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Nitrogen utilization by the raphidophyte *Heterosigma akashiwo*: Growth and uptake kinetics in laboratory cultures

Julian Herndon *, William P. Cochlan

Romberg Tiburon Center for Environmental Studies, San Francisco State University, 3152 Paradise Drive, Tiburon, CA 94920-1205, USA

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Abstract

The nitrogen uptake and growth capabilities of the potentially harmful, raphidophycean flagellate *Heterosigma akashiwo* (Hada) Sournia were examined in unialgal batch cultures (strain CCMP 1912). Growth rates as a function of three nitrogen substrates (ammonium, nitrate and urea) were determined at saturating and sub-saturating photosynthetic photon flux densities (PPFDs). At saturating PPFD (110 μE m^{-2} s^{-1}), the growth rate of *H. akashiwo* was slightly greater for cells grown on NH_4^+ (0.89 d^{-1}) compared to cells grown on NO_3^- or urea, which had identical growth rates (0.82 d^{-1}). At sub-saturating PPFD (40 μE m^{-2} s^{-1}), both urea- and NH_4^+-grown cells grew faster than NO_3^--grown cells (0.61, 0.57 and 0.46 d^{-1}, respectively). The N uptake kinetic parameters were investigated using exponentially growing batch cultures of *H. akashiwo* and the ^{15}N-tracer technique. Maximum specific uptake rates (V_{max}) for unialgal cultures grown at 15 °C and saturating PPFD (110 μE m^{-2} s^{-1}) were 28.0, 18.0 and 2.89×10^{-3} h^{-1} for NH_4^+, NO_3^- and urea, respectively. The traditional measure of nutrient affinity—the half saturation constants (K_s) were similar for NH_4^+ and NO_3^- (1.44 and 1.47 μg-at N L^{-1}), but substantially lower for urea (0.42 μg-at N L^{-1}). Whereas the α parameter ($\alpha = V_{max}/K_s$), which is considered a more robust indicator for substrate affinity when substrate concentrations are low ($<K_s$), were 19.4, 12.2 and 6.88×10^{-3} h^{-1}/(μg-at N L^{-1}) for NH_4^+, NO_3^- and urea, respectively. These laboratory results demonstrate that at both saturating and sub-saturating N concentrations, N uptake preference follows the order: $NH_4^+ > NO_3^- >$ urea, and suggests that natural blooms of *H. akashiwo* may be initiated or maintained by any of the three nitrogen substrates examined.

Keywords: *Heterosigma akashiwo*; *Heterosigma carterae*; Ammonium; Nitrate; Nitrogen uptake kinetics; Raphidophyte; Urea

1. Introduction

Nitrogen (N) is the macro-nutrient thought to limit the growth of natural phytoplankton assemblages in most coastal (e.g., Ryther and Dunstan, 1971) and oceanic studies (e.g., Goldman et al., 1979). The N sources traditionally considered most important for the growth of marine phytoplankton are the most oxidized form – nitrate (NO_3^-) and the more reduced form – ammonium (NH_4^+), with generally an uptake preference for NH_4^+(e.g., McCarthy, 1981). However, both substrates appear to support similar growth rates when supplied as the sole N source under optimal laboratory culture conditions (e.g., see reviews by Antia et al., 1975; Syrett, 1981; Dortch, 1990) and only a few studies have shown enhanced growth rates of cells growing on NH_4^+ versus NO_3^- under saturating growth irradiance (e.g., Thompson et al., 1989 and references therein). Recently, the importance of dissolved organic nitrogen (DON) substrates as N sources for growth, most notably urea, have been demonstrated in both natural assemblages and

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* Corresponding author. Tel.: +1 415 338 3708; fax: +1 415 435 7120.
E-mail address: jherndon@sfsu.edu (J. Herndon).

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laboratory cultures of phytoplankton (see reviews by Kudela and Cochlan, 2000; Berman and Bronk, 2003; Glibert et al., 2006). Of particular interest has been the linkage between DON and the growth of harmful algal bloom (HAB) species, including known toxin producers such as the dinoflagellates *Karenia brevis* (Steidinger et al., 1998 named *Gymnodinium breve*) and *Pfiesteria piscicida* (Lewitus et al., 1999) and red-tide species such as *Lingulodinium polyedrum* (Kudela and Cochlan, 2000), only recently confirmed to produce yessotoxin (Armstrong and Kudela, 2006 and references therein).

Blooms of the fish-killing flagellate *Heterosigma akashiwo* (Hada) Sournia (Raphidophyceae) have been associated worldwide with massive mortality of cultivated (pen-raised) finfish and lesser (but unknown) losses of free-ranging finfish (see reviews by Honjo, 1993; Smayda, 1998). Both the extent and duration of these phytoplankton blooms have increased over the past decade in the Pacific northwestern region of the United States and southwestern British Columbia, Canada (e.g., Taylor and Haigh, 1993; Taylor and Horner, 1994; Horner et al., 1997), and during the summer of 2002 extremely dense blooms of this potentially ichthyotoxic flagellate were observed for the first time in San Francisco Bay ($>3 \times 10^8$ cells L^{-1}; Herndon et al., 2003). Such blooms, however, do not necessarily indicate toxicity to the members of marine ecosystems, as extensive non-toxic blooms of *H. akashiwo* have been observed on the west coast (e.g., Hood Canal, Washington during September 2000, Connell et al., 2001) and east coast of North America (e.g., Narragansett Bay, Rhode Island; Tomas, 1980). At present neither the environmental factors responsible for bloom initiation and maintenance, nor its toxicity are clearly understood.

