

# The role of the picoeukaryote *Aureococcus anophagefferens* in cycling of marine high-molecular weight dissolved organic nitrogen

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## Abstract

Environmental evidence suggests that *Aureococcus anophagefferens* (Pelagophyceae), a eukaryotic picoplankton that blooms in coastal seawaters, can outcompete other organisms because of its ability to use abundant dissolved organic nitrogen (DON). To test this hypothesis, we isolated *A. anophagefferens* in axenic culture and monitored its growth on high-molecular weight (HMW) DON collected from sediment pore waters, a putative source for DON in bays where blooms occur. HMW DON originating from pore water had a substantially higher protein content than surface seawater DON. We found that *A. anophagefferens* could deplete 25–36% of the available nitrogen in cultures with HMW DON as the sole source of nitrogen and that this corresponded well with the protein fraction in pore-water HMW DON. High rates of cell surface peptide hydrolysis and no detectable *N*-acetyl polysaccharide hydrolysis, together with the high percentage of hydrolyzable amino acids compared to hydrolyzable aminosugars present in the HMW DON, pointed to the protein fraction as the more likely source of nitrogen used for growth. Whether or not nitrogen scavenging from protein is a common mechanism in phytoplankton is at present unknown but needs to be investigated.

Intense blooms of *Aureococcus anophagefferens*, or brown tides, were first observed in 1985 at three separate locations along the northeast coast of the United States (Casper et al. 1987; Sieburth et al. 1988; Olsen 1989). The simultaneous occurrence of brown tides in geographically isolated regions along the U.S. East Coast suggested that regional-scale, climatological forcing might play a role in triggering the blooms. Yet, attempts to correlate bloom initiation with rainfall (Casper et al. 1990) or wind stress (Vieira and Chant 1993) have failed to provide a consistent explanation for the bloom. In a review of historical data, LaRoche et al. (1997) hypothesized that brown tide blooms are controlled by interannual variability in the relative supply of dissolved inorganic and organic nitrogen, determined in part by groundwater flow. The supply of inorganic nitrogen through groundwater is greater than any other external source of nitrogen to Long Island bays, and a decrease in groundwater input coupled with a seasonal build-up of DON

concentration appears conducive to brown tide blooms (LaRoche et al. 1997).

Dissolved organic nitrogen (DON) is potentially available to phytoplankton through bacterial degradation to inorganic nitrogen or as simple organic nutrients such as amino acids and urea. Whether more complex forms of DON are also directly available to phytoplankton is not known. The availability of DON will depend on its chemical composition and on the enzymatic hydrolytic capabilities of the phytoplankton. Certain phytoplankton might possess specialized enzymes induced under nitrogen limitation that degrade organic matter (Palenik and Koke 1995). The same enzymatic adaptations might be important both in open ocean and coastal environments where seasonal maxima of labile DON are observed.

## Materials and methods

To determine whether eukaryotic picoplankton can grow directly on high-molecular weight (HMW) DON as a source of nitrogen without the intervention of bacteria, we investigated growth of *A. anophagefferens* (Pelagophyceae) on HMW DON in axenic culture. The HMW DON used in these experiments was collected from surface sediments (0–8 cm) by a grab sampler in West Neck Bay, Long Island (41°04'N, 72°21'W), a site of frequent brown tides. Sediments from multiple grabs were combined in a 114-liter plastic container fitted with wire mesh cylinders. Pore water was allowed to drain overnight into the wire cylinders, where

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Table 1. Chemical characteristics of West Neck Bay pore-water and surface seawater HMW DOM.

	WNB pore water*	Surface seawater†
Total DOC ( $\mu\text{mol L}^{-1}$ )	2,200	70–120
HMW DOC ( $\mu\text{mol L}^{-1}$ )	308	21–36
C:N (Molar ratio)	9	17
Carbohydrate:acetate:lipid‡	74:7:19	80:10:10
% acyl polysaccharide§	50	70
% amino acids	23.5	8–9
% aminosugars	0.82	n.a.

