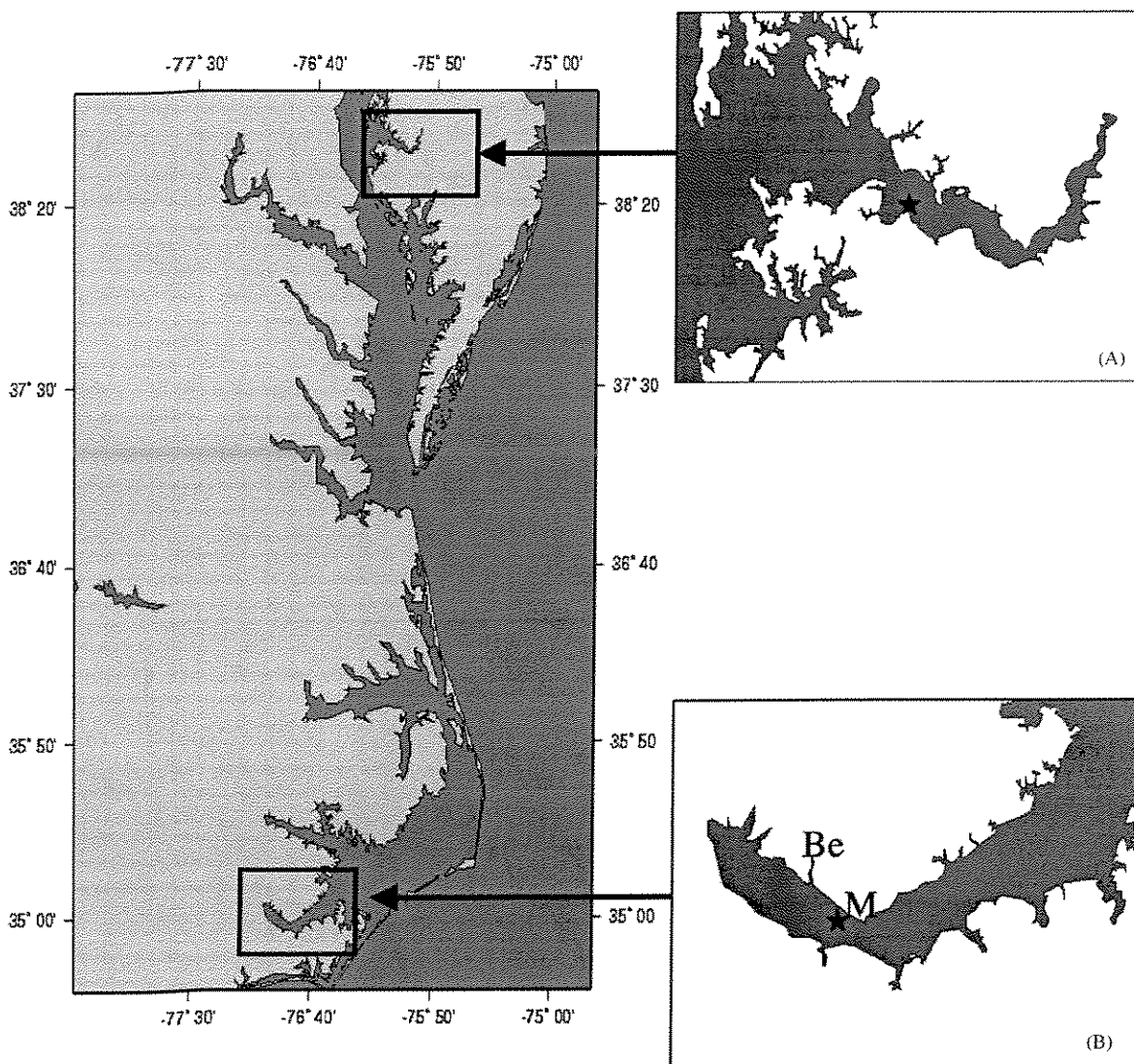


Fig. 1. Locations of the study areas for the natural *P. minimum* bloom in: (A) the Choptank River in Chesapeake Bay, (★: site sampled); and (B) the Neuse Estuary of the Albemarle–Pamlico Estuarine System (lines = patches extending from Beards Creek [Be] down to Minnesott Beach [M], a 5.0 km distance (area covered, 4.0 km²; ★: main bloom site).

cell counts, and to identify the presence of other phytoplankton.

2.2. Neuse Estuary sampling and environmental parameters

In January 1999, a dinoflagellate bloom of mixed composition was detected in the Neuse Estuary

(Fig. 1). The bloom consisted mostly of *Heterocapsa rotundata* (Lohmann) Loeblich (Hansen, 1995; Daugbjerg et al., 2000), *H. triquetra* (Ehrenberg) Stein, and *P. minimum*. Sampling and experimentation were carried out on two successive days. One day was devoted to measuring nutrient concentrations and nitrogen uptake rates in the bloom, while on the second day water from just outside the bloom

was analyzed for comparison. Samples were taken and environmental conditions were characterized at 1300 h, following the procedures given in Glasgow and Burkholder (2000); here, presenting data for the upper water column (<1.5 m). Concentrations of inorganic nutrients, urea, and DFAA were determined as above. Cell densities were quantified using Palmer chambers (Wetzel and Likens, 2000), counting at least 400 cells per sample ($n = 7$; Burkholder and Wetzel, 1989). Size dimensions of dominant Neuse phytoplankton taxa were quantified considering at least 25 cells per taxon. Biovolumes and surface areas were calculated using formulae for solid geometric shapes that most closely matched the cell shape (Smayda, 1978; Burkholder and Wetzel, 1989). Mean cell biovolumes and surface areas were used to calculate the totals contributed by each taxon.

2.3. Culture growth and experimentation

A culture of *P. minimum* previously isolated from Chesapeake Bay by A. Lewitus and maintained in the Horn Point culture collection for several years was used for these experiments. The culture was held at 20 °C, and 120 $\mu\text{mol photons}(\text{m}^{-2} \text{s}^{-1})$ on a 14:10 L:D cycle, and was unialgal but not axenic. Efforts to reduce bacterial growth in this stock culture have previously been described, and measured bacterial levels were $\leq 10\%$ of those determined in the natural estuarine waters from which this culture originated (Fan et al., 2003). This stock culture was transferred to 21 bottles about 1 week before the experiments were initiated. To determine the effects of different growth nitrogenous substrates on nitrogen uptake kinetics, *P. minimum* was grown on three different nitrogen media (NO_3^- , NH_4^+ , and urea). The medium was *f*20 (88 $\mu\text{g atom-N l}^{-1}$) for the nitrogen growth source, and *f*2 standard for other nutrients (phosphate and vitamins; Guillard, 1975). All sub-cultures were grown at 20 °C and 120 $\mu\text{mol photons}(\text{m}^{-2} \text{s}^{-1})$ on a 14:10 L:D cycle, with the light period beginning at 06:00 h. Growth rates were determined by cell counts using a Coulter Counter (Coulter Multisizer II). After 1 week, culture densities reached about 11,000 cells ml^{-1} and were used in the experiments. All cultures were at mid-exponential stage for the uptake experiments.