Despite the ubiquitous coastal distribution of this alga and its extensive study in the field and laboratory (see review by Smayda, 1998), relatively little is known about the nitrogenous nutrition of this species. Determining whether *H. akashiwo* exhibits a preference for a particular source of nitrogen may be an integral part in understanding, predicting and potentially reducing the incidence of such large bloom events, including the devastating commercial losses associated with ichthyotoxic blooms. Published studies to date have provided conflicting results on nitrogen growth preference for NO_3^- or NH_4^+ by *H. akashiwo* at high irradiance (Chang and Page, 1995; Wood and Flynn, 1995) and even less is known about its growth on organic forms such as urea, although it appears that urea cannot be used as effectively as either nitrate or ammonium (e.g., Watanabe et al., 1982). Additionally, the only previous study to examine NO_3^- or NH_4^+ uptake kinetics (Tomas, 1979) used a strain isolated from the east coast of the United States from a nontoxic bloom of *H. akashiwo* (named *Olisthodiscus luteus*, Tomas, 1980). In the present study we have examined the nitrogen growth capabilities of a strain of *H. akashiwo* isolated from a bloom in the Pacific Northwest that was not associated with a fish kill, however this region is well known for toxic blooms of this alga. The present study also provides revised estimates of the ability of *H. akashiwo* to utilize NO_3^- and NH_4^+ as a function of their concentration, and provides the first measure of the urea uptake capability for this potentially toxic alga.

2. Materials and methods

2.1. Cell culturing

Unialagal batch cultures of *H. akashiwo* (culture CCMP1912, isolated from Kalaloch, WA, USA by R. Horner in 1996) were maintained in filter-sterilized (0.2-μm, MediaKap®-25 Hollow Fiber Media Filter, Spectrum®) nutrient-enriched artificial seawater based on ESAW (Harrison et al., 1980); modifications to this artificial seawater are described in detail by Berges et al. (2003) and also include the reduction of the nitrate enrichment, the sole nitrogen source, from 550 to 50 μg-at N L^{-1} (70 μg-at N L^{-1} for the kinetics experiments). Temperature was maintained at 15 °C (±0.3 °C) in a temperature-controlled environmental chamber. Batch cultures were grown in 250-mL borosilicate Erlenmeyer flasks (Pyrex®), and continuously illuminated from one side by four Vita-Lite® Plus Power Twist fluorescent tubes (Duro-Test® Corporation; Color Rendering Index-91); PPFD measured with a 4π collector (Biospherical Instruments QSL-100 quantum scalar irradiance meter) immersed within medium-filled culture vessels, was 110 μE m^{-2} s^{-1}, experimentally determined to be saturating for growth of this strain at 15 °C (Fig. 1). Sterile technique was employed to prevent fungal growth and to minimize bacterial contamination. Transfers and pre-experimental culture manipulation were conducted in a positive pressure, laminar flow hood (Envirco Corporation™ MAC 10® HEPA filtration unit). All glass and polycarbonate flasks used for culturing phytoplankton, and for storing enriched seawater, were soaked in freshly made Hydrochloric acid (10%, v/v) for at least 24 h, rinsed thoroughly with Milli-Q® water and autoclaved prior to use. All experiments were inoculated with cells growing at mid-exponential phase.

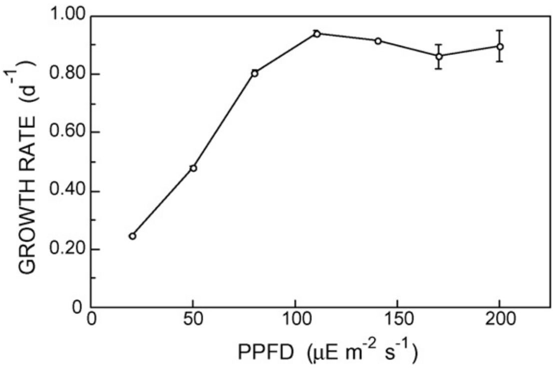

Fig. 1. The specific growth rate (μ) in d^{-1} as a function of photosynthetic photon flux density (PPFD) for *Heterosigma akashiwo* grown in *f*/2 enriched, sterile-filtered seawater. Mean of duplicate ($n = 2$) cultures are plotted; error bars indicate range of duplicates.

2.2. Analytical methods

Samples (1.5 mL) for cell counts were collected and preserved with gluteraldehyde (0.2–0.5% final concentration), and a minimum of 400 cells immediately counted using an etched Palmer-Maloney counting chamber (Hausser Scientific Co.) and a phase contrast microscope (Nikon Eclipse E4000), at 100× total magnification. Immediate counting is necessary to minimize the severe losses associated with preservation time; cell abundance decreases by *ca.* 20% within 0.5 h of the fixative addition and by *ca.* 50% after 24 h (Herndon, 2003).

