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Peptide hydrolysis and the uptake of dipeptides by phytoplankton

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Abstract

Rates of peptide hydrolysis (using the fluorescent substrate, lucifer yellow anhydride-labeled tetra-alanine) and dipeptide uptake (using dually labeled, ^{15}N and ^{13}C, dialanine) were measured in phytoplankton cultures and in natural populations during algal blooms dominated by one or two taxa. During most sampling events, both peptide hydrolysis and dipeptide uptake were greatest in the size fraction containing the dominant phytoplankter, suggesting that phytoplankton contribute substantially to or may even dominate observed extracellular peptide hydrolysis and dipeptide uptake in the environment. These are the first data suggesting that dipeptides may be taken up directly by phytoplankton and this may represent a previously unaccounted-for nitrogen source in aquatic systems. Like many other processes in phytoplankton, peptide hydrolysis appears sensitive to the diel light cycle and the nutrient environment, with rates varying depending on the dominant N source, but with no clear pattern. Uptake of dialanine, the dominant product of the hydrolysis of the peptide tetra-alanine, also varied depending on the dominant taxa and the nutrient regime. Most of the time, it appeared that low production of dialanine by tetra-alanine hydrolysis limited the uptake of the dipeptide. Close coupling between peptide hydrolysis and dipeptide uptake may also help explain the absence of correlations between rates of peptide hydrolysis and the concentration and composition of the free amino acid pool.

In many marine and estuarine systems, nitrogen is thought to limit growth and production. Although inorganic nitrogen species can be quickly depleted during primary production, dissolved organic nitrogen (DON) compounds are rarely depleted and can dominate the N pool in a variety of aquatic systems, including estuaries. At least some DON appears to be available to resident microbes (Berman and Bronk 2003), and this availability appears to be related to the size of the compounds. Although most of the dissolved organic matter (DOM) pool in nature is found in the <1000-Da size fraction, we now know that the high-molecular-weight (HMW) DOM pool, isolated using ultrafiltration, is more biologically reactive than previously thought (Amon and Benner 1996; Guo and Santschi 1997; Benner 2002).

In living cells of marine microorganisms, proteins account for a major fraction of the cellular and intracellular carbon and most of the nitrogen (Nguyen and Harvey 1994; Kirchman 2000). We therefore expect DON released from living organisms to contain protein; the predominance of amide N in the HMW DOM pool seems to confirm this, and in fact, dissolved proteins have been identified in seawater (Tanoue 1995; Tanoue et al. 1996; Yamada and Tanoue 2003). Nuclear magnetic resonance spectroscopy suggests that >90% of the N in HMW DOM is amide N and <10% is as the free amine (McCarthy et al. 1996, 1997; Aluwihare et al. 2005).

Although smaller peptides are believed to be important intermediates in the degradation of protein, the presence of peptides has not been clearly documented in the marine environment (except as isolated natural products), and only 7–11% of total DON and 1–7% of total dissolved organic carbon can be recovered as total hydrolyzable amino acids (THAA) (Yamashita and Tanoue 2003; Jones et al. 2004), the analytical window that would include peptides. In the HMW DOM, amide N represents a somewhat larger portion of the N (16–30%) (McCarthy et al. 1996; Aluwihare and Repeta 1999; Aluwihare et al. 2005). These relatively low contributions to the HMW N pool suggest that (1) protein N is not the dominant form of HMW DON, (2) protein and peptide N are rapidly degraded and consumed in the environment, and (3) analytical methods currently being used underestimate the contribution of proteins to this fraction of the DOM pool.

Hydrolysis of biopolymers like proteins is thought to be a rate-limiting step in microbial uptake of DOM and particulate organic matter (POM) in many aquatic environments (Meyer-Reil and Köster 1992; Hoppe et al. 2002). The current model of protein degradation in aquatic environments assumes that hydrolysis of proteinaceous compounds yields peptides (amino acid sequences with molecular weights <6000 Da) and free amino acids (Hoppe 1991; Hoppe et al. 2002; Nunn et al. 2003). Although larger organisms can consume protein and hydrolyze it internally, marine microbes can only take up smaller compounds, so they must hydrolyze proteins and peptides to smaller substrates outside the cell (Payne 1980). Molecules larger than approximately 600 Da (about the size of a dipeptide) are thought to be too large to be transported across microbial cell membranes for nutrient acquisition (Nikaido and Vaara 1985).

Extracellular hydrolysis occurs by ecto- or extracellular enzymes. These hydrolytic enzymes (and most other cell surface enzymes) have not been characterized or sequenced in the ocean, although numerous studies have detected proteolytic activity in both seawater and sediments (Pantoja and Lee 1999; Sala et al. 2001; Hoppe et al. 2002). Although bacteria have long been thought to be the

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primary producers of protein hydrolases, it is now clear that phytoplankton and cyanobacterial proteases make a substantial contribution to exo- and ectocellular proteolytic activity (Mulholland et al. 2002; Stoecker and Gustafson 2003; Stoecker et al. 2005). It is thought that this capability might be beneficial to phytoplankton competing for scarce nitrogenous resources. Culture (Antia et al. 1991; Lewitus 2006) and field studies (Berman and Bronk 2003; Mulholland et al. 2004) have shown that some phytoplankton can use organic compounds directly. We hypothesize that one possible reason that dipeptides, the primary product of peptide hydrolysis, are not hydrolyzed further is that they are small enough to be taken up directly by cells. Here we test the hypotheses that peptide hydrolysis by phytoplankton may be more widespread than previously thought, and that dipeptides can be taken up by phytoplankton. We describe below a series of experiments that were conducted measuring peptide hydrolysis and dipeptide uptake in cultures and field populations of microbes (phytoplankton and bacteria) during monospecific or multispecies phytoplankton blooms.

Methods

To determine whether peptide hydrolysis and dipeptide uptake are widespread among groups of marine microbes or within particular microbial size fractions, a study was undertaken over several years and across a range of environments including those that experience blooms of a variety of algal taxa known to take up DON. In addition, because field studies suggested that phytoplankton-sized microbes contributed to peptide hydrolysis in nature, cultured populations were examined to determine whether particular phytoplankton were capable of peptide hydrolysis and dipeptide uptake during growth on standard media and on media altered with respect to the N source and its concentration.

Cultures—Phytoplankton cultures were grown using standard media (Tables 1, 2) at salinities and temperatures characteristic of the environments from which they were isolated. The nitrogen source and its concentration were varied during treatment incubations as shown. All cultures were conditioned for at least four generations on the specified growth medium, but in some cases, cultures were conditioned on media with altered N concentrations. N concentration is specified in Table 1 if it was substantially altered from the specified growth medium. All experiments were conducted using exponentially growing batch cultures with the exception of the *Synechococcus* sp. and *Nitzschia* sp. cultures, for which experiments were conducted in steady-state continuous cultures. Cultures were deemed at steady state when biomass was constant for at least four generations.

Natural populations—Sampling of opportunity was undertaken to investigate peptide hydrolysis and the uptake of dipeptides across a range of environmental conditions that included a variety of populations and bloom organisms. Because organic nutrients are thought to be important causative factors in many coastal algal blooms, we concentrated these studies on coastal areas that are prone to blooms. In particular, samples were collected in the Lafayette River, Virginia, as the dominant phytoplankton assemblages changed seasonally. Experiments were conducted at this site during spring and summer dinoflagellate blooms during 2002 and 2003. In addition, blooms of the brown tide pelagophyte, *Aureococcus anophagefferens*, occur during the spring in mid-Atlantic coastal lagoons such as Chincoteague Bay, Virginia. Peptide hydrolysis and dipeptide uptake were examined during a 2002 brown tide bloom at this site (Mulholland et al. 2004; Table 3). In the Gulf of Mexico, red tides plague coastal waters along the West Florida Shelf. Because organic matter is thought to be important in maintaining red tide blooms, we measured rates of peptide hydrolysis during a massive *Karenia brevis* bloom in 2001 (aboard the RV *Suncoaster*). For comparison, we measured peptide hydrolysis during mixed diatom blooms in Florida coastal waters (Gulf of Mexico) in 2003 (aboard the RV *Pelican*), and during slicks of *Trichodesmium* sp. in the Arafura Sea between Australia and New Guinea in 1999 (aboard the RV *Maurice Ewing*). In addition, because *K. brevis*, *Trichodesmium*, and another common dinoflagellate found along the north coast of Australia, *Pyrocystis* sp., are large, we amended filtered seawater with cells isolated from gently collected plankton tows. Water samples were collected using either Niskin bottles mounted on a conductivity–temperature–depth rosette or in acid-cleaned buckets and carboys. Experiments were initiated within 1 h of sample collection.