\* The results of the chemical characterization of the West Neck Bay pore-water HMW DOM are specific to the sample used in the culture experiments. Similar results were obtained from samples collected in West Neck Bay on two other occasions.

† Samples from Georges Bank, Mid-Atlantic Bight, Woods Hole coastal water, Oosterschelde estuary, Scripps Pier, Peru upwelling, Hawaii, the central Equatorial Pacific Ocean, and the central North Pacific gyre (Aluwihare et al. 1997; McCarthy et al. 1997).

‡ Determined by integration of  $^1\text{HNMR}$  according to Aluwihare et al. (1997).

§ Sum of  $\text{CH}_2\text{O}$ -, acetate-, and lipid-carbon as percentage of total available carbon in HMW DOM.

|| Amino acid- or aminosugar-nitrogen as percentage of total available nitrogen in HMW DOM.

it was recovered. Samples were filtered through a precombusted Whatman GF/F filter and a 0.2- $\mu\text{m}$  polysulphone cartridge filter before the HMW DOM was isolated using an Amicon DC-10L ultrafiltration system equipped with two spiral-wound polysulphone filter membranes (1 nm, nominally  $>1$  kDa). The ultrafiltered samples were diafiltered by serial dilution and concentrated 10 times with Milli-Q water to remove salts. The final product was characterized chemically prior to culture experiments as follows. Total carbon and nitrogen content were analyzed using a Fissions model 1108 elemental analyzer. Amino acids released by acid hydrolysis of HMW DOM were analyzed by high-pressure liquid chromatography following derivatization according to Pantoja and Lee (1999). Aminosugars released by hydrolysis of HMW DOM were analyzed in triplicate as alditol acetates and quantified by capillary gas chromatography according to Repeta et al. (2002). HMW dissolved organic matter was characterized by proton nuclear magnetic resonance ( $^1\text{HNMR}$ ) collected on a Bruker 500-MHz NMR spectrometer. For  $^1\text{HNMR}$  spectra, 3–5-mg subsamples were freeze-dried in  $\text{D}_2\text{O}$  (to reduce HDO) and dissolved in  $\text{D}_2\text{O}$  and the solvent suppressed (Bruker PRESAT) during acquisition. Results are reported relative to water at 4.8 ppm.

Filter-sterilized aliquots of HMW DON were dissolved in autoclaved,  $f/2$ -enriched artificial seawater (Goldman and McCarthy 1978) and used as the sole nitrogen source in *A. anophagefferens* cultures. HMW DON was added to a final concentration of 200–220  $\mu\text{mol N L}^{-1}$  and the N:P ratio in the medium varied from 5.5 to 6.1. *A. anophagefferens* was also grown with  $\text{NH}_4^+$  (100  $\mu\text{mol L}^{-1}$ , N:P = 2.8),  $\text{NO}_3^-$  (300  $\mu\text{mol L}^{-1}$ , N:P = 8.3), histidine (300  $\mu\text{mol L}^{-1}$ , N:P = 25 assuming availability of nitrogen atoms in aromatic ring structure; N:P = 8.3 assuming availability of amine nitrogen only), chitobiose (300  $\mu\text{mol L}^{-1}$ , N:P = 16.6), glucosamine (300  $\mu\text{mol L}^{-1}$ , N:P = 8.3), and *N*-acetylglucos-