Chlorophyll *a* (chl *a*) samples were collected on Whatman® GF/F filters, extracted for *ca.* 24 h in 8 mL of 90% acetone (−20 °C) and analyzed for chl *a* and phaeopigments by *in vitro* fluorometry using a recently calibrated Turner Designs 10-AU fluorometer (Parsons et al., 1989). Samples for nutrient analyses were filtered through combusted (450 °C for 4.5 h) Whatman® GF/F filters into previously acid-washed, MQ-rinsed polyethylene bottles. Duplicate samples for $NO_3^- + NO_2^-$ were analyzed using a LaChat Instruments automated ion analyzer (8000 series) according to the QuickChem® Method 31-107-04-1-E procedure (Smith and Bogren, 2001). Samples for NH_4^+ analysis, were collected in duplicate using 60-mL low density polyethylene (LDPE) centrifuge tubes (Corning®) and stored refrigerated after addition of the phenolic reagent; the addition of the phenolic reagent binds NH_4^+ and eliminates the need to freeze samples (Degobbis, 1973). The remaining reagents were added within 24 h, and the samples manually analyzed using a spectrophotometer equipped with a 10-cm cell (Solórzano, 1969). Urea samples were collected as above and frozen at −20 °C, and subsequently thawed at room temperature before manual analysis using the diacetyl monoxime thiosemicarbizide technique (Price and Harrison, 1987), modified to account for a longer (72 h) and lower digestion temperature (22 °C). Although others have found that freezing may cause decreases in urea concentration (Mulvenna and Savidge, 1992), tests conducted by Cochlan and Bronk (2001) with seawater standards of known concentrations demonstrated no such losses, in agreement with Price and Harrison (1987).

2.3. Experimental procedures

Three nitrogen substrates (NO_3^-, NH_4^+ and urea) were used to characterize the differential, exponential growth rates of *H. akashiwo*. Cells (*ca.* 350 cells mL^{-1} final conc.) were aseptically transferred to borosilicate (Pyrex®) 50-mL culture tubes fitted with polypropylene screw-caps and containing ESAW medium enriched with an initial concentration of 50 μg-at N L^{-1} of NO_3^-, NH_4^+ or urea as the yield limiting nutrient. A minimum of two transfers were conducted to acclimate the cells to the various N substrates and lighting conditions before the actual preference experiments were conducted and growth rates reported. All N growth experiments were conducted in triplicate at two different irradiances: a sub-saturating PPFD of 40 μE m^{-2} s^{-1} and a saturating PPFD of 110 μE m-$^{-2}$ s^{-1} determined previously (Fig. 1).

Cultures were generally monitored twice daily for a minimum of 7 days for *in vivo* fluorescence using a Turner Designs 10AU fluorometer. Fluorescence readings were obtained by inserting the whole culture tube into the fluorometer after gentle mixing by multiple inversions. Specific growth rates were calculated from a least-squares linear regression analysis of the exponential growth phase, determined from plots of the natural log of *in vivo* fluorescence versus time ($r^2 \geq 0.99$) using the following equation:

$$K_e = \frac{\ln(N_1/N_0)}{t_1 - t_0} \quad (1)$$

where K_e is the growth constant (in units of d^{-1}) and N_1 and N_0 are the raw fluorescence units at time 1 (t_1) and time 0 (t_0), respectively (Guillard, 1973). The specific growth rates reported were determined over a minimum of 4 days of exponential growth, prior to any N depletion.

Cells for the N uptake kinetic experiments were grown on NO_3^- in 2-L polycarbonate suspension culture flasks (Nalgene®), continuously stirred by Teflon®-coated magnetic stir bars at *ca.* 55–60 rpm. Cultures were sampled daily, using sterile, disposable serological pipettes to monitor cell abundance and ambient nitrogen concentrations. Kinetic experiments were initiated immediately after the ambient NO_3^- + NO_2^- concentration in the cultures was below detection limits (*ca.* 0.05 μg-at N L^{-1}). Growth rates for both cultures averaged 1.0 d^{-1} during the 4–5 days prior to N depletion and throughout the period needed to conduct the kinetic experiments. Timing was important, because NO_3^- in the medium must be depleted, but the condition of the cells must not be N starved to avoid non-linearity in uptake that has been observed for flagellates, including *H. akashiwo* (French and Smayda, 1995). Sub-samples (30-mL) were transferred to a series of 50-mL borosilicate (Pyrex®) culture tubes equipped with polypropylene screw caps and inoculated with ^{15}N-ammonium chloride (98.85 at.%; Cambridge Isotopes), ^{15}N-sodium nitrate (98.25 at.%) or ^{15}N-urea (98.2 at.%) at a range of initial substrate concentrations: 0.1, 0.2, 0.4, 0.8, 1.6, 2.4, 4.2, 6.0 and 12 μg-at N L^{-1}, each conducted in duplicate. Incubations were terminated after 10 min by filtration (50–70 mm Hg) onto 5.0-μm silver membrane filters (Osmonics Inc.) and frozen in polypropylene cryovials; the filtration period was always less than 30 s. Heterotrophic bacteria in the filtrate and cultures were enumerated by epifluorescence microscopy according to Cochlan (2001). Silver filters were employed in these uptake experiments rather than glass fiber filters to minimize the loss of ^{15}N from the particulate fraction to the filtrate; potential losses due to extensive cell lysis are routinely observed during 'tortuous path' filtration when using glass fiber filters (Herndon, 2003). After thawing/drying (<50 °C for minimum of 24 h) the filters were analyzed for isotopic enrichment with a Europa Scientific Robo-Prep Tracermass mass spectrometer.

