

## Peptide hydrolysis, amino acid oxidation, and nitrogen uptake in communities seasonally dominated by *Aureococcus anophagefferens*

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### Abstract

Elevated levels of dissolved organic nitrogen (DON) and dissolved inorganic nitrogen (DIN) are among the factors implicated in the initiation of algal blooms. However, the degree to which phytoplankton augment their autotrophic metabolism with heterotrophic uptake of organic carbon that is associated with DON is unknown. We evaluated the relative importance of peptide hydrolysis, amino acid oxidation, and amino acid uptake over a seasonal cycle in an embayment on Long Island, New York, that had high concentrations of dissolved organic matter (DOM) and a bloom of the brown tide pelagophyte, *Aureococcus anophagefferens*. Amino acids were a significant component (up to 50%) of the total N uptake, particularly during the late summer. About half of the associated amino acid C was also taken up. Amino acid oxidation rates were an order of magnitude lower than free amino acid uptake rates, but still supplied up to 32.5% of the  $\text{NH}_4^+$  taken up. Up to 75% of the amino acid oxidation was in the bacterial size fraction ( $<1.2 \mu\text{m}$ ), and rates were significantly correlated with bacterial densities. Peptide hydrolysis rates were high, and most (up to 72%) occurred in the brown tide size fraction (1.2–5  $\mu\text{m}$ ). The high rates of peptide hydrolysis and amino acid uptake measured in cultures of *A. anophagefferens* confirm that this species can readily hydrolyze peptides and take up N and C from amino acids. Laboratory findings and size-fractionation studies in the field suggest that *A. anophagefferens* plays a major role in consumption of both C and N from DOM.

Increases in the occurrence of harmful algal blooms have been attributed to dissolved organic nitrogen (DON) enrichment of estuarine waters in the United States mid-Atlantic region (Paerl 1988; Lewitus et al. 1999; Glibert et al. 2001), including embayments on Long Island, New York (Berg et al. 1997; Gobler and Sañudo-Wilhelmy 2001a), where the pelagophyte *Aureococcus anophagefferens* has reached densities in excess of  $10^9$  cells  $\text{L}^{-1}$  (Casper et al. 1990). Phytoplankton that can use dissolved organic matter (DOM) as an N source may have a competitive advantage in organically enriched environments where dissolved inorganic N (DIN) is in short supply. A variety of species can use dissolved organic N (DON) to meet their N needs (Antia et al. 1991), and heterotrophic uptake of dissolved organic C

(DOC) has been observed in a number of dinoflagellates (Lewitus and Caron 1991) and chrysophytes (Wheeler et al. 1977; Kristiansen 1990). However, the size of individual organic compounds may preclude their transport into cells.

Brown tide blooms of *A. anophagefferens* have been occurring in estuaries of the mid-Atlantic region for nearly two decades (Casper et al. 1990; Bricelj and Lonsdale 1997). *A. anophagefferens* can cooccur with other phytoplankton species (Nuzzi and Waters 1989; Smayda and Villareal 1989) but can also form fairly monospecific blooms, during which it represents  $>90\%$  of the total cell and chlorophyll biomass (Nuzzi and Waters 1989; Bricelj and Lonsdale 1997; Gobler and Sañudo-Wilhelmy 2001a). The scale and density of brown tide blooms have been related to the magnitude of preceding phytoplankton blooms and  $\text{NO}_3^-$  inputs, although populations of *A. anophagefferens* typically flourish only after  $\text{NO}_3^-$  concentrations have been depleted (Gobler and Sañudo-Wilhelmy 2001a) or in years when groundwater  $\text{NO}_3^-$  inputs are greatly reduced (LaRoche et al. 1997) and DON is elevated relative to DIN (Lomas et al. 1996; Berg et al. 1997; Glibert et al. 2001).

Based on uptake kinetics, *A. anophagefferens* has a high affinity for  $\text{NH}_4^+$  and urea but not for  $\text{NO}_3^-$  (Lomas et al. 1996; Berg et al. 1997). This is similar to other species that grow in low nutrient environments (e.g., Caperon and Meyer 1972). However, it is unclear how DON stimulates growth of these species relative to cooccurring taxa. Culture studies have shown that *A. anophagefferens* can take up organic

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compounds (Dzurica et al. 1989), and organic carbon inputs appear to stimulate *A. anophagefferens* growth rates in the field (Gobler and Sañudo-Wilhelmy 2001b). This capacity may supplement autotrophic C uptake from photosynthetic CO<sub>2</sub> fixation. However, the relative uptake of C versus N from various DOM pools by *A. anophagefferens* has not been measured.

The contribution of dissolved free amino acids (DFAAs) and dissolved combined amino acids (DCAAs) to mixotrophic growth has not been broadly assessed, although these compounds generally account for about 50% of the bacterial N demand (Keil and Kirchman 1991, 1993; Middelboe et al. 1995) and about 25% of the bacterial C demand in estuarine and coastal systems (Middelboe et al. 1995). While DFAA concentrations are fairly low in marine and estuarine systems, their turnover can be quite high (Keil and Kirchman 1991; Middelboe et al. 1995), and their production has been correlated with in situ primary productivity (Sellner and Nealley 1997; Bronk et al. 1998). DCAA concentrations are higher than those of DFAAs, and estuarine DCAAs can account for up to 13% of total DON (Keil and Kirchman 1991). DCAA lability can vary greatly, since DCAAs are comprised of a mixture of peptides, proteins, and other amino acids that require acid hydrolysis to release them from their chemical or physical matrix. DFAAs are used preferentially over DCAAs until concentrations and turnover times of DFAAs become low (Keil and Kirchman 1993; Middelboe et al. 1995). In seawater, most microbes and phytoplankton can take up only inorganic or small organic compounds (Nikaido and Vaara 1985; Antia et al. 1991). Therefore, extracellular hydrolysis of proteins and peptides or oxidation of amino acids is required before the N or C can be used by organisms for growth.

A number of methods have been developed to detect proteolytic activity in seawater and sediments. Peptide-like fluorogenic compounds such as leucine-methylcoumarinylamide (Leu-MCA) have been used to measure aminopeptidase activity (Hoppe 1983). Combined hydrolysis and uptake of radio-labeled proteins and peptides have also been measured (Hollibaugh and Azam 1983; Keil and Kirchman 1992; Taylor 1995). A fluorescently labeled lucifer yellow anhydride (LYA)-peptide was recently synthesized and tested in seawater and sediments (Pantoja et al. 1997; Pantoja and Lee 1999). This compound has the advantage of allowing analysis of both the parent compound that is being hydrolyzed and the products formed by the hydrolysis reaction; it also competes with a range of peptides of different compositions for extracellular hydrolysis.

Extracellular amino acid oxidation has been observed in a variety of phytoplankton species (Palenik and Morel 1990b, 1991) and systems (Pantoja and Lee 1994; Mulholland et al. 1998). Amino acid oxidation results in the liberation of NH<sub>4</sub><sup>+</sup> from amino acids, which is then available for uptake. This oxidation pathway has been quantified using the fluorescently labeled amino acid, LYA-lysine (Pantoja and Lee 1994; Mulholland et al. 1998).

To determine whether amino acids are important in fueling the growth and development of brown tides, we conducted a seasonal study in Quantuck Bay, Long Island, New York. In addition to standard nutrient, urea, DOC, DON, and DIN

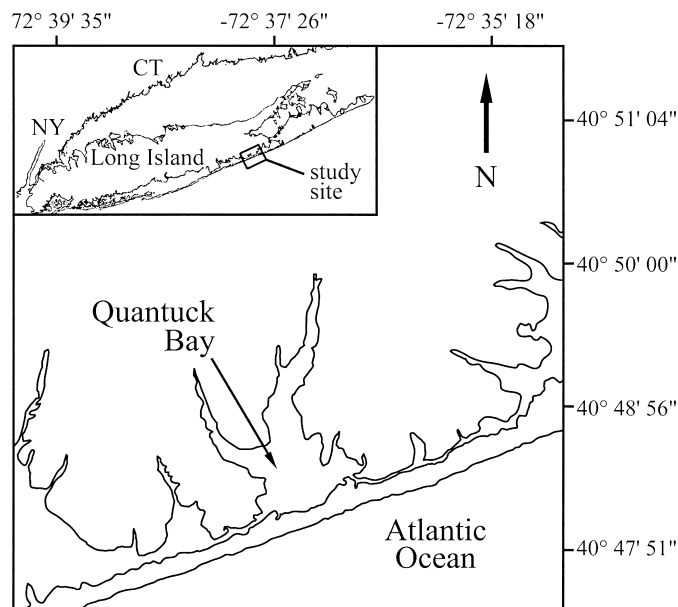


Fig. 1. Map of the Quantuck Bay, Long Island, New York.

concentrations, we measured concentrations of free and combined amino acids and uptake of amino acid N and C relative to the uptake of DIN and urea. In addition, we used LYA-lysine and LYA-peptides to measure rates of amino acid oxidation and peptide hydrolysis to assess the importance of these pathways both in the field and in cultures of *A. anophagefferens*.

## Methods

**Sampling site**—Quantuck Bay is a small (~5 km<sup>2</sup>), shallow (~4 m) lagoon along the south shore of Long Island, New York, which tidally exchanges with Moriches Bay to the west and with Shinnecock Bay to the east (Fig. 1). Brown tide blooms have recurred seasonally in this and other Long Island embayments for over 15 yr (Cosper et al. 1990; Gobler and Sañudo-Wilhelmy 2001b). Water was collected from a nearshore station in the southeast portion of Quantuck Bay on a monthly basis from April through November 2000 to compare rates of N and C uptake, amino acid oxidation, and peptide hydrolysis over a seasonal cycle as the population changed and a brown tide bloom formed and decayed. Water was collected from just below the surface with a high-density polyethylene bucket and transferred to 20-liter polyethylene carboys for transport to the laboratory. Carboys, plastic buckets, and all other materials associated with the sampling, handling, and storage of seawater during this project were soaked in 10% HCl between sampling dates and rinsed liberally with distilled-deionized water before use. The shallow (~4 m), well-mixed nature of estuaries along the south shore of Long Island (Wilson et al. 1991) ensured that sample water collected was representative of the entire water column. Experiments were initiated in the laboratory within 30 min of sample collection.

