

# Evidence for dissolved organic nitrogen and phosphorus uptake during a cyanobacterial bloom in Florida Bay

P. M. Glibert<sup>1,\*</sup>, C. A. Heil<sup>2,3</sup>, D. Hollander<sup>2</sup>, M. Revilla<sup>1</sup>, A. Hoare<sup>2</sup>,  
J. Alexander<sup>1</sup>, S. Murasko<sup>2</sup>

<sup>1</sup>University of Maryland Center for Environmental Science, Horn Point Laboratory, PO Box 775, Cambridge, Maryland 21613, USA

<sup>2</sup>College of Marine Science, University of South Florida, 140 7th Avenue South, St. Petersburg, Florida 33701, USA

<sup>3</sup>*Present address:* Florida Fish and Wildlife Conservation Commission, Florida Marine Research Institute, 100 Eighth Avenue South, St. Petersburg, Florida 33701, USA

**ABSTRACT:** Florida Bay, a shallow, seagrass-dominated bay in southern Florida, USA, receives significant nutrient inputs and has experienced seagrass losses and microalgal blooms within the last several decades. Inorganic nutrient inputs have been well characterized, but the role of organic nutrients, specifically of dissolved organic nitrogen (DON) and organic phosphorus (DOP), in supporting microbial processes in the bay is unknown. In this study various techniques were used to assess the importance of these nutrients along a transect in Florida Bay when a cyanobacterial bloom occurred in the central region in November 2002. These techniques included measurements of ambient particulate and dissolved nutrients, enzyme (urease and alkaline phosphatase) activities, and experiments to determine rates of <sup>15</sup>N uptake (nitrate, ammonium, urea, and amino acids over a period of 0.5 h) and long-term (48 h) changes in microbial biomass and <sup>15</sup>N natural abundance in enrichment bioassays. The cyanobacterial bloom in central Florida Bay was associated with the highest concentrations of DON and DOP, whereas the microflagellate- and diatom-dominated eastern bay region was associated with the highest concentrations of inorganic nutrients. The zeaxanthin:chlorophyll *a* ratio (an indicator of the relative contribution of cyanobacteria to phytoplankton biomass) was positively correlated with the rate of uptake of urea, and negatively correlated with the rate of uptake of inorganic nitrogen. The opposite pattern was observed for the fucoxanthin:chlorophyll *a* ratio (indicative of relative diatom biomass) and the peridinin:chlorophyll *a* ratio (indicative of relative photosynthetic dinoflagellate biomass), suggesting that different algal groups were using different N substrates. Biomass responses in the bioassay experiments showed that phytoplankton (as chlorophyll *a*) responded to DON additions in the western region and to DOP additions in the eastern region, but heterotrophic bacteria, in contrast, responded to DOP additions in the west and DON additions in the east. These findings thus demonstrate the potential for different sources of N, including DON, to stimulate different components of the algal community, and for the phytoplankton and bacteria to respond differently to N and P.

**KEY WORDS:** Florida Bay · Cyanobacteria · Organic N · Organic P · Nutrient limitation · Urease · Alkaline phosphatase · Bioassay · Nutrient uptake rates

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## INTRODUCTION

Florida Bay, a subtropical estuarine system located between the mainland of southern Florida and the

Florida Keys, has been the recent focus of scientific and management concern because of significant ecological changes that have been hypothesized to be associated with ongoing eutrophication and land-use

changes. Since the onset of industrialization in the 1880s, the health of the Florida Bay ecosystem has been negatively impacted on both decadal (e.g. increasing eutrophication) and centennial (e.g. changes in land use and water management practices within southern Florida) time-scales (Fourqurean & Robblee 1999). These anthropogenic changes have led to a significant alteration in freshwater flow patterns within the Everglades, causing declines in seagrass distribution and abundance (Zieman et al. 1989, Robblee et al. 1991, Fourqurean et al. 1993), increases in pelagic algae blooms (Butler et al. 1995, Phlips et al. 1995, Phlips & Badylak 1996), decreases in coral health (Chiappone & Sullivan 1994, Szmant & Forrester 1994), and economically important fisheries (e.g. Tortugas shrimp), (Nance 1994, Costello & Allen 1996), sport fisheries (Tilmant 1989) and manatees (McIvor et al. 1994) within the bay. Although Florida Bay receives its freshwater flow from the Everglades, this flow results from managed discharge rather than from natural hydrological conditions (Rudnick et al. 1999). Long-term management plans for the Southern Florida area call for restoration of the natural flow conditions within the Everglades. Thus the key questions of concern to both scientists and ecosystem managers are to what extent do nutrients originating from the Everglades contribute to eutrophication in Florida Bay, and what are the greatest impacts of these nutrients?

The central region of Florida Bay has experienced frequent microalgal blooms in the past decade (Phlips & Badylak 1996, Hitchcock et al. 1998, Phlips et al. 1999), typically dominated by the cyanobacterium genus *Synechococcus* (Phlips & Badylak 1996, Phlips et al. 1999). The relationship between such blooms and potential eutrophication originating from the Everglades watershed has been the topic of much debate (e.g. Lapointe & Clark 1992, Boesch et al. 1993, Zieman et al. 1999). Whereas the eastern bay region is generally considered to be severely phosphorus-limited (Fourqurean et al. 1992, Hitchcock et al. 1998, Lavrentyev et al. 1998), the central bay generally has sufficient total dissolved nitrogen and phosphorus to support such blooms (Boyer et al. 1999, Fourqurean & Robblee 1999). The sources of these central bay nutrients (e.g. the Everglades via Shark River Slough inputs, or local benthic or pelagic regeneration) are complex (Rudnick et al. 1999). One of the major unknowns is the role of organic nutrient forms in supporting these blooms.

With the impending restoration of Florida Bay and the proposed return of sur-

face flow through the Everglades, the forms, amount and delivery of dissolved nutrients to the bay are expected to change. In particular, an increase in dissolved organic and inorganic nitrogen (DON and DIN) is expected. As stated in a recent synthesis of Florida Bay research and needs, 'Measurements of the composition and bioavailability of Everglades DON to Florida Bay's microbial communities (pelagic, epiphytic, and benthic) are essential in order to assess the functional relationship of Florida Bay and its watershed.' (Florida Bay Science Program 2003, p 5–32). As part of a larger study of the sources and fates of inorganic and organic nutrients in Florida Bay (Project Key LARGO, Limitation of Algal Rates and Growth by Organic nutrients), we assessed the *in situ* uptake rates and biomass responses to DON and dissolved organic phosphorus (DOP) during a cyanobacterial bloom event in November 2002, and present the results herein.