2.4. Calculations

Nitrogen specific uptake rates (V [h^{-1}] N taken up per unit PN) were estimated from the accumulation of ^{15}N in the particulate material, and calculated according to a constant specific uptake model (Eq. (6) of Dugdale and Wilkerson, 1986). Absolute uptake rates (ρ [μg-at N L^{-1} h^{-1}]) were calculated by multiplying specific rates by the mean particulate nitrogen (PN) concentrations; cell normalized rates were calculated by dividing these volumetric rates by the cell abundance at the beginning of individual kinetic experiments. Rates were not corrected for the effects of isotopic dilution (Glibert et al., 1982) as these are expected to be minimal due to the extremely short incubation times and lack of micrograzers in the unialgal cultures.

Curve fitting was completed using a computerized, iterative non-linear least-squares technique (Kaleidograph®; Abelbeck Software) which utilizes the Levenberg-Marquardt algorithm (Press et al., 1992). Data were initially linearized and plotted using a double reciprocal Hanes–Woolfe method (Dowd and Riggs, 1965), and these derived values were entered into the curve fitting package. The N kinetics data were fitted directly to the Michaelis–Menten formulation:

$$V = \frac{V_{\max} S}{K_s + S} \tag{2}$$

where V is the PN specific uptake rate (h^{-1}), $V_{\max}$ the maximal specific uptake rate, S the substrate concentration (μg-at N L^{-1}), and K_s is the half-saturation constant for the N substrate (μg-at N L^{-1}). The substrate affinity at low concentrations (i.e., $S < K_s$) was determined from the initial slope (α) of the Michaelis–Menten plot, and was calculated as $\alpha = V_{\max}/K_s$; the derivative of Eq. (2), with respect to S, as S approaches zero (Button, 1978; Healey, 1980; Cochlan and Harrison, 1991a). The kinetic parameters have been calculated separately for the individual cultures, as well as for the data treated together (Table 1). All other data processing and statistical analyses were conducted using Microsoft® Excel and MINITAB™ 13 Statistical Software.

3. Results

3.1. Growth rates

In vivo fluorescence provides an accurate estimate of cellular growth rate during the exponential growth phase of *H. akashiwo*; linear regressions of cell abundance (determined microscopically) versus raw fluorescence units (RFU) yield coefficient of determination (r^2) values of ≥0.99, and both RFU and cell abundance also are strongly correlated ($r^2 \geq 0.99$) with extracted chl. *a* (data not shown). Despite these strong correlations, cell counts determined over time yield a slightly higher growth rate than RFU by 6–9%, possibly related to the uncoupling of chloroplast replication from cell division and the highly variable chlorophyll content per cell observed by others for *H. akashiwo* (see review by Smayda, 1998).

The specific growth rates of *H. akashiwo* determined in the N growth preference experiments at saturating PPFD averaged 0.82–0.89 d^{-1} during exponential

Table 1
Kinetic parameters for N uptake by duplicate cultures of N-replete *Heterosigma akashiwo*

Culture no.	Cell and PN concentration		NO_3^-				NH_4^+				Urea			
	$\times10^6$ L^{-1}	µM	V_{max}	K_s	α	r^2	V_{max}	K_s	α	r^2	V_{max}	K_s	α	r^2
1	16.6	47.3	17.1 (2.28)	1.68 (0.48)	10.2	0.92 (12)	27.2 (3.55)	2.23 (0.81)	12.2	0.80 (18)	2.89 (0.24)	0.42 (0.16)	6.88	0.67 (18)
2	10.1	40.3	18.1 (1.30)	1.35 (0.31)	13.4	0.92 (18)	30.6 (1.78)	1.17 (0.23)	26.1	0.94 (18)	–	–	–	–
1 + 2 (combined)			18.0 (1.08)	1.47 (0.25)	12.2	0.92 (30)	28.0 (2.17)	1.44 (0.35)	19.4	0.81 (36)	–	–	–	–

Rates of NH_4^+, NO_3^- and urea uptake are reported in units of $\times10^{-3}$ h^{-1}. Half-saturation constants (K_s) and PN concentrations are reported in µg-at N L^{-1}. PN concentrations are the average of the six samples enriched at <0.5 µg-at N L^{-1} for each uptake kinetic experiment after 10-min incubation. Substrate affinity ($\alpha = V_{max}/K_s$) is reported in units of $\times10^{-3}$ h^{-1}/(µg-at N L^{-1}). Estimated standard error (S.E.) values of parameters are given in parentheses. The r^2 column provides the coefficient of determination and the sample size (n). Cell abundances were determined on fresh samples.

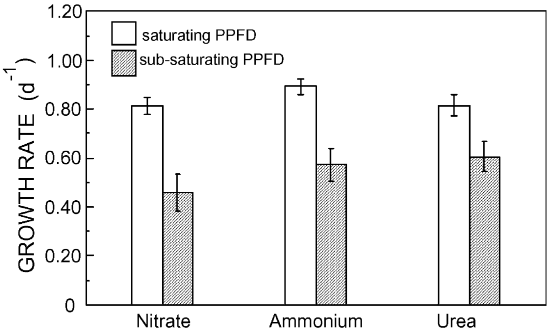

Fig. 2. *H. akashiwo* growth rates as a function of PPFD and nitrogen source (50 µg-at N L^{-1}) at 15 °C. Means of triplicate ($n = 3$) cultures at sub-saturating (40 µE m^{-2} s^{-1}) and saturating (110 µE m^{-2} s^{-1}) irradiance are shown; error bars are ±1 standard deviation. The mean growth rates for high and low PPFD are NO_3^-: 0.82 ± 0.035 and 0.46 ± 0.078 d^{-1}; NH_4^+: 0.89 ± 0.032 and 0.57 ± 0.064 d^{-1}; urea: 0.82 ± 0.043 and 0.61 ± 0.059 d^{-1}.