*Amino acid, nutrient, and biomass measurements*—In the laboratory, samples for nutrient analyses were filtered through precombusted (450°C for 2 h) GF/F filters or acid-washed 0.2- $\mu\text{m}$  polycarbonate filters. Duplicate acid-cleaned bottles were filled with between 5 and 50 ml of water and frozen until analysis. Filters and preserved samples (1% glutaraldehyde) were collected for biomass determination.

Dissolved free amino acids were measured in duplicate as the individual compounds by high-performance liquid chromatography (HPLC) (Lindroth and Mopper 1979; Cowie and Hedges 1992) and/or fluorometrically as total DFAAs (Parsons et al. 1984). Total hydrolysable amino acids were measured in duplicate after vapor phase hydrolysis (Tsugita et al. 1987; Keil and Kirchman 1991).

Ammonium, urea, and  $\text{NO}_3^-$  were analyzed colorimetrically (Newell et al. 1967; Jones 1984; Parsons et al. 1984), all in duplicate. Recoveries (mean  $\pm$  1 standard deviation [SD]) of SPEX Certi-Prep<sup>INC</sup> inorganic nutrient standard reference material at environmentally representative concentrations were  $98 \pm 7\%$  for nitrate and  $103\% \pm 8\%$  for ammonium. Total dissolved nitrogen concentrations (TDN) were determined in duplicate using persulfate oxidation (Valderrama 1981). Recoveries of SPEX Certi-Prep<sup>INC</sup> organic nutrient standard reference material at environmentally representative levels were  $94 \pm 12\%$  for total nitrogen. DON was calculated as the difference between TDN and dissolved inorganic nitrogen (nitrate, nitrite, ammonium). Precombustion of glassware and acid-cleaned plasticware provided adequately low blanks for chemical analyses (<10% of lowest sample).

*A. anophagefferens* abundance was quantified in triplicate, glutaraldehyde-preserved samples by direct counts using an epifluorescence microscope after reacting cells that were gently filtered (<5 kPa) onto a 0.8- $\mu\text{m}$  black polycarbonate filter with an immunofluorescent antibody label (Anderson et al. 1989). The original technique was modified by increasing the amount of primary and secondary antibody by a factor of two. A minimum of 100 cells were counted per sample in at least 10 fields to yield a relative standard deviation of 9% for replicate counts of the same sample ( $n = 6$ ) at cell densities of  $2 \times 10^5$  cells  $\text{ml}^{-1}$ , approximating average densities during blooms. To ensure accurate results, the immunofluorescent technique was compared to counts performed with a hemacytometer on a light microscope. The two techniques yielded statistically identical results using *A. anophagefferens* clone 1708 at cell densities of  $2 \times 10^5$  cells  $\text{ml}^{-1}$ , the approximate mean densities found in Quantuck Bay during this study.

Bacteria were enumerated according to Porter and Feig (1980) using the fluorochrome 4,6-diamidinophenylindole (DAPI). Chlorophyll *a* was measured in triplicate by standard fluorometric methods (Parsons et al. 1984). The amount of Chl *a* due to the presence of brown tide in samples was estimated by assuming a constant Chl *a* per cell value for *A. anophagefferens* ( $0.035 \pm 0.003$  pg cell<sup>-1</sup> for nutrient replete cultures) and multiplying this value by *A. anophagefferens* densities. While such calculations could introduce bias from variability of cellular chlorophyll concentrations, they have been used successfully to compare *A. anophagefferens* biomass to that of the total algal community

(Schaffner 1999; Gobler and Sañudo-Wilhemly 2001b; Gobler et al. 2002).

*Oxidation and hydrolysis rate measurements*—During a 1999 pilot study, we observed no enzyme activity in the <0.2- $\mu\text{m}$  size fractions in Quantuck Bay. This observation was consistent with our other estuarine studies (Mulholland et al. pers. comm.). Therefore, we examined activity in the <1.2- $\mu\text{m}$  size fraction (e.g., bacterial sized), the <5.0- $\mu\text{m}$  size fraction (brown tide sized), and whole water (larger plankton). Rates of amino acid oxidation and peptide hydrolysis were measured in the lab in triplicate by placing 25 ml of size-fractionated or whole water into an acid-cleaned polycarbonate incubation bottle. Either 100 nM LYA-lysine (amino acid oxidation) or 95 nM LYA-tetraalanine (peptide hydrolysis) was added to incubation bottles. Samples were taken at time zero and subsequently at intervals ranging from 30 min to 4–6 h. Samples were filtered (0.2  $\mu\text{m}$ ) and frozen until analysis by HPLC.

LYA-lysine and LYA-ala<sub>4</sub> and their products were separated and quantified using a Shimadzu HPLC system equipped with a refrigerated autosampler, a McPherson fluorometric detector (424 nm excitation and 476 nm emission), and a 250 mm Beckman C18 column. For the analysis of LYA-lysine and its products, we ran a gradient using 0.05 M  $\text{KH}_2\text{PO}_4$  (pH 4.5) and a mixture of 50:50 acetonitrile:0.05 M  $\text{KH}_2\text{PO}_4$  (pH 4.5) (Pantoja et al. 1993; Pantoja and Lee 1994; Mulholland et al. 1998). For the analysis of LYA-tetraalanine and its products (LYA-alanine, LYA-dialanine, and LYA-trialanine), we used a gradient of 0.05 M  $\text{KH}_2\text{PO}_4$  (pH 4.5) and methanol (Pantoja et al. 1997).

First-order rate constants for amino acid oxidation and peptide hydrolysis were calculated based on the disappearance of parent compounds during the time-course incubations (Pantoja et al. 1997). Both rate constants and maximum rates are reported. Maximum rates were calculated by multiplying the first-order rate constant by the pool of DFAAs for amino acid oxidation, or DCAAs for peptide hydrolysis. Because the calculated rate is directly proportional to the size of the substrate pool (Pantoja et al. 1997), and the availability of the substrate pools is unknown but undoubtedly less than 100% of that measured, we consider the calculated rates to be maximum rates.

*Uptake rate measurements*—Uptake of <sup>15</sup>N-labeled compounds ( $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , urea) and dually labeled, <sup>13</sup>C and <sup>15</sup>N glutamate and alanine was measured on size-fractionated water incubated in acid-cleaned polycarbonate bottles. N atoms were uniformly labeled in all substrates used. Both C and N atoms were uniformly labeled for the dually labeled amino acids. Experiments were initiated by adding tracer concentrations (10% or 0.03  $\mu\text{mol L}^{-1}$ ) of the highly enriched (96–99%) labeled substrate, with the exception of the kinetic experiment described below. After an incubation period of less than 1 h, experiments were terminated by gentle filtration (<5 mg Hg) through precombusted GF/F filters (450°C for 2 h); these filters were rinsed with filtered seawater and frozen until analysis.

Incubations for uptake and enzyme rate measurements were conducted at temperatures within 2°C of ambient levels

Table 1. Temperature and nutrient concentrations in water from Quantuck Bay, Long Island, and in cultures of *A. anophagefferens*. Numbers in parentheses are standard deviations.

Date	Temp. (°C)	Nitrate (μM)	Ammonium (μM)	Urea (μM)	DON (μM)	DFAA (μM)	DCAA (μM)	Phosphate (μM)	DIN : DIP	DIN : DON
Quantuck Bay										
17 Apr 00	9	1.30 (0.04)	0.10 (0.05)	0.10 (0.05)	13.6 (0.39)	0.28	1.98	0.15 (0.04)	9.15	0.10
24 May 00	21	0.67 (0.07)	0.62 (0.40)	0.19 (0.02)	21.6 (1.04)	0.42 (0.007)	1.99	0.09 (0.02)	14.1	0.06
29 Jun 00	24.1	0.31 (0.03)	1.74 (0.04)	0.33 (0.16)	30.0 (2.23)	0.99 (0.002)	3.95 (0.14)	0.58 (0.03)	3.50	0.07
26 Jul 00	21.4	0.14 (0.08)	1.03 (0.31)	0.46 (0.01)	31.5 (0.17)	1.22 (0.22)	1.07 (0.08)	1.90 (0.09)	0.61	0.04
30 Aug 00	22.8	0.20 (0.13)	1.02 (0.14)	0.17 (0.06)	36.1 (0.97)	1.12 (0.06)	1.28 (0.01)	1.21 (0.08)	1.01	0.03
26 Sep 00	16.7	0.16 (0.03)	0.18 (0.17)	0.82 (0.08)	30.2 (0.62)	1.95 (0.02)	1.15 (0.01)	1.03 (0.09)	0.33	0.01
24 Oct 00	13.3	0.11 (0.12)	0.70 (0.31)	0.22 (0.20)	25.2 (2.42)	0.47 (0.00)	0.81 (0.10)	0.63 (0.31)	1.28	0.03
29 Nov 00	6.4	13.54 (0.49)	4.94 (0.13)	0.35 (0.08)	23.1 (3.11)	0.50 (0.01)	0.33 (0.01)	0.33 (0.02)	55.7	0.80
Cultures										
Exponential	20	916	0.63	0.03		0.21 (0.02)	3.20			
Late exp.	20	153	0.54	0.03		0.84	13.8			

in Quantuck Bay and under  $70 \mu\text{E m}^{-2} \text{s}^{-1}$  of light. The average incoming solar radiation during daylight hours to Long Island during our study dates was  $1,500 \mu\text{E m}^{-2} \text{s}^{-1}$  (V. Cassella, Brookhaven National Lab, pers. comm.), and the average extinction coefficient in the Quantuck Bay water column during experiments was 1.71. Therefore, the light levels used in our incubations were equivalent to the light levels found at 1.8 m in the water column of Quantuck Bay during this period, or  $\sim 5\%$  of incident radiation.

