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## Interannual Differences in Nutrient Dynamics During a Brown Tide Bloom (*Aureococcus anophagefferens*) and the Interaction of *A. anophagefferens* with Heterotrophic Bacteria

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**INTERANNUAL DIFFERENCES IN NUTRIENT DYNAMICS DURING A  
BROWN TIDE BLOOM (*AUREOCOCCUS ANOPHAGEFFERENS*) AND THE  
INTERACTION OF *A. ANOPHAGEFFERENS* WITH HETEROTROPHIC  
BACTERIA**

by

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A Dissertation Submitted to the Faculty of  
Old Dominion University in Partial Fulfillment of the  
Requirement for the Degree of

DOCTOR OF PHILOSOPHY

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OLD DOMINION UNIVERSITY  
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## ABSTRACT

### INTERANNUAL DIFFERENCES IN NUTRIENT DYNAMICS DURING A BROWN TIDE BLOOM (*AUREOCOCCUS ANOPHAGEFFERENS*) AND THE INTERACTION OF *A. ANOPHAGEFFERENS* WITH HETEROTROPHIC BACTERIA

George Eric Boneillo  
Old Dominion University, 2010  
Director: Dr. Margaret Mulholland

Blooms of *Aureococcus anophagefferens* (Brown Tides) in Chincoteague Bay were observed over a six-year period (2002-2007) during which interannual differences in nitrogen and carbon uptake and concentrations of dissolved constituents were compared at two sites, one in Maryland and the other in Virginia. Overall, I observed an increase in bloom intensity and duration over time. No single nitrogen compound was responsible for fueling blooms. Instead, *A. anophagefferens* demonstrated the ability to use a wide range of nitrogen compounds to meet its nutritional demands. Results show that  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea, and DFAA were taken up simultaneously during blooms and the dominant source of N varied between years. Although photosynthesis was the dominant form of carbon acquisition, organic carbon uptake contributed up to 30% of the total carbon uptake.

The contribution of *A. anophagefferens* and heterotrophic bacteria to total carbon and nitrogen uptake rates was also examined by using flow cytometry. Results demonstrated that it is possible to distinguish and quantify taxon-specific uptake of C and N by *A. anophagefferens* versus heterotrophic bacteria during incubations of natural assemblages using stable isotopes as tracers coupled with flow cytometry. Bacteria and *A. anophagefferens* cell-specific uptake rates reported here confirm that *A.*

*anophagefferens* uses a wide range of N sources during blooms including  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea, and DFAA-N and it, and not bacteria, are the dominant consumers of these resources in the environment. This finding has important implications for bacterial productivity studies that assume bacteria are the primary consumers of the amino acids.

C and N uptake was also examined over many diel light cycles to determine if dark C and N uptake augments photosynthetic C uptake and DIN uptake by *A. anophagefferens* during the day. Results demonstrated that *A. anophagefferens* actively takes up both organic C and organic and inorganic N during the day and night. This finding is critical for understanding the N and C nutrition of this organism because current dogma is that C uptake by photoautotrophs is limited to daylight hours and N uptake at night is low and limited to particular N compounds and environmental conditions.

This dissertation is dedicated to my parents, George and Roxane Boneillo, for all their love and support.

## **ACKNOWLEDGMENTS**

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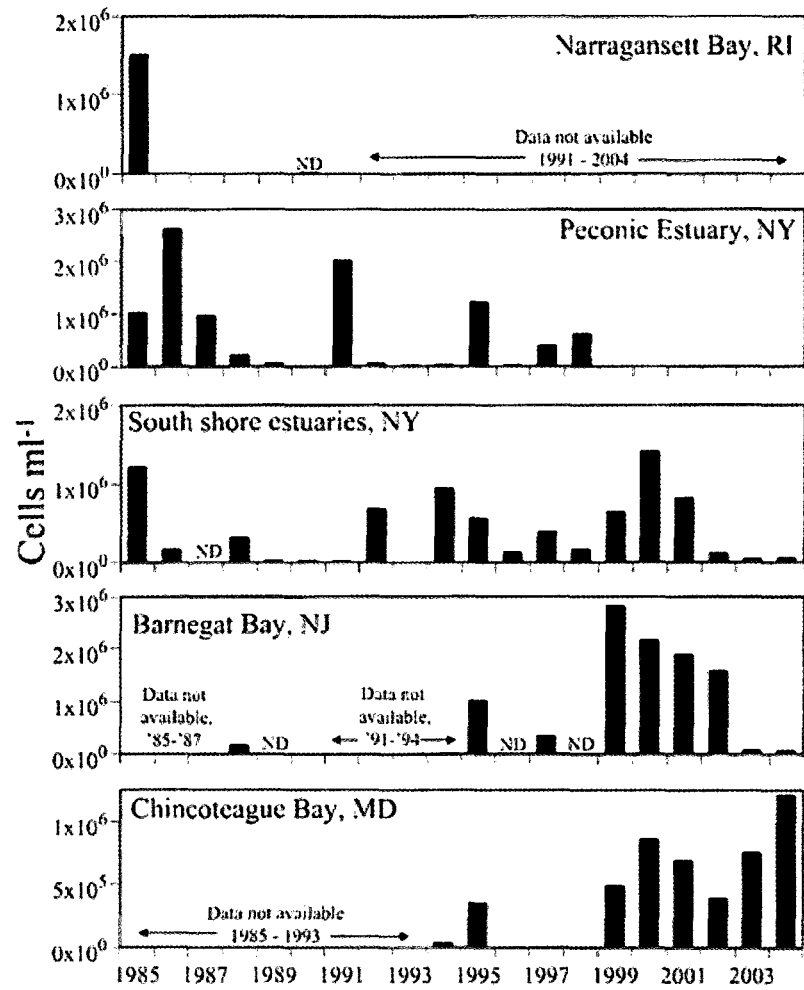
## CHAPTER I