2.4. Nitrogen uptake kinetics: field experiments

For the experiments with the naturally occurring *P. minimum* population in the Choptank River, water samples were dispensed into 50 ml clear polycarbonate flasks immediately after being transported to the laboratory. Four nitrogen substrates (NO_3^- , NH_4^+ , urea, and an amino acids mixture, Cambridge Isotope Laboratories, all 99 at.% of ^{15}N) were used to characterize nitrogen uptake kinetics. The amino acids mixture consisted of (%): alanine (7.3), arginine (6.8), aspartic acid (9.5), glutamic acid (10.4), glycine (6.2), histidine (1.9), isoleucine (4.0), leucine (10.6), lysine (13.7), methionine (1.0), phenylalanine (4.5), proline (6.5), serine (4.1), threonine (4.6), tyrosine (3.9), and valine (5.1). Each ^{15}N substrate was added at eight levels ranging from 0.4 to 30.0 $\mu\text{g atom-N l}^{-1}$ at final concentration. Due to the number of treatments, complete replication was not possible. To obtain insight into sample handling variability, one treatment, the urea addition at eight concentrations was replicated in the first experiment (7th May). There was no significant difference between all replicates (*t*-test, $P = 0.68$).

After addition of ^{15}N nitrogen substrates, flasks were immediately transferred to the dock, and were incubated for 0.5 h in a culture incubator with flowing surface water to maintain in situ temperature. Experiments were conducted at ca. 60% of average incident irradiance by using one layer of neutral density screen. Incubations were terminated by gentle filtration (pressure <100 mm Hg) onto pre-combusted (500 °C, 2 h) GF/F filters that were placed in a drying oven (50 °C, 24 h) overnight and readied for isotope excess analysis (below).

The nitrogen uptake experiments in the Neuse Estuary were conducted on a residential dock at Carolina Pines, and followed identical protocols except for use of six (rather than eight) different concentrations of ^{15}N substrate additions (0.4–30.0 $\mu\text{g atom-N l}^{-1}$). Separate kinetic data were not obtained for each of the three dominant dinoflagellate species because available techniques could not be used to separate them for study. Therefore, the data reported for Neuse Estuary are more reflective of general nutritional ecology of these three mixed dinoflagellate species rather *P. minimum* alone.

2.5. Nitrogen uptake kinetics: laboratory experiments

For the laboratory culture study, on the morning on which each experiment was conducted, the *P. minimum* culture was concentrated by photo-attraction and gently washed twice in dilute Sargasso seawater (salinity 15) before final dilution to 21 bottles to minimize carry-over of nitrogen from the initial culture medium. The culture was then dispensed into 50 ml polycarbonate tissue culture flasks. Four ^{15}N substrates (NO_3^- , NH_4^+ , urea, and an amino acids mixture) were added at final concentrations ranging from 0.4 to 30.0 $\mu\text{g atom-N l}^{-1}$. The amino acids mixture was the same as that used in the experiments with natural samples. Immediately following addition of ^{15}N substrates, subsamples were returned to the growth chamber and incubated under 20 °C and 120 $\mu\text{mol (m}^{-2} \text{s}^{-1})$ for 0.5 h. Incubations were terminated by filtration onto pre-combusted (500 °C for 2 h) GF/F filters at <100 $\mu\text{m Hg}$. Filters were dried overnight at 50 °C for isotope analysis as described below.

2.6. Isotope enrichment analysis

All filters for ^{15}N isotope enrichment analyses were prepared using the general methods of Fiedler and Proksch (1975). Samples were ground with copper oxide (Baker #1820-05, prepared for use by combusting at 600 °C for 3 h), placed into Pyrex glass ampoules (pre-combusted at 450 °C for 1 h) with copper metal accelerator (Alpha Resources Inc.), evacuated and sealed. Samples were combusted at 550 °C for 2.5 h and then analyzed for isotopic enrichment by a Nuclide mass spectrometer using the methods of Glibert et al. (1991). Each sample was analyzed in triplicate. Precision of triplicate samples was about 0.001 at.%, and standard yielded a 99.7% recovery of calculated standard additions.

2.7. Calculations

Nitrogen-specific uptake rates (V (h^{-1})) were calculated according the equation of Dugdale and Goering (1967), and were not corrected for isotope dilution as little would have been expected at the

relative high concentration and short incubation period used (Glibert et al., 1982). Absolute uptake rates (ρ) per volume per cell were calculated by multiplying V by the particulate nitrogen (PN) to yield ρ_{volume} ($\mu\text{g atom-N (l h)}^{-1}$), and then normalized to the cell-absolute uptake rate by dividing by the cell density to yield ρ_{cell} (f ($\text{g atom-N (cell h)}^{-1}$)).

Parameters for nitrogen uptake kinetics were based on the Michaelis–Menten equation:

$$\rho = \frac{\rho_{\text{max}} S}{K_s + S}$$

where ρ is the uptake rate in volumetric or cellular units, ρ_{max} the maximal uptake rate, S the substrate concentration ($\mu\text{g atom-N l}^{-1}$), and K_s the concentration ($\mu\text{g atom-N l}^{-1}$) at which uptake is half-maximal. These parameters were obtained by using an iteratively hyperbolic curve-fitting technique (Jandel Scientific SigmaPlot).

The affinity coefficient α was calculated as the ratio of ρ_{max}/K_s (Healey, 1980). For this calculation, ρ_{max} was expressed in volumetric units, $\rho_{\text{max-volume}}$ ($\mu\text{g atom-N (l h)}^{-1}$), rather than the cellular units, $\rho_{\text{max-cell}}$ (f ($\text{g atom-N (cell h)}^{-1}$)); thus the unit of α was reduced to h^{-1} .