phase (Fig. 2). These rates do not differ from those obtained on enriched natural seawater (Herndon, 2003), and are within the range reported for other *H. akashiwo* strains grown under similar environmentally controlled (temperature, light and salinity) conditions. For example, the growth rate of a number of Japanese strains range from ~1 to 1.4 d^{-1} (reviewed by Honjo, 2003), growth rates of a well-studied Spanish strain range from 0.7 to 1.2 d^{-1} (Wood and Flynn, 1995; Clark and Flynn, 2002), the New Zealand strain used by Chang and Page (1995) grows between 0.69 and 1.39 d^{-1} (interpreted from their 3D figures), the long-maintained Long Island Sound strain grows at 0.65 d^{-1} (Cattolico et al., 1976) and the Narragansett strain, although highly dependent on temperature and salinity, grows at *ca.* 0.9 d^{-1} at 30 ppt and 15 °C (Tomas, 1978). The maximum growth rate observed in our experiments during exponential growth phase was 1.1–1.2 d^{-1} at 15 °C and 110 µE m^{-2} s^{-1} which, like those reported above, is considerably less than the remarkable rates reported for outdoor cultures of wild *H. akashiwo* from Sagami Bay (Honjo and Tabata, 1985). Here they report maximum rates of 2.4 d^{-1} during exponential growth (average = *ca.* 1 d^{-1}) and 3.5 d^{-1} in rapid growth, whereas their laboratory cultures grew exponentially at rates similar to ours (*ca.* 0.7–2.3 d^{-1}).

In the growth rate experiments, a two-way ANOVA ($\alpha = 0.05$) reveals that both light ($p < 0.0001$, $f = 138.6$) and N substrate ($p = 0.021$, $f = 5.4$) individually have significant effects on the growth rate of *H. akashiwo* (Fig. 2). Irradiance had a strong effect; cells grown at high light grew faster than cells grown at low light for all N substrates. At saturating PPFD (110 µE m^{-2} s^{-1}), cells supplied with NH_4^+ grew 9%

faster (0.89 d^{-1}) than cells grown on either urea or NO_3^- (both 0.82 d^{-1}; Fig. 2). At sub-saturating PPFD (40 µE m^{-2} s^{-1}), NH_4^+- and urea-maintained cells grew 24 and 33% faster than NO_3^--grown cells (0.57, 0.61 and 0.46 d^{-1}, respectively). The interactive effect of N substrate and light on growth, i.e. does irradiance affect which substrate supports the highest growth rate, is difficult to discern ($p = 0.087$, $f = 3.0$). Although urea supports the greatest growth rate at low PPFD and the lowest growth rate (but equal to NO_3^-) at high PPFD, there are not sufficient data to confirm the relationship between light and N substrate. Increasing the number of replicates for each light/substrate combination in future experiments will likely demonstrate a significant interaction effect of light and substrate. Examination of the growth rates with Student's *t*-tests indicates that there is only a statistical difference between NH_4^+ and NO_3^- at high PPFD (*t*-test $p = 0.0052$), whereas there are no statistical differences between the growth rates of urea, NO_3^- and NH_4^+ at low PPFD.

3.2. Nitrogen uptake kinetics

The uptake rates of NO_3^-, NH_4^+ and urea by *H. akashiwo* can be related to their external substrate concentration following Michaelis–Menten type kinetics (Table 1, Fig. 3). In this study, during 10-min incubations, the specific NH_4^+- and NO_3^--uptake rates can be effectively described as a hyperbolic function of their concentration, and saturate at concentrations at or below the highest N concentration employed (12 µg-at N L^{-1}), although it is not known if higher concentrations would reveal nonsaturating (linear) or biphasic uptake kinetics as found elsewhere (e.g., Collos et al., 1997; Lomas and Glibert, 1999). The highest urea concentration used (12 µg-at N L^{-1}) may have been insufficient to fully saturate specific urea uptake by *H. akashiwo* (based on one datum), but a trend toward possible saturation rates is apparent. Greater urea concentrations would be required to clearly demonstrate non-saturable kinetics. A comparison of the maximum uptake rates (V_{max}) indicated that the substrate-saturated uptake rates of NH_4^+ were greatest, followed by uptake rates of NO_3^- and urea, with average (pooled means from replicate cultures) PN-specific rates of 28.0, 18.0 and 2.89 × 10^{-3} h^{-1} for NH_4^+, NO_3^- and urea, respectively. The average K_s values for NH_4^+ and NO_3^- are 1.44 and 1.47 µg-at N L^{-1}, respectively, and are indistinguishable, whereas a considerably lower K_s value for urea was derived from the sole urea experiment, 0.42 µg-at N L^{-1}. The α values for NO_3^- and NH_4^+ range from 10.2 to 26.1 × 10^{-3} h^{-1}/(µg-at N L^{-1}) and are two to four times greater than the α value for urea (6.88 × 10^{-3} h^{-1}/(µg-at N L^{-1})) indicating that the