### INTRODUCTION

#### Introduction

*Aureococcus anophagefferens* is a 2-3  $\mu\text{m}$  spherical pelagophyte that can cause harmful algal blooms. Blooms of *A. anophagefferens*, frequently referred to as brown tides, were first observed in 1985 in Great South Bay and Peconic Bay, New York (Nuzzi and Water 1989); Narragansett Bay, Rhode Island (Sieburth et al. 1988); and Barnegat Bay, New Jersey (Olsen 1989). Since then, brown tide blooms have occurred regularly in Long Island coastal waters and other coastal embayments along the Northeast coast of the US (Fig. 1) (Milligan and Cisper 1997; Bricelj and Lonsdale 1997). Surveys of *A. anophagefferens* abundance have detected at background concentrations (1-200 cells  $\text{mL}^{-1}$ ) as far north as Maine (Anderson et al. 1993) and as far south as Florida (Popels et al. 2003). Blooms of *A. anophagefferens* have also been documented outside the United States in Saldanha Bay, South Africa (Pitcher and Calder 2000; Probyn et al. 2001; Probyn et al. 2010).

Although *A. anophagefferens* has been detected all along the US east coast, harmful blooms (concentrations  $>35,000$  cells  $\text{mL}^{-1}$ ; Gastrich and Wazniak 2002) have been limited to the mid-Atlantic and Northeast regions. Recently category 3 brown tides (concentrations  $>200,000$  cells  $\text{mL}^{-1}$ ; Gastrich and Wazniak 2002) occurred in Chincoteague Bay, MD, with cell concentrations reaching over  $0.7 \times 10^6$  cells  $\text{mL}^{-1}$  in 2001 (Maryland DNR, [www.dnr.state.md.us/coastalbays](http://www.dnr.state.md.us/coastalbays)) and  $1.2 \times 10^6$  cells  $\text{mL}^{-1}$  in 2002 (Mulholland et al. 2009). During 2002, blooms were not observed in the Virginia



**Fig. 1** Maximum annual abundances of *A. anophagefferens* in Narragansett Bay, Rhode Island, Peconic Estuary, New York, South shore estuaries of Long Island, New York, Barnegat Bay, New Jersey, and Chincoteague Bay, Maryland from 1985 to 2003. ND indicates no data available for a given location and year (From Gobler et al. 2005)

(southernmost) part of Chincoteague Bay. This changed in 2003, when the first documented bloom occurred in Virginian waters, with *A. anophagefferens* concentrations approaching  $0.5 \times 10^6$  cells  $\text{mL}^{-1}$ . Although the first observed bloom occurred in MD during 2001, HPLC pigment records have shown that *A. anophagefferens* has been present in Chincoteague Bay since at least 1993 and a category 3 bloom ( $>200,000$  cells  $\text{mL}^{-1}$ ) was detected in 1995 (Trice et al. 2004). Additionally, the presence of the chemotaxonomic marker (Z)-24-propylidenecholesterol in Peconic Bay sediments suggest that *A. anophagefferens* was present in Long Island waters at least 120 years ago (Giner et al. 2004).

Despite having no known toxin associated with it, *A. anophagefferens* blooms can have negative impacts on aquatic ecosystems due to their high biomass and their ability to negatively affect food web dynamics. Brown tide blooms have recently been classified as ecosystem disruptive algal blooms (Sunda et al. 2006) because of the wide spread impact they can have on the environment. *A. anophagefferens* have been shown to impact microzooplankton and mesozooplankton grazing rates (Gobler et al. 2002; Caron et al. 2004; Sieracki et al. 2004) and may be responsible for a zooplankton community shift (Deonarine et al. 2006).

At bloom concentrations, *A. anophagefferens* scatters or blocks light (Bricelj and Lonsdale 1997) resulting in light limitation for other phytoplankton, seagrasses, and benthic algae. Low light conditions associated with brown tides have a detrimental effect on eelgrass (*Zostera marina*) (Cosper et al. 1987). The loss of eelgrass beds in Peconic Bay due to brown tides has resulted in high mortality rates (64-82%) (Bricelj and Lonsdale 1997) for scallops (*Argopecten irradians*), resulting in a 2 million dollar a year

loss for the fishery (Kahn and Rockel 1988).

In addition to reducing light penetration, blooms of *A. anophagefferens* inhibit gill ciliary activity and thereby feeding in several species of shellfish including *Mytilus edulis*, *Crassostrea virginica*, *Ostrea edulis*, *Modiolus modiolus* (Gainey and Shumway 1997) and *Mercenaria mercenaria* (Gainey and Shumway, 1997; Bricelj et al. 2001; Greenfield and Lonsdale 2002; Wazniak and Glibert 2004). *A. anophagefferens* has also been shown to affect the growth but not survivorship of *M. mercenaria* larvae (Padilla et al. 2006). In Maryland, *A. anophagefferens* concentrations of only 20,000 cells mL<sup>-1</sup> had a negative impact on juvenile *M. mercenaria* growth rates (Wazniak and Glibert 2004). Since *A. anophagefferens* has been found to be nutritionally adequate for bivalves (Bricelj et al. 1989), the reduction of gill ciliary activity is most likely a result of the exocellular polysaccharide-like layer associated with *A. anophagefferens* (Sieburth et al. 1988; Gainey and Shumway, 1997).