3. Results

3.1. General features of the natural blooms

3.1.1. Choptank River bloom

Although *P. minimum* blooms are relatively common in Chesapeake Bay (Tyler and Seliger, 1981; Stoecker et al., 1997), the late spring bloom of 1998 was unusual both for its high density and long temporal extent. The measurements made during this study were analyzed in the context of other measurements available from the Chesapeake Bay monitoring program (station ET5.2; <http://www.chesapeakebay.net/data/index.htm>), and from previously reported nutrient values (Glibert et al., 2001). During the pre-bloom period, water temperatures were below 15 °C, chlorophyll *a* concentrations were below 15 $\mu\text{g l}^{-1}$, and ambient NO_3^- , NH_4^+ , and urea concentration were high (Fig. 2). A high river flow was observed roughly 1 month before the bloom developed, then during the early phase of the bloom, another, but

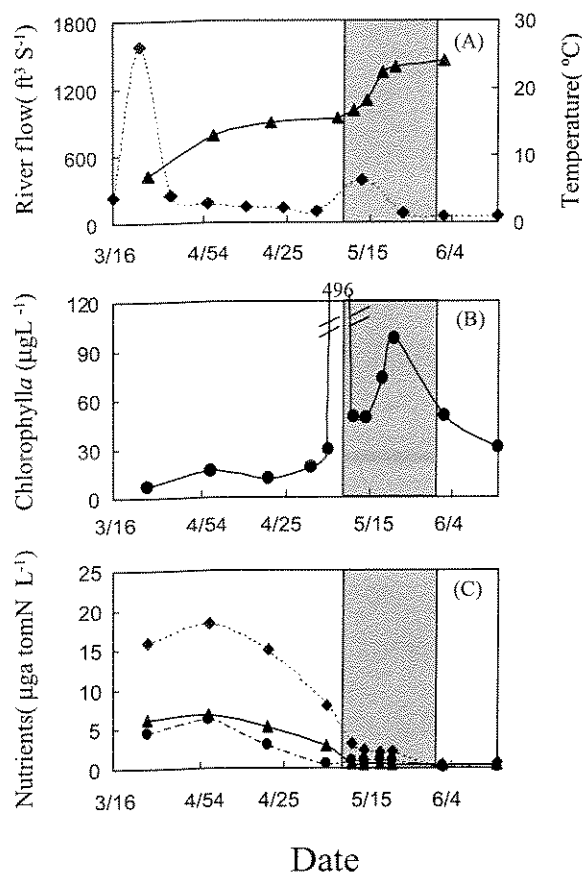


Fig. 2. (Panel A) The (▲) temperature, (◆) flow; (Panel B) (●) chlorophyll *a* concentrations; and (Panel C) ambient (◆) NO₃⁻, (▲) NH₄⁺, and (●) urea concentrations in near-surface waters of the Choptank River during the 1998 *P. minimum* bloom. The gray shading represents the period of this study. The data before and after the bloom were from a water quality monitoring station (ET5.2) of Chesapeake Bay Program (<http://www.chesapeakebay.net/data/index.htm>) in the Choptank River. Urea concentrations were obtained from the study of Glibert et al. (2001).

smaller, period of elevated river flow was again noted (Fig. 2A).

The bloom was first observed when pigment concentrations were sufficient to discolor the water. In the first bloom sampling date, chlorophyll *a* concentration reached roughly 500 µg l⁻¹ (Fig. 2B). Physical concentration processes had, most likely, contributed to this unusually high biomass. The remaining period of the bloom was characterized by chlorophyll

a that increased from about 40 to 90 µg l⁻¹. These concentrations were roughly 4–5-fold higher than mean chlorophyll *a* concentrations during the same period in non-bloom years, and 6–10-fold higher than pre-bloom concentrations (Fig. 2B).

During the sampling period, surface water temperatures ranged from 15.7 to 23.0 °C (Fig. 2A). Surface salinity was ca. 5.5–7.0, and pH was 8.5–9.0 (data not shown). Dissolved inorganic nitrogen concentrations were relatively low during the blooms, potentially reflecting high demand for nutrients from the dense dinoflagellate populations (Table 1), but were significantly higher in the weeks leading up to the bloom (Fig. 2C). The nitrogen form in highest concentration was generally NO₃⁻ (mean: 1.94 µg atom-N l⁻¹), followed by NH₄⁺ (mean: 0.94 µg atom-N l⁻¹). Concentrations of urea during this bloom were considerably lower (mean: 0.41 µg atom-N l⁻¹), as were concentrations of DFAAs (mean: 0.14 µg atom-N l⁻¹). Phosphate was very low (<0.3 µg atom-P l⁻¹) during the bloom period.

During the recorded bloom maximum, *P. minimum* represented >93% of the total phytoplankton cells ($n = 6$) and the cell densities reached 1.16×10^5 cells ml⁻¹. The surface chlorophyll *a* concentrations were correlated with *P. minimum* cell densities.

3.1.2. Neuse Estuary bloom

The bloom during late January 1999 occurred as patches mostly near the north shore from Beards Creek to Minnesott Beach (Fig. 1), and was notable for its early occurrence and relatively short duration (ca. 23 days). During the 2-day sampling period, water temperatures ranged from 11.2 to 13.2 °C (11.9 ± 0.7 °C), salinity from 2.6 to 5.9 (3.6 ± 1.2) and incident irradiance from 360 to 850 µmol photons (m⁻² s⁻¹) (670 ± 270 µmol photons (m⁻² s⁻¹)). As in the Choptank River bloom, dissolved inorganic nitrogen concentrations were lower within bloom patches, likely reflecting demand for nutrients from the dense dinoflagellate populations (Table 1). NO₃⁻ concentrations were highest (20.9 ± 4.0 µg atom-N l⁻¹). Other nitrogen forms (NH₄⁺, urea, and DFAA) were <1 µg atom-N l⁻¹. Phosphate averaged 0.45 ± 0.03 µg atom-P l⁻¹ (Table 1).

The mixed dinoflagellate assemblage consisted mostly of *H. rotundata* (ca. 40%; $21,500 \pm 3,500$ cells ml⁻¹), *H. triquetra* (ca. 22%; $9,500 \pm 500$

Table 1
Ambient nutrient concentrations for sampling in the natural blooms indicated

Estuary	NO ₃ ⁻	NH ₄ ⁺	Urea	Dissolved free amino acids	PO ₄ ³⁻
Choptank River					
7 May	0.31	0.64	0.16	0.15	0.29
11 May	3.14	1.03	0.50	0.12	0.17
14 May	2.24	0.95	0.44	0.12	0.15
18 May	1.91	1.08	0.53	0.12	0.17
21 May	2.10	0.98	0.44	0.17	0.28
Neuse Estuary ^a					
Bloom patches	20.85 ± 4.01	0.64 ± 0.57	0.52	0.42	0.45 ± 0.03
Outside patches	36.07 ± 4.57	4.57 ± 0.29	0.31	0.31	0.81 ± 0.06

All nitrogen data are in the units of (μg atom-N l⁻¹). All phosphate data are in the units of (μmol-P l⁻¹).

^a Means ± 1 S.E. (n = 21) for all nutrient variables except urea.

cells ml⁻¹) and *P. minimum* (ca. 21%; 9,400 ± 600 cells ml⁻¹) (n = 3). These dinoflagellates formed ca. 80% of the total phytoplankton cells, 97% of the total biovolume (ca. 11% as *H. rotundata*, 47% as *H. triquetra*, and 39% as *P. minimum*), and 98% of the total cell surface area (ca. 21% as *H. rotundata*, 39% as *H. triquetra*, and 38% as *P. minimum*). Other phytoplankton present (17% of the total cells) were mostly small cryptomonad, prymnesiophyte, and green flagellates. Chlorophyll *a* averaged 170 ± 55 μg l⁻¹ in bloom patches with *P. minimum*. In contrast, chlorophyll *a* outside bloom patches was 20 ± 4 μg l⁻¹ (n = 3).