Because we were not aware of any previous studies using dually labeled amino acids to estimate C and N uptake by phytoplankton, we conducted an intensive kinetic study during May 2000. For this experiment, we added 0.03, 0.1, 0.3, 1.0, 3.0, 10, or  $30 \mu\text{mol L}^{-1}$  of either dual  $^{15}\text{N}/^{13}\text{C}$ -labeled alanine or glutamate to replicate bottles filled with whole water from Quantuck Bay. For each substrate and each level of substrate addition, we filtered samples after 0.17, 0.33, 0.67, 1, or 1.58 h to measure uptake of  $^{15}\text{N}$  or  $^{13}\text{C}$  from amino acids. From these experiments, we plotted specific uptake rates (V in units of  $\text{h}^{-1}$ ) versus substrate concentration.

Carbon and nitrogen isotopic ratios in samples were analyzed on a Europa Scientific ANCA-SL 20–20 isotope ratio mass spectrometer (IRMS), and uptake rates were calculated using the equations of Glibert and Capone (1993) with one exception. Uptake of amino acids was calculated using dissolved free amino acids (DFAAs) as the relevant ambient amino acid pool. While the relative lability of individual amino acids is unknown, we assumed that all amino acids were equal. To calculate the atom percent enrichment (both C and N) of the DFAA pool for the dually labeled amino acids ( $^{15}\text{N}$  and  $^{13}\text{C}$ ), we estimated the atom C:N ratio of the ambient DFAA pool. We did this by quantifying the concentrations and C:N ratios of individual amino acids and appropriately weighting these to estimate the C:N ratio of the total pool. Such calculations established a 3.7:1 C:N ratio for the DFAA pool during this study.

*Culture experiments*—For comparison with results from natural waters, amino acid oxidation, peptide hydrolysis, and N uptake rates were examined in exponential and late-exponential phase cultures of *Aureococcus anophagefferens*. Axenic cultures of *A. anophagefferens* were not available to

us at the time of these experiments. Clone CCMP1706 was grown on L1 artificial seawater (30‰) containing 1 mM  $\text{NO}_3^-$  at  $20^\circ\text{C}$  and  $100 \mu\text{E m}^{-2} \text{s}^{-1}$  supplied on a 14:10 light:dark cycle. Cultures were bubbled with  $0.2\text{-}\mu\text{m}$  filtered air. Light levels were maintained with cool white fluorescent lamps. Rates were measured in triplicate samples over time courses both in whole culture and in the  $<1.2\text{-}\mu\text{m}$  filtrate. Experiments were initiated and terminated in a manner identical to the studies with natural waters. Rates reported here are the difference between the whole and  $<1.2\text{-}\mu\text{m}$  fraction, and thus should exclude most bacteria present in the culture (Dzurica et al. 1989).

## Results

*Microbial and nutrient dynamics in Quantuck Bay*—Dynamic changes in temperature and nutrients (Table 1) and phytoplankton biomass and bacterial densities (Table 2) were observed during our 8-month study of Quantuck Bay. While Chl *a*, *A. anophagefferens* cell densities, DON, and temperature were low in April, all of these parameters increased by a factor of two during May. Small cells dominated the phytoplankton community throughout the study, with an average of  $89 \pm 12\%$  (1 SD) of chlorophyll in the  $<5\text{-}\mu\text{m}$  size fraction (Table 2). An intense phytoplankton bloom occurred during the month of June, as chlorophyll, POC, PON, and *A. anophagefferens* concentrations reached their highest levels of our study ( $30 \mu\text{g L}^{-1}$ ,  $438 \mu\text{M}$ ,  $55 \mu\text{M}$ , and  $4.8 \times 10^5 \text{ cells ml}^{-1}$  respectively; Table 2). *A. anophagefferens* accounted for 55% of phytoplankton biomass during this phytoplankton bloom, and DON levels exceeded  $30 \mu\text{M}$ . Bacterial densities were also elevated during this bloom ( $1.5 \times 10^7 \text{ cells ml}^{-1}$ ; Table 2). By July, the bloom had dissipated, chlorophyll levels decreased threefold, and *A. anophagefferens* densities dropped by an order of magnitude (Table 2). In contrast, bacterial densities remained high ( $1.7 \times 10^7 \text{ cells ml}^{-1}$ ) during July, and PC and PN decreased by only 25% (Table 2).

During August and September, the brown tide population rebounded and cell densities exceeded  $2.0 \times 10^5 \text{ cells ml}^{-1}$  during both months. During this time, *A. anophagefferens*

Table 2. Particulate organic C and N (PC, PN), chlorophyll *a* concentrations (whole water and <5  $\mu\text{m}$ ), bacterial densities, abundance of brown tide cells (BT), and relative abundance of brown tide in the phytoplankton community (% BT) in water from Quantuck Bay, Long Island, and in cultures of *A. anophagefferens*. Numbers in parentheses are standard deviations.

Date	PN ( $\mu\text{M}$ )	PC ( $\mu\text{M}$ )	C:N	Chl <i>a</i> ( $\mu\text{g L}^{-1}$ )	Chl <i>a</i> < 5 $\mu\text{m}$ ( $\mu\text{g L}^{-1}$ )	Bacteria cells $\text{ml}^{-1}$ ( $\times 10^7$ )	BT cells $\text{ml}^{-1}$ ( $\times 10^5$ )	% BT
Quantuck Bay								
17 Apr 00	11.1 (2.3)			3.55 (0.94)	2.84 (0.61)	0.23 (0.01)	0.04 (0.01)	3.75
24 May 00	11.7 (0.99)	99.5 (6.1)	8.50	7.08 (0.50)	6.53 (0.74)	0.69 (0.17)	0.40 (0.12)	19.76
29 Jun 00	50.5 (0.4)	438 (6.5)	8.67	30.3 (2.19)	30.6 (1.91)	1.57 (0.79)	4.76 (0.46)	54.92
26 Jul 00	37.5 (0.4)	335 (7.2)	8.93	9.54 (0.78)	9.26 (2.54)	1.75 (0.05)	0.48 (0.10)	17.55
30 Aug 00	38.7 (1.2)	324 (26)	8.37	10.8 (0.84)	10.1 (0.40)	1.12 (0.05)	2.04 (0.30)	66.24
26 Sep 00	34.2 (0.05)	229 (3.7)	6.70	11.7 (0.31)	11.0 (0.96)	1.49 (0.17)	2.58 (0.36)	77.36
24 Oct 00	22 (0.5)	173 (1.2)	7.86	4.73 (0.13)	4.13 (0.14)	0.76 (0.03)	0.23 (0.17)	16.67
29 Nov 00	12.8 (0.5)	356 (8)	27.8	2.33 (0.24)	1.5 (0.09)	0.29 (0.03)	0.06 (0.04)	9.02
Cultures								
Exponential	91.3 (2.3)	632 (14)	6.92				37.6*	100
Late exp.	323 (10)	2367 (60)	7.33				133*	100

\* Estimated based on an average of 0.34 pg N cell<sup>-1</sup> (Gobler 1995).

accounted for 66% (August) and 77% (September) of phytoplankton biomass in Quantuck Bay (Table 2). At the same time, bacterial cell densities exceeded  $1 \times 10^7$  cells  $\text{ml}^{-1}$  and DON levels were  $>30 \mu\text{M}$  (Table 1). During October and November, temperature, DON, chlorophyll, brown tide, and bacterial cell densities all decreased markedly (Tables 1 and 2). By contrast, the bay was enriched in both nitrate and ammonium during November (14 and 5  $\mu\text{M}$ ; Table 1). Organic nitrogen dominated the dissolved N pool of Quantuck Bay throughout the study period. DON levels were at least an order of magnitude (10- to 90-fold) greater than DIN levels, except in November when DON and DIN were more similar (Table 1). On average,  $12 \pm 5\%$  (mean  $\pm 1$  SD) of the DON pool consisted of the three labile components we measured, DCAAs, DFAAs, and urea, usually in that relative order.

We observed DFAA concentrations between 0.28 and 1.95  $\mu\text{M}$  during our study period, with the highest levels ob-

Table 3. First-order rate constants for amino acid oxidation and peptide hydrolysis in <1.2  $\mu\text{m}$ , <1.2–5.0  $\mu\text{m}$ , and whole water from Quantuck Bay, Long Island, and in cultures of *A. anophagefferens*.

Date	AAO ( $\text{d}^{-1}$ )			Peptide hydrolysis ( $\text{d}^{-1}$ )		
	<1.2 $\mu\text{m}$	<5.0 $\mu\text{m}$	Whole	<1.2 $\mu\text{m}$	<5.0 $\mu\text{m}$	Whole
Quantuck Bay						
17 Apr 00	0	0	0	0.30	0.35	0.66
24 May 00	0.34	1.25	1.32	1.54	2.40	3.80
29 Jun 00	3.58	4.58	4.78	1.92	6.99	7.13
26 Jul 00	1.54	3.75	4.26	0.60	2.33	2.40
30 Aug 00	1.31	2.81	2.91	0.50	2.37	3.31
26 Sep 00	2.05	2.98	2.85	0.29	1.66	2.41
24 Oct 00	0.29	0.42	0.65	0.14	0.40	0.46
29 Nov 00	0	0	0	0	0.09	0.55
Cultures						
Exponential	0.4		2.22	1.25		58.3
Late exp.	2.97		11.42	0.57		179.1

served during summer months (Table 2). Summer concentrations of DFAAs were higher than those observed in some other estuarine systems but comparable to those we have measured in a similar organically enriched Chesapeake Bay subestuary (Mulholland et al. pers. comm.). For comparison, DFAA concentrations averaging between 0.14 to 0.47  $\mu\text{M}$  have been reported for the mesohaline Chesapeake Bay (Bronk et al. 1998). DFAA concentrations averaged 0.18 to 0.22  $\mu\text{M}$  in Flax Pond, New York (Jørgensen et al. 1993), and were between 0.3 and 0.7  $\mu\text{M}$  in the Delaware Estuary (Middelboe et al. 1995). DCAA concentrations were between 0.81 and 3.95  $\mu\text{M}$  during our study period, generally consistent with other estuarine studies (Keil and Kirchman 1991, 1999; Mulholland et al. pers. comm.).