amine (300  $\mu\text{mol L}^{-1}$ , N:P = 8.3) as sole sources of nitrogen. The medium was modified by substituting Tris (Tris-hydroxymethyl-aminomethane) buffer with boric acid ( $\text{H}_3\text{BO}_3$ ) and by adding selenious acid ( $\text{H}_2\text{SeO}_3$ ) to a final concentration of 1  $\mu\text{mol L}^{-1}$ . The complete medium was filter sterilized through 0.22- $\mu\text{m}$  Millipore single-use filter disks into culture vials. Cultures were grown at 17°C under fluorescent cool-white light (60  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) on a 12:12 L:D (LD) cycle. With each transfer, bacterial contamination was assessed by transferring culture aliquots to marine broth (MB2216, Difco) and by direct cell counting following staining with acridine orange. No bacterial contamination was observed. Determination of *A. anophagefferens* biomass was made at 24-h intervals by monitoring in vivo fluorescence using a Turner Designs model 10 fluorometer (5–60 blue excitation and 2–64 red emission filters) after mixing. Samples for particulate organic nitrogen and carbon (PON, POC) were filtered through precombusted (450°C, 1 h) GF/F filters and analyzed on a Carlo Erba CHN analyzer. The filtrate was used to determine total dissolved nitrogen (TDN) and dissolved inorganic nitrogen, DIN ( $\text{NH}_4^+$ ,  $\text{NO}_3^- + \text{NO}_2^-$ ) concentrations according to Grasshoff et al. (1999). Contamination with DIN in the HMW DON medium was  $<6\%$  of the total dissolved nitrogen and was subtracted from estimates of HMW DON availability. Because of the complexity of HMW DON as a nitrogen source, a mass balance of nitrogen utilization was carried out to demonstrate that PON accumulation reflected the decrease in TDN. In subsequent experiments, only PON was measured.

## Results and discussion

Recent studies show that HMW DOM in seawater is remarkably constant in its chemical characteristics (Aluwihare et al. 1997; McCarthy et al. 1997). However, HMW DOM recovered from Long Island surface sediments used in the present investigation was chemically distinct from seawater HMW DOM (Table 1). Proton NMR spectra of HMW DOM recovered from West Neck Bay sediment showed major resonances for carbohydrates (5.5–4.9 ppm, anomeric H; and 4.5–3.0 ppm), bound acetate (hereafter acetate, 2.2–1.7 ppm), and lipid (1.5–0.8 ppm), superimposed on a baseline with a broad maximum at 2.3 ppm (Fig. 1). Seven neutral sugars—rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose—previously observed to be the major sugars in marine HMWDOM (Aluwihare et al. 1997) were also the most abundant sugars in the West Neck Bay pore-water sample (Repeta et al. 2002). However, the ratio of carbohydrate:acetate:lipid determined from  $^1\text{HNMR}$  after baseline subtraction was 74:7:19, different from the typical surface seawater ratio of 80:10:10. Moreover, carbohydrate, acetate, and lipid resonances accounted for 50% of the total HMW dissolved organic carbon (DOC) as determined by  $^1\text{HNMR}$  spectra, lower than the 70% total HMW DOC observed for surface seawater (Table 1). The broad baseline and lower contribution of carbohydrate, acetate, and lipid resonances to the total HMW DOC in West Neck Bay DOM could be due to a higher percentage of proteinaceous material or unidentified nitrogen-containing substrates. This is

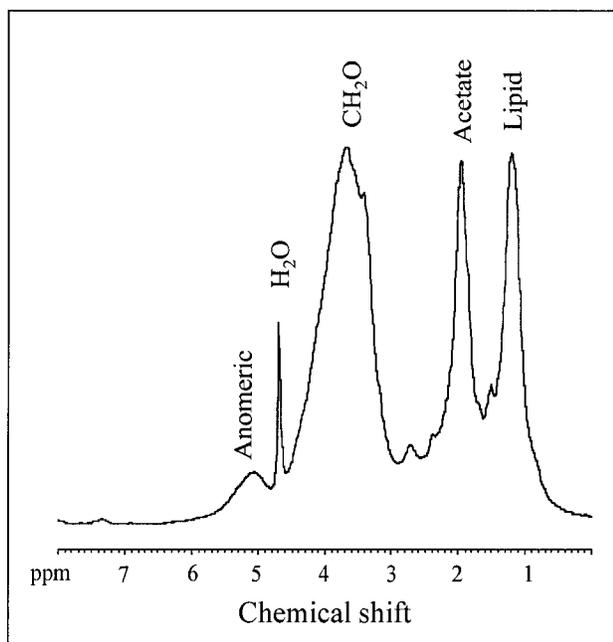


Fig. 1.  $^1\text{H-NMR}$  spectrum of HMW DOM from West Neck Bay pore water showing major resonances from carbohydrate: 5.5–4.9 (anomeric), 4.5–3.0 ( $\text{CH}_2\text{O}$ ), 2.2–1.7 (acetate), 1.5–0.8 (lipid). The spectrum was collected in  $\text{D}_2\text{O}$  with water presaturation at 4.8 ppm (sharp peak).

consistent with the lower C:N ratio of West Neck Bay HMW DOM (C:N = 9) compared to that of typical seawater ratios (C:N = 15–17).