Dissolved oxygen (DO) concentrations may also be impacted by blooms of *A. anophagefferens*. The fact that *A. anophagefferens* blooms occur in shallow, well mixed estuaries may prevent drastic decreases in DO concentrations. In addition, the small size of *A. anophagefferens* may prevent sinking and associated increase in sediment biological oxygen demand (Briceli and Lonsdale 2001). However, since *A. anophagefferens* blooms are becoming more intense and lasting longer in some areas (see Chapter II), an increase in biological oxygen demand may be associated with these blooms. Large die-offs of *Zostera marina* (eelgrass) and shellfish may contribute to oxygen demand and low DO concentrations.

Embayments where blooms of *A. anophagefferens* have been observed are

typically shallow lagoons with high salinities and long residence times that are depleted in dissolved inorganic nitrogen (DIN) but have high dissolved organic N (DON) concentrations and high DON:DIN ratios (Lomas et al. 2004). This may not always be the case however. During a 2002 *A. anophagefferens* bloom in Chincoteague Bay, Mulholland et al. (2009) compared a bloom site to a non bloom site. Both sites had elevated DON:DIN due to depleted DIN concentrations (Mulholland et al. 2009).

One goal of this dissertation was to undertake a multiyear comparison of bloom dynamics within Chincoteague Bay, including comparisons between sites. While there are numerous studies reporting results from sampling during blooms, there are few that have examined interannual differences in bloom dynamics. By comparing physical, chemical, and biological data from multiple years (Chapter II), I hoped to determine the common factors contributing to and promoting bloom formation and persistence. Since most studies focus on a single bloom event, I hoped that a multiyear comparison might provide a better understanding of common features of *A. anophagefferens* blooms.

Since the first recorded *A. anophagefferens* bloom in North America over 25 years ago, numerous studies have been carried out to determine the causes of these blooms, including the unique environmental and nutrient conditions during blooms, the physiological aspects of this species, the nutrient uptake during blooms, and grazing control of blooms. Despite two decades of research, many questions remain unanswered. Although there are monitoring results that report nutrient concentrations and *A. anophagefferens* abundances, there have been few process-oriented studies comparing C and N uptake in natural systems and none that have done this at the same site over multiple years. This study was designed to address some of these unresolved questions

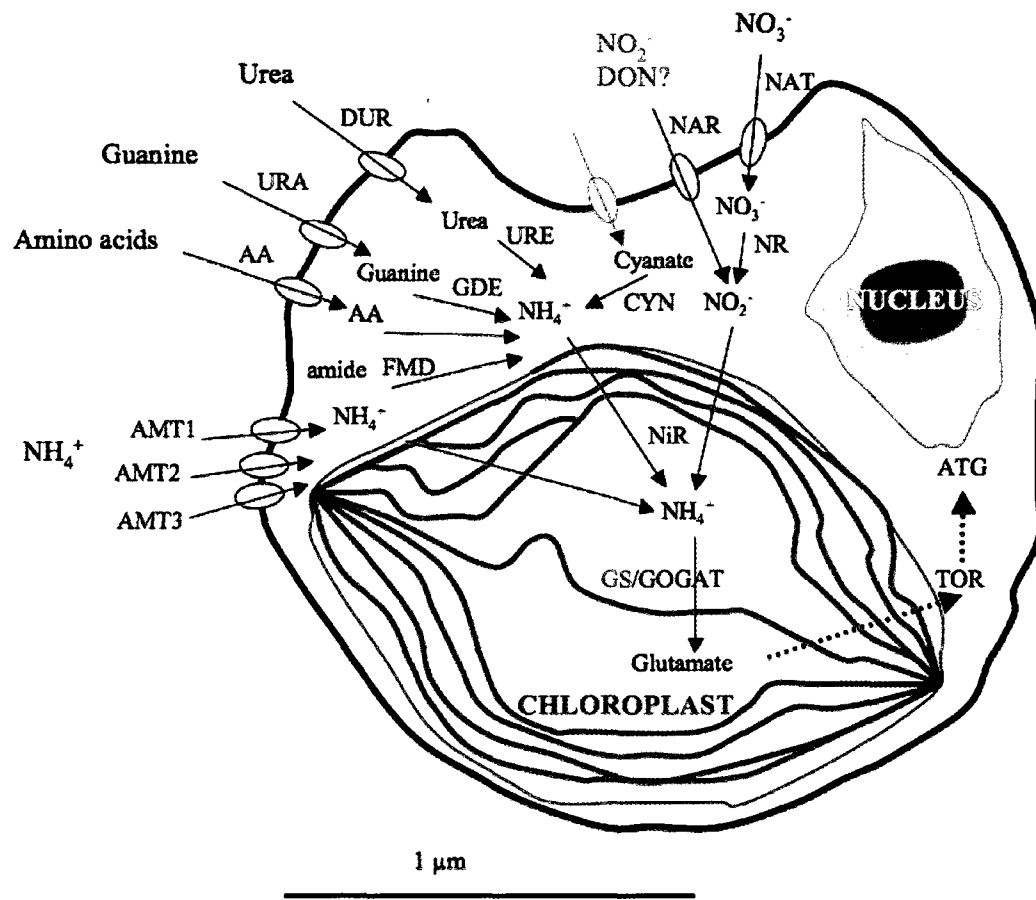


and to examine them over a multiyear period.