Nutrient and amino acid analysis—Concentrations of dissolved free amino acids (DFAA) were measured by high-performance liquid chromatography (HPLC) (Cowie and Hedges 1992). THAA were measured after modified vapor-phase hydrolysis (Kuznetsova and Lee 2002). Dissolved combined amino acids (DCAA) were calculated as the difference between THAA and DFAA and include peptides, proteins, and amino acids that are free but are in or adsorbed to humic and fulvic acids or clays, and that can be released upon acid hydrolysis.

Inorganic nutrient concentrations (nitrate, nitrite, phosphate) were measured on an Astoria Pacific autoanalyzer using colorimetric methods (Parsons et al. 1984). Ammonium and urea concentrations were determined using the manual phenol hypochlorite method (Solorzano 1969) and the monoxamine method (Price and Harrison 1987), respectively. Total dissolved N (TDN) was measured after persulfate oxidation (Valderrama 1981) and DON calculated as the difference between TDN and the summed inorganic N. Chlorophyll *a* (Chl *a*) concentrations were estimated by fluorometry after extraction of cells in acetone (Welschmeyer 1994).

Uptake rate measurements—Uptake of ^{15}N-labeled compounds (NH_4^+, NO_3^-) and dually labeled (^{13}C and ^{15}N) urea, DFAA (mixed amino acids, glutamate, or alanine), and a dipeptide (dialanine) (custom-synthesized by Cambridge Isotope Laboratory) were measured after incubating water samples in acid-clean polycarbonate

Table 1. Summary of culture conditions used in this study, biomass estimates (as Chl *a*, PN, and PC), measured or estimated DCAA concentrations, measured rate constants for peptide hydrolysis, and rates of dipeptide uptake in whole culture samples. Standard deviations of two or more replicate samples are in parentheses. Empty fields indicate that no data are available.

Species	Growth medium	N source	Salinity	Temp (°C)	Chl *a* (μg L^{-1})	PN (μmol N L^{-1})	PC (μmol C L^{-1})	DCAA (μmol L^{-1})	Peptide hydrolysis (h^{-1})	Dipeptide uptake (μmol N L^{-1} h^{-1})
Cyanophyte:										
Synechococcus (CCMP 1334)	f/20	1 μmol L^{-1} NO_3^-	32	22	20.4 (2.6)	47.2 (12.6)	320.0 (90.7)	0.42 (0.17)	0.28 (0.02)	1.68 (0.28)
Synechococcus (CCMP 1334)	f/20	1 μmol L^{-1} NO_3^-	32	22	16.3 (0.3)	55.7 (11.5)	427.8 (140.9)	0.71 (0.07)	0.16 (0.01)	1.51 (0.21)
Pelagophyte:										
Aureococcus anophagefferens (CCMP 1785)	L1	20 μmol L^{-1} NO_3^-	28	18	10.9 (3.3)	101.3 (3.4)	1211.0 (144.8)	0.47	0.03 (0.01)	0.40 (0.17)
Aureococcus anophagefferens (CCMP 1785)	L1	20 μmol L^{-1} NH_4^+	28	18	11.4 (1.7)	80.8 (4.5)	1124.8 (70.9)	0.44	0.28 (0.01)	0.44 (0.17)
Aureococcus anophagefferens (CCMP 1785)	L1	20 μmol L^{-1} urea	28	18	17.9 (4.4)	86.3 (2.3)	1453.0 (10.2)	0.29	0.25 (0.02)	0.22 (0.02)
Dinoflagellates:										
Prorocentrum minimum (D-5) isolated from the Choptank River, MD	f/2-Si	NO_3^-	15	15		58.2 (3.5)	258.0 (39.9)	0.5*	0.07 (0.01)	2.38 (0.36)
Prorocentrum minimum (CCMP 699)	L1, f/2-Si	20 μmol L^{-1} NO_3^-	28	18	21.6 (4.4)	173.9 (12.8)	850.2 (50.9)	0.55	0.20 (0.01)	0.24 (0.11)
Prorocentrum minimum (CCMP 699)	L1, f/2-Si	20 μmol L^{-1} NH_4^+	28	18	15.4 (1.5)	129.0 (2.7)	739.5 (31.3)	0.36	0.23	0.09 (0.07)
Prorocentrum minimum (CCMP 699)	L1, f/2-Si	20 μmol L^{-1} urea	28	18	12.6 (1.5)	83.0 (2.4)	630.8 (23.8	1.38	0.18	1.16 (0.11)
Scrippsiella trochoidea (CCMP 1331)	f/2-Si	20 μmol L^{-1} NO_3^-	28	18	11.9 (2.8)	56.9 (6.4)	316.0 (34.0)	0.76	0.33	0.57 (0.02)
Scrippsiella trochoidea (CCMP 1331)	f/2-Si	20 μmol L^{-1} NH_4^+	28	18	13.5 (1.1)	49.4 (2.6)	235.4 (13.0)	0.27	0.33 (0.008)	0.27 (0.04)
Scrippsiella trochoidea (CCMP 1331)	f/2-Si	20 μmol L^{-1} urea	28	18	1.1 (0.7)	19.2 (0.2)	55.4 (3.9)	1.64	0.31 (0.05)	0.48 (0.14)
Katodinium rotundatum (CCMP 1542)	f/2-Si	20 μmol L^{-1} NO_3^-	15	18	4.8 (0.7)	19.1 (3.6)	158.1 (32.2)	0.73		0.06 (0.04)

Table 1. Continued.

Species	Growth medium	N source	Salinity	Temp (°C)	Chl *a* (μg L^{-1})	PN (μmol N L^{-1})	PC (μmol C L^{-1})	DCAA (μmol L^{-1})	Peptide hydrolysis (h^{-1})	Dipeptide uptake (μmol N L^{-1} h^{-1})
Katodinium rotundatum (CCMP 1542)	f/2-Si	20 μmol L^{-1} NH_4^+	15	18	3.2 (0.4)	14.0 (1.2)	111.8 (14.6)	0.55		0.08 (0.02)
Katodinium rotundatum (CCMP 1542)	f/2-Si	20 μmol L^{-1} urea	15	18	2.2 (0.4)	12.6 (1.0)	117.2 (18.7)	0.91		0.07 (0.04)
Cryptophytes:										
Rhodomonas sp. (CCMP 767)	f/2-Si	20 μmol L^{-1} NO_3^-	15	18	7.0 (1.4)	23.3 (0.8)	239.7 (18.2)	1.25	0.36 (0.02)	0.04 (0.03)
Rhodomonas sp. (CCMP 767)	f/2-Si	20 μmol L^{-1} NH_4^+	15	18	5.0 (0.8)	16.5 (1.3)	199.8 (18.2)	0.22	0.16 (0.03)	0.01 (0.001)
Rhodomonas sp. (CCMP 767)	f/2-Si	20 μmol L^{-1} urea	15	18	3.3 (0.3)	11.8 (0.04)	120.7 (3.1)	1.42	0.14 (0.02)	0.06 (0.02)
Diatom: (continuous culture)										
Nitzschia sp. (CCMP2144)	L1/10	88 μmol L^{-1} NO_3^-	30	22	33.2 (4.9)	38.2 (3.8)	252.2 (25.9)	1.87 (0.88)	0.69 (0.01)	1.10 (0.11)
Nitzschia sp. (CCMP2144)	L1/10	88 μmol L^{-1} NO_3^-	30	22	134.7 (4.8)	66.8 (6.6)	469.5 (61.8)	1.30 (0.14)	1.46 (0.11)	0.42 (0.03)

* DCAA estimated as 0.5 μmol L^{-1}

Table 2. Summary of culture conditions along with measured rate constants for peptide hydrolysis in cultures of unknown cell density. These results suggest that other taxa may similarly hydrolyze peptides under nutrient-replete growth conditions. Standard deviations of two or more replicate samples are in parentheses.