3.2. Nitrogen uptake kinetics of the natural blooms

During the Choptank River bloom, relationships between cell-specific nitrogen uptake rates and substrate concentration varied both with substrate and time (Fig. 3). At all measured substrate concentrations, highest uptake rates were observed for NH₄⁺, followed by the amino acids mixture and urea. In all experiments, lowest uptake rates were found for NO₃⁻. In the Neuse Estuary, absolute cell-specific uptake rates for all substrates were 10–100-fold lower than those measured over the Choptank River bloom. Again, highest uptake rates were observed for NH₄⁺, while uptake rates across the measured concentration range of the other three substrates did not significantly differ and were considerably lower (Fig. 4). Nitrogen uptake rates in bloom patches were significantly higher than uptake rates outside patches for all nitrogenous substrates (*t*-test, *P* < 0.05; Fig. 4) supporting the

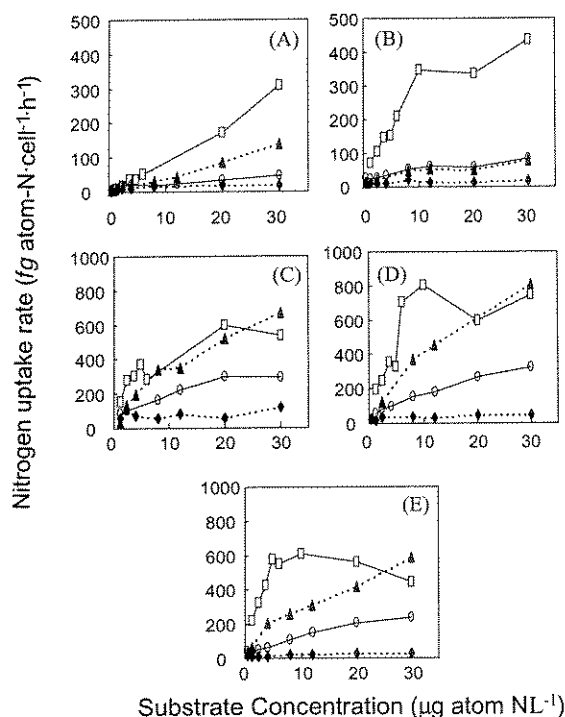


Fig. 3. Cell-specific kinetic relationships for uptake of: (◆) NO₃⁻, (□) NH₄⁺, (○) urea, and (▲) amino acids by phytoplankton collected from the Choptank River during the *P. minimum* bloom in late spring 1998. Data for each of the five experimental dates are provided: (A) 7th May, (B) 11th May, (C) 14th May, (D) 18th May, and (E) 21st May. Values for urea in the 7th May experiment were replicated, and variation was less than the size of the symbol on the graph.

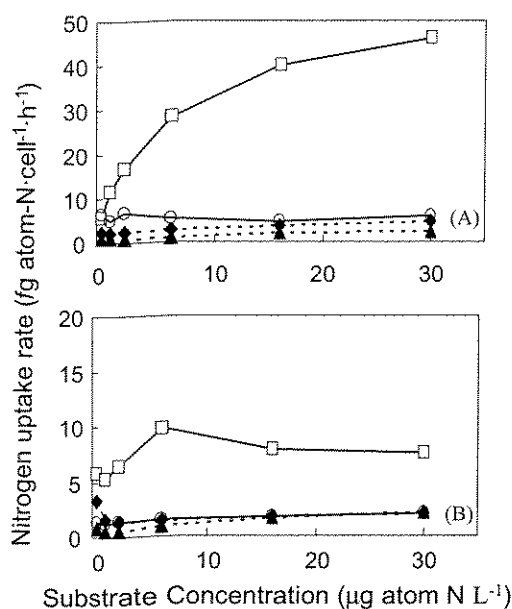


Fig. 4. Cell-specific kinetic relationships for uptake of: (◆) NO_3^- , (□) NH_4^+ , (○) urea, and (▲) amino acids by phytoplankton collected from the Neuse Estuary during a mixed-species dinoflagellates bloom in January 1999. Panel (A) represents data obtained inside the bloom patch at Minnesott Beach; and Panel (B) shows data from outside the bloom patch in the same general area.

premise that depressed inorganic nutrient concentrations within patches (Table 1) had resulted from uptake by the dense phytoplankton.

Calculated parameters for uptake kinetics of the four nitrogen substrates showed the same patterns (Table 2). For the Choptank River study, the inorganic nitrogen substrates (NH_4^+ and NO_3^-) generally had lower K_s values, 5.34 and 3.54 $\mu\text{g atom-N l}^{-1}$, respectively, than the organic nitrogen substrates (urea and amino acids mixture), with mean values of 11.7 and 18.8 $\mu\text{g atom-N l}^{-1}$, respectively. Moreover, highest cell-specific $\rho_{\text{max-cell}}$ values were observed for NH_4^+ and the amino acids mixture. The $\rho_{\text{max-cell}}$ of urea was generally lower than that for NH_4^+ and amino acids, but was always considerably higher than the $\rho_{\text{max-cell}}$ for NO_3^- in all the sample dates (Table 2).

The Neuse Estuary phytoplankton had significantly lower mean cell specific ρ_{max} values for all four nitrogen substrates than those measured for the Choptank River (Table 2). The $\rho_{\text{max-cell}}$ for NH_4^+ was

Table 2

Calculated cell-specific parameters for uptake kinetics of NO_3^- , NH_4^+ , urea, and amino acids by the field assemblages and the *P. minimum* cultures