## MATERIALS AND METHODS

Sampling was conducted from November 6 to 12, 2002, at 7 stations in Florida Bay (Fig. 1). One station was sampled each day. The stations were located along a general west–east gradient, and encompassed several previously classified regions, i.e. the Gulf transition region: Stn 1 (Sprigger Bank); the west–central region: Stn 2 (Rabbit Key); the central region: Stn 3 (Barnes Key), and 4 (Rankin Bight); and the eastern region: Stns 5 (Little Madeira), 6 (Duck Key), and 7 (Sunset Cove). Sunset Cove was the only site located off Florida Keys; the other sites were either in the main

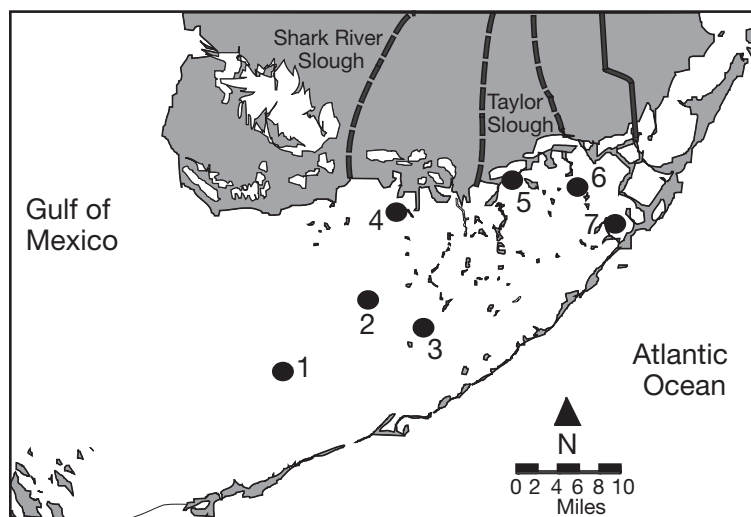


Fig. 1. Map of Florida Bay, showing sampling locations. Stn 1: Sprigger Bank; 2: Rabbit Key; 3: Barnes Key; 4: Rankin Bight; 5: Little Madeira; 6: Duck Key and 7: Sunset Cove

part of the bay, or were located closer to the Florida mainland (Fig. 1).

Samples were collected from 10 cm below the surface in 20 l carboys, and returned for processing and experimentation to the National Park Service Florida Bay Interagency Science Center at Key Largo within 1 h of collection. The samples were then immediately divided into 3 aliquots for different types of analysis: Aliquot 1 was used for determination of ambient dissolved and particulate nutrients and other indices of algal and bacterial biomass, Aliquot 2 for short-term incubation experiments of  $^{15}\text{N}$  uptake of several N forms, and Aliquot 3 for longer-term 48 h enrichment bioassays.

**Aliquot 1.** Replicates of the first aliquot of sample were filtered through precombusted (450°C, 4 h) GF/F filters to yield subsamples for analysis of dissolved and particulate nutrients. Filtrates were frozen for subsequent analysis of dissolved nutrients. Concentrations of dissolved inorganic nutrients ( $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NH}_4^+$ ,  $\text{PO}_4^{3-}$ ,  $\text{Si}(\text{OH})_4$ ) were determined in triplicate using autoanalysis techniques (Atlas et al. 1971, Gordon et al. 1994). Concentrations of total dissolved nitrogen (TDN) were determined in duplicate using persulfate oxidation followed by autoanalysis using the calibration recommendations of Bronk et al. (2000). Total dissolved phosphorus (TDP) concentrations were determined in duplicate by the method of Solórzano & Sharp (1980). Both DON and DOP were determined as the difference between the mean dissolved total and inorganic fractions. Concentrations of urea were determined in triplicate using the urease method described by Parsons et al. (1984), modified for small sample volumes. The filters, which were kept frozen, were used for particulate analysis. Concentrations of particulate nitrogen (PN), and particulate carbon (PC) were determined using a control equipment CHN analyzer, while those of particulate phosphorus (PP) were determined according to Solórzano & Sharp (1980). Chlorophyll *a* (chl *a*) concentrations were determined in duplicate on the total, bulk fraction (GF/F filter), as well as on a <3  $\mu\text{m}$  fraction prepared by filtering the original sample through a 3.0  $\mu\text{m}$  Nuclepore filter followed by filtration onto a GF/F. Both sets of samples were frozen over desiccant and analyzed within 1 wk by the fluorometric method of Holm-Hansen et al. (1965). An additional GF/F filter of the unfractionated particulates was stored on liquid  $\text{N}_2$  for subsequent analysis of phytoplankton pigments using a Hewlett Packard high-performance liquid chromatograph (Model 1100) system, according to the method of Van Heukelem et al. (1994) and Van Heukelem & Thomas (2001).

Samples for bacterial enumeration were preserved in glutaraldehyde and subsequently analyzed using a FACS caliber Becton Dickson flow cytometer accord-

ing to the method of del Giorgio et al. (1996). A 100 ml sample aliquot was also preserved using Lugol's preservative for phytoplankton community composition and enumeration. Phytoplankton were enumerated and identified to the species level where possible using an Olympus Axiovert25 inverted microscope. Samples were gently mixed and 3 ml were transferred to a settling chamber and allowed to settle undisturbed for 24 h. Triplicate samples for picoplankton enumeration were counted using a Brightline haemocytometer and a Nikon optiphot epifluorescent compound microscope. A Shannon-Wiener diversity index value ( $H'$ ) was calculated for each station sample according to the following formula:

$$H' = -\sum_{i=1}^s p_i \log_2 p_i \quad (1)$$

$$p_i = N_i/N \quad (2)$$

where  $N_i$  is the number of individuals of species  $i$ ,  $N$  is the number of individuals in the sample,  $p$  is the proportion of individuals in the  $i$ th species and  $s$  is the number of species.

Activities of the enzyme alkaline phosphatase (APA) were determined by measuring changes in sample fluorescence over time after the addition of a methyl fluorescein phosphate substrate, according to Perry (1972). Changes in fluorescence upon substrate additions were followed at 15 min intervals for 2 h with a Turner Designs fluorometer to confirm the linearity of the response. Activities of the enzyme urease were determined within 2 wk of collection on samples filtered on GF/F filters, and stored frozen in liquid  $\text{N}_2$ . The method of Peers et al. (2000) was used, with the buffer and sample volume modifications of Fan et al. (2003) and C. Solomon et al. (unpubl.).

**Aliquot 2.** The second aliquot was used to determine rates of uptake of dissolved N substrates using  $^{15}\text{N}$  tracer techniques (Glibert & Capone 1993). At 6 of the 7 stations (samples from Stn 6 were not collected for this analysis), uptake rates of  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea, glycine, and glutamic acid were measured over an enrichment gradient from 0.1 to 10  $\mu\text{g-at N l}^{-1}$  to determine response kinetics; however, here we report only the results for the lowest enrichment level reflecting ambient (or 'trace'), uptake rates. Incubations were done in 1 l Nalgene bottles, under 60% natural irradiance (achieved by neutral-density screening) and ambient temperature for approximately 0.5 h. All incubations were initiated during mid-to-late morning, and were terminated after incubation by filtration onto precombusted GF/F filters. Filters were dried and subsequently analyzed by mass spectrometry.

**Aliquot 3.** Bioassay experiments were initiated at the same 6 stations for Aliquot 2, coincident with station sample processing, and samples were incubated for

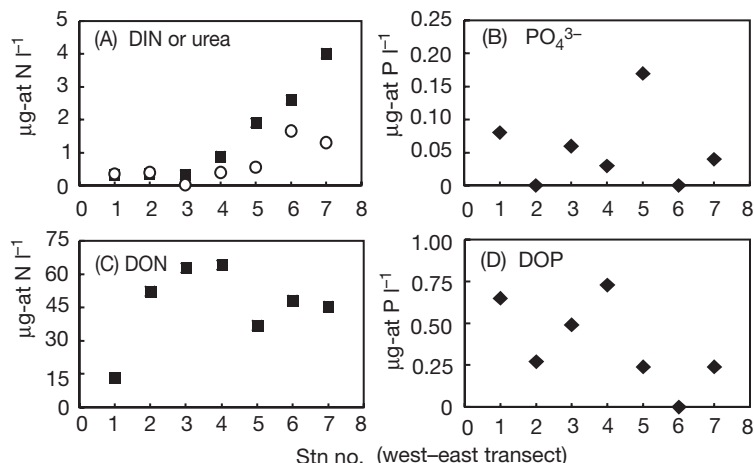


Fig. 2. Dissolved nutrient pools along general west–east gradient in Florida Bay. (A) Dissolved inorganic nitrogen ( $\text{NO}_3^- + \text{NO}_2^- + \text{NH}_4^+$ ; ■) and urea (○); (B) dissolved inorganic phosphorus (◆); (C) dissolved organic nitrogen (■); (D) dissolved organic phosphorus (◆). Data represent duplicate or triplicate analyses; error bars ( $\pm$ SD) not shown, as smaller than symbols on the graphs