A higher proportion of nitrogen in pore-water DOM relative to surface seawater DOM suggests the presence of a labile nitrogen fraction. Most of the available nitrogen may be bound as amide with a minor portion existing as free amines, as indicated by nitrogen-nuclear magnetic resonance ( $^{15}\text{N-NMR}$ ) spectra of ultrafiltered seawater (McCarthy et al. 1997). Two of the most common amide-containing biopolymers in marine environments include protein and *N*-acetyl aminosugars. Whereas in protein, the amide linkage forms the backbone of the polymer primary structure, in *N*-acetyl aminosugar polymers, such as chitin, the amide linkage is part of the functional group and is not an integral part of polymer structure. In the latter case, breaking the amide bond is relatively easy under mild denaturing conditions. Acid hydrolysis demonstrates that 50% of amide nitrogen in surface seawater HMW DOM is easily hydrolyzable (Aluwihare and Repeta unpubl. data), suggesting that the other half might be bound as proteinaceous material. However, current estimates of recoverable protein and aminosugars from both surface seawater and pore water do not correspond with  $^{15}\text{N-NMR}$  analysis. In the samples from West Neck Bay, amino acids and aminosugars released by the hydrolysis of HMW DOM comprised 24 and 0.8%, respectively, of the available nitrogen (Table 1). Still, the recoverable amino acid content was double or more compared with previous estimates of protein-derived matter in HMW DON extracted from sediments (Pantoja and Lee 1999) and surface seawater (McCarthy et al. 1997).

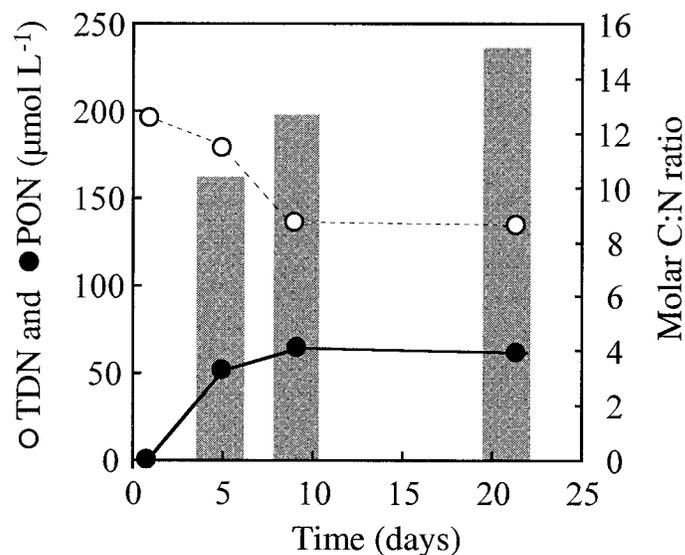


Fig. 2. Time course of depletion of total dissolved nitrogen (open circle) and accumulation of particulate organic nitrogen (solid circle) in an axenic culture of *A. anophagefferens* grown on sterile-filtered HMW DON in artificial seawater medium as the sole source of nitrogen. Grey bars represent the molar C:N ratio of the PON. Precision for triplicate TDN analysis was better than  $\pm 2.6\%$ , and precision for duplicate PON analysis was better than  $\pm 3.0\%$ . Mass balance of dissolved and particulate nitrogen concentrations was within 90%. Nitrogen availability of the HMW DOM was 25% after subtraction of inorganic nitrogen contamination in the medium.