Location and substrate	$\rho_{\text{max-cell}}$ (f (g atom-N (cell h) $^{-1}$))	K_s (μg atom-N l $^{-1}$)	n
Choptank River			
7th May			
NO_3^-	18.37 (4.32)	2.82 (1.71)	8
NH_4^+	N/A	N/A	8
Urea	38.15 (16.88)	9.9 (4.42)	16
Amino acids	N/A	N/A	8
11th May			
NO_3^-	25.84 (10.93)	1.36 (0.22)	8
NH_4^+	564.4 (66.87)	9.83 (2.63)	8
Urea	83.11 (16.14)	6.64 (3.98)	8
Amino acids	84.22 (12.92)	4.79 (2.27)	7
14th May			
NO_3^-	51.52 (14.11)	4.6 (0.44)	8
NH_4^+	327 (172.5)	4.07 (4.27)	8
Urea	188.5 (135.8)	7.24 (1.75)	8
Amino acids	562.2 (55.8)	21.21 (4.05)	8
18th May			
NO_3^-	53.77 (5.66)	7.12 (5.54)	7
NH_4^+	868.6 (159)	5.09 (2.23)	8
Urea	492.6 (71.5)	16.84 (4.87)	8
Amino acids	1516 (134)	26.56 (4.08)	7
21st May			
NO_3^-	37.32 (4.64)	1.8 (1.39)	8
NH_4^+	602.3 (67.44)	2.38 (0.27)	8
Urea	379.4 (55.76)	17.89 (5.1)	7
Amino acids	970.3 (198.7)	22.51 (8.39)	7
Neuse Estuary			
NO_3^-	3.98 (1.58)	0.54 (0.08)	6
NH_4^+	52.9 (1.7)	4.91 (0.48)	6
Urea	5.77 (0.49)	0.37 (0.08)	6
Amino acids	2.28 (0.46)	2.26 (1.72)	6
Laboratory			
NO_3^- grown culture			
NO_3^-	115.5 (11)	5.18 (1.46)	8
NH_4^+	1070 (120.5)	6.23 (0.47)	8
Urea	149.2 (12.3)	0.87 (0.38)	8
Amino acids	335.3 (75.7)	14.12 (6.8)	8
NH_4^+ grown culture			
NO_3^-	341.2 (90.7)	23.3 (11.1)	8
NH_4^+	1217 (88.7)	2.48 (0.8)	8
Urea	480.5 (13.4)	0.86 (0.16)	8
Amino acids	517.1 (125.8)	22.6 (9.9)	8
Urea grown culture			
NO_3^-	95.8 (5.5)	0.68 (0.23)	8
NH_4^+	1208 (33.2)	6.08 (0.47)	8
Urea	152.8 (11.1)	1.82 (0.59)	8
Amino acids	271.6 (35.6)	16.4 (4.3)	8

Data for each experiment are given as the mean (\pm 1 S.E., standard error for curve fitting).

the highest ($52.9 \text{ fg atom-N (cell h)}^{-1}$) while values for all other substrates were $<6 \text{ fg atom-N (cell h)}^{-1}$. Values for K_s also were lower in the Neuse study. Lowest values were observed for urea and NO_3^- ($<0.55 \text{ } \mu\text{g atom-N l}^{-1}$), while values for amino acids and NH_4^+ were 2.26 and $4.91 \text{ } \mu\text{g atom-N l}^{-1}$, respectively (Table 2).

3.3. Nitrogen uptake kinetics in cultured *P. minimum*

The laboratory culture of *P. minimum*, like the field assemblages, showed highest cell-specific uptake rates for NH_4^+ across all substrate levels examined, and all nitrogen growth substrates (Table 2, Fig. 5). Uptake rates of all other substrates were lower and comparable (Fig. 5).

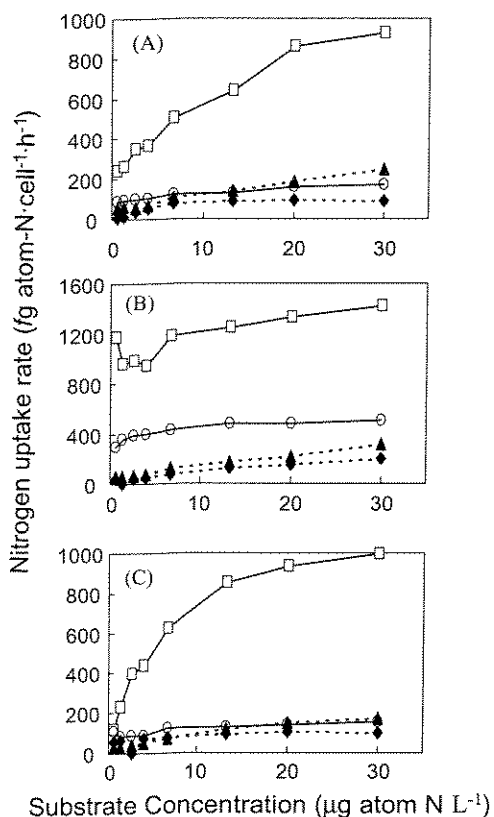


Fig. 5. Cell-specific kinetic relationships for uptake of: (◆) NO_3^- , (□) NH_4^+ , (○) urea, and (▲) amino acids by *P. minimum* laboratory cultures that were grown on: (A) NO_3^- , (B) NH_4^+ , and (C) urea.

4. Discussion

There is growing evidence that increases in frequency and extent of some harmful bloom species are related to nutrient enrichment in coastal waters and inland seas (Smayda, 1990; Anderson, 1989; Burkholder, 1998; Anderson et al., 2002). Not only are coastal waters receiving increased quantities of industrial, agricultural, and sewage effluents, but the composition of these discharges has also been altered in recent years (reviewed in Anderson et al., 2002). The challenge is to understand the relationships between these sources and the potential to stimulate growth of harmful algal species. This requires knowledge of the physiological pathways by which cells acquire their nutrients, nutritional preferences, and the factors that may influence these nutritional pathways and mechanisms. Here, the nutritional preferences and variations in uptake and affinity for different substrates by the natural assemblages and laboratory cultures of *P. minimum* are explored.

4.1. Nutritional preferences

One metric that has been used in assessing nutrient preference is the relative preference index (RPI; McCarthy et al., 1977). This index compares the rate of uptake for each nitrogen substrate at ambient concentration levels, to that of the other available substrates. Although this index commonly has been applied in the literature (McCarthy et al., 1977; Glibert et al., 1982, 1995), there are few examples wherein organic substrates have also been included in the calculations and comparisons. Consistently, the RPI values for NH_4^+ were >1 for both field studies (Table 3). For urea, values were >1 for the Neuse Estuary assemblage, and were near 1.0 for the Choptank River *P. minimum* population on the first three sampling dates. For amino acids, values were >0.5 in the Choptank study, but lower in the Neuse Estuary. With exception of the first sampling date, all RPI values for NO_3^- were <0.6 for the Choptank River *P. minimum* population.

4.2. Kinetic variability

Applications of kinetic parameters for nutrient uptake are useful in understanding phytoplankton physiological ecology (Dugdale, 1967; Eppley and

Table 3
Calculated ambient uptake rates for each nitrogen substrate (f (g atom-N (cell h)⁻¹)) and relative preference index of NO₃⁻, NH₄⁺, urea, and amino acids by the phytoplankton assemblages of the two natural blooms

Location and date	Ambient uptake rate				Relative preference index			
	NO ₃ ⁻	NH ₄ ⁺	Urea	Amino acids	NO ₃ ⁻	NH ₄ ⁺	Urea	Amino acids
Choptank River								
7th May	2.02	6.20	0.98	0.67	0.83	1.23	0.80	0.57
11th May	14.23	53.52	7.98	1.36	0.28	3.22	0.99	0.76
14th May	79.38	121.7	21.22	5.70	0.58	2.10	0.80	0.85
18th May	30.35	181.8	14.97	6.82	0.25	2.62	0.44	0.88
21th May	13.63	250.5	9.01	7.27	0.09	3.36	0.27	0.56
Neuse Estuary								
In bloom patches	3.27	4.97	5.33	0.34	0.34	3.29	3.46	0.26
Outside patches	1.5	6.22	0.97	0.88	0.11	2.42	1.06	0.69