48 h each. A suite of 6 duplicate bioassay treatments involving both inorganic and organic nutrient enrichments were used to assess potential, rather than *in situ* responses. For the purposes here, we report only those treatments which were enriched with  $10 \mu\text{g-at N l}^{-1}$  DON (prepared as  $5 \mu\text{g-at N l}^{-1}$  urea,  $2.5 \mu\text{g-at N l}^{-1}$  arginine and  $2.5 \mu\text{g-at N l}^{-1}$  glutamine;  $\delta^{15}\text{N} = -3.61$ ), DOP (prepared as  $2.5 \mu\text{g-at P l}^{-1}$  glycerophosphate), and humic acids (prepared as  $4 \text{ mg l}^{-1}$  from Sigma-Aldrich humic acid sodium salt;  $\delta^{15}\text{N} = 1.65$ ). All station water used for bioassays was filtered through  $154 \mu\text{m}$  mesh prior to initial enrichment to remove large grazers. Incubations were conducted in 4 l Cubitainers, at 60% of natural radiance (neutral-density screening) and ambient temperature. Station measurements served as initial sample measurements for all bioassays, and each bioassay experiment was subsampled at 24 and 48 h to determine total chl *a*, chl *a* in the  $<3 \mu\text{m}$  fraction, and bacterial abundance, as described for Aliquot 1.

Natural  $^{15}\text{N}$  abundance of the particulate material in each bioassay was sampled at time zero and after 48 h. Replicate bioassay treatments from each station were pooled to provide a sufficient sample volume to measure a  $\delta^{15}\text{N}$  signal (only data for Stns 2 and 5 are reported here. Stable isotope analysis of particulate material was accomplished using a Finnigan-Mat delta plus coupled to a

Carlo-Erba element analyzer. Natural abundance results are presented using delta ( $\delta$ ) notation:

$$\delta^{15}\text{N} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000 \quad (3)$$

where  $R$  (relative abundance) =  $^{15}\text{N}/^{14}\text{N}$ .

## RESULTS

### Nutrient and biomass characteristics (Aliquot 1)

Concentrations of DIN were  $<1 \mu\text{g-at N l}^{-1}$  in the western and central regions of the bay, but increased to  $4 \mu\text{g-at N l}^{-1}$  in the eastern region (Fig. 2A). Concentrations of urea also varied from  $<0.5 \mu\text{g-at N l}^{-1}$  in the western and central regions to  $1.4 \mu\text{g-at N l}^{-1}$  in the eastern region (Fig. 2A). Concentrations of DON exceeded  $30 \mu\text{g-at N l}^{-1}$  at all sites except at the Gulf transition (Stn 1), but in contrast to DIN and urea, concentrations of DON were substantially higher in the central bay relative to the eastern bay region (Fig. 2C). Concentrations of dissolved inorganic phosphorus ( $\text{PO}_4^{3-}$ ) were  $<0.2 \mu\text{g-at P l}^{-1}$  at all stations, while concentrations of DOP were highest in the western and central bay regions (Fig. 2B,D). A pronounced chl *a* maximum of  $8.6 \mu\text{g l}^{-1}$  was observed in the central bay (Stn 3; Fig. 3A). Along the entire transect, virtually all the chl *a* was in the  $<3 \mu\text{m}$  fraction (Fig. 3A). Concentrations of heterotrophic bacteria were highest in the central bay, but their peak abundance was

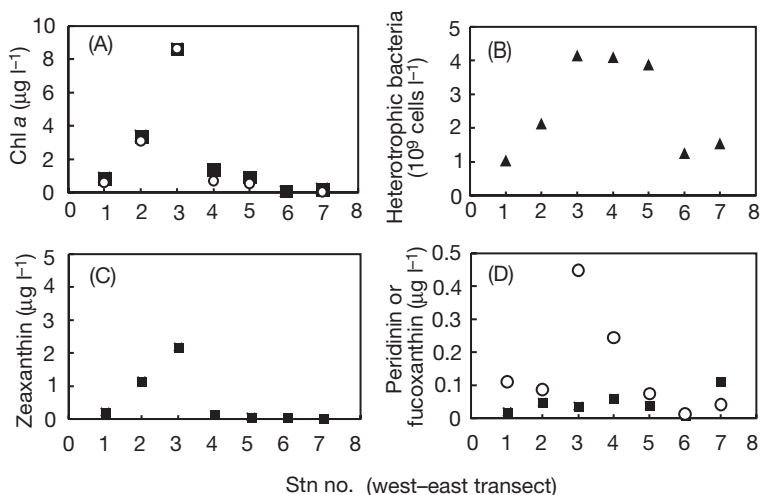


Fig. 3. Biomass parameters along general west–east gradient in Florida Bay. (A) concentration of total chl *a* (■) and chl *a* in  $<3 \mu\text{m}$  fraction (○); (B) abundance of heterotrophic bacteria (▲); (C) zeaxanthin concentrations (■); and (D) concentration of peridinin (■) and fucoxanthin concentrations (○)

shifted to the east relative to the chl *a* maximum (Fig. 3B).

Accessory pigments and microscopic enumerations were used to indicate the presence of dominant groups of algae. Although unambiguous interpretation is difficult using only pigment data, in conjunction with microscopic identifications, it is possible to use zeaxanthin as an indicator of cyanobacteria, fucoxanthin as an indicator of diatoms and some chrysophytes, and peridinin as an indicator of photosynthetic dinoflagellates (e.g. Jeffrey & Wright 1994, Jeffrey & Vesik 1997, Ansotegui et al. 2001). Concentrations of the accessory pigment zeaxanthin were highest at Stn 3, indicating that the chl *a* maximum was dominated by cyanobacteria (Fig. 3C). The highest concentration of fucoxanthin (e.g. diatoms or chrysophytes) was observed in the chl *a* maximum (Fig. 3D). Peridinin-containing organisms (e.g. dinoflagellates) were detected in the easternmost station

(Fig. 3D), and their proportional abundance was highest there also. The numerically dominant phytoplankton present at each station are listed in Table 1. The eastern bay (Stns 5 and 7) was characterized by a diverse ( $H'$  values of 0.986 and 0.737, respectively) community dominated by flagellate species. The dinoflagellate *Pyrodinium bahamense* was present at both stations, but its toxicity is unknown. Stations in the central region (Stns 2 and 3) were dominated by a bloom of *Synechococcus elongatus* which comprised >99.9% of the numerical abundance at both stations. Diversity indices for these stations were 0.00005 and 0.00052, respectively. The western bay was dominated by a mix of microflagellates and diatom species, with a diversity index of 0.666.

