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Uptake of *Spartina*-derived humic nitrogen by estuarine phytoplankton in nonaxenic and axenic culture

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Abstract

Humic substances are a collection of colored organic acids characterized by high molecular weight and low nitrogen (N) content that are thought to be biologically recalcitrant. We examined a suite of nonaxenic estuarine phytoplankton isolates to determine their ability to take up ^{15}N-labeled humic substances formed in the laboratory and supplied as the sole N source. All 17 estuarine and coastal strains took up the added humic N, but the one polar isolate did not. Two of the coastal isolates (*Heterosigma akashiwo* and *Fibrocapsa japonica*) could take up the humic N in nonaxenic culture but not in axenic culture, suggesting that bacterial remineralization played a role in making humic N accessible to these species. The ability of nonaxenic phytoplankton isolates to use humics of different ages (1 week to 1 yr old) was tested using three strains capable of taking up humic N at high rates. Younger, fresher humics were taken up by the phytoplankton strains at higher rates than older, more fulvic-like compounds, and at rates higher than inorganic N uptake run in parallel. Time-course results indicate that while uptake of the inorganic N forms was sustained, high rates of humic N uptake declined after the first few hours of incubation. Additional humic substances were labeled with both ^{15}N and ^{13}C, and the relative incorporation of N versus carbon (C) was used to infer potential uptake mechanisms. None of the isolates took up humic C, suggesting that uptake of the humic N followed breakdown of the humic molecule by bacteria or via extracellular enzyme cleavage of humic N. Regardless of the mode of uptake, the observation that humic N can be rapidly used by phytoplankton suggests that the importance of humic N as a source of phytoplankton N nutrition should be reevaluated.

On average, approximately 7.3×10^{12} g of dissolved organic nitrogen (DON) is discharged into the coastal ocean annually (Meybeck 1982; Sarmiento and Sundquist 1992), with 40–80% of this nitrogen (N) considered to be humic in nature (Beck et al. 1974; Thurman 1985). However, the ability of estuarine and coastal phytoplankton strains to use humic substances as an N source has not been thoroughly investigated. Owing to their size and chemical composition, humics have traditionally been perceived as biologically recalcitrant, largely because they are high molecular weight (HMW), carbon (C)-rich compounds with N making up only 0.5–6% of the humic molecule (Rashid 1985; Thurman 1985; Hedges and Hare 1987). Furthermore, only 50% of humic N is in the amine form and therefore likely to be labile (Schnitzer 1985). More recently, however, the refractory nature of humic substances has been challenged, and evidence is accumulating that indicates that coastal phytoplankton may have the ability to take up humic N, either directly or after remineralization by bacteria (Carlsson et al. 1995, 1998, 1999). More recently, the uptake of laboratory produced ^{15}N-labeled humic compounds into the >0.7-μm size fraction has been observed in both riverine and coastal ecosystems (Bronk et al. unpubl. data), humic substances were shown to be a potential source of C and/or N to the toxic dinoflagellate *Alexandrium catenella* (Doblin et al. 2000), and growth of the toxic dinoflagellate *Alexandrium tamarense* was shown to increase when exposed to humic substances (Gagnon et al. 2005). There is also evidence that humic substances are not as N poor in natural waters as currently believed. It has been shown that during the process of isolating humic substances with macroporous resins N is stripped from the humic structure, resulting in

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Table 1. Coastal isolates used for the uptake experiments.

Taxa	Strain	Origin	Culture salinity
Chlorophyceae			
Ankistrodesmus sp.	HP9101	Choptank River (estuary), Maryland	16
Selenastrum sp.	SCAEL010524-NF	Kiawah Island (brackish pond), South Carolina	5
Chrysophyceae			
Ochromonas sp.	SCAEL970626	North Inlet (estuary), South Carolina	30
Cryptophyceae			
Storeatula major	HP9001	Choptank River (estuary), Maryland	16
Cyanophyceae			
Anabaenopsis elenkini	SCAEL010524-1C3	Kiawah Island (brackish pond), South Carolina	5
Limnothrix sp.	HP9101	Choptank River (estuary), Maryland	16
Synechococcus sp.	HP9101	Choptank River (estuary), Maryland	16
Bacillariophyceae			
Nitzschia sp.	SCAEL940210	North Inlet (estuary), South Carolina	30
Phaeodactylum sp.	HP9101	Choptank River (estuary), Maryland	16
Thalassiosira cf. *miniscula*	HP9101	Choptank River (estuary), Maryland	16
Dinophyceae			
Amphidinium carterae	CCMP1314	Falmouth Great Pond (brackish), Massachusetts	30
Prorocentrum minimum	SCAEL010403-1A3	Murrells Inlet (estuary), South Carolina	30
Haptophyceae/Prymnesiophyceae			
Phaeocystis cf. *antarctica*	CCMP1871	Bellingshausen Sea, Antarctica	36
Prymnesium parvum	SCAEL010524-1B2	Kiawah Island (brackish pond), South Carolina	5
Pavlova sp.	HP9101	Choptank River (estuary), Maryland	16
Raphidophyceae			
Chattonella subsalsa	CAAE1662X	Kiawah Island (brackish pond), South Carolina	20
*Fibrocapsa japonica**	CAAE1661X	Kiawah Island (brackish pond), South Carolina	20
*Heterosigma akashiwo**	CAAE1665X	Neuse River (estuary), North Carolina	20

* Both axenic and nonaxenic strains were examined.
Culture collection codes: HP = Horn Point Laboratory; SCAEL = South Carolina Algal Ecology Laboratory; CCMP = Provasoli-Guillard National Center for Culture of Marine Phytoplankton; CAAE = Center for Applied Aquatic Ecology.

an apparent C : N ratio of isolated humic substances that is likely lower than those same humics had in natural waters prior to isolation (See and Bronk 2005).