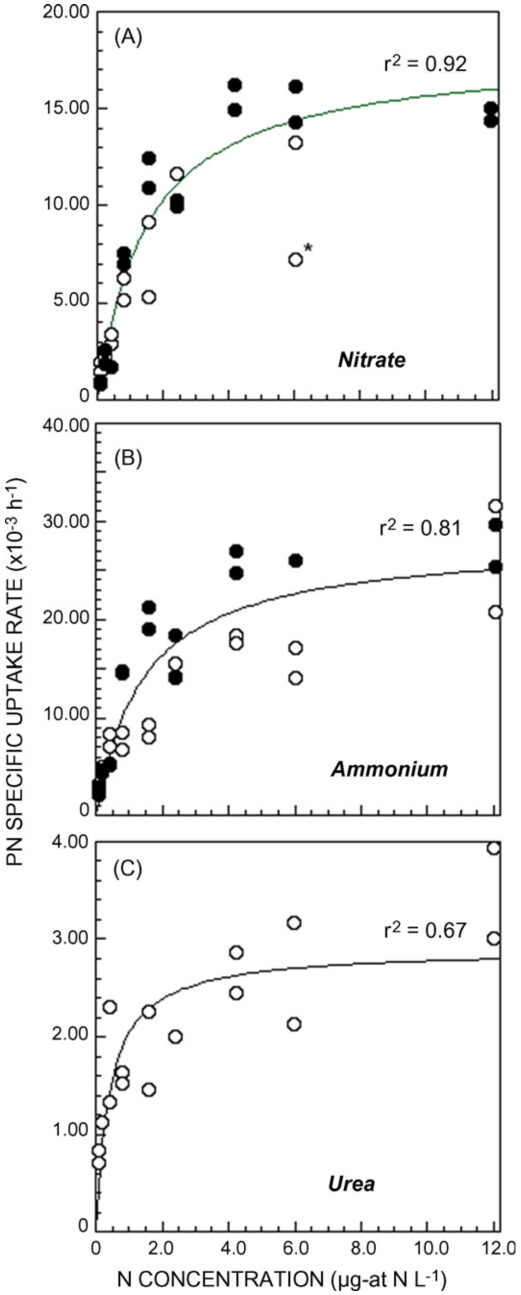

Fig. 3. Specific uptake rates (h^{-1}) of NO_3^- (A), NH_4^+ (B), and urea (C) for duplicate, nitrate-replete cultures of *H. akashiwo* plotted vs. substrate concentration calculated at the beginning of each 10-min incubation period. Rate estimates for culture one (○) and culture two (●) are fitted together to the Michaelis–Menten equation (see text for details). Note the change in scale of ordinal axes. Asterisk (*) in panel A denotes a datum not used in curve-fitting calculations.

affinity of *H. akashiwo* for urea is less than either of the inorganic N substrates. The K_s and α values for the duplicate NO_3^--enriched cultures agreed well with each other, but poorer agreement was found between the affinity estimates for ammonium-enriched cultures. Although not quantified, it is possible that the discrepancy between these estimates is due to a loss of labeled isotope during filtration from the cells into the surrounding medium. Despite utilizing a very low vacuum pressure differential (50–70 mm Hg), and employing smooth filters, some cell disruption may have occurred given the fragile nature of *H. akashiwo* cells.

An underlying assumption in the use of the Michaelis–Menten equation for the estimation of kinetic uptake parameters is that uptake remains constant over the duration of the experiment. Non-linearity in uptake rates in such experiments may result as a consequence of elevated (surge) uptake response in N-deficient cells (e.g., Conway et al., 1976; Goldman and Glibert, 1982) or substrate exhaustion (e.g., Goldman et al., 1981; Fisher et al., 1981). In our 10-min incubations, cells very recently depleted of N were used, so it is unlikely that the non-linearity in uptake rates that has been reported for N-deficient flagellates (e.g., Cochlan and Harrison, 1991b) including *H. akashiwo* (French and Smayda, 1995) would occur over the incubation periods. Based on the isotopic enrichment and concentration of the ^{15}N-substrate added, and the at.% excess and PN concentration collected at the end of the short incubations, it was calculated that on average (±1 S.D.) $5.3 \pm 5.1\%$ ($n = 36$), $5.0 \pm 4.0\%$ ($n = 30$), and $1.4 \pm 2.1\%$ ($n = 18$) of the ^{15}N-substrate was used during the $^{15}NH_4^+$, $^{15}NO_3^-$ and ^{15}N-urea kinetic experiments, respectively. Even at the lowest N substrate enrichment (0.10 μg-at N L^{-1}), only an average of $10.8 \pm 5.9\%$ ($n = 10$) was used. So despite the elevated concentrations of phytoplankton biomass (average PN = 47.3 and 40.3 μg-at N L^{-1} for cultures 1 and 2), substrate depletion was not considered a problem in these N-uptake experiments, even at the lowest N enrichments. Bacterial N uptake (e.g. see review by Kirchman, 1994) was not a complicating issue in these experiments as the 5.0-μm pore-size filters employed in this study retained less than 1% of the heterotrophic bacteria present in the cultures. Bacterial N biomass (estimated using 5.6 fg-at N $cell^{-1}$; Lee and Fuhrman, 1987) captured by the filters are only *ca.* 0.02% of the PN collected for isotopic enrichment, and thus bacterial N uptake is considered insignificant in the resultant uptake rates reported.