Molar ratios of the dissolved inorganic N:P (DIN:DIP) pools deviated significantly from Redfield stoichiometric proportions (16:1; Fig. 4A). At 2 stations, Rabbit Key (2) and Sunset Cove (7), the DIN:DIP ratio

Table 1. Phytoplankton community composition at each of Florida Bay stations sampled, showing numerically dominant species or groups for each station in order of decreasing abundance. Shannon-Wiener diversity index calculated using base<sub>10</sub>log. For station positions see Fig. 1; Stn 6 (Duck Key) was not sampled for this analysis

Station Dominant species/group	Concentration (cells l <sup>-1</sup> × 10 <sup>3</sup> )	% of total community	Shannon-Wiener diversity index
Sprigger Bank (Stn 1)			
Microflagellates	65.0	63.4146	0.66574
<i>Nitzschia</i> spp.	12.5	12.1951	
Prymnesiophyte	7.5	7.31707	
<i>Cylindrotheca clostridium</i>	4.2	4.06504	
Rabbit Key (Stn 2)			
<i>Synechococcus elongatus</i>	8.16 <sup>a</sup>	99.989	0.00052
<i>Chlorella</i> sp.	150.0	0.0018	
<i>Lyngbya</i> sp.	29.7	0.0004	
<i>Nitzschia</i> spp.	7.3	0.00004	
Barnes Key (Stn 3)			
<i>Synechococcus elongatus</i>	38.1 <sup>a</sup>	99.9984	0.00005
<i>Gymnodinium</i> sp.	241.7	0.00063	
<i>Nitzschia</i> spp.	71.0	0.00019	
Rankin Bight (Stn 4)			
Flagellates	49.2	41.089	0.91628
Microflagellates	31.7	18.812	
<i>Nitzschia</i> spp.	15.0	8.9109	
<i>Chlorella</i> sp.	9.2	5.4455	
Little Madeira (Stn 5)			
<i>Prorocentrum micans</i>	10.0	16.575	0.98559
Ciliates	9.7	16.022	
<i>Pyrodinium bahamense</i>	9.0	14.917	
<i>Nitzschia</i> spp.	4.0	6.6298	
Sunset Cove (Stn 7)			
Flagellates	32.7	40.833	0.73671
Ciliates	24.7	30.833	
<i>Scrippsiella</i> sp.	9.7	10.394	
<i>Pyrodinium bahamense</i>	5.0	5.3763	
<i>Nitzschia</i> spp.	4.3	4.6595	

<sup>a</sup>Cells l<sup>-1</sup> × 10<sup>8</sup>

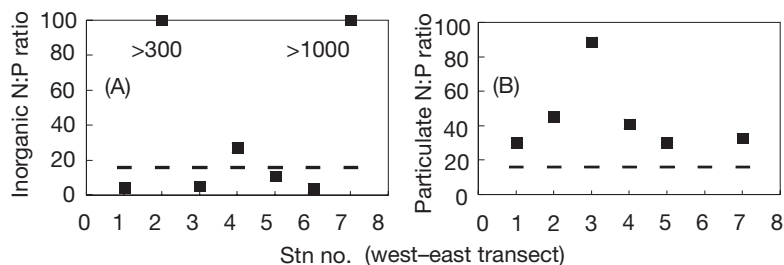


Fig. 4. Nitrogen:phosphorus (N:P) molar ratio of (A) dissolved inorganic and (B) particulate nutrients along general west-east gradient in Florida Bay. Dissolved inorganic nitrogen values comprise  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and  $\text{NH}_4^+$ . Dashed line: Redfield values

exceeded 300, indicative of strong P limitation. At Rankin Bight (4), the DIN:DIP ratio exceeded Redfield proportions by  $<2$ , suggesting modest, if any P limitation. The other stations had DIN:DIP ratios  $<16:1$ , implying limitation by N. In contrast, the N:P stoichiometric ratios of the particulate material in every case was significantly elevated above Redfield proportions (Fig. 4B), with the highest N:P ratios at Stn 3, coincident with the chl *a* maximum, demonstrating that particulate material was P-limited along the entire transect.

Activities of the enzymes urease and APA differed, depending on whether values were normalized on a volumetric basis or on a biomass (chl *a*) basis (Fig. 5). On a volumetric basis, both enzymes had higher rates of activity in the western-central or central bay regions; both had low activities at Sprigger Bank (Stn 1) and at Sunset Cove (Stn 7; Fig. 5A,C). Highest urease and APA activities ( $\text{l}^{-1}$  basis) were found at the station with the highest chl *a* biomass (Stn 3). When both enzyme activities were normalized on a chl *a* basis, highest activities were found at Stn 7 (Fig. 5B,D).

#### Rates of N uptake (Aliquot 2)

Rates of N uptake were highest at the central bay stations (Fig. 6), consistent with the highest biomass in this region. Although DIN uptake accounted for virtually all the N uptake at Sprigger Bank (Stn 1), in the central bay at Stns 2 and 3 the contribution of urea and amino acid uptake to the total N uptake rate was substantial (Fig. 6).

Pigment ratios were compared to the use of different forms of N. The zeaxanthin:chl *a* ratio was positively related

to percent uptake of urea, while both peridinin:chl *a* and fucoxanthin:chl *a* were negatively correlated with percent uptake of urea (Fig. 7A–C). In contrast, the zeaxanthin:chl *a* ratio was negatively correlated with the percent uptake of DIN, while the other ratios were positively correlated (Fig. 7D–F). The peridinin:chl *a* was also positively correlated with percent amino acid uptake (data not shown), but the fucoxanthin:chl *a* ratio was not.

#### Bioassay responses (Aliquot 3)

The potential of different fractions of the plankton community to respond to organic substrates was examined in bioassay experiments. In both the total chl *a* and the  $<3 \mu\text{m}$  fractions, a proportionately greater response to DON was observed at the Gulf transition station (Stn 1) and in the western and central bay stations (Stns 2 to 4) relative to the eastern bay stations (Stns 5 to 7; Fig. 8A). The opposite pattern was observed for the bioassay experiments enriched with both DOP and with humic acids: a greater chl *a* response was observed in the eastern bay (Fig. 8B,C). The relative response by bacteria after 48 h incubation in the enriched bioassays also differed from the chl *a* response, with the greatest response to DON in the eastern bay (Fig. 8D), and to DOP and humic acids in the western and central bays (Fig. 8E,F).

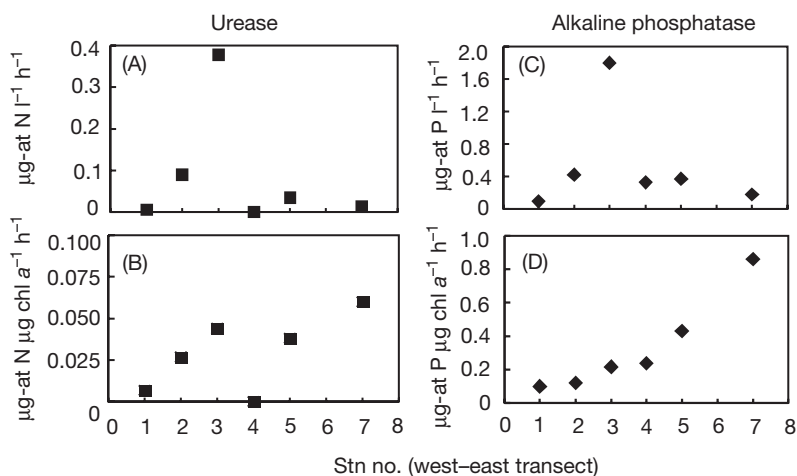


Fig. 5. Activities of (A,B) urease and (C,D) alkaline phosphatase in whole water samples from each station along a general west-east gradient in Florida Bay. Activities in (A) and (C) expressed on a per volume basis ( $\mu\text{g-at N or P l}^{-1} \text{h}^{-1}$ ), and in (B) and (D) normalized to chlorophyll *a* concentration ( $\mu\text{g-at N or P chl a}^{-1} \text{h}^{-1}$ ). Data are average of duplicate analyses; error bars ( $\pm$  SD) not shown, as smaller than symbols