*A. anophagefferens* has shown the capacity to take up a wide range of nitrogen (N) compounds to support its growth (Fig. 2). A recent study of the *A. anophagefferens* genome has determined that *A. anophagefferens* can utilize at least eight different forms of N (Berg et al. 2008). *A. anophagefferens* has a high affinity for ammonium ( $\text{NH}_4^+$ ) and urea (Lomas et al. 1996; Berg et al. 1997) but can obtain a significant amount of its nitrogen through the uptake of dissolved free amino acids (DFAA) (Mulholland et al. 2002) and nitrate ( $\text{NO}_3^-$ ) (Mulholland et al. 2009) when these compounds are available. Since *A. anophagefferens* can take up both DIN and DON, it may have a competitive advantage over phytoplankton species that can use only DIN. This advantage may allow it to form monospecific blooms in estuaries where N sources are diverse and N is recycled many times before it is exported to the coastal ocean or sediments.

In addition to N, studies have shown that while *A. anophagefferens* performs photosynthesis, it can also acquire carbon from dissolved organic carbon (DOC) compounds. Dzurica et al. (1989) showed that cultures of *A. anophagefferens* were capable of taking up  $^{14}\text{C}$  labeled glucose and glutamic acid. Field studies using dually labeled  $^{15}\text{N}$  and  $^{13}\text{C}$  organic compounds as tracers demonstrated that *A. anophagefferens* takes up both the carbon and nitrogen from amino acids (Mulholland et al. 2002). Other field studies have shown that the addition of DOC stimulates the growth of *A. anophagefferens* (Gobler and Sanudo-Wihelmy 2001a) and that during intense monospecific blooms, there can be a significant drawdown the DOC pool (Gobler et al. 2004), suggesting that *A. anophagefferens* is using DOC.

Despite the recognition that this species is mixotrophic (it acquires C both auto-



**Fig. 2** Schematic of partial N transport and assimilation network present in *A. anophagefferens* (From Berg et al. 2008)

and heterotrophically) it is unclear to what extent it manifests this ability in nature. Also it is not known how the ratio of autotrophic versus heterotrophic uptake changes over the diel light cycle or over the course of blooms as nutrients and CO<sub>2</sub> are drawn down. At the onset of blooms, light is probably not limiting for photosynthetic C uptake and most carbon may be acquired via photosynthesis. However, as blooms progress, and biomass increases, light may become increasingly limited due to self-shading. If *A.*

*anophagefferens* can take advantage of DOC to augment photosynthetic C acquisition, it might be able to outcompete strictly autotrophic species that may become light limited when cell densities are high. Similarly, if *A. anophagefferens* can take up DOC during the day or night, it could supplement its photosynthetic C uptake during the day. In addition to C, taking up DOM could give *A. anophagefferens* a competitive advantage over other phytoplankton because it can supply nitrogen, phosphorus, and other elements that are unavailable to strict autotrophs.

Although *A. anophagefferens* can take up both organic and inorganic N and C, it is not known to what degree *A. anophagefferens* competes with heterotrophic bacteria for these compounds in the environment. Heterotrophic bacteria use a wide range of nitrogen compounds, including DIN and DON, but as heterotrophs, rely on organic C to meet their C demand. Bacteria have been shown to take up NH<sub>4</sub><sup>+</sup> (Wheeler and Kirchman 1986; Keil and Kirchman 1991; Lipschultz 1995; Hoch and Kirchman 1995; Tungaraza et al. 2003; Fouilland et al. 2007), NO<sub>3</sub><sup>-</sup> (Horrigan et al. 1988; Harrison and Wood 1998; Kirchman and Wheeler 1998; Kirchman et al. 1994; Lipschutz 1995; Middleburg and Nieuwenhuize 2000; Allen et al. 2002; Fouilland et al. 2007), urea (Middleburg and Nieuwenhuize 2000; Tungaraza et al. 2003; Jørgensen 2006; Fouilland

et al. 2007; Sanderson et al. 2008; Bradley et al. 2010), DFAA (Wheeler and Kirchman 1986; Keil and Kirchman 1991; Jørgensen et al. 1993; Kirchman et al. 1994), dissolved combined amino acids (DCAA) (Jørgensen et al. 1993; Kroer et al. 1994), and other organic nitrogen compounds such as DNA (Jørgensen et al. 1993). Studies have also shown that *A. anophagefferens* takes up the inorganic N compounds listed above (Dzurica 1989; Lomas et al. 1996; Berg et al. 1997; Mulholland et al. 2002). In addition, like many bacteria, *A. anophagefferens* appears capable of extracellular peptide hydrolysis and amino acid oxidation (Mulholland et al. 2002; 2004; 2009). The fact that both *A. anophagefferens* and heterotrophic bacteria are capable of using the same carbon and nitrogen compounds to support their growth leads us to question whether these two groups may be competing for the same nutrient resources in the environment.

Direct competition between bacteria and *A. anophagefferens* was examined in one previous study. Berg et al. (2002) showed that three strains of bacteria, isolated from an *A. anophagefferens* culture, had higher mean hydrolysis rates for urea and acetamide than *A. anophagefferens*. However, *A. anophagefferens* was able to hydrolyze aminopeptide and chitobiose at higher rates than the bacterial strains. These observations were among the first to demonstrate that *A. anophagefferens* has diverse metabolic capabilities allowing them to use a variety of organic N compounds. Recent genomic evidence confirms the capacity of *A. anophagefferens* to exploit diverse N resources (Berg et al. 2008).