Coatsworth, 1968; McCarthy, 1981). The underlying hypothesis is that species are selected on the basis of their ability to compete for nutrients characteristic of their habitat (MacIsaac and Dugdale, 1969). Among uptake kinetic parameters, K_s is considered to be an index of species potential competitive ability at low nutrient concentrations. From consideration of published values together with this study, two broad generalizations can be drawn (reviewed in Smayda, 1997). First, dinoflagellates generally show a higher K_s value than diatoms for inorganic nitrogen substrates, suggesting that dinoflagellates would not have a competitive advantage over diatoms if inorganic nitrogen were the only nitrogen source. Second, differences in the K_s values for NO₃⁻ and NH₄⁺ uptake are not large, rarely exceeding a factor of two, suggesting that at low nutrient levels species are poised to take up either inorganic substrate equally efficient. As discussed below, there may be more variability in K_s for some species, such as *P. minimum*, than previously thought.

The parameter ρ_{max} is also useful in assessing substrate affinity, but lesser attention has been given to this parameter than to K_s , because nitrogen is generally considered to be available in limiting rather than saturating levels. Furthermore, little appreciation has been given to kinetic curves that do not saturate over the experimental range, typically (as in this study) ranging from 0 to 30 $\mu\text{g atom-N l}^{-1}$ (Dortch et al., 1991; Collos et al., 1992, 1997; Lomas and Glibert, 1999b). Non-saturable (linear) uptake kinetics recently has been found to be a common feature in

the spring diatom assemblages of temperate estuaries (Lomas and Glibert, 1999b), and may reflect simple diffusion into the cells. In this study, during 0.5 h uptake measurements, non-saturable kinetics (over the examined substrate concentration range) were found for NH₄⁺ during the first Choptank River experiment, and for the amino acids mixture and urea on several other sampling dates. Not only do these relationships suggest very high uptake rates for these substrates at higher concentration levels, but they also indicate a potential adaptation to acquisition of high substrate levels of these nutrients with time.

Variability in K_s within a single species has previously been suggested on theoretical grounds (Goldman and Glibert, 1982). Variations in K_s for NH₄⁺ as a function of time, on the scale of min–h, have been previously reported for the diatom *Chaetoceros simplex* and the chlorophyte *Dunaliella tertiolecta* (Goldman and Glibert, 1982), and for natural assemblages of Chesapeake Bay phytoplankton (Wheeler et al., 1982), but there have been virtually no reports of such variations in the uptake of other N substrates either with incubation duration or physiological state. Variations in ρ_{max} have also been documented as a function of short-term time courses in natural assemblages (e.g. Glibert and Goldman, 1981).

In this study, the calculated kinetic parameters determined for the *P. minimum*-dominated and mixed-species field assemblages and the cultures grown on different N sources varied considerably (Table 2). Thus, variations in uptake kinetics and affinity for substrate by the field and laboratory populations

are here explored as a function of state of the bloom, temperature, growth N source, and relative growth rate.

4.3. Variations in affinity with bloom duration and temperature

The α , or the initial slope of the uptake curve, provides an indicator of efficiency at low ambient substrate concentrations (Healey, 1980). In the Choptank River bloom, the affinity coefficient α varied differentially with time for each of the nitrogen substrates (not shown). Generally, NH_4^+ had the highest α value in all the sampling dates, indicating that it was the most efficiently used substrate. With time, the affinity for NO_3^- declined sharply, that for NH_4^+ increased nearly four-fold, while the affinity for urea remained relatively constant. The affinity for DFAAs also appeared to increase with bloom duration, but the extent of the increase was significantly less than that for NH_4^+ .

As the water temperature rose during the course of the bloom, these relationships also held as a function of temperature (Fig. 6): the affinity for NO_3^- declined as temperature increased, that for NH_4^+ and DFAAs increased, while that for urea remained relatively constant. The rate of uptake of NO_3^- by nat-

ural phytoplankton assemblages has previously been shown to be a negative function of temperature over this temperature range (Lomas and Glibert, 1999a), and the temperature optimum for nitrate reductase is in the range of 18–20 °C for *P. minimum* (Lomas and Glibert, 2000). Thus, as temperatures rapidly warmed, a decrease in NO_3^- uptake was expected. Similarly, increases in NH_4^+ uptake as a function of temperature over this temperature range have previously been observed (Glibert et al., 1982; Lomas and Glibert, 1999a). It has also been shown that for *P. minimum*, urease, the enzyme involved in urea assimilation, appears not to be sensitive to temperature over the range from <20 to 50 °C (Fan et al., 2003). The flat response as a function of temperature is consistent with the response of this enzyme. The affinity constants calculated for the assemblage growing under the cooler bloom temperatures of the Neuse Estuary are also generally consistent with these temperature relationships (Fig. 6). At 12 °C, the temperature of that bloom, the affinity for NO_3^- was higher than that observed in the 20–25 °C range for the Choptank River, but somewhat lower than that found for 15 °C in the Choptank. This may suggest a temperature optimum for affinity for NO_3^- , or, a variable response due to the variable responses of the other species in the Neuse Estuary

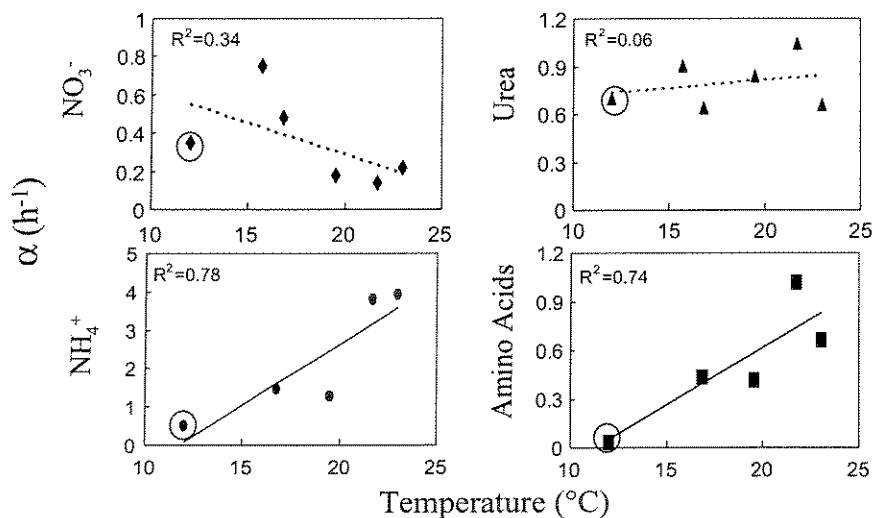


Fig. 6. The variation in α (h^{-1}) as a function of water temperature for each of the N substrates measured during the natural blooms of *P. minimum* in the Choptank River and mixed dinoflagellates in the Neuse Estuary. The Neuse Estuary data are indicated by the circled symbols. Regression lines for all data are indicated; those that were significant ($P < 0.05$) are drawn with (—); those that were not significant are drawn with (---).

assemblage. The affinities for NH_4^+ and DFAA for the Neuse Estuary were considerably lower than the values determined for the Choptank River assemblages, but consistent with the positive temperature response (Fig. 6). Furthermore, even at the cool temperature of the Neuse Estuary bloom, the affinity for urea was roughly equal to that found for the Choptank bloom.