The current study had four objectives: (1) to determine the prevalence of humic N uptake capability among several estuarine phytoplankton strains, (2) to determine how the uptake of humic N compares to that of dissolved inorganic N (DIN), (3) to determine how changes in age and structure of the humic compound affect the uptake of humic N, and (4) to determine what mechanisms are likely to occur in the estuarine environment that would allow phytoplankton to access humic N.

Materials and methods

Culture selection—Seventeen phytoplankton isolates from estuarine environments and one polar clone (*Phaeocystis* cf. *antarctica*) were tested to determine their ability to access humic N as an N source (Table 1). *P. antarctica* was used because it is a species not typically exposed to estuarine humic substances. The cultures were maintained in 0.2-μm filtered f/2-enriched Sargasso seawater (Guillard 1983), diluted with deionized water (DIW) to the appropriate salinity when necessary. All coastal cultures were grown on a 12 : 12 light : dark (L : D) cycle under fluorescent light at 20°C; the polar strain was grown under constant light at −1.5°C. The cultures were not axenic, but bacterial biomass was 5% ± 6% of the total biomass in the cultures as determined by the DAPI (4′, 6-diamidino-2-phenylindole) method of Porter and Feig (1980).

Several months following the initial survey, humic uptake was measured in two of the isolates (*Fibrocapsa japonica* and *Heterosigma akashiwo*) under axenic and nonaxenic conditions to determine whether the presence of bacteria was necessary for humic N uptake. Each strain was maintained under identical conditions to the coastal isolates used in the earlier experiments.

Preparation of ^{15}N-labeled humics from Spartina alterniflora—Humics labeled with ^{15}N were produced in the laboratory by growing *Spartina alterniflora* plants with ^{15}N-labeled ammonium chloride (NH_4Cl) added to the surrounding sediment. *S. alterniflora* was chosen as the source for humic formation because it is the dominant primary producer within most marsh ecosystems of the southeastern United States and can be responsible for 80% or more of marsh primary production (Alberts and Filip 1994). Small *S. alterniflora* plants were collected at the Skidaway Institute of Oceanography (SkIO), grown in buckets over a period of 3 months (April–June), and watered regularly with a 4.0 mmol L^{-1} $^{15}NH_4Cl$ solution (Cambridge Isotope Laboratories; ^{15}N, 98+%). The ^{15}N label was added to the plants in a series of 32 treatments (approximately every third day). *S. alterniflora* plants were harvested, dried in an oven for 1 week at 40°C, and shredded in a Wiley mill (60 mesh). The shredded *S.*

Table 2. Humic label used for uptake experiments.

Age	Concentration (mg humic C L^{-1})	Atomic C : N ratio	Atom % enrichment
1 week	129.7±1.0	38.1	7.4
2 weeks	87.2±2.2	20.7	8.1
1 month	77.8±3.9	23.7	9.0
3 months	90.1±6.4	23.2	9.8
6 months	127.3±0.8	25.5	10.0
1 yr	64.11±0.6	18.8	6.0
Dual-labeled	14.35±0.2	14.0	C: 2.73 N: 17.96

alterniflora (8 g) was then added to 1 liter of coastal seawater collected from SkIO, from which the humic material had been extracted (*see following*), and spun in the dark with a magnetic stir bar. To determine the effects of aging on bioavailability, subsamples were removed from the dark at 1 week, 2 weeks, 1 month, 3 months, 6 months, and 1 yr (Table 2). At each time point, the aged humic substances were isolated onto DAX-8 resin (*see following*), neutralized, and frozen until used in the uptake experiments.

Preparation of dual-labeled (13C and 15N) humics—To prepare dual-labeled humic substances, *S. alterniflora* plants were grown under identical conditions described above for the ^{15}N-labeled humics with the following exceptions. In a series of three treatments, ^{13}C-labeled sodium bicarbonate (Isotec; ^{13}C, 98+%) was added to the surrounding atmosphere. Three grams of ^{13}C-labeled sodium bicarbonate was added to a beaker, and both the plant and beaker were enclosed in a clear nylon bag (Reynolds Metals Company). Hydrochloric acid (HCl, 6 mol L^{-1}) was then added to the beaker to release ^{13}C-labeled carbon dioxide (CO_2) into the atmosphere. All dual-labeled humic substances were extracted onto DAX-8 after 3 months.

Isolation of humic substances—Humic substances were extracted onto Supelite DAX-8 resin as previously described by Aiken (1988) for Amberlite XAD-8. DAX-8 is an acrylic ester resin, and both Supelite DAX-8 and Amberlite XAD-8 resins have been shown to isolate comparable bulk humic solutes from aquatic sources, producing mixtures with similar chemical compositions (Peuravuori et al. 2002). Because humic substances adsorb to the DAX-8 resin in the protonated form, each sample was acidified with 6 mol L^{-1} HCl to a pH <2 and passed through a glass column (2.5 cm $\times$ 50 cm) packed with acidified DAX-8 resin. The resin was then rinsed with DIW until the eluate reached a pH >5 to remove any remaining salts from the resin. Following the rinse, the column was backflushed with two bed volumes of 0.2 mol L^{-1} sodium hydroxide (NaOH) to elute the bound humic substances from the resin.

XAD-8 resins have previously been shown to bleed small amounts of organic molecules with the eluate (Aiken 1988). Therefore, prior to the extraction of humics, the DAX-8 resin was cleaned over several days via a Soxhlet extraction procedure (solvents include ether, acetonitrile, and methanol) followed by extensive rinses of HCl, NaOH, and DIW (Thurman 1985). Prior to sample extractions, DIW was passed through the columns to establish baseline levels of DON, ammonium (NH_4^+), nitrate (NO_3^-), and dissolved organic C (DOC) that may leach from the DAX-8 resin.