During the development of spring brown tide blooms, bacteria and *A. anophagefferens* concentrations appear to increase in tandem, however, peak bacteria concentrations continue to increase after the bloom (Gobler and Sanudo-Wihelmy 2001a;

Mulholland et al. 2002). In 2000, bacterial cell densities increased as *A. anophagefferens* concentrations increased in Quantuck Bay, New York, with the highest bacterial cell concentrations observed after the peak in the brown tide bloom in June, 2000 (Mulholland et al. 2002). Similarly, in West Neck Bay, NY, in 1998, bacterial populations reached their maximum cell densities immediately after *A. anophagefferens* began to die off (Gobler and Sanudo-Wihelmy 2001a). Similar dynamics were observed during a 2002 *A. anophagefferens* bloom in Chincoteague Bay, MD when bacterial abundance and DOC concentrations peaked after the *A. anophagefferens* bloom began to decline (Mulholland et al. 2009).

It is possible that *A. anophagefferens* and heterotrophic bacteria are directly competing for nutrients in the environment. The most practical way to test this hypothesis is to simultaneously measure nutrient uptake rates for both *A. anophagefferens* and bacteria under natural environmental conditions. However, this is difficult because *A. anophagefferens* and bacteria are similar in size, and size fractionation techniques fail to completely separate the two groups. Further, filters can easily clog, so even if there were big differences between the two groups in cell size, small cells can be retained on filters, thereby making it difficult to attribute uptake to either group. In order to overcome the problems associated with size fractionation, I used flow cytometry to sort bacteria and *A. anophagefferens* to estimate taxa-specific uptake of N and C using stable isotopes as tracers. Results are reported in Chapter III.

Because *A. anophagefferens* is capable of photosynthesis, I hypothesized that uptake of organic compounds would be low during the day when cells have sufficient light to perform photosynthesis. In contrast, when light reaching the cells is limited due

to self shading or during the night, cells may augment photosynthetic C uptake with the uptake of organic C.

Studies have demonstrated that *A. anophagefferens* can take up nutrients and grow at low light levels (Dzurica 1989; Lomas et al. 1996; Milligan and Cosper 1997). Further, *A. anophagefferens* is adapted to low light environments (Yentsch et al. 1989; MacIntyre et al. 2004) and is prone to photoinhibition when light levels are high (MacIntyre et al. 2004). Cultures of *A. anophagefferens* have been shown to survive in the dark for 30 days (Popels and Hutchins 2002). In this study, I examined not only how carbon and nitrogen uptake vary over the course of blooms but also how carbon and nitrogen uptake vary over diurnal light cycles. While previous studies demonstrated that cultures of *A. anophagefferens* can take up organic carbon during both the light and dark periods (Dzurica 1989), most studies of N and C uptake by natural populations have been conducted during the day when cells should be primed to photosynthesize. If *A. anophagefferens* can incorporate inorganic carbon during the day and organic carbon at night, it would have a significant advantage over organisms that rely solely on photosynthesis for carbon acquisition. To test this hypothesis, nutrient uptake experiments were conducted over several diel cycles and during multiple years and during different stages of bloom development to determine whether there were differences in nutrient uptake dynamics over 24-diel light cycles during bloom initiation, peak bloom, or during bloom demise. Results are reported in Chapter IV.