4. Discussion

4.1. Growth rates

Preference for a particular N source can be assessed by comparing maximum uptake rates (V_{max}) or maximum growth rates (μ_{max}) for one N substrate in the absence of other N substrates (e.g., Dortch, 1990). The reader is cautioned not to confuse these measures of N preference with the relative preference index (RPI, McCarthy et al., 1977), which compares the relative utilization of a particular N substrate to the relative availability of that N substrate in the water. As pointed out by others (Dortch, 1990 and references within; Stolte and Riegman, 1996), the RPI has several problems which limit its ecological relevance and contribute to its misinterpretation, and was originally introduced as a measure of N sufficiency of field environments, or competitive interaction between N substrates (McCarthy, 1981) rather than an index of physiological N preference by algae.

The substrate growth preference experiments in this study demonstrate that NH_4^+, NO_3^- and urea all serve as good N sources for *H. akashiwo,* although NH_4^+ supports faster exponential growth rates than NO_3^- or urea at high light levels. However, at low light, cells supported by NO_3^- grew slower than either NH_4^+ or urea-grown cells. Lack of definitive N preference for inorganic N nutrition has been reported previously for Japanese *Heterosigma* strains obtained from Gokasho Bay (Iwasaki and Sasada, 1969; named *H. inlandia*), the Fukuyama coast (Iwasaki et al., 1968; named *Entomosigma* sp), Osaka Bay (Watanabe et al., 1982) and Tokyo Bay (Hosaka, 1992). In these studies, actual growth rates were not always reported, but growth yields after specific growing periods generally showed little or no preference for NH_4^+ over NO_3^- at the low N concentrations (50–70 μg-at N L^{-1}) utilized, and sometimes slight NO_3^- preference at higher concentrations (>700–2000 μg-at N L^{-1}) likely due to NH_4^+ toxicity. In contrast, Wood and Flynn (1995; named *Heterosigma carterae*) found that the maximum cell-specific growth rates for NH_4^+-grown cells isolated from Spain were at least 20% greater than NO_3^--grown cells, and that these differences were statistically significant at high PPFDs (200 and 300 μE m^{-2} s^{-1}), but not at 50 μE m^{-2} s^{-1}. Chang and Page (1995; named *Heterosigma carterae*) also report the differential effect of light on N preference for a New Zealand isolate. At the high PPFDs employed (160 and 80 μE m^{-2} s^{-1}), growth was highest with NO_3^-, intermediate with NH_4^+ and lowest with urea, whereas at the lower PPFD (40 μE m^{-2} s^{-1}) the growth rates of NO_3^--grown cells were lower than those of either NH_4^+- or urea-grown cells, although it is unclear if these differences were statistically significant.

The utilization of urea for growth by *H. akashiwo* has been less studied, but generally well-illuminated cultures use urea less effectively for growth than NO_3^- or NH_4^+ (Iwasaki and Sasada, 1969; Watanabe et al., 1982; Chang and Page, 1995) or not at all (Hosaka, 1992). In the present study, urea served as a good N substrate for growth at both high and low PPFDs, and the cells exhibited no evidence of urea toxicity such as reduced growth, morphological distortion or cell inflation that have been observed by others at higher (>400 μg-at N L^{-1}) concentrations (e.g., Chang and Page, 1995).

The differential growth rates as a function of N seen in this study, but not always by others, may be due to physiological differences in the various strains utilized and/or subtle differences in the experimental design employed, including varying N concentrations, PPFDs, photoperiod, seawater medium, and temperature for culture maintenance. Temperature, for example, has been shown to greatly influence N preference for growth: <17 °C, nitrate supports 30–100% higher growth rates in *H. akashiwo* batch cultures, but >17 °C, NH_4^+ supports 20–40% higher growth rates than nitrate (Miki, 1983 as cited by Okaichi, 2003). Given the important regulatory role of temperature in controlling physiological processes supporting algal growth, one may expect nutrient requirements, N preferences and concentration dependency to change as a function of bloom development under field conditions.

4.2. Nitrogen uptake kinetics

The kinetic parameters of N uptake can be used to assess the relative preference (and affinity) of one N substrate over another in the low and high N environments representative of oligotrophic and eutrophic areas, respectively. In the present study, the V_{max}-NH_4^+ for N-sufficient *H. akashiwo* cultures were ~1.6-fold greater than the V_{max}-NO_3^-, but ~10-fold greater than V_{max}-urea. It has been suggested that based on the level of amino acid synthesis by *H. akashiwo*, as indicated by the ratio of glutamine to glutamate, NO_3^--grown cells are stressed relative to NH_4^+-grown cells (Wood and Flynn, 1995). Nitrogen deficiency or stress may elicit short-term elevated (surge) uptake rates of NH_4^+ in N-deficient or –starved phytoplankton (e.g., Conway et al., 1976; Goldman and Glibert, 1982; Cochlan and Harrison, 1991b) including *H. akashiwo* (French and Smayda, 1995). However, it is unknown if such transient elevated uptake velocities contributed to measured V_{max}-NH_4^+ reported in the present study, where exponentially growing cells recently depleted of NO_3^- were used in short-term (10-min) incubations.