Uptake experiments: Coastal phytoplankton isolates—Prior to the incubation of estuarine strains with the laboratory-formed ^{15}N-labeled humics, each isolate was transferred a minimum of two times into amended f/2-enriched seawater containing commercial humic acid salts (Aldrich humic acids) at a concentration of 833 μmol L^{-1} humic C. The concentration of NO_3^- in the media was also reduced to ensure that N-limiting conditions would occur (10 : 1 N : P). Culture growth was monitored daily by in vivo chlorophyll *a* (Chl *a*) fluorescence on a Turner 10 AU fluorometer. Solid Chl *a* standards were used to convert in vivo Chl *a* fluorescence to actual Chl *a* concentration for the uptake experiments. Uptake experiments were initiated only after each individual culture had depleted NO_3^- to concentrations less than 1 μmol N L^{-1}.

To measure uptake of humic N, 15 mL of each isolate was dispensed into six 25-mL culture tubes. Two culture tubes received ^{15}N-labeled humic substances (2 weeks old, final concentration 10 μmol L^{-1} humic N). Two culture tubes were used as killed controls to correct for adsorption of the labeled humics to phytoplankton cells and filter. The killed controls received a 15-μL aliquot of a saturated mercuric chloride (HgCl) solution, were mixed by hand, and then left for 5 min prior to the addition of ^{15}N-labeled humics (final concentration 10 μmol L^{-1} humic N). The remaining two culture tubes received 0.13 μmol L^{-1} $K^{15}NO_3$ (^{15}N, 98+%) and were used to ensure that cultures were active when the uptake experiments were started.

Samples were incubated for 3 h in an incubator under fluorescent light at the culture maintenance light and temperature levels (11.6 μmol quanta m^{-2} s^{-1} and 20°C for coastal isolates and 56.5 μmol quanta m^{-2} s^{-1} and −1.5°C for the polar strain). Following incubation, phytoplankton cells were filtered onto a 25-mm Whatman GF/F filter (0.7-μm nominal pore size) and frozen until mass spectrometric analysis.

Uptake experiments: Time course—Time-course experiments were conducted to determine whether the uptake of humic N was sustainable, or whether only a small fraction of the bound N was bioavailable. The use of a time course also allowed for the monitoring of changes in the ratio of C

uptake to N uptake for the dual-labeled humics. In these time-course experiments, three of the phytoplankton isolates that took up humic N (*Synechococcus* sp., *Amphidinium carterae*, and *Thalassiosira* cf. *miniscula*) were examined to determine how changes in humic age would affect N uptake rates. While these isolates did not possess the highest normalized uptake rates (nmol humic N μg Chl a^{-1} h^{-1} or nmol humic N μg $cell^{-1}$ h^{-1}), they did exhibit high bulk uptake. In addition, *Synechococcus* sp. (Cyanophyceae) was selected because its small size likely restricts uptake mechanisms to extracellular or cell-surface enzymes, one mechanism phytoplankton may use to cleave amino groups from humic molecules. *A. carterae* was selected because it has shown evidence of increased growth when exposed to high concentrations of humics (Granéli and Moreira 1990). Finally, *T. miniscula* was selected because we were unaware of a diatom found to possess cell-surface amino oxidases (Palenik and Morel 1990); some diatoms, however, do appear to possess a high affinity for organic molecules (Lewin and Lewin 1960, 1967).

In the time-course experiments, parallel sets of the three strains received 40 μmol N L^{-1} of either ^{15}N-labeled humic N (aged 1 week to 1 yr), dual-labeled humics, $^{15}NH_4Cl$ (98+ atom %), or $K^{15}NO_3$ (98+ atom %). The 40 μmol N L^{-1} concentration was used to ensure that the added substrate would not be exhausted during the experiment. Half of the tubes that received labeled humics were used as killed controls as described above. Samples were then incubated for 24 h in an environmental chamber under fluorescent light on a 12 : 12 L : D cycle. At 1, 3, 12, and 24 h, duplicate samples of each treatment were filtered onto 25-mm Whatman GF/F filters and frozen until analysis.

Determination of uptake rate—Uptake filters were dried at 50°C and wrapped in tin discs for analysis on an isotope ratio mass spectrometer (Europa Geo 20/20 with ANCA sample preparation unit). The uptake rates of humic N and NO_3^- were calculated with the equations of Dugdale and Goering (1967) using bulk humic N as the source pool for the humic N uptake calculations. Adsorption of the humic label to phytoplankton cells and filter was corrected at the atom percentage level with the killed controls subtracted prior to calculation of the uptake rate using the following equation:

$$\text{atom\%xs}_{\text{Corrected}} = \left(\frac{\frac{(\text{PN}_{\text{Live}})(\text{atom \%xs}_{\text{Live}})}{100} - \frac{(\text{PN}_{\text{Killed}})(\text{atom \%xs}_{\text{Killed}})}{100}}{\text{PN}_{\text{Live}}} \right) \times 100$$

where atom $\%xs_{Corrected}$ is the atom percentage excess value (atom percentage value over natural abundance) used for calculating uptake, PN_{Live} is the μmol particulate N L^{-1} in the live samples, atom $\%xs_{Live}$ is the atom percentage excess for the live sample, PN_{Killed} is the μmol particulate N L^{-1} for the poisoned samples, and atom $\%xs_{Killed}$ is the atom percentage excess for the poisoned control samples. Contaminant NH_4^+ was detected in the humic label used for the initial uptake experiment. The NH_4^+ in the humic label was isolated via solid phase extraction (Cochlan and Bronk 2001) and determined to have an isotopic ratio similar to the humic label (8.15 atom %). Uptake rates were corrected for potential $^{15}NH_4^+$ uptake by assuming that all of the NH_4^+ in the culture was taken up. This assumption results in a more conservative estimate of humic N uptake. All contaminant NH_4^+ was removed from the humic label prior to the time-course experiments by degassing with a SpeedVac concentrator (see 2003).