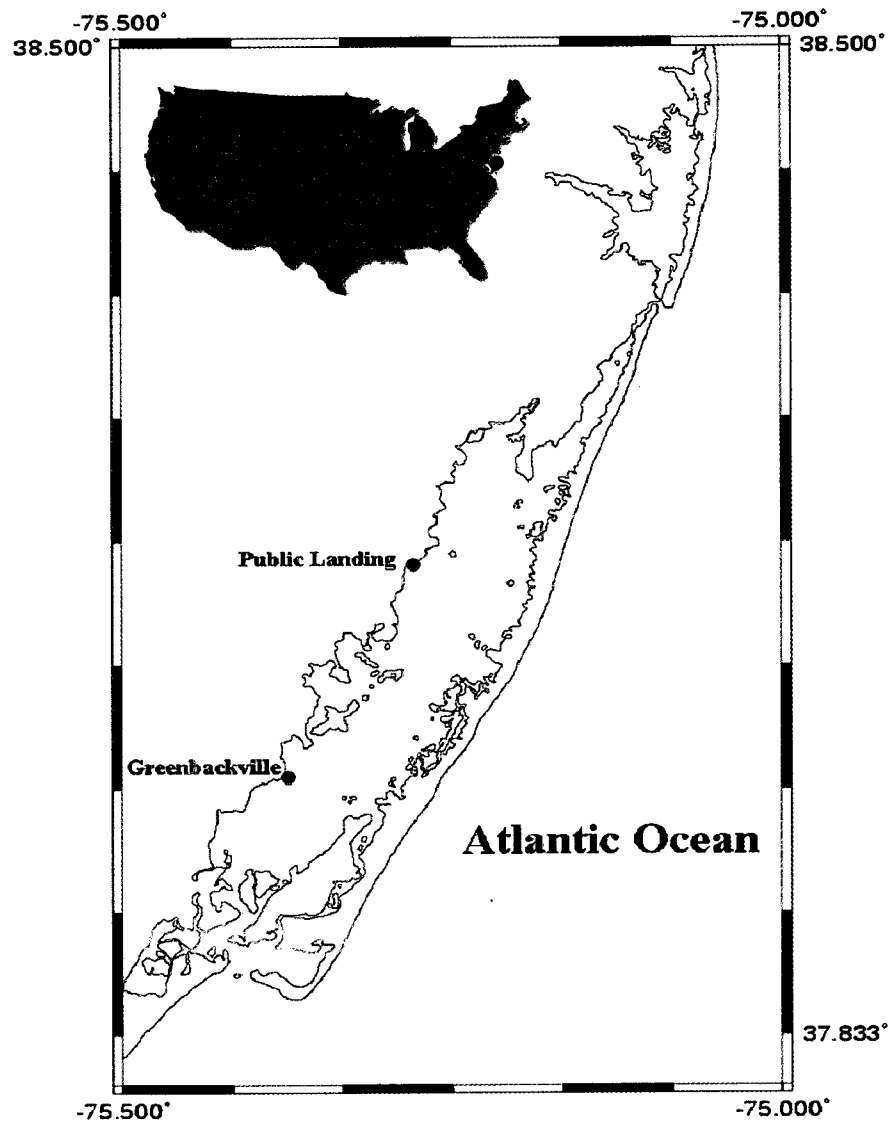
#### **Study area: Chincoteague Bay**

All experiments for this study were performed in Chincoteague Bay where *A. anophagefferens* blooms have been documented since 1995 (Trice et al. 2004).

Chincoteague Bay is a shallow (average depth 1.22 meters) lagoon located on the mid-Atlantic North American continental shelf between 37.5° and 38.2°N latitude (Fig. 3, Boynton 1996). This embayment extends from Maryland to Virginia, has a surface area of 377 km<sup>2</sup>, and has two small inlets at its southern and northern ends that allow it to exchange water with the Atlantic Ocean. This configuration causes the bay to have a fairly long residence time (about 63 days; Pritchard 1960). However, because of the lack of riverine input, the salinity in Chincoteague Bay is close to that of seawater and ranged between 21-33 ppt during this study.

The watershed for the bay is approximately 316 square km and is made up mostly of forested areas and wetlands. One third of the watershed is made up of agricultural lands (Bratton et al. 2009) to which fertilizers that now contain a high percentage of urea are commonly applied (Glibert et al. 2006). Total nitrogen loading for Chincoteague Bay during a year with average rainfall is 576,470 Kg N yr<sup>-1</sup> with 55% of that load coming from diffuse sources, 45% from atmospheric source and a small percent from point sources (Boynton 1996).

Since there is little river input, a major source of freshwater coming into Chincoteague Bay is groundwater. Groundwater is either rapidly discharged near the shoreline or it enters Chincoteague Bay from a subestuarine semi-confined flow system that is recharged by onshore aquifers (Bratton et al. 2009). NO<sub>3</sub><sup>-</sup> concentrations in groundwater collected in 2000 at Public Landing were 137 μM (Dillow et al. 2002). Dillow et al. (2002) also determined the age of this groundwater to be less than 30 years old. Nutrient concentrations of pore water collected offshore of Public Landing (depth 48cm) showed that NH<sub>4</sub><sup>+</sup> was the dominant form of nitrogen in pore waters with



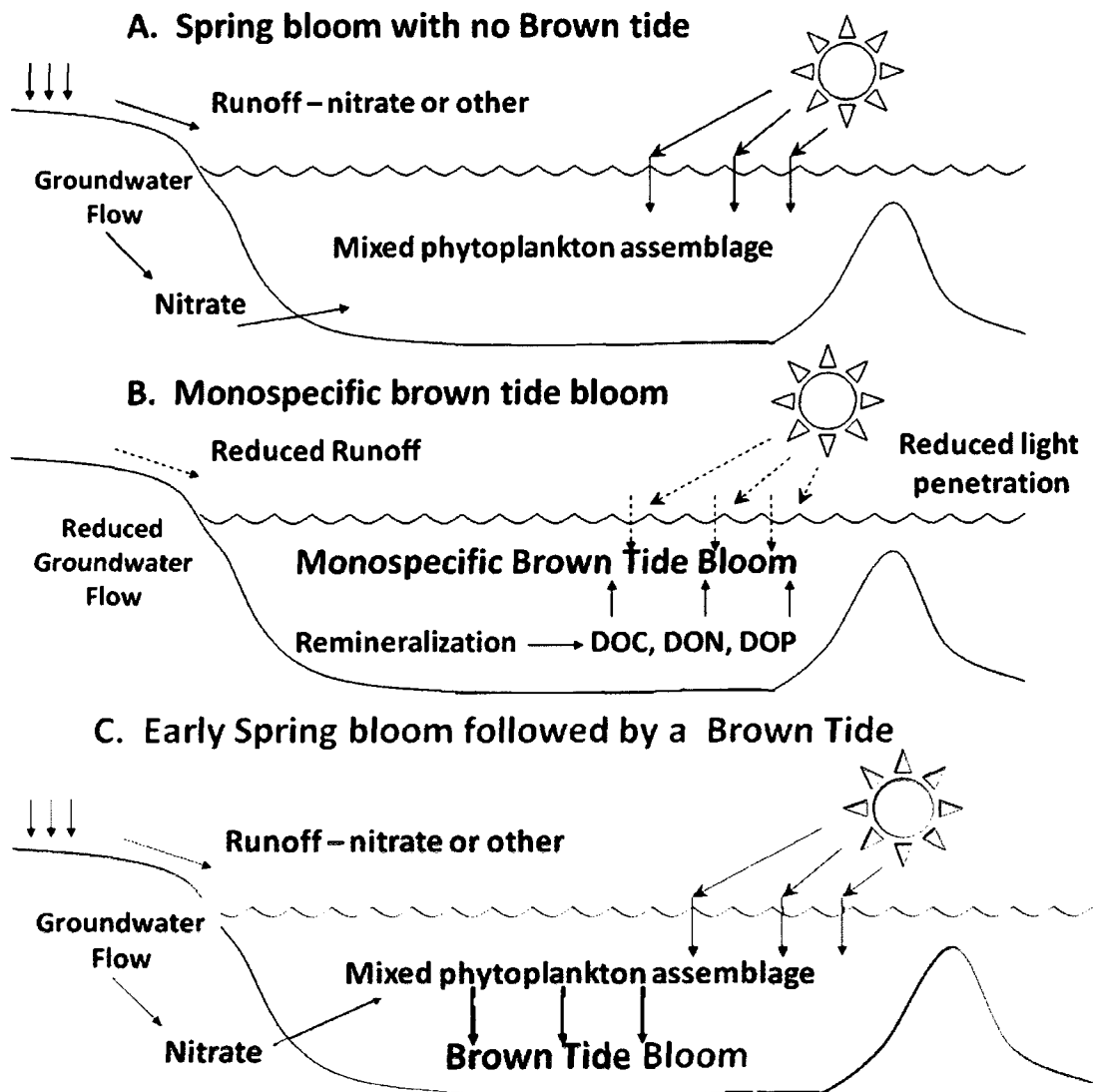
**Fig. 3** Map of the study sites at Public Landing (PL), MD, and Greenbackville (GB), VA, in Chincoteague Bay, a mid-Atlantic coastal lagoon