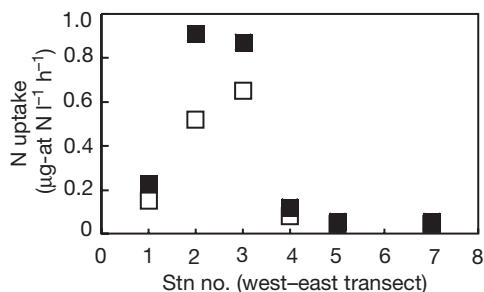


Fig. 6. Uptake rates of sum of all measured substrates (■) and inorganic substrates only (□) along a general west–east gradient in Florida Bay. Inorganic N substrates comprised  $\text{NO}_3^-$  and  $\text{NH}_4^+$ , measured substrates  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea, glycine and glutamic acid

The isotopic composition of the particulate matter in the bioassays also changed to a varying extent after 48 h enrichment with organic substrates; 2 example stations illustrate this. At Rabbit Key (Stn 2), one of the stations high in zeaxanthin and dominated by cyanobacteria, the  $\delta^{15}\text{N}$  of particulate material in all treatments, including controls, became lighter with time; this change ranged from  $-2$  to  $-2.8\text{‰}$  (Fig. 9A). In contrast, at Little Madeira (Stn 5), which had a higher proportion of heterotrophic bacteria, the  $\delta^{15}\text{N}$  of particulate material decreased in the control and in the +DON enrichment over the 48 h incubation (by as much as  $-5\text{‰}$ ), but increased in the +DOP ( $+1.9\text{‰}$ ) and in the +humic treatments ( $+7.7\text{‰}$ ; Fig. 9B).

## DISCUSSION

Herein we have taken a cross-cut approach, employing a range of methods to assess the extent to which organic nutrient sources may have contributed to phytoplankton and bacterial nutrition during November 2002 in Florida Bay: trends in ambient concentrations and ratios, direct uptake rates, enzyme assays, and bioassay responses. The results collectively show that the responses to different fractions of N and P varied among phytoplankton groups and for the bacterial fraction. They also indicate that not only did the microbial community have the potential to respond to organic inputs, but it was using organic substrates *in situ*. We shall describe these results in the context of previous findings and the implications for management.

The bloom encountered in the fall of 2002 is consistent with previous observations of *Synechococcus* sp. blooms in Florida Bay in the fall in the north central region (e.g. Philips et al. 1999). Such blooms have been hypothesized to be related to the hydrography of the area, its shallow depth and enhanced internal nutrient cycling, as well as to seasonal winds. In particular, fall cold-fronts may be responsible for shifting the prevailing wind direction from east/southeast to northeast (Philips et al. 1999), which may bring water from the north into the more central region, in turn leading to increased resuspension and nutrient flux. Other environmental characteristics of this sampling period are also consistent with long-term data sets

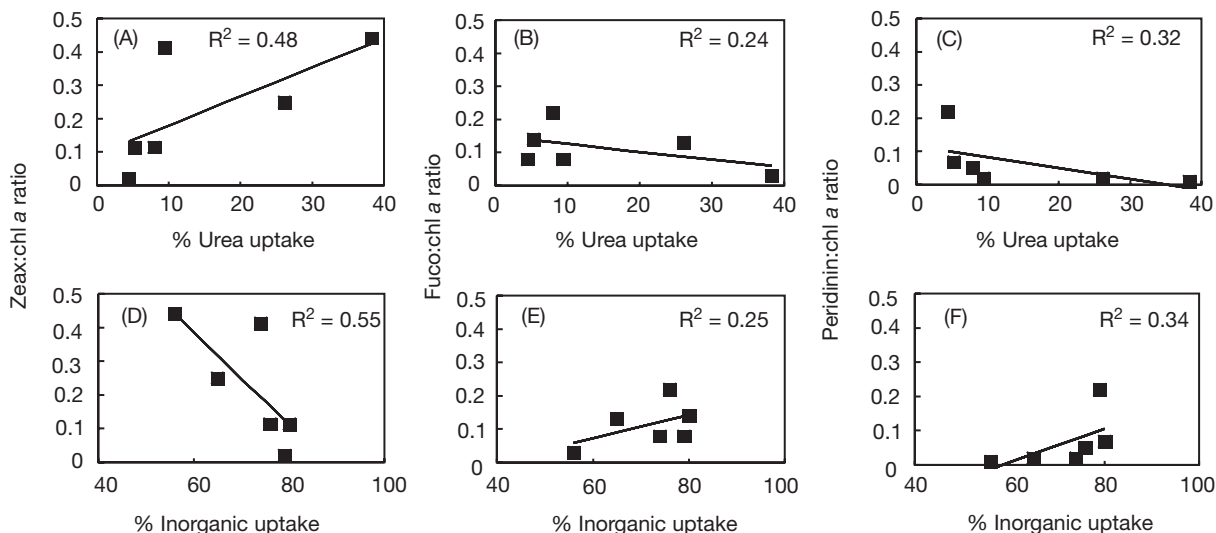


Fig. 7. Relationships between percent contribution of different N substrates to total phytoplankton N uptake and composition of the phytoplankton community as indicated by pigment ratios. (A–C) Relationships between uptake of urea fraction and (A) zeaxanthin:chlorophyll a ratio, (B) fucoxanthin:chlorophyll a ratio and (C) peridinin:chlorophyll a ratio; (D–F) same relationships relative to uptake of inorganic nitrogen fraction

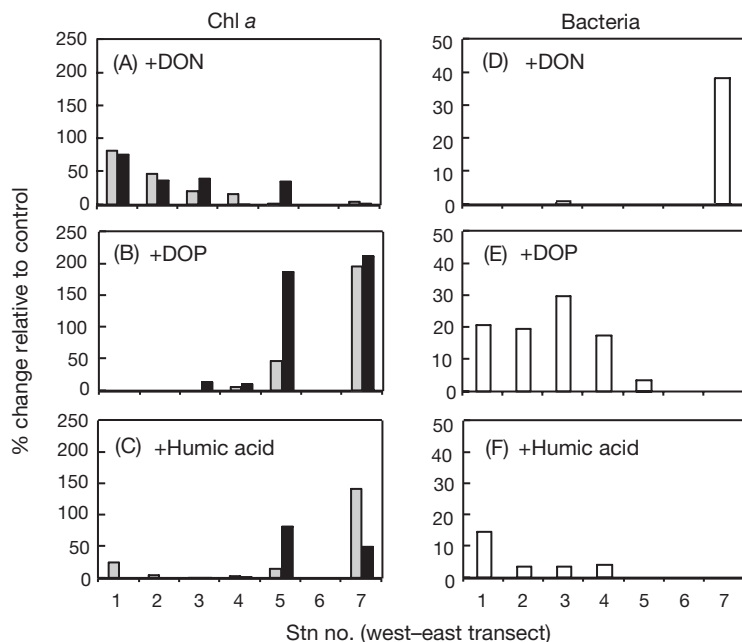


Fig. 8. Relative change in chl *a* concentration and bacterial abundance in enriched bioassay treatments along general west-east gradient in Florida Bay stations. Composition of enrichments is described in 'Materials and methods'. Response expressed as percent change in either chl *a* concentration or bacterial abundance after 48 h incubations relative to change in controls. In (A)–(C) gray bars = bulk chl *a* concentration; black bars = chl *a* concentration in <3  $\mu\text{m}$  fraction

from Florida Bay. Boyer et al. (1999), for example, examined trends in water quality from 1989 to 1997, and reported DON to be significantly higher (median value of 80.8  $\mu\text{M}$ ) in the central bay than in the eastern and western regions, and DIN in the form of  $\text{NO}_3^-$  to be higher in the eastern bay. They also reported molar DIN:DIP ratios in excess of 100 in the eastern and central bay regions (Boyer et al. 1999). Thus, our results on nutrient concentrations and ratios (Figs. 2 & 4) appear to be typical for the season and the regions of the bay studied.