Species	Growth medium	N source	Salinity	Temp (°C)	Peptide hydrolysis (h^{-1})
Cyanophyte:					
Trichodesmium IMS101	YBCII	None	32	28	0.03 (0.004)
Dinoflagellates:					
Karenia brevis (Piney Pt Isolate B4)	GP	NO_3^-	35	28	0.38 (0.004)
Pfiesteria piscicida (NCSU113-3)	SW	None	15	20	1.90 (0.29)
Pyrodinium bahamense (020501-1 B5 isolate)	ESDK	NO_3^-	36	28	0.13 (0.03)
Karlodinium micrum (CCMP 2283 – S Carolina)	GP	NO_3^-	30	22	1.48 (0.31)
Raphidophyte:					
Heterosigma akashiwo (JW010423 – S Carolina)	GP	NO_3^-	25	22	0.85 (0.27)
Cryptophytes:					
Rhodomonas sp. (CCMP 768)	SW + f/2 N − Si	NO_3^-	15	20	0.06 (0.003)

incubation bottles (Mulholland et al. 2002). Uptake experiments were initiated by adding tracer concentrations (most often 0.03 μmol L^{-1}; additions were always >1%, but usually ≤10% of the ambient concentration) of highly enriched (96–99%) labeled substrates. After an incubation period (less than 1 h at in situ temperature and light levels), experiments were terminated by gentle filtration through precombusted (450°C for 2 h) GF/C (nominal pore size ~1.2 μm) or GF/F (nominal pore size ~0.7 μm) filters. Filters were then frozen until analysis. Time-course incubations were conducted periodically to determine that appropriate incubation times were used (data not shown).

Particulate carbon and nitrogen masses and isotopic ratios in samples were analyzed on a Europa Scientific 20-20 isotope ratio mass spectrometer equipped with an automated nitrogen and carbon analyzer–SL prep unit. Uptake rates were calculated using the following equations (Mulholland et al. 2006):

$$^{15}\text{N uptake} = \frac{(\text{atom \% PN})_{\text{final}} - (\text{atom \% PN})_{\text{initial}}}{(\text{atom \% N source pool} - \text{atom \% PN})_{\text{initial}} \times \text{time}} \times [\text{PN}] \quad (1)$$

$$^{13}\text{C uptake} = \frac{(\text{atom \% PC})_{\text{final}} - (\text{atom \% PC})_{\text{initial}}}{(\text{atom \% C source pool} - \text{atom \% PC})_{\text{initial}} \times \text{time}} \times [\text{PC}] \quad (2)$$

where PN was the particulate nitrogen and PC was the particulate carbon collected on the filter either before or at the end of incubations. The source pool was the relevant dissolved pool that was enriched.

To calculate the atom percentage enrichment (both C and N) of the DFAA and DCAA pools for the dually labeled amino acids and dialanine (^{15}N and ^{13}C), the C : N ratio of the ambient DFAA pool was calculated on the basis of the concentrations of individual amino acids from HPLC runs (Mulholland et al. 2002). If all DCAA were potentially dipeptides, the maximum dipeptide pool would be the DCAA pool divided by two. Therefore, the initial dipeptide pool was estimated as half of the measured DCAA pool. This is obviously an overestimate because some of the peptide linkages may be in other forms or may not be easily hydrolyzed. Further, the DCAA pool in nature may be a ''residual'' pool if dipeptide production and uptake are tightly coupled and so labile forms don't accumulate in nature.

Peptide hydrolysis rate measurements—Peptide hydrolysis rates were measured using lucifer yellow anhydride (LYA)-labeled tetra-alanine (Pantoja et al. 1997; Mulholland et al. 2002). Rates of peptide hydrolysis were measured in the <1.2-μm size fraction (i.e., bacteria-sized), the <10- or 20-μm size fraction (bacteria and small phytoplankton), and in whole water. During blooms, the dominant phytoplankton species was targeted for exclusion from the smaller size fractions. Differences among size fractions were used to assess the contribution of specific groups of organisms when possible. During previous studies, little enzyme activity was observed in the <0.2-μm size fractions (abiotic controls) (Mulholland et al. 2002, 2003), and so this size fraction was often excluded in this study.

Rates of peptide hydrolysis were measured in triplicate samples placed in acid-cleaned 25-mL polycarbonate incubation bottles. Assays were initiated by adding LYA-tetra-alanine (LYA-ala4) at a concentration of ~100 nmol L^{-1}. We estimated this to be a ''tracer'' addition (~10%) assuming that DCAA concentrations were on the order of 1 μmol L^{-1} in these environments, that all DCAA was peptide, and that turnover of the substrate would then reflect turnover times of the entire dipeptide pool. Subsamples were collected from incubation bottles at time zero and subsequently at intervals ranging from 30 min to 2 h. Samples were filtered (0.2 μm) and the filtrate frozen until analysis by HPLC. LYA-ala4 and the products of its hydrolysis were separated and quantified using a Shimadzu HPLC system (Pantoja et al. 1997; Mulholland et al. 2002, 2003). First-order rate constants for peptide hydrolysis were calculated on the basis of quantification of parent compounds and products during

Table 3. Chl *a*, PN, and PC biomass, physical properties, nutrient concentrations, rate constants for peptide hydrolysis, and dipeptide uptake during sampling periods. Sampling events were during "blooms" or periods of elevated Chl *a* when one or two species dominated the population. For dates on which there were multiple sampling events, the time is indicated next to the dominant taxa. Standard deviations from replicate samples are in parentheses, where available. BDL indicates that the analyte was below the limit of analytical detection. Blank cells indicate that no data are available.