*Amino acid oxidation and peptide hydrolysis*—First-order rate constants (the reciprocal of turnover times) were calculated for amino acid oxidation and peptide hydrolysis in size-fractionated water samples taken throughout our study (Table 3). In whole water samples, the DFAA pool turned over in 0.21 to 1.5 d due to amino acid oxidation, while the DCAA pool turned over in 0.14 to 2.2 d due to peptide hydrolysis. For comparison, turnover times for DFAAs were between 0.03–0.29 d in the northern Sargasso Sea (Keil and Kirchman 1999) and 0.013–0.14 d in the Mississippi River plume (Hopkinson et al. 1998). DCAA turnover times were similar to those reported for the protein pool (0.38 and 3.42 d) in the Sargasso Sea (Keil and Kirchman 1999). Rate constants were combined with DFAA or DCAA concentrations (Table 1) to estimate maximum rates.

Amino acid oxidation and peptide hydrolysis rates were generally higher during warm, summer months and lower during the cooler months of our study (Table 4; Fig. 2A,B). For example, mean amino acid oxidation rates were  $0.22 \pm 0.27$  (1 SD)  $\mu\text{M N d}^{-1}$  during April, May, October, and November but averaged  $4.8 \pm 1.1 \mu\text{M N d}^{-1}$  from June to September (Fig. 2A). Similarly, peptide hydrolysis rates averaged  $0.62 \pm 0.60$  (1 SD)  $\mu\text{M N d}^{-1}$  during April, October, and November but were an order of magnitude greater from

Table 4. Rates of amino acid oxidation and peptide hydrolysis in <1.2  $\mu\text{m}$ , <1.2–5.0  $\mu\text{m}$ , and whole water and amino acid and  $\text{NH}_4^+$  uptake in <5.0  $\mu\text{m}$  and whole water from Quantuck Bay, Long Island, and cultures of *A. anophagefferens*. Numbers in parentheses are standard deviations.

Date	AAO ( $\mu\text{M}$ DFAA $\text{d}^{-1}$ )			Peptide hydrolysis ( $\mu\text{M}$ DCAA $\text{d}^{-1}$ )			Amino acid uptake ( $\mu\text{M}$ N $\text{d}^{-1}$ )		$\text{NH}_4^+$ uptake ( $\mu\text{M}$ N $\text{d}^{-1}$ )		Total N uptake ( $\mu\text{M}$ $\text{d}^{-1}$ )	Percent AA uptake
	<1.2 $\mu\text{m}$	1.2–5.0 $\mu\text{m}$	>5.0 $\mu\text{m}$	<1.2 $\mu\text{m}$	1.2–5.0 $\mu\text{m}$	>5.0 $\mu\text{m}$	<5.0 $\mu\text{m}$	>5.0 $\mu\text{m}$	>5.0 $\mu\text{m}$	<5.0 $\mu\text{m}$		
Quantuck Bay												
17 Apr 00	0	0	0	0.59	0.1	0.61	1.2 (0.1)	2.7 (0.4)	0.24 (0.02)	0 (0.01)	4.5	85.8
24 May 00	0.14	0.38	0.03	3.1	1.71	2.79	1.8 (0.4)	2.1 (0.6)	18.6 (2.3)	4.03 (0.5)	30.7	12.7
29 Jun 00	3.54	0.99	0.2	7.58	20	0.55	12.9 (0.5)	11.3 (0.5)	80.9 (2.8)	0 (0.5)	117.3	20.6
26 Jul 00	1.87	2.69	0.62	0.64	1.85	0.07	24.1 (0.3)	0 (1.2)	68.0 (6.7)	0 (0.5)	115.3	20.9
30 Aug 00	1.47	1.68	0.11	0.64	2.39	1.20	38.4 (0.6)	0 (0.9)	88.3 (1.9)	2.88 (0.2)	151.6	25.3
26 Sep 00	4.0	1.81	0	0.33	1.57	0.86	53.3 (2.6)	25.3 (0.4)	17.9 (0.1)	1.10 (0.1)	158.3	49.7
24 Oct 00	0.14	0.06	0.11	0.11	0.21	0.05	1.4 (0.1)	0.4 (0.2)	53.9 (2.8)	10.2 (0.7)	58.4	3.1
29 Nov 00	0	0	0	0	0.09	0.09	0.05 (0.01)	0.02 (0.002)	0.26 (0.09)	0 (0.09)	0.41	17.7
Cultures												
Exponential	0.08	0.47		3.9	184			22.7 (0.5)		44.8 (1.4)	71.9	31.6
Late exp.	2.51	9.61		7.9	2,500			64 (20)		84.6 (3.9)	152.5	42.0

May through September ( $9.1 \pm 10.8$  [1 SD]  $\mu\text{M}$  N  $\text{d}^{-1}$ ; Fig. 2B). Rates of amino acid oxidation were high during all of the summer months and were correlated with temperature ( $r^2 = 0.73$ ;  $P < 0.01$ ) (Fig. 2A) and not Chl *a* or brown tide abundance (Fig. 2C). However, rates of peptide hydrolysis were more closely correlated with Chl *a* ( $r^2 = 0.89$ ;  $P < 0.001$ ) and brown tide abundance ( $r^2 = 0.70$ ;  $P < 0.001$ ) (Fig. 2D) and not well correlated with temperature (Fig. 2B).

Almost half of the amino acid oxidation was in the bacterial size fraction (<1.2  $\mu\text{m}$ ; 49  $\pm$  16% of total activity May–November; Fig. 3A), while peptide hydrolysis was primarily in the brown tide-sized size fraction (1.2–5.0  $\mu\text{m}$ ; 56  $\pm$  16% of total activity [May–November; Fig. 3B]), particularly during the June bloom (71%). Consistent with our size-fractionated sample data, regression analysis indicated that amino acid oxidation rates were highly correlated with bacterial densities ( $r^2 = 0.91$ ;  $P < 0.001$ ), while peptide hydrolysis rates were correlated with Chl *a* concentrations and *A. anophagefferens* cell densities (see above).

**Nitrogen uptake**—In Quantuck Bay, most of the N uptake was in the <5.0- $\mu\text{m}$  size fraction, and  $\text{NH}_4^+$  was the primary form of N taken up during most of the year (Fig. 4). During the June brown tide event when *A. anophagefferens* represented 55% of the biomass,  $\text{NH}_4^+$  uptake accounted for nearly 60% of the total N uptake. Similarly, during August, *A. anophagefferens* accounted for 66% of algal biomass, and 58% of N uptake was from  $\text{NH}_4^+$  (Fig. 4). However, in September, when *A. anophagefferens* was 77% of the total biomass,  $\text{NH}_4^+$  uptake accounted for just 18% of the total N uptake (Fig. 4), since concentrations of  $\text{NH}_4^+$  were near the limit of analytical detection (Table 1). At that time, uptake of amino acid (50%) (Table 4) and urea (36%) N was 86% of the total N uptake (Fig. 4). The relative contribution of urea to N uptake was comparable to that of amino acids during May, June, July, and October (e.g., Table 4). Amino acid uptake was higher than urea uptake in April, August, and September (Fig. 4), when DFAA concentrations were high relative to urea (Table 1). A comparison of amino acid oxidation and

$\text{NH}_4^+$  uptake rates shows that amino acid oxidation could at times contribute substantially to  $\text{NH}_4^+$  uptake (<1–32.5 %) and could therefore account for rates of PN turnover of up to 0.17  $\text{d}^{-1}$  (Table 5).

**Amino acid N and C uptake**—Uptake rates of DFAA-N in this study ranged from 75 to 3,275  $\text{nM}$  N  $\text{h}^{-1}$  and represented between 3.2% (November) and 78% (September) of the total N uptake (Fig. 4). Total DFAA-N uptake rates were higher, and amino acid uptake a larger portion of total N supply, than in similar studies in Chesapeake Bay (1–7%; Glibert et al. 1991) and during a brown tide bloom in another Long Island embayment in July 1995 (11–16%; Berg et al. 1997).

Measured rates of DFAA-C uptake ranged from 41 to 110  $\mu\text{M}$  C  $\text{d}^{-1}$  between June and September in whole water samples from organic-rich Quantuck Bay, but were lower in October (Table 6). These rates are at least 100 times higher than those observed in a study in Long Island Sound, using  $^3\text{H}$  tracer (Fuhrman 1987) and 10 to 100 times the rate measured in Flax Pond, a tidal embayment on Long Island using  $^{14}\text{C}$  tracer (Jørgensen et al. 1993).

The turnover of PN and PC pools due to amino acid uptake ranged from 0.08 to 2.3  $\text{d}^{-1}$  and 0.02 to 0.48  $\text{d}^{-1}$ , respectively (Table 7). Similar to the DFAA pool, PN and PC turnover times were shorter between June and September. Turnover of PN from amino acid uptake increased from 0.48  $\text{d}^{-1}$  in June to 2.3  $\text{d}^{-1}$  in September. Similarly, rates of PC turnover from amino acid uptake increased from 0.11  $\text{d}^{-1}$  to 0.48  $\text{d}^{-1}$  over the same interval. The growth rate of *A. anophagefferens* in another Long Island embayment was estimated to be 0.3  $\text{d}^{-1}$  (Berg et al. 1997).