The data reported here add to the growing body of evidence that algae can and do take up DON and DOP, and that uptake of these organic fractions contributes disproportionately to different algae groups. In a recent review of DON utilization by phytoplankton (Berman & Bronk 2003), it was clearly shown that not only can many species utilize DON, but many types of DON are utilized by phytoplankton. Phytoplankton groups differ in their requirements for, and in their ability to utilize, both inorganic and organic forms of nitrogen. The utilization of varying substrates depends on the physiologi-

cal ability of the cells to use specific substrates and on their physiological state (nutrient status, growth rate, etc.) at the time of nutrient supply. The occurrence of many fast-growing diatoms has been found to be highly correlated with large and/or frequent additions of  $\text{NO}_3^-$  (e.g. Goldman 1993, Lomas & Glibert 1999), and the positive correlation between fucoxanthin:chl *a* and DIN uptake found herein (Fig. 7) is consistent with this. In contrast, recent studies in enriched coastal areas have shown that while productivity may increase quantitatively with increasing overall nitrogen availability, the DON component may contribute disproportionately to the uptake by dinoflagellates, pelagophytes and cyanobacteria, many of which are considered to be harmful algal bloom species (Paerl 1988, Berg et al. 1997, LaRoche et al. 1997, Lomas et al. 2001, Glibert et al. 2001). In fact, urea has been previously implicated as an important nitrogen source for *Synechococcus* spp. in both fresh and marine systems (Berman & Chava 1999, Collier et al. 1999, Sakamoto & Bryant 2001). Other cyanobacteria have also been shown to respond positively to organic nitrogen additions: *Aphanizomenon ovalisporum* used DON as its nitrogen source in Lake Kinneret, Israel (Berman 1997, 2001), and DON (urea and amino acids) contributed significantly to a filamentous cyanobacterial bloom in the Gulf of Riga, Baltic Sea (Berg et al. 2001, 2003). The correlation reported here between zeaxanthin and percent urea uptake (Fig. 7) is consistent with these findings.

The results reported herein also show that DOP contributed to the nutrition of the microbial community of Florida Bay, but that different fractions of the microbial assemblage responded to DOP additions

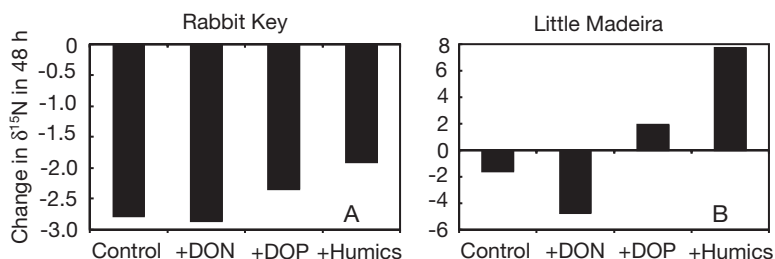


Fig. 9. Change (%) in  $\delta^{15}\text{N}$  of particulate matter in bioassay experiments for Rabbit Key (Stn 2) and Little Madeira (Stn 5) after 48 h incubations relative to controls (time zero).  $\delta^{15}\text{N}$  of particulate matter at time zero at Rabbit Key was 4.2‰, that at Little Madeira was 8.63‰

than to DON additions. As shown by the bioassay results, the chl *a* in the western and central bays responded to DON, but in the eastern bay it responded to DOP (Fig. 8). The heterotrophic bacteria of the western and central regions, however, responded to DOP. Cotner et al. (2000) also previously observed that heterotrophic bacteria in Florida Bay were limited by P; however, their study focused on inorganic P, rather than on DOP.

Interestingly, in the bioassay experiments, both the patterns in chl *a* response and the relative change in  $\delta^{15}\text{N}$  were similar for the +DOP and +humic acids enrichments, but different for the control and +DON treatments at Little Madeira (Stn 5), where heterotrophic bacterial abundance was high. The contrasting patterns in  $\delta^{15}\text{N}$  between Rabbit Key (Stn 2) and Little Madeira (Stn 5) (Fig. 9) indicate that the sources of the DON used at these stations were possibly different. The decrease in  $\delta^{15}\text{N}$  in all treatments (including controls) at Rabbit Key suggests that not only was the added DON being utilized, but that ambient DON was also probably serving as an N source. This would be consistent with the high ambient concentrations of DON, the elevated activity of urease, and the highest percentage uptake of urea at this station. At Little Madeira, in contrast, the control and +DON treatments became progressively lighter with time, suggesting utilization of DON, but the  $\delta^{15}\text{N}$  enrichment in the +humic acids and +DOP treatments increased over time, indicating the possible release of N in these treatments. The uptake rates of  $^{15}\text{N}$  substrates suggested a proportionately low uptake of organics at Little Madeira. These results also demonstrate the potential for shifts in the community structure upon longer-term exposure to organic substrates.

The enzyme activity data (Fig. 5) further shows that different fractions of the community were poised to use urea and DOP. High activities of urease were observed at Barnes Key (Stn 3) where cyanobacteria dominated. In cyanobacteria, including *Synechococcus* sp. (Clone WH 7805; Collier et al. 1999), several urease genes have been well characterized, and higher rates of urease activity have been found for cells grown on urea than for cells grown on  $\text{NH}_4^+$  (Collier et al. 1999). There is some evidence that urease may be constitutively expressed in some cyanobacteria, dinoflagellates and diatoms (Collier et al. 1999, Peers et al. 2000, Fan et al. 2003). Relatively high rates of urease activity have been previously reported for the dinoflagellates *Alexandrium fundyense* and *A. catenella* (Dyrhrman & Anderson 2003), as well as for *Prorocentrum minimum* (Fan et al. 2003). Fan et al. (2003) found that urease content per cell was significantly higher for the dinoflagellate *P.*

*minimum* than for either the diatom *Thalassiosira weissflogii* or the pelagophyte *Aureococcus anophagefferens*; however, on a per cell volume basis, *A. anophagefferens* had the highest activity. In this study the easternmost bay site (Stn 7) was characterized by the highest rate of biomass-normalized urease activity and was composed of a higher relative proportion of peridinin-containing dinoflagellates, which would be consistent with the above relationships.

In contrast to urease, APA is a well-studied enzyme that is known to be inducible upon limitation by DIP and is often indicative of P-limitation (Healey & Hendzel 1979, Chrost 1991, Cotner & Wetzel 1991). Higher rates of APA have previously been observed in the south-central region of Florida Bay compared to the northwest and north-central regions (Cotner et al. 2000), and the long-term median rates (volumetric basis) of APA have been shown to be higher in the central region than in the eastern and western basin (Boyer et al. 1999). The values reported by both Boyer et al. (1999) and Cotner et al. (2000) for summer and winter are similar to those found in this study. In the Northern Gulf of Aqaba, another P-limited system, APA was shown to be correlated with abundance of *Synechococcus* sp. (Li et al. 1998). APA can also be found in particle-free water, as it has a long lifetime when released from its extracellular location on phytoplankton cells (Li et al. 1998); thus, in dynamic systems, a strong relationship to chl *a* would not be expected.