Date	Dominant taxa	Salinity	Temp (°C)	Chl *a* (μg L^{-1})	PN (μmol N L^{-1})	PC (μmol C L^{-1})	Dissolved inorganic N (μmol N L^{-1})	DON (μmol N L^{-1})	DCAA (μmol L^{-1})	Peptide hydrolysis (h^{-1})	Dipeptide uptake (μmol N L^{-1} h^{-1})
Choptank River, MD:											
10 May 02	*P. minimum*	11.5	23.0	49.7	35.5 (1.1)		3.3		0.69	0.30 (0.01)	
Lafayette River, VA:											
19 Apr 02	*P. minimum*	21.2	19.8	21.1 (1.6)	17.3 (2.2)	98.5 (15.7)	7.5	34.9	1.46	0.03 (0.004)	0.08 (0.002)
08 May 02	*P. minimum*	20.7	21.3	26.0 (0.6)	13.5 (1.9)	92.9 (12.9)	11.2		2.36*	0.03 (0.002)	0.02 (0.003)
13 May 02	*A. sanguinea*	20.0	20.0	27.0	95.8 (12.0)	602.7 (61.6)	1.1		2.36	0.05 (0.01)	0.73 (0.04)
15 May 02	*A. sanguinea*	20.0	19.5	22.4 (7.4)	67.8 (7.8)	453.8 (35.0)	5.4		3.96	0.10 (0.03)	0.59 (0.07)
16 May 02	*A. sanguinea* and *S. costatum*	20.0	18.5	16.1	37.9 (3.1)	301.3 (39.2)	7.5		3.96*	0.16 (0.01)	0.38 (0.02)
06 Aug 02	*A. sanguinea*	22.0	28.0	17.4 (1.1)	14.3 (1.0)	120.2 (9.0)	8.9	24.6	1.40	0.09 (0.02)	0.39 (0.02)
05 Feb 03	*Heterocapsa* sp.	17.0	5.0	18.0 (0.4)	40.7 (3.7)	247.0 (17.9)	3.6	42.6	1.0†	0.08 (0.004)	0.07 (0.002)
03 Apr 03	*P. minimum*	12.0	14.4	17.1 (0.2)	27.7 (1.3)	198.6 (9.1)	2.2	17.4	1.0†	0.03 (0.004)	0.04 (0.01)
11 Apr 03	*P. minimum*	8.0	10.0	13.6 (0.7)	14.7 (1.1)	126.3 (9.0)	7.4	20.4	1.0†	0.05 (0.01)	0.03 (0.001)
18 Apr 03	*Heterocapsa* sp.	18.0	14.8	9.0 (0.2)	15.5 (2.2)	107.9 (12.2)	6.9	23.4	1.0†	0.05 (0.004)	0.05 (0.01)
28 Apr 03	*P. minimum*	10.0	17.8	23.2 (0.9)	16.0 (2.6)	97.0 (12.6)	3.1	27.5	1.0†	0.03 (0.004)	0.06 (0.01)
30 Apr 03	*P. minimum* – 1200	11.0	19.0	23.2 (0.6)	32.9 (2.8)	302.1 (16.8)	1.0	31.5	0.24		0.03 (0.00)
30 Apr 03	*P. minimum* – 1600	10.0	22.0	187.7 (30.2)	371.7 (27.6)	3585.0 (218.7)	1.0	32.0	1.22		1.27 (0.08)
30 Apr 03	*P. minimum* – 2000	10.0	20.0	10.2 (2.9)	26.2 (2.7)	319.9 (45.7)	1.6	40.9	0.63		0.04 (0.01)
30 Apr 03	*P. minimum* – 2400	10.0	19.8	5.2 (1.3)	17.7 (3.8)	194.7 (21.5)	1.0	23.4	0.58		0.02 (0.01)
01 May 03	*P. minimum* – 0400	10.0	19.8	25.2 (0.6)	121.8 (7.5)	409.6 (13.3)	0.5	20.3	0.46	0.06 (0.00)	0.24 (0.05)
01 May 03	*P. minimum* – 0800	10.0	19.0	17.3 (1.3)	28.7 (3.1)	205.7 (24.0)	0.5	29.1	0.55		0.07
06 May 03	*P. minimum* – 1200	13.0	18.0	9.5 (1.5)	22.7 (2.1)	234.2 (22.4)	6.1	27.7	0.15	0.02 (0.00)	0.02 (0.02)
06 May 03	*P. minimum* – 2400	12.0	18.0	3.5 (0.2)	11.3 (1.4)	89.3 (9.8)	3.2	36.0	0.03	0.04 (0.00)	0.001 (0.001)
08 May 03	*P. minimum* – 1000	14.0	20.6	12.8	47.9 (6.0)	406.2 (48.7)	3.9	42.4	0.12	0.04 (0.02)	0.04 (0.03)
08 May 03	*P. minimum* – 2200	13.0	20.6	8.8 (1.0)	27.5 (3.8)	252.9 (26.9)	6.7	46.2	0.50	0.05 (0.01)	0.05 (0.04)
17 Jun 03	*A. sanguinea* – 1200	12.0	21.8	159.4 (25.7)	234.2 (13.9)	1149.4 (125.2)	1.4	52.3	0.11		0.13 (0.01)
17 Jun 03	*A. sanguinea* – 2200	12.0	21.7	21.3 (7.5)	37.8 (9.4)	250.9 (52.3)	2.8	42.9	0.35	0.01 (0.01)	0.04 (0.00)
19 Jun 03	*A. sanguinea* – 1000	9.0	22.9	112.0 (1.1)	339.8 (42.8)	1509.3 (185.7)	4.4	22.5	0.10	0.05 (0.02)	0.18 (0.001)
19 Jun 03	*A. sanguinea* – 2200	10.5	23.2	25.5 (0.4)	35.7 (8.2)	239.9 (52.7)	3.2	27.8	0.11	0.05 (0.02)	BDL
17 Jul 03	*A. sanguinea*	13.0	26.0	49.6 (1.7)	95.6 (10.5)	495.4 (59.6)	2.9	30.9	1.01	0.11 (0.01)	0.79 (0.002)
22 Jul 03	*A. sanguinea* – 1000	14.0	25.6	33.7 (0.1)	45.3 (9.6)	226.3 (43.8)	2.9	21.9	1.54‖	0.05 (0.01)	0.45 (0.01)
22 Jul 03	*A. sanguinea* – 2200	16.0	25.6	10.8 (0.2)	18.5 (2.9)	93.4 (16.5)	3.0	33.7	1.54‖	0.81 (0.30)	0.21 (0.01)
26 Aug 03¶	*A. sanguinea* and *Scrippsiella* sp.	17.0	26.6	21.7 (2.4)	14.5 (2.6)	75.7 (11.7)	0.4	28.1	1.58	0.17 (0.07)	0.09 (0.01)
30 Sep 03¶	*Cochlodinium* sp.	12.0	21.4	24.9 (1.3)	31.3 (4.6)	144.4 (19.4)	1.4	18.4	1.42	0.06 (0.03)	0.12 (0.001)
Mid-Atlantic coastal lagoon:											
12 Jun 02	*A. anophagefferens*§	32.6	24.8	19.5 (1.5)	35.6 (1.8)	495.1 (25.5)	BDL	29.7	1.0†	0.05 (0.02)	0.23 (0.04)

* Used DCAA data from previous day.
† Estimated as 1.0 μmol L^{-1}.
§ Mulholland et al. 2004.
‖ DCAA are from a single sample.
¶ Nutrient data are from 28 Aug and 25 Sep 2003.

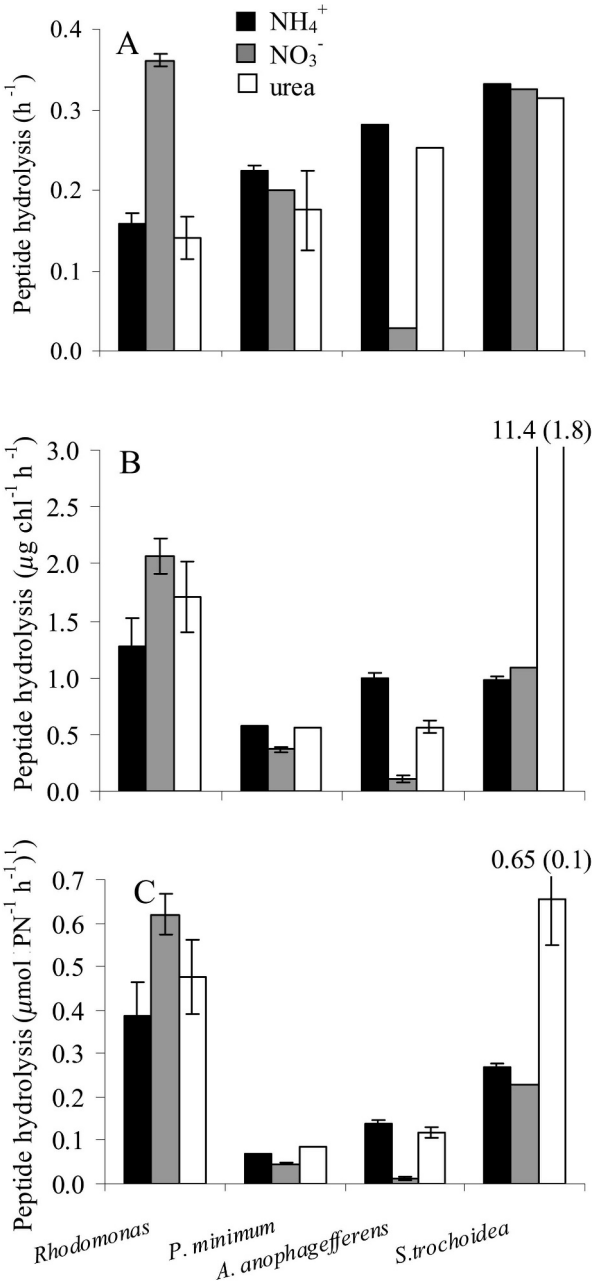

Fig. 1. Peptide hydrolysis (h^{-1}) in cultures conditioned on media containing 20 μmol L^{-1} NH_4^+, NO_3^-, or urea as their only N source. Turnover times are expressed in units of inverse time (A) before and (B) after being normalized to Chl *a* contained in the incubation bottle.

the time-course incubations (Pantoja et al. 1997). Rates of peptide hydrolysis were compared with estimates of dipeptide uptake rates and the uptake of other N and C compounds.