To investigate the nitrogen availability of DOM produced in sediments, we grew *A. anophagefferens* on ultrafiltered pore water isolated from West Neck Bay in Long Island. We found that *A. anophagefferens* grew well on HMW DON over a period of 1 yr, as evidenced in stock cultures maintained by successive transfers in HMW DON medium. In more detailed, larger volume experiments, 25 and 36% of the HMW DON was available to *A. anophagefferens* (Figs. 2, 3, respectively) after subtraction of the inorganic nitrogen contamination from the medium. These values closely corresponded with the amount of recoverable amino acids in the HMW DON (Table 1). Although the efficiency of HMW DON utilization was one third of the efficiency of  $\text{NH}_4^+$  utilization, it was comparable to the utilization of  $\text{NO}_3^-$  in culture (Fig. 3), implying that DON supplied from surface sediments may support *A. anophagefferens* blooms in coastal regions like Long Island. For example, in West Neck Bay (~4 m deep), decline of algal blooms preceding the brown tide might contribute labile organic matter to the sediments which is rapidly converted to DON through benthic microbial processes. This DON, redistributed throughout the shallow water column via diffusion, wind mixing, and tidal advection, might be readily available to *A. anophagefferens* (Gobler et al. 2002). In contrast, *A. anophagefferens* did not grow on HMW DON with a C:N ratio of 17 isolated from Hawaii surface seawater (data not shown). Lending support to the importance of benthic DON input, mesocosm studies have demonstrated that the intensity of brown tide blooms are related to the presence of benthos (Keller and Rice 1989; Nixon et al. 1994). Further comparisons between West Neck

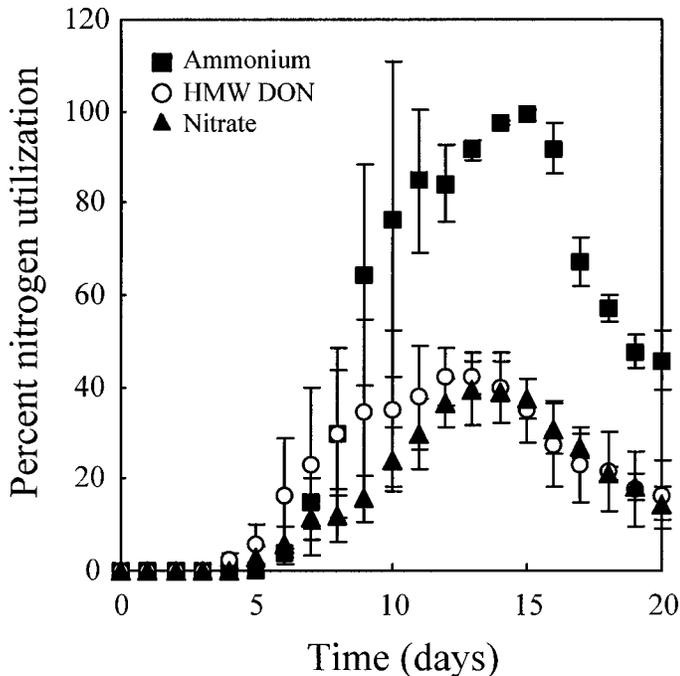


Fig. 3. Percent nitrogen utilization =  $[\text{PON}]_t / [\text{TDN}_0] \times 100$  in *A. anophagefferens* cultures, where PON, is the concentration of particulate organic nitrogen estimated at a given time point based on the relationship between in vivo fluorescence (F) and PON ( $\mu\text{mol L}^{-1}$ ),  $F = 0.9157(\text{PON}) + 10.68$  ( $r^2 = 0.93$ ,  $n = 15$ ,  $p < 0.001$ ), and  $\text{TDN}_0$  is the total available nitrogen concentration at the start of the time course. Nitrogen availability of the HMW DON was 36% after subtraction of inorganic nitrogen contamination in the medium from the highest mean PON value. Means and standard deviations are shown from two successive transfers of triplicate cultures. Cultures were adapted to each nitrogen source for two transfers prior to the experiment.