The finding that DON and DOP may contribute differentially to the proliferation of cyanobacteria and other microbes in Florida Bay has important management implications. Our findings do not permit us at this point to identify the source of the organic nutrients, and in fact the variation in the  $\delta^{15}\text{N}$  values of particulate matter at the start of the bioassay experiments (Fig. 9) suggests that a combination of terrestrial (Everglades runoff), sedimentary (i.e.  $\text{N}_2$  fixation) and internal sources may exist. Differentiating these sources will be important, as some may ultimately be controllable, such as runoff from the Everglades, while other sources may be more difficult to control, such as sedimentary sources. In Florida Bay a systematic, west–east shift of 12‰ in the  $\delta^{15}\text{N}$  composition of seagrass and macroalgae has been speculated to reflect regional difference in the sources and utilization of dissolved nitrogen sources and the importance of groundwater circulation in the eastern region of the Bay (Corbett et al. 1999). Nevertheless, our results demonstrate the potential for algal blooms to be stimulated by this source of N. Thus, if organic N fluxes should increase, the potential for increased algal blooms exists.

**Acknowledgements.** Financial support for this research was provided by grants from the NOAA South Florida Ecosystem Research and Monitoring Program (SFP) to C.A.H., D.H. and P.M.G. M.R. was supported by a Fulbright grant from the Spanish Ministry of Education and by the European Social Fund. We thank the US National Park Service and the Florida Bay Interscience Agency for the use of research facilities at the Key Largo Ranger Station, and J. Cornwell and M. Owens for assistance with sampling logistics and boat use. Analytical assistance for this project was provided by L. Lane, S. Rhoades, L. Van Heukelem, C. Shoemaker, C. Fan and D. Harris. The experimental design and scope of this research benefited greatly from discussions with J. Cornwell of the Horn Point Laboratory, C. Madden, D. Rudnick and S. Kelly of the South Florida Water Management District, and G. Vargo and M. B. Neely of the University of South Florida. We also thank several anonymous reviewers, whose comments strengthened this paper. This is contribution # 3771 from the University of Maryland Center for Environmental Science.

#### LITERATURE CITED

- Anotegui A, Trigueros JM, Orive E (2001) The use of pigment signatures to assess phytoplankton assemblage structure in estuarine waters. *Estuar Coast Shelf Sci* 52: 689–703
- Atlas EL, Gordon LI, Hager SW, Park PK (1971) A practical manual for use of the Technicon AutoAnalyzer in seawater nutrient analysis (revised). Tech Rep Dep Oceanogr Oregon St Univ Corvallis, 215:1–49
- Berg GM, Glibert PM, Lomas MW, Burford M (1997) Organic nitrogen uptake and growth by the chrysophyte *Aureococcus anophagefferens* during a brown tide event. *Mar Biol* 129:377–387
- Berg GM, Glibert PM, Jorgensen NOG, Balode M, Purina I (2001) Variability in inorganic and organic nitrogen uptake associated with riverine nutrient input in the Gulf of Riga, Baltic Sea. *Estuaries* 24:204–214
- Berg GM, Balode M, Purina I, Bekere S, Bechemin C, Maestrini SY (2003) Plankton community composition in relation to availability and uptake of oxidized and reduced nitrogen. *Aquat Microb Ecol* 30:263–274
- Berman T (1997) Dissolved organic nitrogen utilization by an *Aphanizomenon* bloom in Lake Kinneret. *J Plankton Res* 19:577–586
- Berman T (2001) The role of DON and the effect of N:P ratios on occurrence of cyanobacterial blooms: implications from the outgrowth of *Aphanizomenon* in Lake Kinneret. *Limnol Oceanogr* 46:443–447
- Berman T, Bronk DA (2003) Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. *Aquat Microb Ecol* 31:279–305
- Berman T, Chava SAF (1999) Algal growth on organic compounds as nitrogen sources. *J Plankton Res* 21:1423–1437
- Boesch DF, Armstrong NE, D'Elia CF, Maynard NG, Paerl HW, Williams SL (1993) Deterioration of the Florida bay ecosystem: an evaluation of the scientific evidence. Report to the Interagency Working Group on Florida Bay. Department of the Interior, National Park Service, Washington, DC
- Boyer JN, Fourqurean JW, Jones RD (1999) Seasonal and long-term trends in the water quality of Florida Bay (1989–1997). *Estuaries* 22:417–430
- Bronk DA, Lomas MW, Glibert PM, Schukert KJ, Sanderson MP (2000) Total dissolved nitrogen analysis: comparisons between the persulfate, UV and high temperature oxidation methods. *Mar Chem* 69:163–178
- Butler MH, Hunt JH, Herrkind WF, Childress MH and 5 others (1995) Cascading disturbances in Florida Bay, USA: cyanobacterial blooms, sponge mortality, and implications for juvenile spiny lobsters *Panulirus argus*. *Mar Ecol Prog Ser* 129:119–125
- Chiappone M, Sullivan KM (1994) Ecological structure and dynamics of nearshore hard-bottom communities in the Florida Keys. *Bull Mar Sci* 54:747–756
- Chrost RJ (1991) Microbial enzymes in aquatic environments, Springer-Verlag, Berlin
- Collier JL, Brahamsha B, Palenik B (1999) The marine cyanobacterium *Synechococcus* sp. WH7805 requires urease (urea amidohydrolase, EC 3.5.1.5) to utilize urea as a nitrogen source: molecular-genetic and biochemical analysis of the enzyme. *Microbiology UK* 145:447–459
- Corbett DR, Chanton J, Burnett W, Dillon K, Rutkowski C, Fourqurean JW (1999) Patterns of groundwater discharge into Florida Bay. *Limnol Oceanogr* 44:1045–1055
- Costello TJ, Allen DM (1996) Migrations and geographic distributions of pink shrimp, *Penaeus duorarum*, of the Tortugas and Sanibel ground, Florida. *Fish Bull Fish Wildl Serv US* 65:449–459
- Cotner JB, Wetzel RG (1991) Uptake of dissolved inorganic and organic phosphorus compounds by phytoplankton and bacterioplankton. *Limnol Oceanogr* 37:232–243
- Cotner JB, Sada RH, Bootsma H, Johengen T, Cavaletto JF, Gardner WS (2000) Nutrient limitation of heterotrophic bacteria in Florida Bay. *Estuaries* 23:611–620
- del Giorgio PA, Bird DF, Prairie YT, Planas D (1996) Flow cytometric determination of bacterial abundance in lake plankton with the green nucleic acid stain SYTO 13. *Limnol Oceanogr* 41:783–789
- Dyhrman S, Anderson DA (2003) Urease activity in cultures and field populations of the toxic dinoflagellate *Alexandrium*. *Limnol Oceanogr* 48:647–655
- Fan C, Glibert PM, Alexander J, Lomas MW (2003) Characterization of urease activity in three marine phytoplankton species. *Mar Biol* 142:949–958
- Florida Bay Science Program (2003) A synthesis of research on Florida Bay, April 2003. US Geological Survey, Gainesville, FL, p 5–32
- Fourqurean JW, Robblee MB (1999) Florida Bay: a history of recent ecological changes. *Estuaries* 22:345–357
- Fourqurean JW, Zieman JC, Powell GVN (1992) Phosphorus limitation of primary production in Florida Bay: evidence from C:N:P ratios of the dominant seagrass *Thalassia testudinum*. *Limnol Oceanogr* 37:162–171
- Fourqurean JW, Jones RD, Zieman JC (1993) Processes influencing water column nutrient characteristics and phosphorus limitation of phytoplankton biomass in Florida Bay, FL, USA: inferences from spatial distributions. *Estuar Coast Shelf Sci* 36:295–314
- Glibert PM, Capone DG (1993) Mineralization and assimilation in aquatic, sediment, and wetland systems. In: Knowles R, Blackburn TH (eds) Nitrogen isotope techniques. Academic Press, New York, p 243–272
- Glibert PM, Magnien R, Lomas MW, Alexander J, Fan C, Haramoto E, Trice TM, Kana TM (2001) Harmful algal blooms in the Chesapeake and Coastal Bays of Maryland, USA: comparisons of 1997, 1998, and 1999 events. *Estuaries* 24:875–883
- Goldman JC (1993) Potential role of large oceanic diatoms in new primary production. *Deep-Sea Res* 40:159–168
- Gordon LI, Jennings JC, Ross AA, Krest JM (1994) A suggested protocol for continuous flow automated analyses of seawater nutrients (phosphate, nitrate, nitrite, silicic acid) in the WOCE Hydrographic Program and the Joint Global