4.4. Variations in affinity with growth on N sources

Nutritional history is a significant factor in determining the rate at which cells will utilize nitrogen. For *P. minimum*, when grown on different N sources, as in the culture experiments herein, kinetic parameters also varied. Pooling all data from the field and the laboratory, the affinity, α , for each nitrogen substrate was examined as a function of the percent of the nutrient pool was provided by each of the growth N sources. For all data combined, when *P. minimum* was grown on NO_3^- , the affinity for each nitrogen substrate generally decreased as the percent of NO_3^- increased (Fig. 7). This same relationship held when the data were examined as a function of total NO_3^- availability (data not shown). With higher nutrient availability, the cells apparently did not need to maintain a high affinity to acquire this nutrient. While the affinities for NO_3^- and NH_4^+ varied little across the gradient of percent NH_4^+ availability, the affinity for urea increased slightly. The relationship between uptake affinity for all nitrogen substrates and percent urea availability was not significant.

4.5. Variations in affinity with relative growth rate

As previously reviewed two decades ago (Goldman and Glibert, 1982), variation in the affinity of nitrogen uptake is a function of several possible factors, including time of exposure to the limiting nutrient and relative growth rate. As the affinity coefficient α is composed of two parameters (ρ_{\max} and K_s), its variation may either be a function of variation in ρ_{\max} , K_s or both.

Cell physiological state may influence the saturation kinetic response of a cell in several ways. As demonstrated by McCarthy and Goldman (1979) using cultures of the diatom *Thalassiosira pseudonanna* (3H) grown in chemostat at various growth rates, the

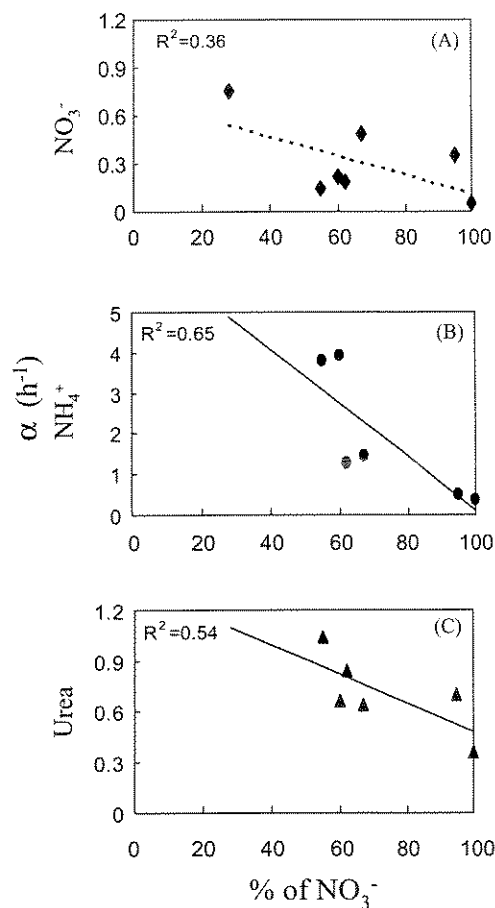


Fig. 7. The correlation between α (h^{-1}) for each of the N substrates measured in the field and in the laboratory cultures as a function of the percentage of NO_3^- . Regression lines for all data are indicated; those that were significant ($P < 0.05$) are drawn with (—); those that were not significant are drawn with (- -).

uptake of NH_4^+ decreased as growth rate increased, matching the nitrogen demand only at μ_{\max} . Thus, a higher ρ_{\max} would be associated with a slower relative growth rate. Goldman and Glibert (1982) further hypothesized that the relationship between ρ_{\max} and K_s might change with growth rate in one of two ways. On the one hand, ρ_{\max} might change with growth rate, while K_s remains constant (Fig. 8A), or, both may change together (Fig. 8B).

Plotting the family of curves for each of the field experiments, it is apparent that the uptake of different N substrates followed different scenarios proposed

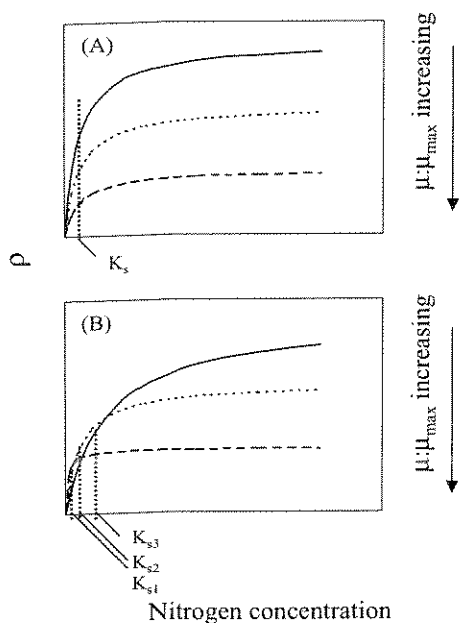


Fig. 8. Conceptual relationship between ρ_{\max} and K_s for a single species at different cell physiological states, or relative growth rates ($\mu:\mu_{\max}$). In Panel A, the family of curves suggests variation in ρ_{\max} , but little variation in K_s . In Panel B, the family of curves suggests co-variation in K_s and ρ_{\max} with varying $\mu:\mu_{\max}$. Adapted from Goldman and Glibert (1982).

by Goldman and Glibert (1982; Fig. 9). For all of the substrates, the lowest ρ_{\max} values were noted early in the bloom, and ρ_{\max} increased with time. Although it is difficult to determine actual growth rates for the field populations, these patterns would be consistent with the notion that the highest relative growth rates occurred early in the bloom, and declining relative growth rates were found as the bloom progressed. For NO_3^- , a broad range in variation in K_s was found, leading to a family of curves that more closely resembled the second alternative proposed by Goldman and Glibert (1982; Fig. 9A). For NH_4^+ and urea, the range in variation in K_s was less than that for NO_3^- , and these values were higher than those for NO_3^- (Fig. 9B and C). The highest variation in K_s was noted for DFAA uptake (Fig. 9D). The values for the Neuse Estuary experiments follow the same trends as for the Choptank River experiments, with the exception of the fact that ρ_{\max} were lower. In the Goldman and Glibert (1982) model, the lower rates would suggest a higher

relative growth rate. However, with the depressed temperatures of the Neuse Estuary, the maximum growth rate would also be depressed.