Alanine and glutamate uptake was linear over time at each substrate concentration tested (0.03 to 30  $\mu\text{M}$ ) (data not shown). However, hyperbolic saturation responses were not obtained for C and N uptake from either amino acid during the amino acid uptake kinetics experiments (Fig. 5). These results precluded measurements at low amino acid concentrations but were similar to the results found for  $\text{NH}_4^+$  in an

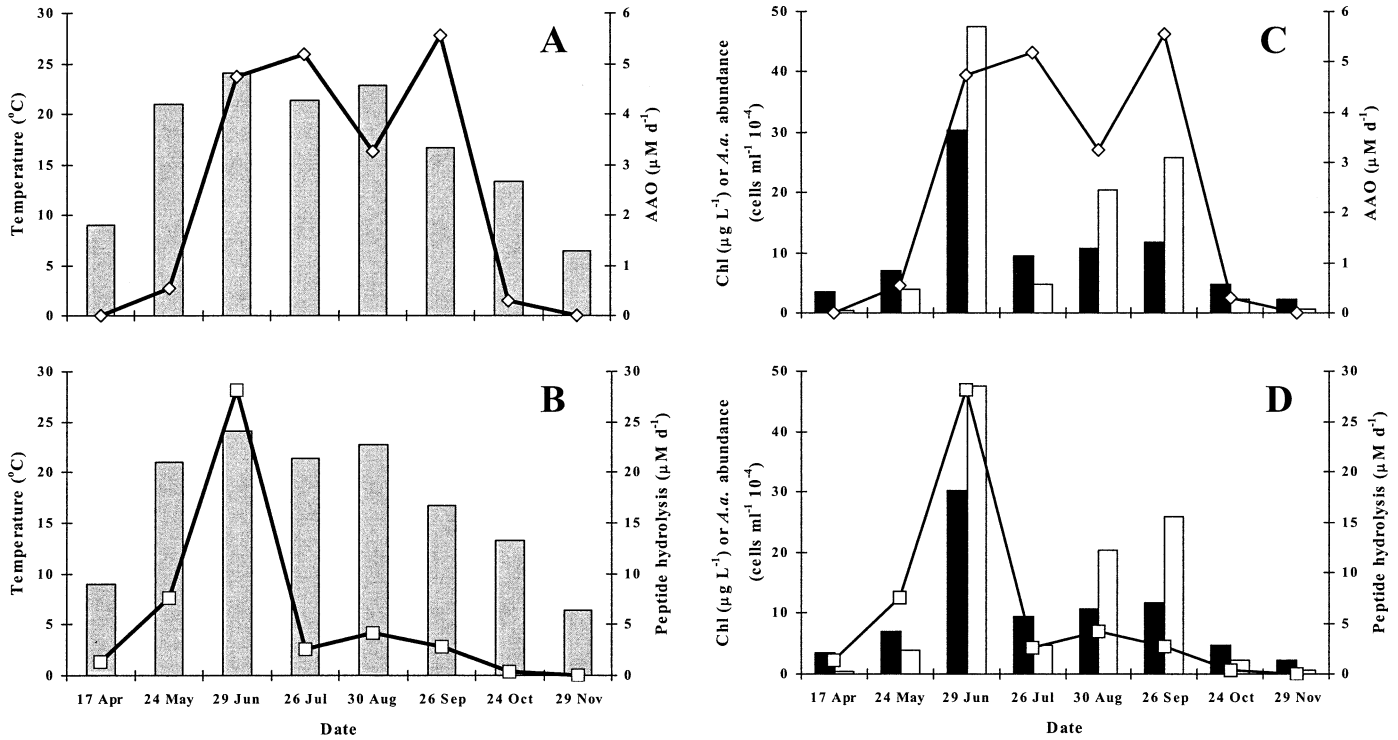


Fig. 2. First-order rate constants for amino acid oxidation (lines in A and C) and peptide hydrolysis (lines in B and D) as a function of temperature (bars in A and B) and as a function of chlorophyll biomass (black bars in C and D) and *A. anophagefferens* abundance (white bars in C and D) over the course of a seasonal cycle.

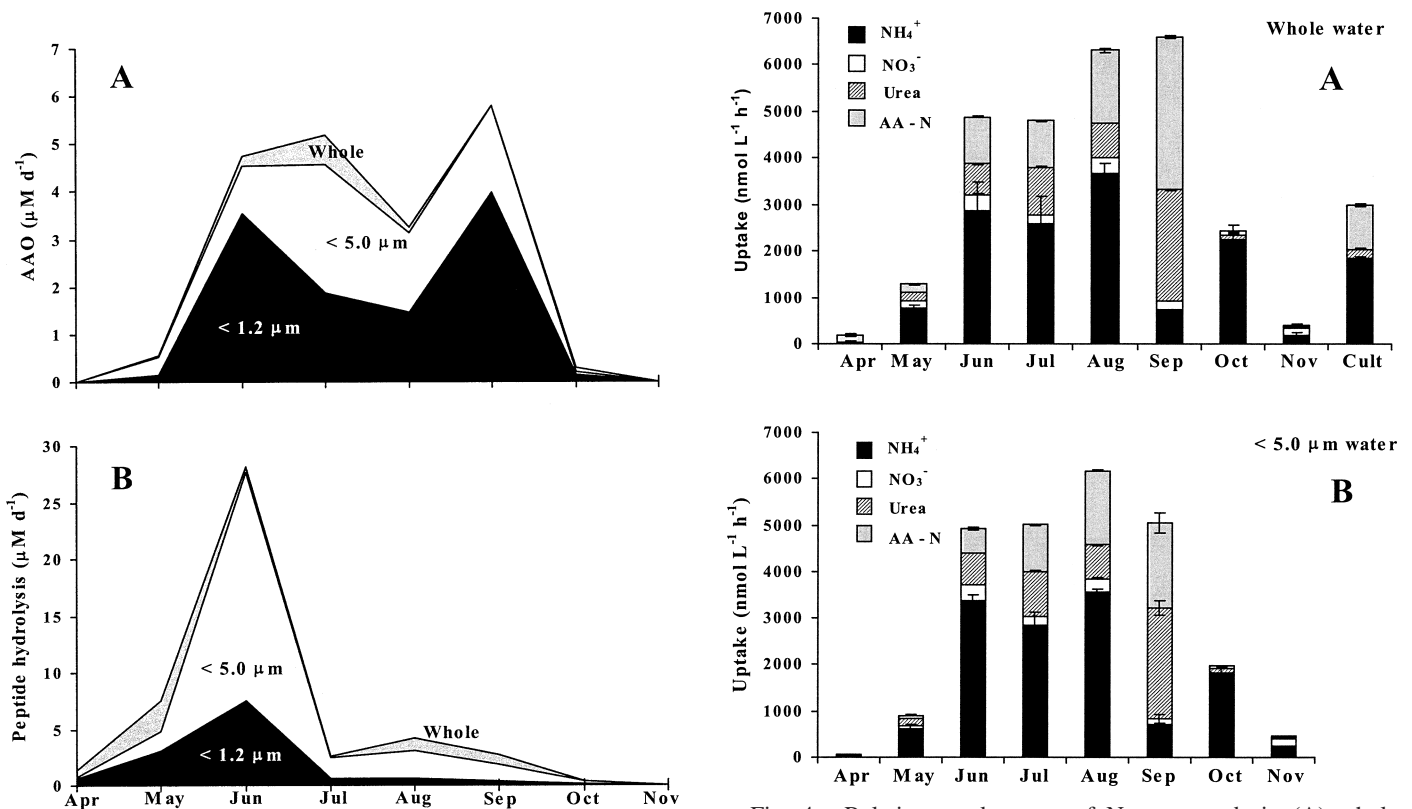


Fig. 3. Distribution of (A) amino acid oxidation and (B) peptide hydrolysis among size fractions over the course of a seasonal cycle.

Fig. 4. Relative uptake rates of N compounds in (A) whole water and cultures and (B)  $< 5.0 \mu\text{m}$  water collected from Quantuck Bay, monthly, over a seasonal cycle.

Table 5. AAO, PH, and  $\text{NH}_4^+$  uptake in whole water samples from Quantuck Bay and cultures of *A. anophagefferens*. Also shown are the percent contribution of  $\text{NH}_4^+$  from AAO to total  $\text{NH}_4^+$  uptake, the relative percent of amino acids oxidized and taken up, and the rate constants for particulate N turnover from AAO-derived N.

Date	AAO ( $\mu\text{M}$ DFAA $\text{d}^{-1}$ )	PH ( $\mu\text{M}$ DCAA $\text{d}^{-1}$ )	$\text{NH}_4^+$ uptake ( $\mu\text{M}$ N $\text{d}^{-1}$ )	Percent AAO/ $\text{NH}_4^+$ uptake	Percent AAO/AA uptake	PN turnover from AAO ( $\text{d}^{-1}$ )
Quantuck Bay						
17 Apr 00	0.0	1.3	0.24	0.0	0.0	0.0
24 May 00	0.55	7.6	22.6	2.4	14.1	0.05
29 Jun 00	4.73	28.1	80.9	5.8	19.5	0.09
26 Jul 00	5.18	2.56	68.0	7.6	21.5	0.14
30 Aug 00	3.26	4.23	91.2	3.6	8.5	0.08
26 Sep 00	5.81	2.76	19.0	30.6	7.4	0.17
24 Oct 00	0.31	0.37	64.1	0.5	17.2	0.01
29 Nov 00	0.0	0.18	0.26	0.0	0.0	0.0
Cultures						
Exponential	0.55	187.9	44.8	1.2	2.4	0.01
Late exp.	12.1	2,508	84.6	14.3	18.9	0.04

earlier study conducted during a brown tide bloom (Lomas et al. 1996). For these reasons, we did not calculate  $V_{\max}$  or  $K_s$  values for either amino acid tested. However, we speculate that they would be on the order of a micromolar based on the responses observed. Phytoplankton amino acid uptake rates have not been routinely measured, so there are few data to compare with these. Literature values for  $K_s$  suggest that the affinity for amino acids is low in phytoplankton (Antia et al. 1991).