Results

Turnover times for hydrolysis of our model peptide varied from 0.03 to 1.48 h^{-1} among taxa in cultured phytoplankton growing on N-replete culture media (Tables 1, 2), and from 0.01 to 0.81 h^{-1} in field populations dominated by different phytoplankton species (Table 3). Cultures of the diazotrophic cyanobacteria *Trichodesmium* sp. had the longest turnover times for our model peptide, whereas the dinoflagellates *Pfiesteria piscicida* and *Karlodinium micrum* (*veneficum*), the raphidophyte *Heterosigma akashiwo*, and the diatom *Nitzschia* sp. had the shortest turnover times (Tables 1, 2). For cultures (Table 1) and natural whole water populations (Table 3) for which rates could be biomass normalized, peptide hydrolysis ranged from 0.01 to 0.87 μmol PN^{-1} h^{-1} and 0.01 to 2.60 μmol PN^{-1} h^{-1}, respectively.

Some of the differences in peptide hydrolysis between cultures could be due to differences in cell densities in the different cultures tested. Cell biomass (Chl *a*, PN, or PC) was not always measured during these "survey" experiments and so results could not consistently be normalized to cell number or Chl *a* concentrations. During the more detailed culture studies, we made these comparisons. Peptide hydrolysis in phytoplankton cultures grown on low N media varied depending on the preconditioning N source (Fig. 1). For example, *A. anophagefferens* had very low rates of peptide hydrolysis, and *Rhodomonas* sp. had higher rates of peptide hydrolysis when growing on nitrate relative to cells grown on ammonium or urea (Fig. 1A). Although cell densities differed between cultures grown on different N sources, these differences could not account for the observed differences in peptide hydrolysis by *Rhodomonas* (Fig. 1B,C). In contrast, volumetric turnover times for peptide hydrolysis did not differ greatly in cultures of *Prorocentrum minimum* and *Scrippsiella trochoidea* (Fig. 1A). However, when normalized to Chl *a* or PN biomass, peptide hydrolysis was lower in *P. minimum* cultures grown on NO_3^- and was higher (although biomass lower) in *S. trochoidea* cultures grown on urea (Fig. 1B,C). When data from cultures were pooled, there was no relationship between PN and rates of peptide hydrolysis, suggesting species-specific differences or differences related to the preconditioning growth conditions (data not shown).

Studies involving size fractionation suggest that in nutrient-replete cultures, very little (e.g., in *Rhodomonas* cultures) to almost all (in *P. minimum* D-5 cultures) enzyme activity was associated with the <1.2-μm size fraction, the size fraction that contained the bulk of bacterial contaminants and virtually no phytoplankton (Fig. 2). In *P. minimum* (D-5) cultures, peptide hydrolysis was faster during the night than it was during the day (Fig. 3), suggesting that the light cycle may also be important in regulating this process. This was observed in cultures growing on media with different N concentrations and sources (NO_3^- at two concentrations vs. NH_4^+). However, in these cultures, the bulk of the peptide hydrolysis was accomplished by the <1.2-μm size fraction that excluded *P. minimum* (*see* Fig. 1) but could have contained microbial contaminants and free enzyme, so rates were not normalized to biomass.

In field incubations where <0.2-μm-filtered seawater was amended with cells of the dinoflagellates *Pyrocystis* sp. and *K. brevis*, or the colonial cyanobacterium *Trichodesmium* sp., rate constants for hydrolysis of our model

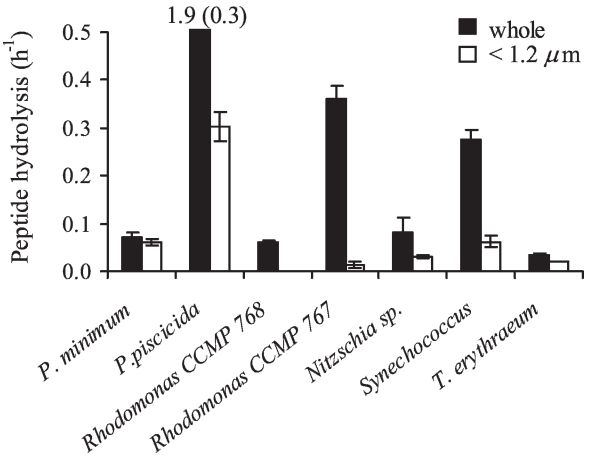

Fig. 2. Relative contribution to peptide hydrolysis (h^{-1}) of contaminating microbes and free enzymes (<1.2 μm; white bars) in whole cultures (black bars) of various phytoplankton species.

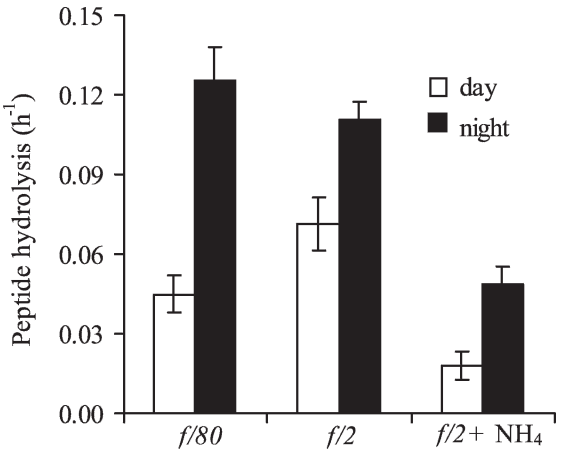

Fig. 3. Peptide hydrolysis (h^{-1}) in whole cultures of *Prorocentrum minimum* (D-5) growing on different culture media during daytime (white bars) vs. at nighttime (black bars). Most hydrolytic activity was due to microbes <1.2 μm.

peptide were 3–10 times higher than in unamended filtered seawater, suggesting that the presence of these organisms contributes to peptide hydrolysis (Fig. 4). During natural surface water "slicks" of *Trichodesmium* in the Arafura and Coral seas, whole water containing *Trichodesmium* usually had the highest rates of peptide hydrolysis compared with smaller size fractions that excluded *Trichodesmium* trichomes (Fig. 5). However, this is a case when we observed high peptide hydrolysis in the <0.2-μm size fraction. This may be due to the presence of free enzyme, the presence of small microbes, or the high temperature and requires further examination. We are still exploring ways to identify which microbes are responsible for extracellular peptide hydrolysis, whether activity is always confined to cells, and what controls these rates.

During monospecific or mixed blooms in subtributaries of the Chesapeake Bay, Chincoteague Bay (a mid-Atlantic coastal bay), the Gulf of Mexico, and the north coast of Australia, cells greater than 1.2 μm in size contributed

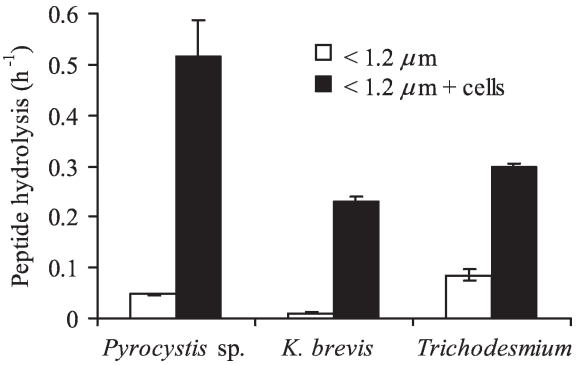

Fig. 4. Peptide hydrolysis (h^{-1}) in 0.2 μm seawater and in 0.2 μm seawater amended with 100 *Pyrocystis* sp. cells (N coast of Australia), concentrated *K. brevis* (Gulf of Mexico), or 20 *Trichodesmium* colonies (N coast of Australia). Water temperatures exceeded 30°C during all of these experiments.

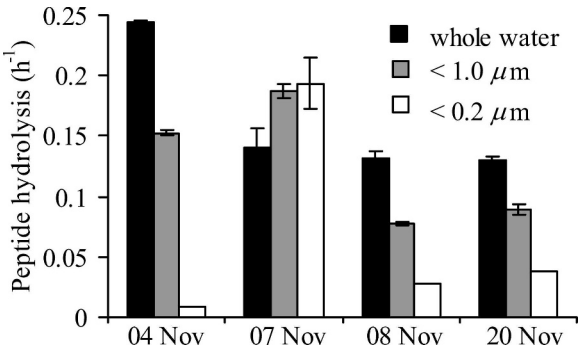

Fig. 5. Peptide hydrolysis in size-fractionated surface water from the north coast of Australia during a cruise in 1999 when water temperatures were nearly 30°C.