To calculate actual uptake rates, ambient concentrations of each substrate in solution are necessary. For NO_3^-, ambient concentrations were determined on an Alpkem autoanalyzer (Parsons et al. 1984). However, due to a lack of culture volume, ambient concentrations of humic N were not quantified prior to incubation. Although grown in media amended with humic acids, it was assumed that the concentration of bioavailable humic N remaining in the cultures at the time of the uptake experiments was negligible. This assumption is based on the cultures being grown in the humic-amended media for a minimum of 7 d prior to the addition of the labeled humic substances. This assumption results in a higher estimate for the atom percentage enrichment of the humic substrate and, therefore, a more conservative estimate of uptake. Results of the time-course experiments showed that most, if not all, of the bioavailable humic N had been used within 3 to 12 h (see Fig. 2).

Cell counts and statistics—Cells were fixed in formalin (final concentration of 5%) and counted under a microscope using either a Bright Line or Nageotte counting chamber (Hausser Scientific). For each sample, a minimum of 200 cells was counted. Bacterial cells were stained with DAPI and counted via epifluorescence microscopy (Porter and Feig 1980). Uptake rates were compared via one-way analysis of variance (ANOVA) using SAS statistical analysis software and deemed significant at the $p < 0.05$ level.

Results

Uptake of humic N—All cultures took up NO_3^-, indicating that they were active at the time of the experiments (data not shown). Corrected uptake rates of humic N for all of the nonaxenic estuarine and coastal isolates examined were significantly greater than zero, indicating that they were capable of taking up the ^{15}N-labeled humic N (Fig. 1). In contrast, the polar strain *P. antarctica* did not take up the humic N. When uptake was later measured in axenic strains of *F. japonica* and *H. akashiwo*, humic N uptake was also not detected. To determine whether the observed uptake in the nonaxenic strains could be attributed to uptake by bacteria retained on the GF/F filter, the potential uptake rates for bacteria alone in the cultures was calculated from bacterial cell counts (data not shown) assuming bacterial retention on the GF/F filters of 61% and bacterial uptake rates of 0.056 μmol N L^{-1} h^{-1} (Wheeler and Kirchman 1986; Lee et al. 1995). It is estimated that the bacteria alone could take up approximately 0.07 fmol $cell^{-1}$ h^{-1}, which is two