The highest specific uptake rates in the kinetic studies were observed at low addition levels. One reason for this may be the high ambient amino acid concentrations during this period (1.07  $\mu\text{M}$ ; alanine and glutamate concentrations were between 5 and 7% of the DFAA pool). Other investigators have measured high uptake rates when the atom percent enrichment of the substrate pool is substantially lower than 10% (Glibert pers. comm.). However, in this study, the decrease in  $V$  was observed for both substrates over addi-

tions ranging from 0.03 to 3.0  $\mu\text{M}$  and not just at low atom percent enrichments. The high uptake rates observed at low concentrations could also be due to a rapid uptake response such as that observed for  $\text{NH}_4^+$  under  $\text{NH}_4^+$  limitation. Further studies are needed to investigate the observed relationship under a variety of nutrient conditions.

Alanine and glutamate uptake were highest in the incubations that were shortest in duration (Fig. 5). This suggests that amino acid regeneration may affect measured uptake rates when incubation times are long and argues for use of short incubation times.

The molar C:N uptake ratio from free glutamic acid and alanine averaged  $1.9 \pm 0.3$  (1 SD; Fig. 6). We can compare this with the theoretical C uptake expected if all of the C from amino acids was assimilated, based on measured N uptake (Table 6). In making this calculation, we subtracted C released from amino acid oxidation, since N can be taken up independently from C during this process. Measured up-

Table 6. Rates of N and C uptake from amino acids, extracellular release of C from AAO, C uptake calculated based on N uptake, C uptake corrected for extracellular C release from AAO, and the percent C unaccounted for after uptake and AAO in whole water samples from Quantuck Bay and in cultures of *A. anophagefferens*. Numbers in parentheses are standard deviations.

Date	AA N uptake ( $\mu\text{M}$ N $\text{d}^{-1}$ )	AA C uptake ( $\mu\text{M}$ C $\text{d}^{-1}$ )	Calculated C released from AAO*	Maximum C uptake from AA N uptake*	Maximum C uptake corrected for AAO†	Percent C imbalance
Quantuck Bay						
17 Apr 00	3.9 (0.4)		0	14.6	14.6	
24 May 00	3.9 (0.6)		2.06	14.6	12.5	
29 Jul 00	24.2 (0.5)	49.3 (1.6)	17.7	90.5	72.8	32.3
26 Jul 00	24.1 (1.2)	41.0 (4.9)	19.4	90.1	70.8	42.1
30 Aug 00	38.4 (0.9)	65.9 (3.1)	12.2	144	131	49.7
26 Sep 00	78.6 (0.4)	110 (3.4)	21.7	294	272	59.6
24 Oct 00	1.8 (0.15)	4.1 (0.9)	1.16	6.7	5.6	26.8
29 Nov 00	1.8 (0.05)	4.3 (1.9)	0	6.7	6.7	35.8
Cultures						
Exponential	22.7 (0.4)	51.3 (3.1)	2.06	84.9	82.8	38.0
Late exp.	64 (12)	1,061 (64)	45.3	239	194	-447

\* Calculated based on N released from AAO or taken up and an average C:N ratio of the DFAA pool of 3.74.

† Assumed all N from AAO was taken up and N uptake was uncoupled from C uptake.

Table 7. Rate constants for DFAA, DCAA, PN, and PC turnover resulting from amino acid uptake by natural populations from Quantuck Bay and cultures of *A. anophagefferens*.

Date	DFAA-N turnover (d <sup>-1</sup> )	DFAA-C turnover (d <sup>-1</sup> )	PN turnover (d <sup>-1</sup> )	PC turnover (d <sup>-1</sup> )
Quantuck Bay				
17 Apr 00	13.9		0.35	
24 May 00	9.30		0.33	
29 Jun 00	24.4	13.3	0.48	0.11
26 Jul 00	19.8	9.02	0.64	0.12
30 Aug 00	34.3	15.7	0.99	0.20
26 Sep 00	40.3	15.1	2.30	0.48
24 Oct 00	3.86	2.35	0.08	0.02
Cultures				
Exponential	108.1	224.3	0.25	0.08
Late exp.	76.2	1263	0.20	0.45

take of C from amino acids was always less than the calculated theoretical C uptake, except for late-exponential phase cultures (*see below*). Since measurements of C uptake were net measurements, the deficit C taken up may have been respired, although respiration rates were not measured during this study.

**Culture experiments**—Exponentially growing cultures of *A. anophagefferens* exhibited very high rate constants for peptide hydrolysis relative to those of natural populations (58 and 179 d<sup>-1</sup> in exponential and late-exponential phase cultures, respectively; Table 3). However, rate constants for

amino acid oxidation were not elevated relative to natural populations (Table 3). DCAA concentrations in exponentially growing cultures were comparable with those observed during the June brown tide bloom in Quantuck Bay, whereas levels in late-exponential cultures were very high (13.8 μM; Table 1). DFAA concentrations in exponential and late-exponential cultures were not elevated relative to concentrations observed in Quantuck Bay. Consequently, calculated rates of peptide hydrolysis were much higher in cultures of *A. anophagefferens* than in the bay, while rates of amino acid oxidation were more similar (Table 4).

In cultures grown on NO<sub>3</sub><sup>-</sup>, uptake of NH<sub>4</sub><sup>+</sup> and DFAAs, presumably recycled in the medium, represented the majority (>90%) of the total N uptake (Fig. 7). In addition, C uptake from amino acids represented a significant source of C for growth, particularly during late-exponential phase. Net C uptake from glutamate (molar C:N ratio of five) and alanine (molar C:N ratio of three) was measured in exponential and late-exponential phase cultures of *A. anophagefferens* (Fig. 8). The molar C:N ratio of net amino acid uptake averaged 2.45 in exponentially growing cultures but increased to 18.5 in late-exponential cultures (Fig. 7).

In cultures of *A. anophagefferens*, turnover times of the DFAA and DCAA pools from amino acid oxidation and peptide hydrolysis, respectively, were shorter than those observed in natural populations. For example, the DCAA pool turned over with rate constants of 179 d<sup>-1</sup>, more than 10 times faster than in the natural populations (Table 3). Similarly, rate constants for uptake of both DFAA-C and DFAA-N were higher in cultures than in natural populations

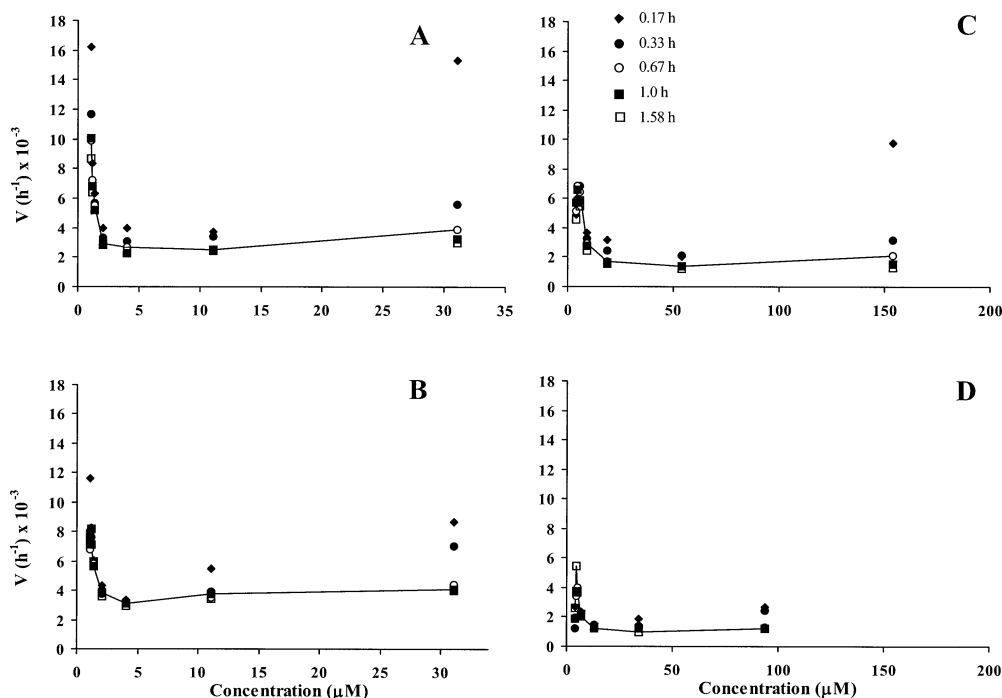


Fig. 5. Nitrogen (A and C) and carbon (B and D) uptake kinetic curves for dual <sup>13</sup>C/<sup>15</sup>N-labeled glutamate (A and B) and alanine (C and D). The symbols are the uptake rates determined by mass spectrometry for experiments using incubations ranging from 0.17 to 1.58 h in duration. The solid line represents the best fit for the 0.67 h set of incubations.

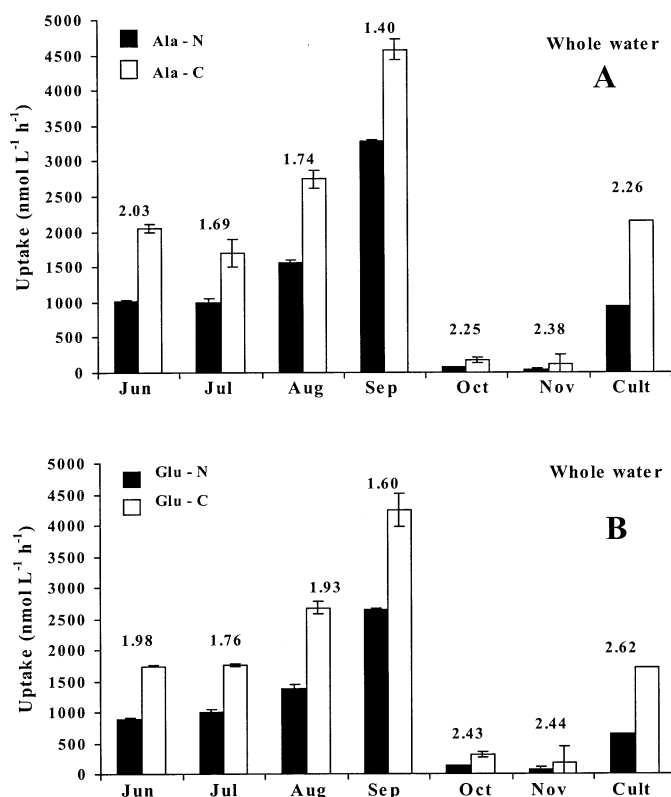


Fig. 6. Relative rates of uptake of amino acid N versus C from (A) alanine and (B) glutamic acid by whole water collected from Quantuck Bay, monthly, over a seasonal cycle. The C:N molar uptake ratio (at the top of each pair of bars) ranged from 1.4 to 2.43 in natural populations.

from Quantuck Bay (Table 7). Turnovers of PN and PC biomass in cultures of *A. anophagefferens* due to amino acid uptake were 0.25 and 0.08 d<sup>-1</sup>, respectively, during exponential phase and 0.20 and 0.45 d<sup>-1</sup>, respectively, during late-exponential phase growth (Table 7). The highest rates of amino acid C uptake were observed during late-exponential growth when cell densities were higher and light penetration lower. Uptake rates were comparable in cultures and natural populations during brown tide blooms.