maximum concentrations of 760  $\mu\text{M}$  (Bratton et al. 2009). The age of this water was over 50 years old (Bratton et al. 2009) suggesting that current nutrient loading of groundwater will have a long term effect on bay.

Initially it was hypothesized that on Long Island, brown tides occurred during years in which precipitation and groundwater discharge were low (LaRoche et al. 1997). Since groundwater on Long Island typically has high  $\text{NO}_3^-$  concentrations (Capone and Bautista 1985; Gobler and Sanudo-Wihemy 2001b), high discharge would result in an increase in the  $\text{NO}_3^-$  concentrations in the water column and a decrease in the DON:DIN ratio. It has been hypothesized that this  $\text{NO}_3^-$  would be available to both non-brown tide phytoplankton and *A. anophagefferens*, but favor non-brown-tide cell growth (Gobler and Sanudo-Wihemy 2001b) (Fig. 4A). When discharge is low,  $\text{NO}_3^-$  levels in the water column decrease and DON:DIN ratios increase. Since *A. anophagefferens* appears to take up a wide range of nitrogen sources, including organic N, this would allow *A. anophagefferens* to outcompete any non brown tide phytoplankton species for organic N compounds (Fig. 4B). Gobler et al. (2001b) provided a conceptual model of how elevated  $\text{NO}_3^-$  concentrations could also lead to a brown tide bloom (Fig. 4C). This model suggests that if a large spring bloom of a non-brown-tide phytoplankton occurs due to  $\text{NO}_3^-$  loading, it would draw down  $\text{NO}_3^-$  and the recycling of the organic matter after the blooms demise would create available DOC, DIN and DON, and DOP that could fuel *A. anophagefferens* growth (Fig. 4C).

An analysis of data from 1996-2004 shows that total dissolved nitrogen (TDN) concentrations have been increasing throughout the coastal bays of Maryland, including Chincoteague Bay (Glibert et al. 2007). While DIN concentrations have remained fairly



**Fig. 4** Conceptual model of brown tide formation. (Modified from Cosper et al. 1989 and Gobler et al. 2001)

constant over this time period, DON concentrations have nearly doubled (Glibert et al. 2007). This has led to an increase in the DON:DIN ratio in Maryland's coastal bays. The high DON to DIN ratio, the long residence times, and the shallow depth of Chincoteague Bay makes this system very similar to other embayments prone to brown tide blooms including Narragansett Bay (Rhode Island), Great South Bay and Peconic Bay (New York), and Barnegat Bay (New Jersey). Chlorophyll concentrations have been also increased in Chincoteague Bay over the past decade. From 1996-2004, mean summertime chlorophyll concentrations have nearly doubled and are positively correlated with DON concentrations (Glibert et al. 2007).

### **Objectives**

The overall objective of this study was to better understand the causal factors promoting brown tide blooms in coastal embayments so that they might be controlled or prevented in the future. This study focused on Chincoteague Bay because it regularly experiences brown tide blooms. With the geographic range of *A. anophagefferens* apparently increasing (Popels et al. 2003), understanding factors contributing to blooms in this system might give important clues regarding what causes, sustains, and terminates brown tide blooms.

The main goals of this study were to:

- 1) Examine the interannual differences in nutrient dynamics during brown tide blooms and to identify common environmental conditions or processes that promote brown tide blooms.