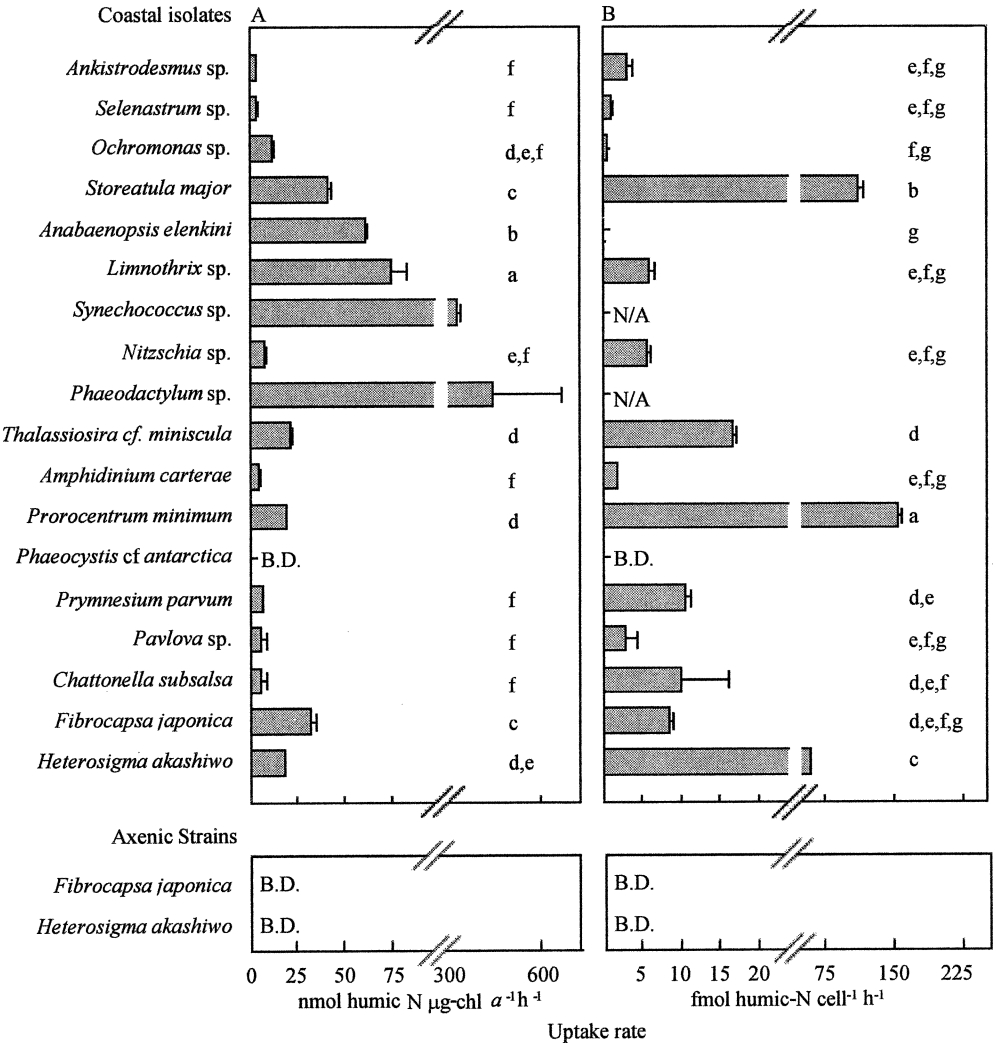

Fig. 1. Humic N uptake rates of 2-week-old humic substances following 3-h incubation (A) normalized to µg Chl *a* (uptake by *Synechococcus* sp. and *Phaeodactylum* were significantly higher than all other isolates and were excluded in the determination of significant difference between the remaining cultures) and (B) normalized to cell abundance. Cell counts for *Synechococcus* sp. and *Phaeodactylum* sp. were not available. Error bars represent 1 standard deviation. B.D., below detection. Values identified by the same letter were not significantly different at $p < 0.05$.

orders of magnitude lower than the overall average uptake observed in the cultures.

When normalized to Chl *a*, the humic N uptake rates for *Synechococcus* sp. (329.9 ± 14.6 nmol humic N µg Chl a^{-1} h^{-1}) and *Phaeodactylum* sp. (445.1 ± 222.0 nmol humic N µg Chl a^{-1} h^{-1}) were significantly greater than those of the other isolates tested (Fig. 1). Note that cell counts were not available for *Phaeodactylum* sp. and *Synechococcus* sp. because of loss of sample.

Time dependant uptake of ^{15}N-labeled humic substances and DIN—In the time-course experiments, uptake rates of humic N in the nonaxenic isolates decreased with increasing incubation time (Figs. 2 and 3). For the shorter time periods (1 h for *Synechococcus* sp. and 1 and 3 h for *A. carterae* and *T. miniscula*), uptake rates of humic N exceeded those obtained for both NH_4^+ and NO_3^- in the incubations in which the younger humic substances (aged 1 week, 2 weeks, and 1 month) were added (Tables 3 and 4). However, these high uptake rates were not sustainable and rapidly dropped below the rates calculated for both NH_4^+ and NO_3^-, which remained relatively constant throughout the incubation. Over the course of 24 h, approximately 30% of bulk humic N was taken up by the estuarine cultures, with the majority of uptake occurring in the first 3 h (Figs. 2 and 3).

Age-dependant uptake of ^{15}N-labeled humic substances—Humic age also played a role in the ability of the phytoplankton isolates to obtain humic N (Figs. 2 and 3, Tables 3 and 4). In general, isolates provided with younger humics (aged 1 week to 1 month) took up the labeled N at a higher rate than when supplied with older humics (3 months to 1 year). The high uptake rates for the N

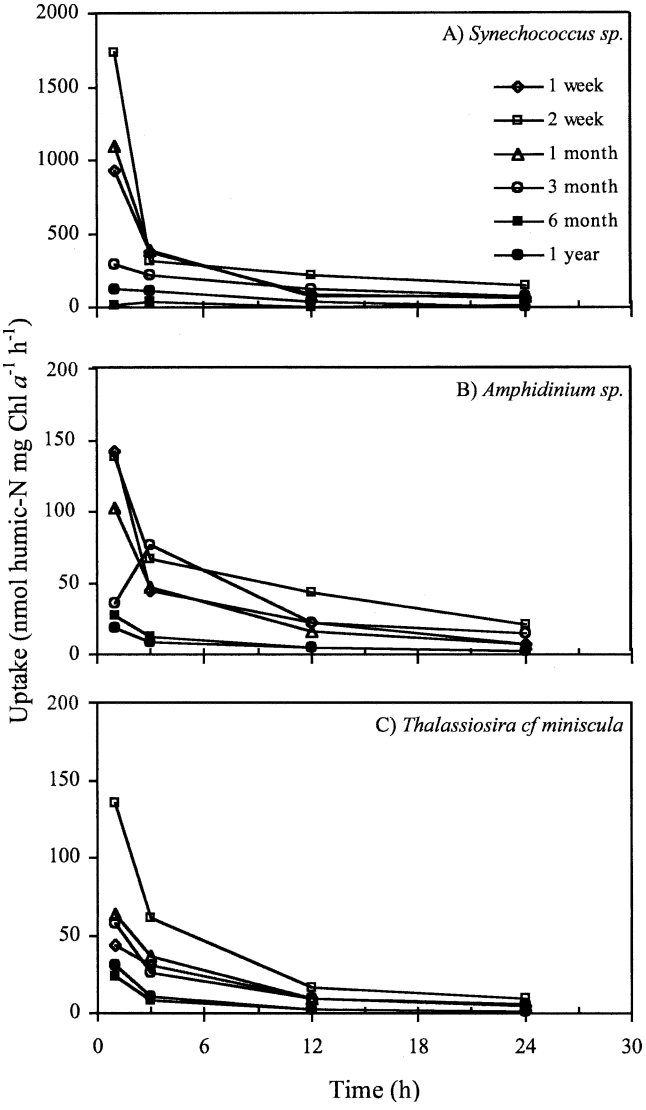

Fig. 2. Uptake rates of humic N with respect to age normalized to Chl *a* content. Uptake is shown for (A) *Synechococcus* sp., (B) *Amphidinium* sp., and (C) *Thalassiosira* cf. *miniscula*. Error bars are not shown to increase clarity, but error measurements can be found in Table 2.