Bay sediments and other coastal sediments are warranted to determine the general lability of benthic HMW DON to phytoplankton.

We further explored the use of DON by *A. anophagefferens* by comparing growth on HMW DON to growth on simpler substrates selected based on our current knowledge of HMW DON composition. These included compounds likely to derive from the degradation of *N*-acetyl polysaccharides and protein. We find that *A. anophagefferens* is able to utilize a number of amino sugars and amino acids as the sole source of nitrogen for growth (Fig. 4), suggesting that at least the products of both protein and *N*-acetyl polysaccharide degradation can contribute equally to growth of the brown tide organism (Fig. 4).

Although phytoplankton generally are known to metabolize simple, low-molecular weight organic compounds intracellularly (Wheeler 1983) few are known to have the capability to break down polymers for nitrogen access at the cell surface (Langheinrich 1995; Sankiewicz and Colepicolo 1999). In the case of protein, this requires breaking the peptide bond to release amino acids, which can be further deaminated at the cell surface (Palenik and Morel 1990, 1991; Mulholland et al. 2002) or transported across the cell membrane. In the case of polymers of *N*-acetyl aminosugars,

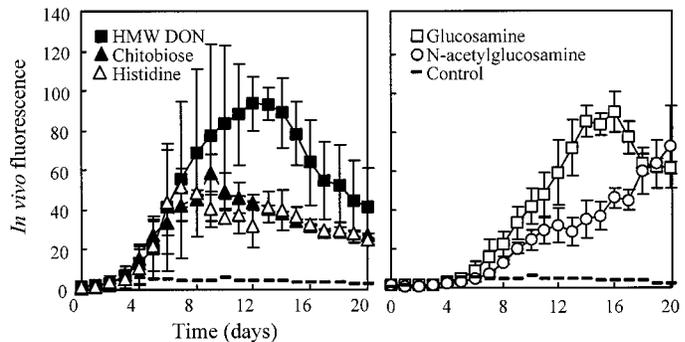


Fig. 4. In vivo fluorescence in *A. anophagefferens* cultures over time. Means and standard deviations are shown as in Fig. 3. The relationship between in vivo fluorescence (F) and cell abundance (cells  $\text{ml}^{-1}$ ),  $F = 2 \times 10^{-5}(\text{cell abundance}) + 8.22$  ( $r^2 = 0.94$ ,  $n = 20$ ,  $p < 0.001$ ), was determined at various time points by direct cell counts on a Zeiss Axioplan epifluorescence microscope following staining with acridine orange.

the (1-4)- $\beta$ -glycosidic bond is broken to release individual aminosugars, followed by deacetylation and linearization prior to deamination (Gooday 1990).

We examined the potential for cell surface protein and chitin polymer breakdown using the fluorogenic substrates L-leucine-4-methylumbelliferylamine HCl (Leu-MUF; Fluka 61888) and 4-methylumbelliferyl-*N*-acetyl- $\beta$ -D-glucosaminide (MUF-GlcNAc; Sigma M2133) according to Berg et al. (2002). *A. anophagefferens* demonstrated no extracellular chitinolytic activity (data not shown). Therefore, nitrogen utilization from *N*-acetyl polysaccharides is probably limited to diffusion of simple aminosugars into the cell (Fig. 4). Given the low concentration of aminosugars (0.82% of the HMW DON pool in West Neck Bay pore water), they are probably not an important fraction of the DOM. In contrast to *N*-acetyl polysaccharides, the protein fraction of HMW DON appears to be directly bioavailable to *A. anophagefferens* on the basis of cell surface peptide hydrolysis measurements (Berg et al. 2002; Mulholland et al. 2002). The aminopeptidase activity in *A. anophagefferens* cultures grown on HMW DON and  $\text{NO}_3^-$  is four times greater than in cultures grown on urea (Fig. 5), indicating that growth substrate affects induction and repression of protein assimilation. The reason aminopeptidase activity is as great when