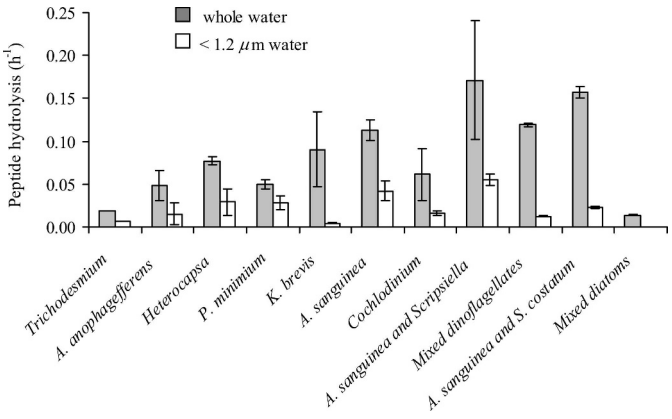

Fig. 6. Peptide hydrolysis in whole water (gray bars) and <1.2-μm filtered water (white bars) collected during algal blooms in the Arafura Sea (*Trichodesmium* sp.), the Gulf of Mexico (*Karenia brevis* and mixed diatoms), a mid-Atlantic coastal bay (*Aureococcus anophagefferens*), and the Lafayette River, Virginia (all others). The dominant bloom-forming organism is indicated.

substantially to peptide hydrolysis (Fig. 6). Further, in the Elizabeth River, turnover of our model peptide was greater within bloom patches than outside bloom patches (Fig. 7). In a visible patch of discolored water, Chl *a* concentrations

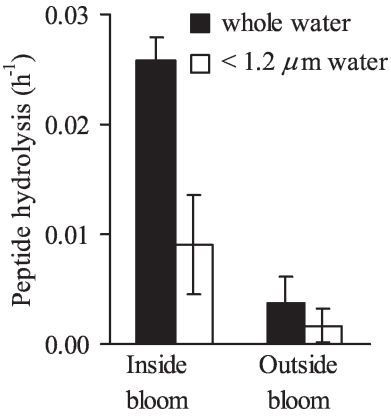

Fig. 7. A comparison of peptide hydrolysis within and outside an area affected by a bloom of *P. minimum* in the Lafayette River, Virginia, on 08 May, 2002.

were 1.4 times higher than in surrounding water, but peptide hydrolysis was 6.9 times higher. Both inside and outside the patch, *P. minimum* was >90% of the phytoplankton community. Although peptide hydrolysis was rapid when Chl *a* concentrations were high and when the population was dominated by a single or a few algal species (e.g., during blooms) (Table 3), they varied over the course of particular blooms such as *P. minimum* (Fig. 8A) and *Akashiwo sanguinea* (Fig. 8B) independently of biomass.

Rates of dialanine uptake were examined in conjunction with the hydrolysis of our model compound, LYA-ala4, and with respect to the uptake of a suite of nitrogenous nutrients in cultured and natural populations (Tables 1, 3). Nitrogen control of peptide hydrolysis and dipeptide uptake was evaluated in cultures preconditioned to 20 μmol L^{-1} ammonium, nitrate, or urea. Volumetric rates of dipeptide uptake were highest in cultures of *P. minimum* grown on urea (Fig. 9A); however, Chl *a*- (Fig. 9B) and PN (Fig. 9C)-normalized dipeptide uptake rates were highest in cultures of *S. trochoidea* grown on urea. As was true for peptide hydrolysis (*see* Fig. 1), normalizing for cell biomass changed the relative outcomes from cultures that grew preferentially on particular substrates; in particular, the culture of *S. trochoidea* did not grow well on urea so cell densities were low.

In continuous cultures of N-replete *Nitzschia* sp. and N-limited *Synechococcus* sp., rates of peptide hydrolysis were high and some of the highest rates of dipeptide uptake were observed. Although ammonium uptake was a higher proportion of the total, dipeptide uptake was 25.0% and 29.9% of the total measured N uptake, 3.1 μmol N L^{-1} h^{-1} and 5.3 μmol N L^{-1} h^{-1}, in the *Nitzschia* and *Synechococcus* cultures, respectively (Table 4). When dipeptide uptake was examined in natural microbial populations during monospecific algal blooms in the Lafayette River, the contribution of dipeptides to total N uptake measured during the day varied (Fig. 10), both with the dominant bloom species, over the course of a bloom, and during different blooms of the same species. Dipeptides contributed between 0.7% and 18.1% to the total measured N uptake. When compared on a diel basis, dipeptide uptake

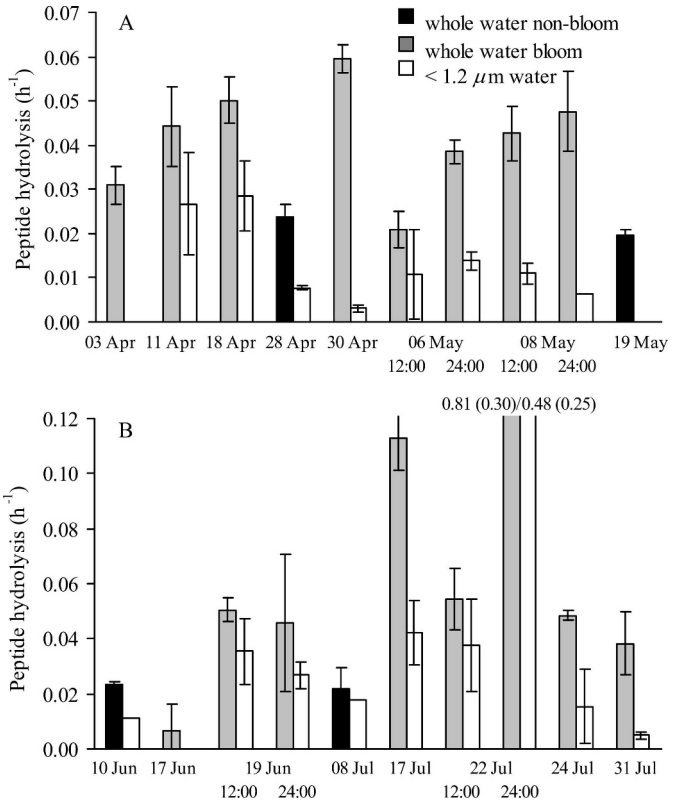

Fig. 8. (A) Peptide hydrolysis during monospecific blooms of *P. minimum* in the Lafayette River, Virginia, during spring 2003, and (B) during monospecific blooms of *A. sanguinea* in the Lafayette River, Virginia, during summer 2003. Samples taken when the bloom algae were present in abundance are depicted in gray. The black bars indicate samples taken when there was no "bloom" or single dominant algal taxa. On some dates, measurements were made at noon (12:00 h) and midnight (24:00 h) to account for possible diel variability.

occurred during the light and dark periods (Fig. 11A) and contributed differentially to total N uptake during the day (Fig. 11B) vs. at night (Fig. 11C). For *A. sanguinea*, dipeptide uptake represented up to 25.6% of the total measured N uptake at night.

Discussion

Hydrolysis and uptake of peptides by phytoplankton—To establish the capacity for phytoplankton to hydrolyze peptides and take up dipeptides, we examined rates of these processes in culture systems dominated by a single algal or cyanobacterial species and in natural populations dominated by a variety of bloom-forming algal taxa. Although the methods used cannot unequivocally distinguish between phytoplankton and bacterial processes, our results show that rates of peptide hydrolysis vary among cultured and natural populations dominated by different phytoplankton species (Tables 1–3; Figs. 1, 2, 4, 6), preconditioned with different nitrogen sources (Figs. 1, 3), and over diel cycles (Figs. 3, 11). Further, peptide hydrolysis in the size fraction that retained most phytoplankton was often greater than that accomplished by size