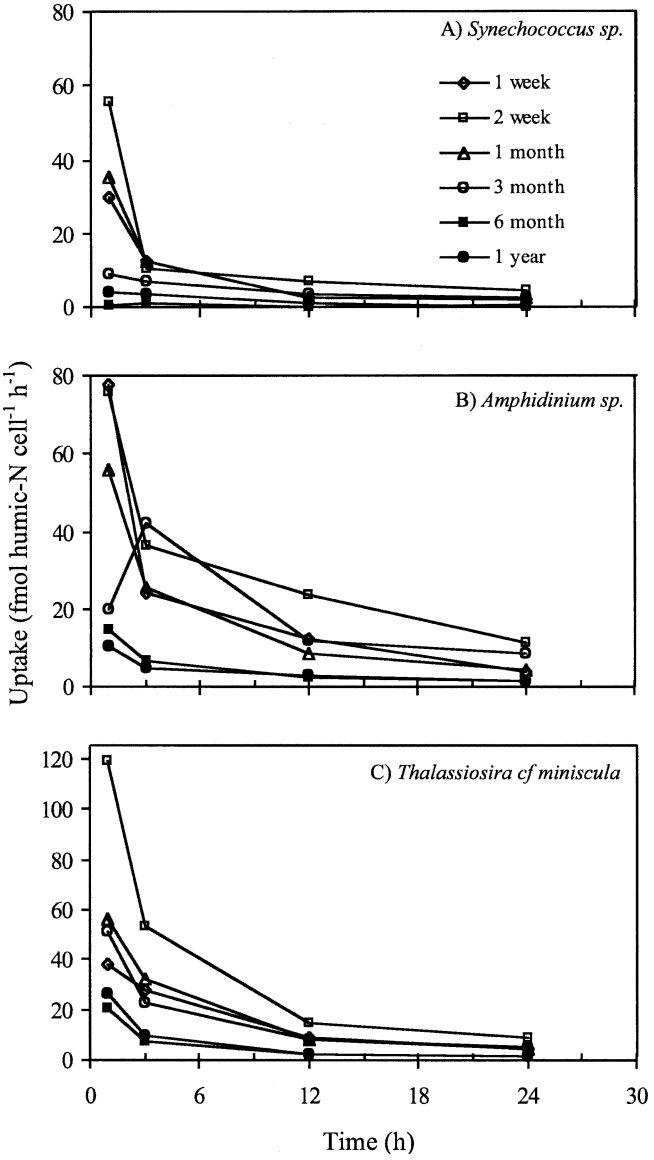

Fig. 3. Uptake rates of humic N with respect to age normalized to cell number. Uptake is shown for (A) *Synechococcus* sp., (B) *Amphidinium* sp., and (C) *Thalassiosira* cf. *miniscula*. Error bars are not shown to increase clarity, but error measurements can be found in Table 3.

bound to younger humics, however, were also not sustainable and quickly dropped off to rates similar to those measured for the older humic substances (Figs. 2 and 3, Tables 3 and 4).

Dual-labeled humic substances—Uptake rates of humics labeled with ^{13}C and ^{15}N followed trends similar to those observed with the ^{15}N-labeled humic substances. Relatively high initial uptake rates of humic N into phytoplankton biomass were followed by a rapid decline in uptake over the course of the experiment, similar to the time-course experiments described above (data not shown). Uptake of ^{13}C from the humic substances, however, was either below detection or not significantly different from zero for all isolates tested at all time points save one (*T. miniscula* at T_{12}, 0.16 ± 0.01 μmol humic C L^{-1} h^{-1}, data not shown).

Discussion

Uptake of humic N by estuarine phytoplankton strains—The widespread ability of the estuarine strains examined in this study to take up humic N suggests that humic N in the estuarine and coastal zones may not be as refractory as traditionally believed. Previous experiments suggest that the addition of humic substances (and soil extract) to phytoplankton strains promotes growth in culture and this stimulatory effect is not solely due to chelation processes (Prakash and Rashid 1968; Prakash et al. 1973). This study supports the previous findings and suggests that N is delivered to the estuarine phytoplankton from the humic substances. Furthermore, the lack of uptake in *P.*

Table 3. Uptake rates of humic N and inorganic N over time. Rates have been normalized to Chl *a*.

Sample	*Synechococcus* (nmol N μg Chl a^{-1} h^{-1})	*Amphidinium* (nmol N μg Chl a^{-1} h^{-1})	*Thalassiosira* (nmol N μg Chl a^{-1} h^{-1})
1-week label			
T_1	928.24±57.61	141.44±0.53	42.94±56.56
T_3	378.67±40.73	44.27±1.96	31.14±1.16
T_{12}	80.04±35.75	22.30±2.65	9.83±0.07
T_{24}	57.77±1.68	6.90±0.00	4.84±0.02
2-week label			
T_1	1,732.25±0.64	138.52±42.25	134.91±0.82
T_3	317.88±21.92	66.97±11.46	60.74±0.52
T_{12}	221.51±45.53	42.94±2.38	16.48±0.19
T_{24}	139.80±2.76	20.81±1.77	9.80±0.27
1-month label			
T_1	1,090.78±504.68	102.05±20.12	63.84±5.27
T_3	390.32±22.96	46.71±17.35	36.13±0.64
T_{12}	77.02±4.12	15.64±2.38	9.50±0.85
T_{24}	69.49±3.49	7.61±2.93	4.84±1.42
3-month label			
T_1	284.37±114.59	36.00±17.88	57.65±5.22
T_3	212.68±21.05	76.57±44.87	25.89±7.99
T_{12}	114.48±0.81	21.96±1.88	9.09±0.98
T_{24}	78.11±9.29	15.18±2.69	5.71±1.17
6-month label			
T_1	12.31±1.79	26.57±3.07	23.44±1.77
T_3	31.64±0.84	12.30±2.20	8.43±0.65
T_{12}	−0.24±4.92	4.69±1.11	2.75±0.25
T_{24}	10.70±4.44	2.29±0.84	1.66±0.21
1-yr label			
T_1	123.64±98.89	18.65±5.54	30.07±1.58
T_3	108.03±36.99	8.53±0.01	10.83±1.03
T_{12}	30.94±9.37	4.94±0.76	2.77±0.01
T_{24}	3.12±1.12	2.66±0.41	1.64±0.12
NH_4^+			
T_1	1,102.01±97.70	50.14±8.28	35.13±0.31
T_3	411.24±24.61	28.25±8.42	19.02±1.96
T_{12}	341.15±13.27	27.29±6.54	8.64±1.13
T_{24}	336.73±5.59	15.27±0.77	9.37±0.24
NO_3^-			
T_1	516.23±39.07	18.19±0.87	10.79±1.79
T_3	407.49±10.69	19.30±0.39	7.92±0.38
T_{12}	262.98±31.43	30.91±0.86	8.13±0.42
T_{24}	321.34±58.14	20.88±0.84	6.69±0.68