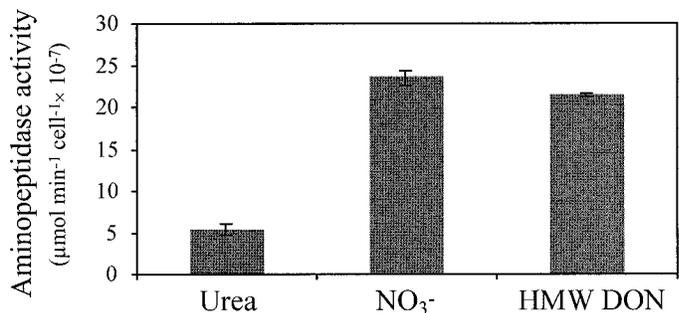


Fig. 5. Aminopeptidase activity in *A. anophagefferens* cultures grown on urea,  $\text{NO}_3^-$ , or HMW DON as the sole source of nitrogen. Means and standard deviations are shown for triplicate cultures.

grown on  $\text{NO}_3^-$  as when grown on HMW DON (Fig. 5) is not clear. However, urease activity is also stimulated in *A. anophagefferens* grown on  $\text{NO}_3^-$  relative to *A. anophagefferens* grown on urea or other reduced nitrogen substrates (Berg and Nissen unpubl. data). One explanation is that *A. anophagefferens* might prefer to assimilate reduced nitrogen, therefore ramping up reduced nitrogen assimilation systems when grown on oxidized nitrogen. Another closely related pelagophyte, *Aureoumbra lagunensis*, is unable to grow on  $\text{NO}_3^-$  as the sole source of nitrogen in culture (DeYoe and Suttle 1994). This might be another species capable of utilizing complex sources of DON such as protein. To date, cell surface peptide hydrolysis has been reported for a freshwater chlorophyte (Langheinrich 1995) and a marine dinoflagellate (Sankievicz and Colepicolo 1999), suggesting that this mechanism of scavenging nitrogen from protein might be more common in algae than previously thought.

We have demonstrated that a eukaryotic phytoplankton can utilize HMW DON under axenic conditions in culture. HMW DON originating from pore water is richer in nitrogen and contains a larger fraction of hydrolyzable amino acids than open ocean DON, capable of supporting eukaryotic plankton blooms in coastal pelagic systems. In the present study, 25–36% of the nitrogen in HMW DON was available for growth, corresponding with the fraction of hydrolyzable amino acids. It appears that the proteinaceous fraction of the HMW DON is a better source of nitrogen to phytoplankton than the *N*-acyl polysaccharide fraction. This could be due to easier accessibility of the amide bond and therefore the nitrogen in protein. The enzymatic adaptations necessary to hydrolyze proteins might be present in open ocean phytoplankton species as well as coastal species. In both coastal and oligotrophic environments, picoplankton unable to utilize  $\text{NO}_3^-$  as a source of nitrogen exist (DeYoe and Suttle 1994 and references therein; Moore et al. 2002), begging the question of whether these species can utilize not only simple organic substrates but also polymers. Despite an increase in blooms of picoeukaryotes that discolor the water and disrupt ecosystem functioning (Casper et al. 1987; Sieburth et al. 1988; DeYoe and Suttle 1994), there is a dearth of information regarding picoeukaryote functionality and physiological capabilities. The ability to grow on a range of reduced forms of nitrogen, including DON and ammonium (Figs. 3, 4), might be a characteristic of many eukaryotic picoplankton. This contrasts with larger eukaryotes, such as diatoms, which dominate  $\text{NO}_3^-$  utilization (Landry et al. 1997; Berg et al. 2003). In the near future, global warming-enhanced stratification could limit diffusion and supply of  $\text{NO}_3^-$  from depth to surface waters, resulting in increased oligotrophic ocean regions (Falkowski et al. 1998; Sarmiento et al. 1998). If this scenario holds, the role of picoeukaryotes and nitrogen fixers in nutrient cycling and primary production could become more important than at present because of their ability to use alternative sources of nitrogen.

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