For the culture experiments, only one growth rate was tested per growth nitrogen source. However, as a function of growth N source, the K_s for NO_3^- uptake varied widely, while that for NH_4^+ and urea showed significantly less variation (Fig. 9E–G).

4.6. Summary and implications for bloom dynamics

These data collectively provide insight into the dynamic physiological response of the dinoflagellate *P. minimum* during growth on a range of conditions. In the field and in the laboratory, the uptake of NO_3^- yielded a direct relationship between K_s and ρ_{\max} , and a decreasing affinity for this substrate with bloom duration (potentially indicative of relative growth rate) and temperature. For NH_4^+ , the range in variation in K_s across all conditions was smaller, and the affinity also increased with increasing temperature. For urea, the K_s values were not indicative of adaptation for low substrate levels, with an affinity that changed little with temperature. Such changes in K_s with growth conditions have implications for comparisons between and among species and for models that describe uptake using a saturating hyperbola and which use a fixed parameter value.

The physiological measurements presented here indicate a preference for NH_4^+ by these *P. minimum* populations and mixed assemblages. The ambient rates of uptake of each of the measured nitrogen substrates show that NH_4^+ uptake was the primary nitrogen source supporting the Choptank River bloom (Fig. 10), and that the contribution of NO_3^- decreased with bloom duration. Furthermore, ambient uptake rates of NH_4^+ increased as the Choptank River bloom progressed. Even with the significant lower ambient concentration compared to NO_3^- , urea contributed up to 10% of the nitrogen taken up in the Choptank River bloom. Physiologically, these cells were well poised to exploit the ambient nutrient pool that was present in the Choptank River leading up to the bloom. In the Neuse Estuary bloom, urea contributed up to 35% of total nitrogen demand.

These differences suggest that a diversity of nitrogen uptake mechanisms may aid the development and

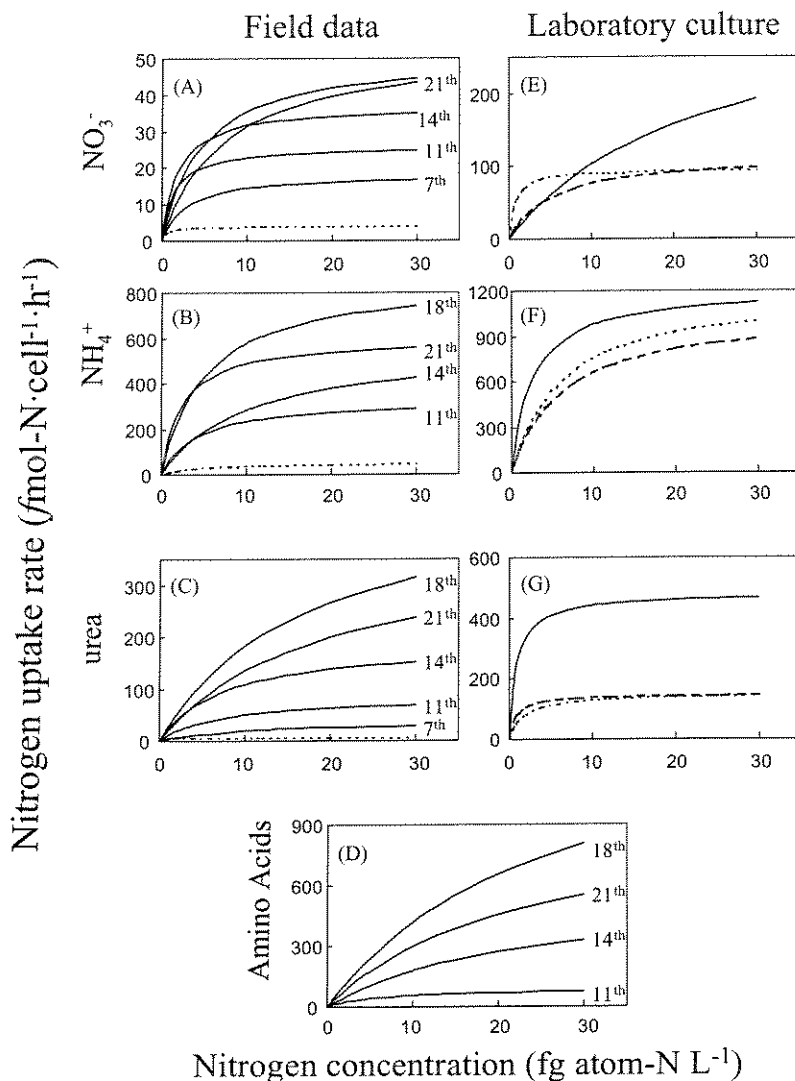


Fig. 9. Kinetic relationships for NO_3^- , NH_4^+ , urea, and amino acids, determined from the field and laboratory experiments conducted herein. The numbers next to each curve indicate the date during May 1998 for which the particular results were obtained from the Choptank River study. Neuse Estuary data are given by the dashed line. For the laboratory culture data: (—) represent data from the NH_4^+ grown culture, (---) data from the urea grown culture, and (- -) data from the NO_3^- grown culture.

maintenance of *P. minimum* blooms. *P. minimum* has the ability to utilize different nitrogen substrates based on the ambient temperature and their nutritional history. The high K_s values (except for that of NO_3^- early in the bloom) further suggest that these cells would not have been good competitors for low levels of dissolved nitrogen if nitrogen-limited conditions prevailed. Vari-

ability in uptake responses as a function of temperature and growth status further suggest that the potential impact of a pulse of nutrient in promoting a species such as *P. minimum* will also depend on when that nutrient is delivered. The dinoflagellate blooms examined here differed in timing, ambient physical conditions, monospecific versus mixed composition, and nutrient

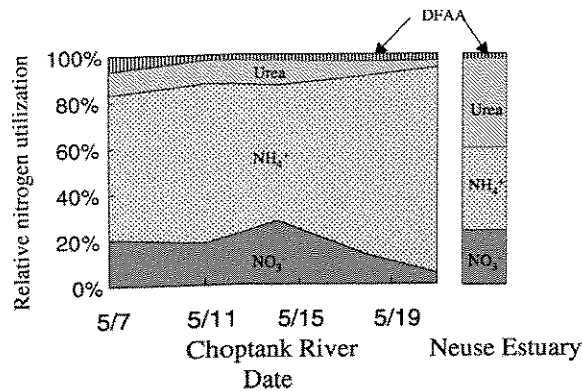


Fig. 10. The percentage of utilization of NO_3^- , NH_4^+ , urea, and DFAA in the Choptank River bloom for the date indicated, and for the Neuse Estuary.

uptake rates, but the uptake kinetics were similarly regulated. The flexibility and diversity of nutrition by *P. minimum* may provide a competitive advantage when appropriate conditions develop.

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