antarctica, the sole strain tested in this experiment not expected to be exposed to humic substances on a regular basis, suggests that the ability to use humic substances as an N source is not present in all environments. Additional comparisons with polar or oligotrophic strains are needed to test this hypothesis.

Humic N uptake relative to uptake of inorganic N—In the time-course experiments, the rapid decline in the uptake rate of humic N suggests that only a fraction of the humic N is bioavailable. This fraction appears to be highly labile such that it is used rapidly. The N that is left behind is likely more integrated into the humic structure or physically protected such that it is not available in short-term incubations. For example, the atom percentage enrichment of cultures given the 1-week-old humic label remained constant over the 24-h experiment, indicating that the majority of the uptake occurred in the first few hours (Fig. 4). In contrast, the atom percentage enrichment of cultures given NH_4^+ rises linearly, suggesting that uptake of the inorganic label was sustained over time (Fig. 4). Additional experiments are needed to determine how the availability of other N substrates (NH_4^+, NO_3^-, urea, and amino acids) would affect uptake rates of humic N.

Changes in lability as humic substances age—Humic substances are comprised of several subcategories, including humic and fulvic acids, based upon their solubility in water at varying pH. Humic acids are higher in molecular weight and more aromatic, while fulvic acids tend to be of lower molecular weight (LMW) and more aliphatic (Thurman 1985). As humics age in the environment, they are degraded and converted into more fulvic-like compounds (Ertel et al. 1984; See and Bronk 2005). An analysis of the structure of the labeled humics used in this study shows that the older humics were more fulvic-like in

Table 4. Uptake rates of humic N and inorganic N over time. Rates have been normalized to cell abundance.

Sample	*Synechococcus* (fmol N cell^{-1} h^{-1})	*Amphidinium* (fmol N cell^{-1} h^{-1})	*Thalassiosira* (fmol N cell^{-1} h^{-1})
1-week label			
T_1	29.95±1.86	77.43±0.29	37.91±49.94
T_3	12.22±1.31	24.24±1.07	27.49±1.02
T_{12}	2.58±1.15	12.21±1.45	8.68±0.06
T_{24}	1.86±0.05	3.78±0.00	4.27±0.02
2-week label			
T_1	55.90±0.02	75.83±23.13	119.11±0.72
T_3	10.26±0.71	36.66±6.27	53.63±0.46
T_{12}	7.15±1.47	23.51±1.30	14.55±0.17
T_{24}	4.51±0.09	11.39±0.97	8.65±0.24
1-month label			
T_1	35.20±16.28	55.87±11.01	56.37±4.65
T_3	12.59±0.74	25.57±9.50	31.90±0.56
T_{12}	2.49±0.13	8.56±1.30	8.39±0.75
T_{24}	2.24±0.11	4.16±1.60	4.27±1.26
3-month label			
T_1	9.18±3.70	19.71±9.79	50.90±4.61
T_3	6.86±0.68	41.92±24.56	22.86±7.05
T_{12}	3.69±0.03	12.02±1.03	8.02±0.86
T_{24}	2.52±0.30	8.31±1.48	5.04±1.03
6-month label			
T_1	0.40±0.06	14.55±1.68	20.69±1.56
T_3	1.02±0.03	6.73±1.20	7.44±0.57
T_{12}	−0.01±0.16	2.57±0.61	2.42±0.22
T_{24}	0.35±0.14	1.25±0.46	1.47±0.18
1-yr label			
T_1	3.99±3.19	10.21±3.03	26.55±1.39
T_3	3.49±1.19	4.67±0.00	9.56±0.91
T_{12}	1.00±0.30	2.70±0.42	2.44±0.01
T_{24}	0.10±0.04	1.46±0.22	1.45±0.11
NH_4^+			
T_1	35.56±3.15	27.45±4.53	31.01±0.27
T_3	13.27±0.79	15.46±4.61	16.79±1.73
T_{12}	11.01±0.43	14.94±3.58	7.63±1.00
T_{24}	10.87±0.18	8.36±0.42	8.27±0.21
NO_3^-			
T_1	16.66±1.26	9.96±0.48	9.53±1.58
T_3	13.15±0.34	10.57±0.21	6.99±0.33
T_{12}	8.49±1.01	16.92±0.47	7.18±0.37
T_{24}	10.37±1.88	11.43±0.46	5.90±0.60