- Ocean Fluxes Study. WOCE Operations Manual Vol. 3. WHP Office Rep WHOP 91-1, WOCE Rep No. 68/91, Part 2, Woods Hole, MA
- Healey FP, Hendzel LL (1979) Fluorometric measurements of alkaline phosphatase activity in algae *Freshw Biol* 9: 429–439
- Hitchcock GL, Vargo GA, Forccuci D, Ortner PB (1998) Phytoplankton biomass in Florida Bay, June 1998–July 1999. In: Brock RJ (ed) State of the Bay, The Condition of Florida Bay in 1998. National Park Service SFNRC Tech Rep, Homestead, FL, p 13–16
- Holm-Hansen O, Lorenzen CJ, Holmes RW, Strickland JDH (1965) Fluorometric determination of chlorophyll. *J Cons Perm Int Explor Mer* 30:3–15
- Jeffrey SV, Vesik M (1997) Introduction to marine phytoplankton and their pigment signatures. In: Jeffrey SW, Mantoura RFC, Wright SW (eds) *Phytoplankton pigments in oceanography: guidelines to modern methods*. UNESCO Paris, p 37–85
- Jeffrey SW, Wright SW (1994) Photosynthetic pigments in the Haptophyta. In: Green JC, Leadbeater BSC (eds) *The haptophyte algae*. Clarendon Press, Oxford, p 111–132
- Lapointe BE, Clark MW (1992) Nutrient inputs from the watershed and coastal eutrophication in the Florida Keys. *Estuaries* 15:465–476
- LaRoche J, Nuzzi R, Waters R, Wyman K, Falkowski PG, Wallace DWR (1997) Brown tide blooms in Long Island's coastal waters linked to interannual variability in groundwater flow. *Glob Change Biol* 3:397–410
- Lavrentyev PJ, Bootsma HA, Johengen TH, Cavaletto JF, Gardner WS (1998) Microbial plankton response to resource limitation: insights from the community structure and seston stoichiometry in Florida Bay, USA. *Mar Ecol Prog Ser* 165:45–57
- Li H, Veldhuis MJW, Post AF (1998) Alkaline phosphatase activities among planktonic communities in the northern Red Sea. *Mar Ecol Prog Ser* 173:107–115
- Lomas MW, Glibert PM (1999) Temperature regulation of nitrate uptake: a novel hypotheses about nitrate uptake and reduction in cool-water diatoms. *Limnol Oceanogr* 44: 556–572
- Lomas MW, Glibert PM, Clougherty DA, Huber DE, Jones J, Alexander J, Haramoto E (2001) Elevated organic nutrient ratios associated with brown tide blooms of *Aureococcus anophagefferens* (Pelagophyceae). *J Plankton Res* 23: 1339–1344
- McIvor CC, Ley JA, Bjork RD (1994) Changes in freshwater inflow from the Everglades to Florida Bay including effects of biota and biotic processes: a review. In: Davis SM, Ogden JC (eds) *Everglades. The ecosystem and its restoration*. St. Lucie Press, Boca Raton, FL, p 117–146
- Nance JM (1994) A biological review of the Tortugas pink shrimp fisheries through December 1993. Galveston Laboratory, Southeast Fisheries Science Center, National Marine Fisheries Service, Galveston, TX
- Paerl H (1988) Nuisance phytoplankton blooms in coastal, estuarine, and inland waters. *Limnol Oceanogr* 33:823–847
- Parsons TR, Maita Y, Lalli CM (1984) *A manual of chemical and biological methods for seawater analyses*. Pergamon Press, New York
- Peers SG, Milligan AJ, Harrison PJ (2000) Assay optimization and regulation of urease activity in two marine diatoms. *J Phycol* 36:523–552
- Perry MJ (1972) Alkaline phosphatase activity in subtropical Central North Pacific waters using a sensitive fluorometric method. *Mar Biol* 15:113–119
- Phlips EJ, Badylak S (1996) Spatial variability in phytoplankton standing crop and composition in a shallow inner-shelf lagoon, Florida Bay, Florida. *Bull Mar Sci* 58:203–216
- Phlips EJ, Lynch TC, Badylak S (1995) Chlorophyll *a*, tripton, color, and light availability in a shallow tropical inner-shelf lagoon, Florida Bay, USA. *Mar Ecol Prog Ser* 127: 223–234
- Phlips EJ, Badylak S, Lynch TC (1999) Blooms of the picoplanktonic cyanobacterium *Synechococcus* in Florida Bay, a subtropical inner-shelf lagoon. *Limnol Oceanogr* 44: 1166–1175
- Robblee MB, Barber TR, Carlson PR, Durako MH and 6 others (1991) Mass mortality of the tropical seagrass *Thalassia testudinum* in Florida Bay (USA). *Mar Ecol Prog Ser* 71: 297–299
- Rudnick DT, Chen Z, Childers DL, Boyer JN, Fontaine TD III (1999) Phosphorus and nitrogen inputs to Florida Bay: the importance of the Everglades watershed. *Estuaries* 22: 398–416
- Sakamoto T, Bryant DA (2001) Requirement of nickel as an essential micronutrient for the utilization of urea in the marine cyanobacterium *Synechococcus* sp. PCC 7002. *Microbes Environ* 16:177–184
- Solórzano L, Sharp JH (1980) Determination of total dissolved phosphorus and particulate phosphorus in natural waters. *Limnol Oceanogr* 25:754–752
- Szmant AM, Forrester A (1994) Sediment and water column nitrogen and phosphorus distribution patterns in the Florida Keys: SEAKEYS. *Bull Mar Sci* 54:1085–1086
- Tilmant JT (1989) A history and an overview of recent trends in the fisheries of Florida Bay. *Bull Mar Sci* 44:3–22
- Van Heukelem L, Thomas CS (2001) Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. *J Chromatogr A* 910:31–49
- Van Heukelem L, Lewitus A, Kana TM, Craft N (1994) Improved separation of phytoplankton pigments using temperature-controlled high performance liquid chromatography. *Mar Ecol Prog Ser* 114:303–313
- Zieman JC, Fourqurean JW, Iverson RL (1989) Distribution, abundance and productivity of seagrasses and macroalgae in Florida Bay. *Bull Mar Sci* 44:292–311
- Zieman JC, Fourqurean JW, Frankovich TA (1999) Seagrass die-off in Florida Bay: long-term trends in abundance and growth of turtle grass *Thalassia testudinum*. *Estuaries* 22: 460–470

Editorial responsibility: Otto Kinne (Editor), Oldendorf/Luhe, Germany

Submitted: September 15, 2003; Accepted: May 18, 2004  
Proofs received from author(s): September 30, 2004