Based on these single substrate experiments, the N preference of *H. akashiwo* follows the order: $NH_4^+ > NO_3^- >$ urea during high ambient N conditions. However the half-saturation constants, which are traditionally used to indicate nutrient affinity under nutrient-limited conditions, are equivalent for NH_4^+ and NO_3^-, but more than triple the K_s for urea. The K_s values could be interpreted as the cells' more effective capability to utilize low concentrations of urea than either NH_4^+ or NO_3^-. However, the interpretation of K_s as measure of affinity is complicated by the functional relationship between V_{max} and K_s, and is not necessarily a reliable indicator of preference at low nutrient concentrations (e.g., Healey, 1980). Determining both parameters and combining them into a ratio ($\alpha = V_{max}/K_s$; the initial slope of the uptake versus substrate concentration curve) emphasizes both factors, and provides a more descriptive picture of nutrient affinity at sub-saturating concentrations ($<K_s$) and when interspecies competition is likely to occur (e.g., Healey, 1980; Harrison et al., 1989; Cochlan and Harrison, 1991a).

In the present study, the average values of the affinity coefficient α for NH_4^+ are 1.6- and 2.8-fold greater than the α values for NO_3^- and urea, respectively. These results demonstrate that *H. akashiwo* can utilize low (sub-saturating) concentrations of NH_4^+ more effectively than equivalent concentrations of NO_3^- and urea, and that N preference under sub-saturating N concentrations would also follow the order $NH_4^+ > NO_3^- >$ urea. Although it is difficult to ascribe the results determined under optimal laboratory conditions to those occurring during natural blooms of *H. akashiwo*, our results suggest that under both conditions of N-limited and N-sufficient growth, NH_4^+ is acquired more readily than NO_3^-, and that urea is the least preferred of the N sources tested during short-term N uptake experiments.

It is not unexpected that despite the three N substrates supporting very similar growth rates (0.8–0.9 d^{-1}), the V_{max} values for the N substrates differ both from each other and from the corresponding growth rates achieved during exponential growth. This apparent lack of agreement likely is the result of a number of factors including (1) the difference in measurement period (days versus minutes) between the N growth experiments and the N uptake experiments, (2) N uptake and cellular growth processes are not necessarily coupled (i.e., not balanced and equal) during the short-term kinetic experiments, and (3) phytoplankton physiology and cell quotas are adaptable over time (e.g., see reviews by McCarthy, 1981; Goldman and Glibert, 1982) resulting in equivalent growth on NH_4^+, NO_3^- and urea over longer time periods. Since each series of kinetic experiments reported here was conducted with cells from a single population with a uniform nutritional and growth history (i.e., all NO_3^--grown and in exponential phase), the effect of growth rates on kinetic parameters shown by others (see Goldman and Glibert, 1982) do not affect the present results.

Recently, N uptake kinetic parameters (both V_{max} and K_s) also have been shown to vary as a function of the N substrate used to precondition cultures of the bloom-forming dinoflagellate *Prorocentrum minimum* (Fan et al., 2003). For example, they found that the affinity for NO_3^- in a NO_3^--grown culture differs from that observed in NH_4^+ and urea-grown cultures. While the well-known effects of NH_4^+ on NO_3^- uptake/assimilation (and to a lesser extent on urea) might be expected to affect their uptake kinetic parameters, it is unclear from their results if the effects of the different growth N substrates on kinetic parameters are a function of growth rates achieved on each substrate (data not given) or solely due to the substrate itself and its effects on the cells' nutritional state. The effect of the different growth N substrates on N uptake kinetics was not examined in the present study.

Despite differences in the strain of *H. akashiwo* used and laboratory methodologies employed, it is appropriate to compare the results of the present N kinetics experiments to those reported by Tomas (1979) for a Narragansett strain. The differences in K_s values are relatively small. In our study, the values are lower by an average of 26 and 27% for NO_3^- and NH_4^+, respectively (1.35–1.68 and 1.17–2.23 μg-at N L^{-1} for NO_3^- and NH_4^+ versus 1.99–2.45 and 1.97–2.33 μg-at N L^{-1} reported by Tomas). There also are no substantial differences in the K_s values for NO_3^- or NH_4^+ within each study. The differences in V_{max} values are more dramatic. In the present experiments, the maximum N uptake rates normalized to cell abundance are 0.81 and 1.20 fg-at N $cell^{-1}$ min^{-1} for NO_3^-, 1.29 and 2.03 fg-at N $cell^{-1}$ min^{-1} for NH_4^+, and 0.14 fg-at N $cell^{-1}$ min^{-1} for urea. Tomas (1979) reports V_{max}-$NO_3^- > V_{max}$-NH_4^+ (urea not tested), and rates that are three orders of magnitude greater than those presented here. However, the difference in rates between the two studies is simply the result of a typographical error during manuscript preparation (Tomas, pers. commun.); the units of N uptake in his original publication (and cited by Smayda, 1998) should be fM $cell^{-1}$ min^{-1}, not pM $cell^{-1}$ min^{-1}. Our study also demonstrates that although *H. akashiwo* can readily utilize urea as a N source for growth, the uptake kinetic parameters indicate that both NH_4^+ and NO_3^- are utilized preferentially when present in either large (N-saturating) or small (N-limiting) concentrations. These results affirm the importance of anthropogenic N sources as potential contributors to the development and/or sustenance of potentially harmful algal blooms in urbanized coastal regions such as San Francisco Bay (USA), Tokyo Bay (Japan) and the Strait of Georgia (Canada) where *H. akashiwo* blooms when reported, are widespread.

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