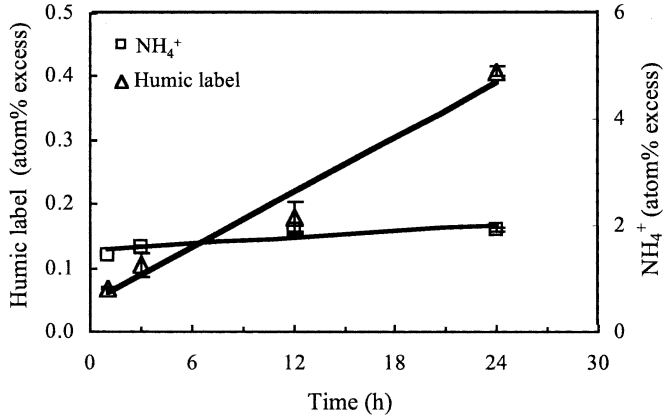

Fig. 4. Atom percentage excess values (atom percentage over natural abundance) for the uptake of the 1-week-old humic label and $^{15}NH_4^+$ for *Thalassiosira* cf. *miniscula*.

nature relative to the younger humics (See 2003; See and Bronk 2005). Based on these observations, it was hypothesized that the N within the older, LMW, more aliphatic, fulvic-like compounds would be more readily available because fewer high energy bonds would need to be broken to access the bound N. This does not appear to be the case, however. The younger "fresher" humic substances were taken up at a higher rate than the older, presumably more degraded, humic substances (Figs. 2 and 3). One explanation may be related to a size-reactivity continuum model that proposes that HMW dissolved organic matter (DOM) is more bioreactive than the LMW counterparts (Amon and Benner 1996*a*). The model reasons that HMW DOM is less diagenetically altered and more closely resembles its source material, making the component compounds within the DOM more susceptible to degradation and enzymatic attack (Amon and Benner 1996*a*). The size-reactivity

continuum model is consistent with our findings that N incorporated into younger, more humic-like compounds was taken up more rapidly than N associated with older, more fulvic-like compounds.

Potential mechanisms of humic N uptake—Phytoplankton could access the N associated with humic substances in a number of ways including direct uptake via pinocytosis or phagocytosis or the uptake of released N after extracellular enzyme cleavage, photochemical breakdown, or bacterial remineralization (reviewed in Bronk 2002). For the dual-labeled humic substances, rates of C and N uptake were compared and used to infer the potential mechanisms employed by three strains for the uptake of humic N. A direct uptake of the entire humic compound via pinocytosis or phagocytosis would result in an uptake of both labeled C and N in the same atomic ratio as the label added to the phytoplankton isolates (14.0, Table 2). Although flagellates and the dinoflagellates *A. catenella*, *Kryptoperidinium foliaceum*, and *Scrippsiella trochoidea* are capable of directly taking up HMW fluorescent dextrans, most likely by pinocytosis (Sherr 1988; Legrand and Carlsson 1998; Lewitus 2006), none of the strains used in this study demonstrated a sustained uptake of ^{13}C, arguing against pinocytosis or phagocytosis as the mechanism of humic N uptake in our experiments. Uptake of labeled N but not labeled C from the humic compounds added, however, would indicate a cleavage of N from the humic compound, most likely by cell-surface enzymes or via photochemical or bacterial breakdown of the humics followed by uptake of the liberated N.

The use of cell surface or extracellular deaminases, capable of cleaving NH_4^+ from amino acids and primary amines, has been demonstrated in several classes of phytoplankton, including dinoflagellates, chlorophytes, and prymnesiophytes (e.g., Palenik and Morel 1990; Pantoja and Lee 1994; Stoecker and Gustafson 2003). While the uptake of labeled N, but not C, in the dual-labeled humic experiments suggests that extracellular deamination is a possible mode of N acquisition in the coastal isolates tested, photooxidative or bacterial breakdown of humics cannot be excluded. In fact, the lack of uptake in the axenic cultures of *F. japonica* and *H. akashiwo* is evidence that N uptake was mediated by bacteria in at least these two species. However, if N is liberated by bacterial remineralization, uptake of ^{15}N would likely also have been observed in *P. antarctica*.

An alternative method for the acquisition of N from humic substances is photooxidation followed by uptake of the N photoproducts. Owing to their aromatic nature, humic substances are photoreactive to ultraviolet (UV) light. Humic materials have been shown to release inorganic N (Bushaw et al. 1996; Bushaw-Newton and Moran 1999; Kieber 2000) as well as small labile organic molecules including amino acids and urea into the surrounding environment when exposed to UV light (Amador et al. 1989; Jørgensen et al. 1998; Bushaw-Newton and Moran 1999). However, it is unlikely that the isolates examined here obtained N released via photooxidation reactions because experiments were performed under fluorescent light in Pyrex test tubes that do not allow UV light to penetrate. Furthermore, if N is liberated by photochemical reactions, uptake of ^{15}N likely would have again been observed in *P. antarctica* and the axenic strains.

Environmental implications—Despite their abundance, humic substances, and DON in general, have been largely ignored in N-loading and eutrophication models (Meybeck 1982; Sarmiento and Sundquist 1992). However, oceanic DOM lacks a strong terrestrial signal, suggesting that this N is used or transformed prior to entering the bulk oceanic DOM pool. Currently, the photochemical breakdown of terrestrial DOM into more labile compounds is believed to be the major process responsible for the removal of this terrestrial signal (e.g., Amon and Benner 1996*b*; Mopper and Kieber 2002). The uptake of humic N by phytoplankton (directly or following photodegradation or bacterial breakdown) is likely another vehicle for removing the terrestrial signal over short time scales (hours to days).

In conclusion, the results from this study indicate that humic N is available to estuarine plankton on the time scale of hours. The ability of phytoplankton to use humic N supports the theory that a large number of estuarine and coastal plankton are capable of obtaining N from organic sources that have traditionally been perceived to be biologically recalcitrant. These data provide an impetus for follow-up studies of differences in humic N uptake potential between estuarine, coastal marine, and open ocean plankton communities and convey the need for a reevaluation of humic N and its potential importance in ecosystem-wide nutrient budgets.

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