## Discussion

During the summer months of our study (June–September), DIN supply rates may have limited phytoplankton growth, since the mean ratio of dissolved inorganic N to P ( $1.36 \pm 1.45$ ) was well below the Redfield ratio, and the mean particulate C:N ratio was 8.2. Such a conclusion is consistent with monthly bioassays conducted from May through October 2000, in which additions of nitrate, ammonium, and urea consistently enhanced phytoplankton growth rates (data not shown). If growth of phytoplankton in Quantuck Bay were limited by N supply, species such as *A. anophagefferens* that have strategies for using larger DON compounds would have a competitive advantage over those that do not, since DON levels are more than an order of magnitude higher than DIN levels at this site (Table 1).

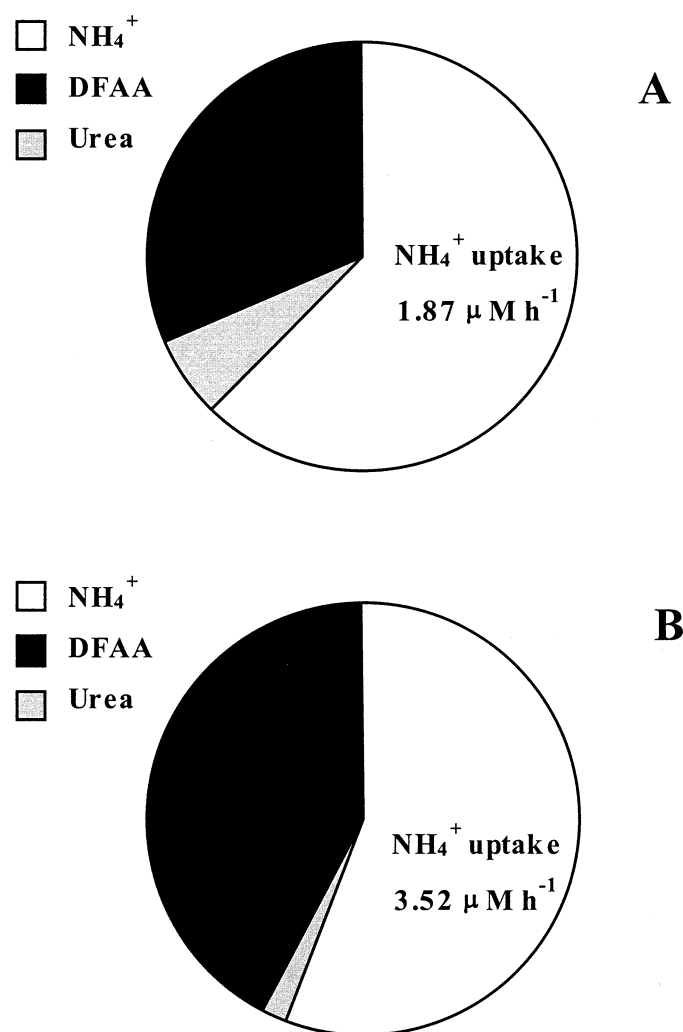


Fig. 7. Relative uptake rates of N compounds in (A) exponential and (B) late-exponential phase cultures of *A. anophagefferens*. There was no measurable NO<sub>3</sub><sup>-</sup> uptake in either culture.

Because amino acids, peptides, and proteins are N rich, they can represent a significant source of N in systems depleted in DIN. In addition, these compounds can be important sources of C for heterotrophic growth. Extracellular peptide hydrolysis facilitates the breakdown of protein and large peptide chains into units small enough to be taken up by cells. Consequently, peptide hydrolysis can support both C and N acquisition by bacteria and phytoplankton mixotrophs. We discuss below, however, our finding that the C incorporated into cells from amino acid uptake is not in the proportion expected based on measured N uptake from these compounds and the C:N ratio of the individual amino acids in the DFAA pool.

One mechanism whereby C and N uptake can be uncoupled is extracellular amino acid oxidation. Amino acid oxidation liberates N as ammonium and C in an organic oxidation product; the amino acid-derived N and C can be taken up independently of one another (and perhaps by different organisms). It has been assumed that organisms possessing extracellular oxidation enzymes take up the NH<sub>4</sub><sup>+</sup> to alleviate

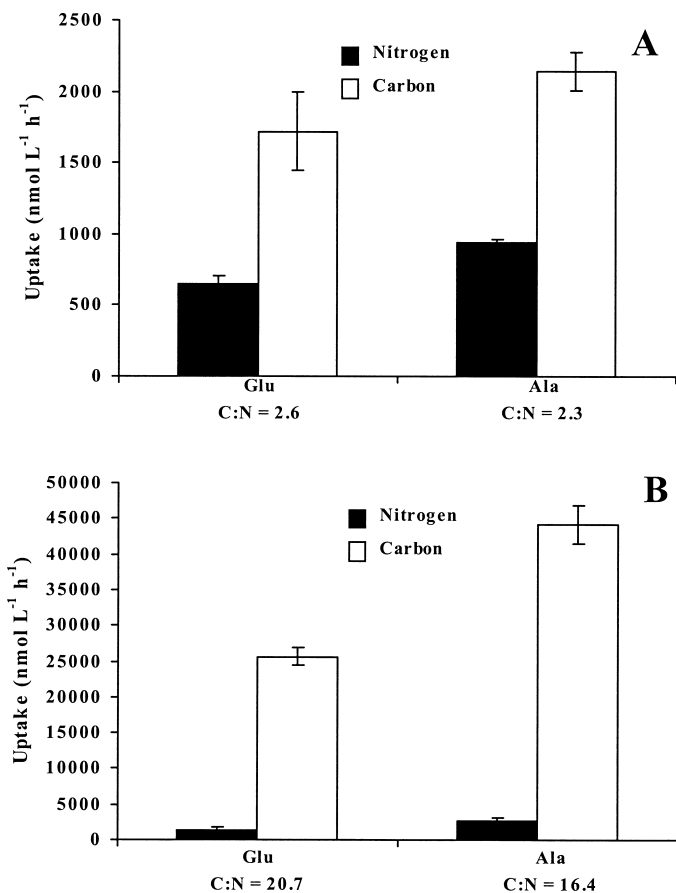


Fig. 8. Relative uptake rates of amino acid N versus C from alanine and glutamic acid by (A) exponential and (B) late-exponential cultures of *A. anophagefferens*. The C:N molar uptake ratio averaged 2.45 in exponential phase cultures and 18.55 in late-exponential phase cultures.

N limitation (Palenik et al. 1988; Palenik and Morel 1990a). Indeed, uptake of  $^{15}\text{NH}_4^+$  liberated from LYA  $^{15}\text{N}$  lysine has been recently demonstrated in a natural estuarine population (Mulholland et al. pers. comm.). In the following discussion, we investigate the relative importance of these enzymatic processes to DFAA and DCAA cycling, and to N cycling in general, in an organically enriched environment that experiences seasonal blooms of *A. anophagefferens*, an algal mixotroph.

**Extracellular amino acid oxidation**—Our results demonstrate that *A. anophagefferens* and communities dominated by this species are capable both of amino acid uptake and extracellular amino acid oxidation. In this study, cells took up amino acids at much higher rates than they oxidized them (e.g., amino acid oxidation was usually less than 20% of the rate of amino acid uptake) (Table 5). Moreover, most oxidation was by bacteria-sized organisms, and rates were highly correlated with bacterial densities. Turnover times of the DFAA pool from amino acid oxidation were short, between 0.21 and 0.34 d, between June and October when *A. anophagefferens* was abundant (Table 3). However, calculations suggest that turnover of the particulate N pool due to amino

acid oxidation alone (0.01 to 0.17 d<sup>-1</sup>; Table 5) would not be high enough to support typical phytoplankton growth rates (e.g., doubling times on the order of 0.2 to 2 d<sup>-1</sup>) or those of *A. anophagefferens* (0.3 d<sup>-1</sup>) measured in another study (Berg et al. 1997). So, we conclude that amino acid oxidation was not the primary mechanism by which cells acquired N in this system.

Rates of amino acid oxidation reported here should be considered maximum values. To estimate amino acid oxidation rates, we assumed that 100% of the DFAA pool was available for oxidation. We multiplied the concentration of DFAAs observed at the beginning of the incubation by the first-order rate constants derived from incubations with the fluorescent tracer (LYA-lysine). If, as is likely, not all the DFAA pool is available for oxidation, then rates are proportionately lower. Enrichment of the substrate pool by added label was not considered for any of the enzymatic measurements, as additions of LYA derivatives were usually <10% of the ambient pools.