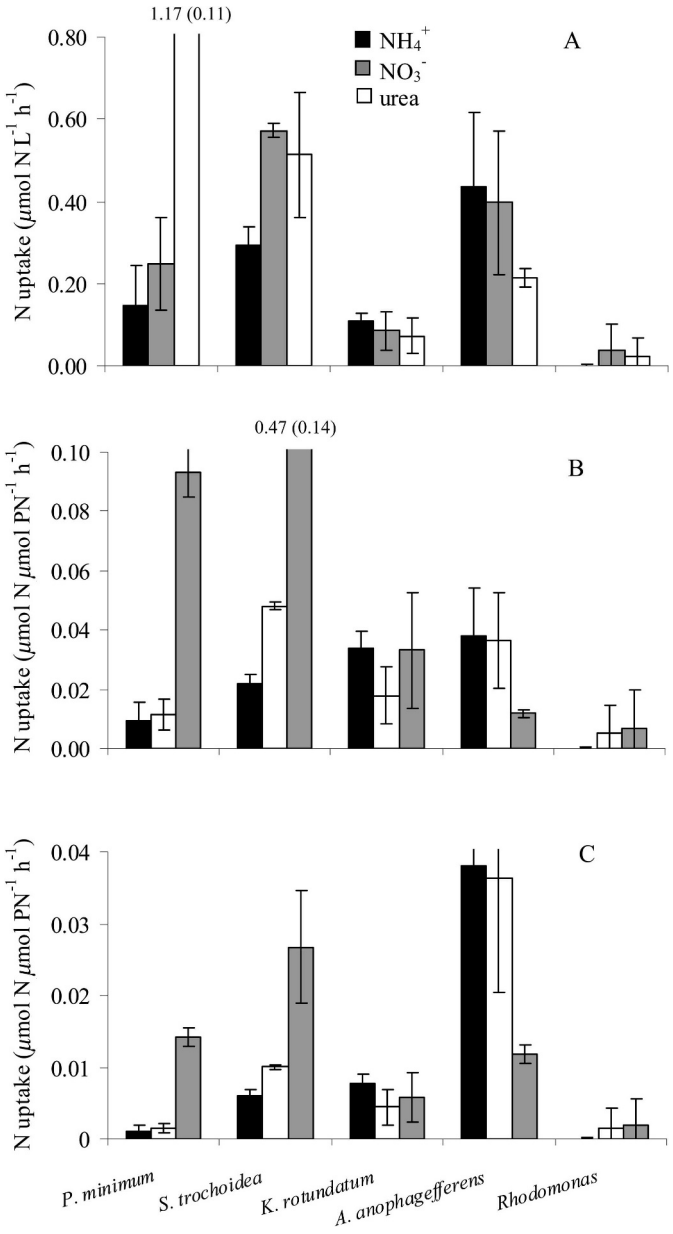

Fig. 9. (A) Volumetric, (B) Chl *a*-normalized, and (C) PN-normalized rates of dipeptide uptake in cultures of *Katodinium rotundatum*, *Rhodomonas* sp., *Prorocentrum minimum*, *Aureococcus anophagefferens*, and *Scrippsiella trochoidea* grown on 20 μmol L^{-1} NH_4^+, NO_3^-, or urea as the sole N source. *See* Table 1 for culture details.

fractions excluding phytoplankton (<1.2 μm), at least in some systems (Figs. 2, 4–8). Direct comparisons with bacterial hydrolysis data from the literature are difficult, as hydrolysis is most often measured as leucine aminopeptidase activity and this may measure a fundamentally different reaction than our LYA-ala4 technique (Pantoja et al. 1997). In addition, size fractionation is imperfect, as many bacteria do not pass through the filters used or are attached to cells, making it difficult to separate phytoplankton and bacteria. Current analytical techniques do not allow the direct measurement of dipeptide concentrations or their production and consumption rates by specific taxa with overlapping size distributions. With the development and application of new taxa-specific methods (Mulholland and Lomas 2008), some of these issues may be resolved in the future.

Table 4. Uptake of various N compounds in steady-state N-limited continuous cultures of *Synechococcus* sp. and N-replete *Nitzschia* sp. Standard deviations from two replicate cultures are in parentheses. *See* Table 1 for culture conditions.

	Uptake rate (μmol N L^{-1} h^{-1})	
N compound	*Synechococcus* sp.	*Nitzschia* sp.
NH_4^+	2.53 (0.40)	1.00 (0.23)
NO_2^-	0.16 (0.03)	0.24 (0.06)
NO_3^-	0.45 (0.01)	0.01 (0.002)
Urea	0.40 (0.03)	0.81 (0.16)
DFAA	0.20 (0.03)	0.23 (0.03)
Dialanine	1.60 (0.35)	0.76 (0.11)

Isotopic enrichment of the >0.7-μm or >1.2-μm particulate pool with dually labeled (^{13}C and ^{15}N) dialanine was significant in both cultures (Fig. 9, Table 4) and the field (Fig. 11), strongly suggesting that the primary product of peptide hydrolysis, dialanine, was taken up directly by phytoplankton or other microbes retained on these filters. That at least some phytoplankton can take up dipeptides is supported by the recent discovery of transporters for dipeptides and tripeptides during the whole genome analysis of the pelagophyte *Aureococcus anophagefferens* (C. Gobler pers. comm.). Like peptide hydrolysis, dipeptide uptake was significant in our study (Table 4) and varied with the dominant phytoplankton species, preconditioning nitrogen source (Figs. 9, Table 4), and over diel light cycles (Fig. 11). However, no attempt was made to systematically define how the N conditions or light cycle affected peptide

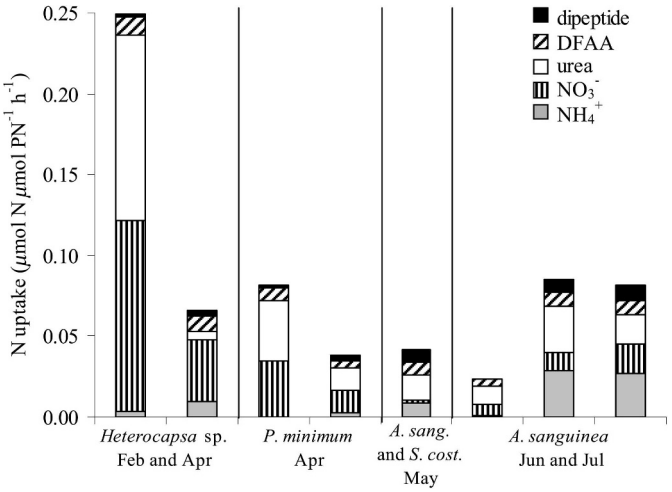

Fig. 10. Relative contribution of dipeptides, DFAA, urea, NO_3^-, and NH_4^+ to total measured N uptake and normalized to PN in natural populations during monospecific or mixed algal blooms in the Lafayette River, Virginia. *See* Table 3 for relative phytoplankton biomass (Chl *a*) estimates for the sampling periods.

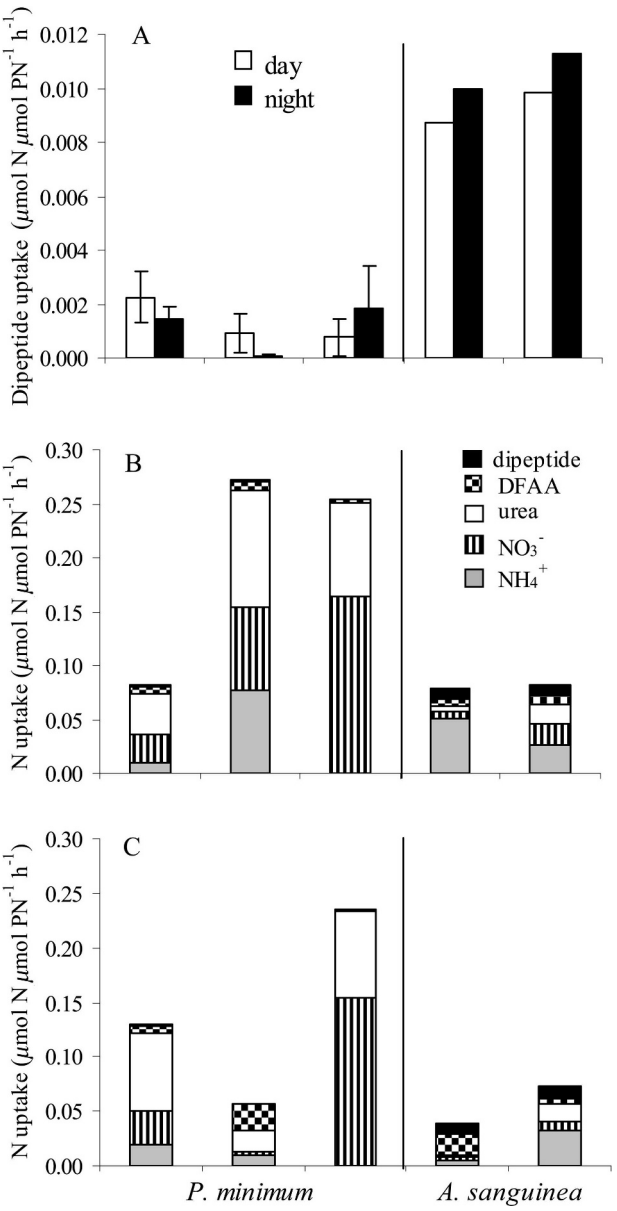

Fig. 11. (A) Diel variability in dipeptide uptake in natural populations dominated by *P. minimum* or *A. sanguinea* as compared with the diel variability in total N uptake by these populations measured (B) during the day or (C) during the night. Dipeptides could represent up to 25.6% of total N uptake during blooms of *A. sanguinea*.

hydrolysis or dipeptide uptake or which taxa specifically were performing these processes.