In earlier studies, amino acid oxidation was elevated in Long Island embayments experiencing blooms of brown tide relative to other coastal and estuarine systems (Pantoja and Lee 1994; Mulholland et al. 1998). In those studies, first-order rate constants for amino acid oxidation were about 0.7 h<sup>-1</sup>. The highest rate constants observed in our study were about 0.4 h<sup>-1</sup> during the June brown tide bloom. Previous studies reported only rate constants or rates of LYA-lysine oxidation; they did not compare oxidation rates with uptake of a suite of nitrogenous compounds to determine the relative importance of this extracellular mechanism. While extracellular amino acid oxidation could contribute to the cycling of DFAAs, amino acid uptake appeared to be a more important pathway of amino acid turnover in this system (Table 4); rates of amino acid oxidation were <21% of amino acid uptake rates and amino acid oxidation supplied up to 32.5% of the  $\text{NH}_4^+$  that was taken up by cells. Since extracellular oxidation of amino acids was primarily accomplished by the bacterial size fraction in this system, bacteria may be important for regenerating  $\text{NH}_4^+$  from amino acids.

**Extracellular peptide hydrolysis**—Bacterial productivity and proteolytic activity appear to be tightly coupled in marine systems (Smith et al. 1992; Hoppe et al. 1993). However, most studies of proteolytic activity have focused on heterotrophic bacteria and not phytoplankton, and consequently the contribution of phytoplankton autotrophs and mixotrophs to proteolytic activity is unknown. During our laboratory studies, we observed that cultures of *A. anophagefferens* hydrolyzed peptides at high rates. In the field, we found that *A. anophagefferens* cell densities were significantly correlated with rates of peptide hydrolysis, a process that would enhance the ability of this species to take up DFAAs and small peptides, the products of peptide hydrolysis. Such a nutritional pathway may be important for growth in embayments such as Quantuck Bay, where N supply may limit phytoplankton growth rates and organic N is abundant.

Rates of peptide hydrolysis measured during the June bloom could potentially supply all of the amino acids taken up at this time and an order of magnitude more amino acids

than were oxidized (Table 5). Rapid hydrolysis of combined amino acids may fuel high growth rates if cells can take up the hydrolysis products. We did not observe production of DFAAs as a result of peptide hydrolysis; DFAA concentrations varied only slightly over time in Quantuck Bay, and we did not measure their concentrations with time in the culture experiments. Since peptide hydrolysis was rapid and there was no buildup of either DFAAs or DCAAs, it is possible that *A. anophagefferens* took up and used the hydrolysis products to grow. However, we can only assume that *A. anophagefferens* is taking up the products of peptide hydrolysis. The uptake of DCAAs has not been measured routinely. In two studies examining the change in concentrations of the DCAA pool, bacterial DCAA uptake was estimated to be between 0 and 55.8 nM N L<sup>-1</sup> h<sup>-1</sup> and represented up to 56% of the total N uptake (Jørgensen et al. 1993, 1994).

Several caveats must be considered with regard to our peptide hydrolysis rate estimates. We assumed that 100% of the DCAA pool was available for hydrolysis, likely an overestimate. We multiplied the DCAA concentration measured at the outset of the incubations by the first-order rate constant calculated for the disappearance of the fluorescent tracer over the time-course incubation to obtain a rate. However, the 4-amino acid LYA analog used here may not be a perfect analog of available peptides and proteins. The composition and length of peptide and protein chains and relative availability of the individual compounds are unknown. Earlier investigations examining hydrolysis of LYA-labeled peptides of different lengths and compositions found the principal product of hydrolysis to be the dipeptide rather than the free amino acid (Pantoja et al. 1997; Pantoja and Lee 1999). Pantoja and Lee (1999) found that peptides containing more than two amino acids were hydrolyzed 10–400 times faster than dipeptides or the fluorogenic substrate Leu-MCA. The production of dipeptides may result from the inhibition of further hydrolysis by the presence of the large fluorescent compound at the end of the residual LYA-di-alanine. Alternatively, dipeptides may be small enough (e.g., <600 Da) to be incorporated directly by microorganisms as pointed out in earlier studies. Studies examining uptake of C and N from compounds larger than individual amino acids are rare (Antia et al. 1991). To date, uptake of dipeptides has been demonstrated only for bacteria (Jørgensen et al. 1993, 1994).

*DIN and DON uptake*—Ammonium and DFAAs were the major forms of N taken up by microbial communities in Quantuck Bay (Fig. 4). Concentrations of NH<sub>4</sub><sup>+</sup> were higher and specific uptake rates were faster between June and August; consequently NH<sub>4</sub><sup>+</sup> uptake fueled much of the N demand for growth during that time. Urea and DFAA concentrations were elevated, and specific uptake rates of these compounds were higher during September; these compounds also contributed more to the N nutrition of resident organisms during that period. In contrast to a previous study in a similar embayment during a brown tide bloom in July 1995 (Lomas et al. 1996; Berg et al. 1997), urea was generally a less important N source for brown tide organisms during most of the sampling periods in this study. During the 1995 study, urea concentrations were lower (e.g., 0.04–0.16 μM)

and uptake rates were potential rates (<sup>15</sup>N additions were saturating [10 μM]); consequently, actual urea uptake may have been overestimated. Relatively low urea uptake rates were observed in this and another culture study of *A. anophagefferens* (Dzurica et al. 1989). Although reduced and recycled, forms of N fueled brown tide-dominated communities throughout the summer, the preferred N source changed over that time. The use of recycled forms of N during the summer is common in estuarine and marine systems where new N inputs are confined to spring NO<sub>3</sub><sup>-</sup> inputs from upwelling events and runoff (Malone et al. 1986; Glibert et al. 1995).

Rates of amino acid uptake reported here should also be considered maximum values. As for amino acid oxidation, we assumed that 100% of the DFAA pool was available for uptake. Unlike the enzymatic rate estimates, if we assume only 50% of the DFAA pool is available for uptake, rates will be reduced by between 55 and 65% because both atom percent enrichment and pool size change with incubation time. Furthermore, not all amino acids may be equally available for uptake. Glutamate and alanine, the compounds used to trace amino acid C and N uptake, were each between 5 and 7% of the DFAA pool during most of the sampling periods.

*C and N coupling*—In addition to being an important source of N, amino acid uptake supplied a substantial portion of the C requirements for growth of *A. anophagefferens* in Quantuck Bay. These results are consistent with laboratory experiments in which nonaxenic *A. anophagefferens* cultures took up <sup>14</sup>C-labeled glutamic acid and glucose (Dzurica et al. 1989) and field studies in which the addition of glucose was observed to significantly enhance *A. anophagefferens* growth rates (Gobler and Sañudo-Wilhelmy 2001b). Moreover, heterotrophic uptake of organic compounds by other phytoplankton has been documented previously (e.g., Wheeler et al. 1977; Lewitus and Caron 1991; Lewitus et al. 1999). However, previous field studies have generally treated uptake of DOM as a heterotrophic process so that uptake of amino acid C by phytoplankton species is rarely measured in the field. In addition, the fate of the C in phytoplankton that use DON has not been assessed in studies using <sup>15</sup>N tracers.

By employing dually labeled amino acids to directly measure both C and N uptake rates from amino acids, we showed that when phytoplankton cells in Quantuck Bay took up amino acids, they incorporated both the N and C from these compounds. The organic C taken up may allow growth in excess of that supported by photosynthetic C acquisition alone. In addition to the advantage of obtaining N, the ability to obtain C from amino acids may offer *A. anophagefferens* a competitive advantage in DOM-enriched environments. If amino acid uptake can also occur in the dark (probable but we only made measurements during the light period), then *A. anophagefferens* can continue to grow during dark periods or when self-shading occurs during dense algal blooms. For example, the 1% light depth in Quantuck Bay during intense brown tide months (June, August, September) was <2 m (based on Secchi disc readings). Under such circumstances, a phytoplankton species possessing the ability to supplement

photosynthetic C fixation with heterotrophic uptake of DOC would have a clear advantage over strictly autotrophic species.

Rates of amino acid C uptake varied seasonally in Quantuck Bay and with growth stage in our culture experiments. We observed that the molar ratio of C:N uptake from amino acids was about two, regardless of the C:N ratio of the substrate used (in this case alanine, C:N ratio of 3:1, or glutamate, C:N ratio of 5:1). But the ratio varied somewhat as the seasons progressed and in cultures varied widely among exponential and late-exponential phase. When we calculated C uptake based on amino acid N uptake (correcting for the amount of N that could be taken up independently after extracellular amino acid oxidation), these calculated rates of C uptake always exceeded the measured rate and, while the difference between calculated and theoretical values increased between June and September, the magnitude of C uptake from amino acids increased (Table 6). This suggests that brown tide cells can alter their nutritional physiology in response to cellular and extracellular parameters. During the period of highest brown tide cell density, the proportion of C uptake from amino acids was highest, and in late-exponential cultures of *A. anophagefferens*, cells took up more of the amino acid C than N, resulting in an N deficit. Physiological changes that allow a shift in the dominant metabolic pathway (e.g., autotrophic versus heterotrophic C uptake) may be important as cell density increases and inorganic nutrients become limiting.

Respiratory losses were not measured during this study and may account for some of the C imbalance; however, cells always retained a large proportion of the amino acid C. In addition, competition from bacterial heterotrophs may have caused a decrease in the proportion of amino acid C available for uptake by brown tide and larger cells. We used GF/F filters (nominal pore size of 0.7–0.8  $\mu\text{m}$ ) in the uptake experiments, so some bacteria probably passed through the filter.

Previous studies demonstrated that amino acid oxidation results in N uptake that is independent from C uptake from amino acids (Mulholland et al. 1998, pers. comm.). Using dual-labeled amino acid tracers, we have demonstrated that amino acid oxidation alone does not account for the imbalance in C and N uptake from amino acids. Thus, N uptake by phytoplankton can be seriously underestimated when C is used as a tracer of amino acid N uptake (e.g., Table 6). Conversely, photosynthetic C uptake measurements may underestimate the total C uptake by phytoplankton when these organisms are capable of taking up organic compounds.

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