Comparison of peptide hydrolysis with dipeptide uptake—Whereas many studies of peptide hydrolysis have involved artificial substrates that measure the hydrolysis of a terminal peptide linkage, the use of fluorescent derivatives of actual peptides as substrates (Pantoja et al. 1997; Pantoja and Lee 1999) allows for the direct measurement of specific hydrolysis products, and thus provides insights into enzymatic hydrolysis pathways and products. Previous studies using these fluorescent peptide substrates have demonstrated that extracellular hydrolysis of peptides is faster than the rate of free amino acid production and uptake (Pantoja et al. 1997; Pantoja and Lee 1999). Indeed, Kuznetsova and Lee (2002) found little correlation between peptide hydrolysis and changes in DFAA concentration. Pantoja and Lee (1999) also found that peptides containing more than two amino acids were hydrolyzed 10–400 times faster than dipeptides or fluorescent dipeptide analogs. Further, fluorescent dipeptides are preferentially produced from the hydrolysis of larger substrates (Pantoja and Lee 1999; Mulholland et al. 2002, 2003). Since dipeptides do not accumulate in seawater, and free amino acids are not being rapidly produced by hydrolysis of dipeptides, dipeptides must be rapidly removed by microorganisms (e.g., taken up) or removed from our analytical window in some other way (e.g., sorption, chemical reaction).

We have endeavored to compare estimates of dipeptide production and uptake by using stable isotopes to label the major product of hydrolysis of our model peptide substrate. For example, DCAA concentrations in cultures of *Synechococcus* sp. were, on average, 0.57 μmol L^{-1} and rates of peptide hydrolysis ranged from 0.16 to 0.28 h^{-1}. Assuming all DCAA were potentially available as dipeptide and that 1 mol DCAA equals 1 mol DCAA-N (e.g., 0.28 μmol N L^{-1} dipeptide), then dipeptides were being produced at a rate of 0.045 to 0.078 μmol N L^{-1} h^{-1}. Since rates of dipeptide uptake were 1.5 to 1.7 μmol N L^{-1} h^{-1}, dipeptides were being produced at lower rates than they were being consumed by *Synechococcus* sp. Thus, the uptake of dipeptides by this species appears to exceed their production by a factor of 20–30, suggesting that peptide hydrolysis might limit dipeptide uptake. Similar calculations for all data presented in Tables 1 and 2 suggest that dipeptide uptake rates exceed dipeptide production rates via peptide hydrolysis most of the time; exceptions were for cultures of *Rhodomonas* and during two bloom sampling dates, one dominated by *P. minimum* and the other by *Akashiwo sanguinea*. Peptide hydrolysis appeared to limit dipeptide uptake more often than not across a wide variety of taxa, suggesting that production and uptake are tightly coupled. This study demonstrates that quantifying standing stocks of dipeptides or DCAA may be insufficient for determining their importance as a nutrient source in marine and estuarine environments.

Ecological role of dipeptide production and uptake by phytoplankton vs. bacteria—Simple comparisons between the amino acid composition of POM (e.g., bacteria and phytoplankton) and that of the HMW DOM and total DOM pools in nature suggest that the amino acid composition of HMW DOM more closely reflects that observed in living organisms (POM), whereas the amino acid composition of bulk DOM reflects the preservation and degradation processes acting on proteins in nature (Aluwihare and Meador 2008). These findings and those of Kuznetsova and Lee (2002) suggest that peptide hydrolysis is important in degrading peptides, but that other processes may be more important in determining the composition of the residual amino acid pool. The assay used in this study to assess peptide degradation was ideal for observing the

primary products of peptide hydrolysis, including dipeptides and free amino acids. In this study, the hydrolysis of our model substrate beyond dialanine was insignificant, and dialanine was directly taken up by the microbial community, including phytoplankton.

Rates of peptide hydrolysis varied seasonally, with microbial community composition, and with availability of labile N and C, as demonstrated here and previously (Mulholland et al. 2002, 2003, 2004), suggesting that there are a variety of controls on peptide hydrolysis ranging from physiological capacity of particular organisms to the physiological status of cells. The results presented here suggest that the same is true for dipeptide uptake. These findings and the observations that the production of dipeptides is faster than amino acid production via other enzymatic pathways may help explain the differences between the compositions of the HMW DOM amino acid pool, wherein peptides are rapidly degraded, and the low-molecular-weight DOM or DFAA pool, which is produced and acted upon more slowly. Because it appears that dipeptides can be consumed as rapidly as they are produced, we propose that peptide hydrolysis limits the uptake of dipeptides.

Since dipeptides do not accumulate in seawater, and dipeptide hydrolysis is so slow as to limit the production of free amino acids, it is more likely that the most abundant terminal products of peptides are dipeptides rather than free amino acids. We propose that dipeptides produced during peptide hydrolysis are taken up directly by microbes, including phytoplankton. Dipeptidases, on the other hand, are either uncommon in nature, or hydrolysis is carried out by nonspecific hydrolases with a low affinity for dipeptides relative to the dipeptide uptake systems of organisms. Uptake of dipeptides by microorganisms was seasonally variable, and rate constants of peptide hydrolysis were generally higher in spring and summer than in fall and winter, consistent with previous studies (Kuznetsova and Lee 2001). Further evidence that DFAA are not the terminal products of peptide hydrolysis can be seen in experiments where protein added to seawater resulted in no change in DFAA concentration or composition, even though protein concentrations decreased (Kuznetsova and Lee 2002).

Because microorganisms are both consumers and producers of dissolved N, their activity leads to complex temporal and spatial patterns of nutrient distribution and turnover. The ecological role of the marine DON reservoir and the factors that control its composition and size remain elusive. Previous studies have shown that some phytoplankton can use organic compounds (Berg et al. 1997; Berman and Bronk 2003; Mulholland and Lomas 2008), and more recent studies have shown that phytoplankton have enzymes for degrading organic nitrogen compounds (Mulholland et al. 2002; Stoecker and Gustafson 2003; Stoecker et al. 2005). However, it has long been thought they do not compete with bacteria in nature on theoretical grounds, although this conjecture has not been widely tested (Kirchman 2000). It has become increasingly clear that this assumption must be re-evaluated as evidence mounts that bacteria and phytoplankton compete for the same N sources in many aquatic systems (Mulholland and Lomas 2008). Uptake of DON by phytoplankton and dissolved inorganic N by bacteria in the upper water column are now widely recognized (reviewed in Mulholland and Lomas 2008). Although bacteria have long been thought to be the primary producers of protein hydrolases, it is clear from this study and others that phytoplankton and cyanobacterial proteases make a substantial contribution to exo- or ectocellular proteolytic activity and the uptake of products generated from this activity. However, little is known about hydrolysis at the molecular level.

To better understand the role of proteins in microbial nutrition in the environment, peptide hydrolysis and dipeptide uptake need to be examined systematically (in the context of total N demand) over a time-dependent gradient of cellular physiological status (e.g., over diurnal cycles or as populations develop, and leading up to, during, and after blooms), and over seasonal cycles of variable nutrient availability, phytoplankton community composition, and temperature. As uptake of organic N compounds is examined more extensively, our view of N uptake and the relative importance of particular N compounds in the environment will continue to change, as will our understanding of the functional roles of phytoplankton and bacteria.

Acknowledgments

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