- 2) Determine the contribution of *A. anophagefferens* versus heterotrophic bacteria to total carbon and nitrogen uptake rates using flow cytometry. This method can distinguish more specifically the nutrient uptake attributable to each of the two groups. I hypothesize that *A. anophagefferens* and heterotrophic bacteria are competing for the same resources.
- 3) To determine whether dark C and N uptake augments photosynthetic C uptake and DIN uptake by *A. anophagefferens* during the day. I hypothesize that *A. anophagefferens* has the ability to utilize carbon throughout the day and night.

## CHAPTER II

### INTERANNUAL DIFFERENCES IN NUTRIENT DYNAMICS DURING BROWN TIDE (*AUREOCOCCUS ANOPHAGEFFERENS*) BLOOMS IN A COASTAL EMBAYMENT

#### Introduction

Since 1985, *Aureococcus anophagefferens* blooms (brown tides) have occurred regularly in coastal lagoons along Long Island, New York and other embayments along the Northeast coast of North America (Milligan and Cosper 1997; Bricelj and Lonsdale 1997). Surveys of *A. anophagefferens* abundance have shown that its geographic distribution has spread along the entire east coast of the United States (US), with background concentrations ( $1\text{-}200\text{ cells mL}^{-1}$ ) being detected as far north as Maine (Anderson et al. 1993) and as far south as Florida (Popels et al. 2003). Although background concentrations have been detected all along the US east coast, harmful blooms (concentrations  $>35,000\text{ cells mL}^{-1}$ ; Gastrich and Wazniak 2002) have been confined to coastal embayments between Massachusetts and Maryland. In this study I report on major brown tides occurring in Maryland and Virginia coastal bays between 2002 and 2007.

Brown tide blooms typically occur in systems that are shallow, have high salinities, and long residence times. The scale and density of brown tide blooms has been related to the magnitude of preceding spring phytoplankton blooms and the nitrate ( $\text{NO}_3^-$ ) inputs fueling them. However, populations of *A. anophagefferens* in NY coastal waters typically flourish only after  $\text{NO}_3^-$  concentrations have been depleted (Gobler and Sañudo-

Wilhelmy 2001a), when dissolved inorganic nitrogen (DIN) concentrations are low (Lomas et al. 2004), or in years when groundwater  $\text{NO}_3^-$  inputs are greatly reduced (LaRoche et al. 1997). Blooms also vary in frequency as well as intensity from year to year.

*A. anophagefferens* has the ability to take up a wide range of nitrogen (N) sources to meet its N requirement. Previous studies have shown that *A. anophagefferens* has a high affinity for ammonium ( $\text{NH}_4^+$ ) and urea (Lomas et al. 1996; Berg et al. 1997). Other studies have shown that *A. anophagefferens* can also obtain a significant amount of its N through the uptake of dissolved free amino acids (DFAA) (Mulholland et al. 2002). Since *A. anophagefferens* can utilize both DIN and dissolved organic N (DON) compounds, this organism may have a competitive advantage over other phytoplankton species that can only use DIN. High concentrations of DON relative to DIN have been linked to blooms of this organism (Lomas et al. 2004).

Previous work has demonstrated that *A. anophagefferens* can also take up dissolved organic carbon (DOC) compounds (Dzurica et al. 1989; Mulholland et al. 2002) and organic C inputs appear to stimulate *A. anophagefferens* growth rates in the field (Gobler and Sañudo-Wilhelmy 2001b). During intense monospecific blooms, there can be a significant drawdown of the DOC pool (Gobler et al. 2004), suggesting that *A. anophagefferens* is taking up compounds from that pool in the environment. The capacity to take up DOC may supplement autotrophic C uptake via photosynthetic  $\text{CO}_2$  fixation and may be advantageous during blooms when cell densities are high (e.g.,  $1.0 \times 10^6$  cells  $\text{mL}^{-1}$ ) and self-shading or depletion of dissolved inorganic C (DIC) limits photosynthetic C uptake (Mulholland et al. 2009a).

Despite recognition that this species is mixotrophic, the proportion of the carbon demand that is obtained through autotrophic uptake of DIC versus heterotrophic uptake of DOC has not been previously evaluated for *A. anophagefferens*. Similarly it is not known how autotrophic and heterotrophic C uptake changes over the course of blooms. Mixotrophic grazing has been shown to be sensitive to light, however, little is known about osmotrophic uptake of DOC by *A. anophagefferens*. While light penetration may be sufficient to allow carbon to be acquired via photosynthesis when cell density is low, as blooms progress and biomass increases, self-shading may limit light penetration, thereby limiting photosynthetic C uptake. If *A. anophagefferens* can compensate for reduced photosynthetic C uptake with heterotrophic C uptake as cell density increases, it may be able to out-compete strictly autotrophic species whose growth may become light or C limited.

In this study, I compared N concentrations with rates of photosynthetic uptake of bicarbonate uptake and the uptake of organic and inorganic N and C during brown tide blooms over the course of several years, between 2002 and 2007, at two sites in Chincoteague Bay, Maryland and Virginia. The two sites are physically similar and contain comparable bacterial and phytoplankton communities (Mulholland et al. 2009a). One site had experienced brown tide blooms since at least 1999, while no blooms had been reported at the other site prior to this study. My goal was to understand nutrient controls of blooms on interannual timescales and to identify possible triggers for blooms in order to identify points at which interventions or management actions might be taken to prevent the initiation of potentially damaging blooms. In addition, I measured photosynthetic versus heterotrophic C uptake over the course of these blooms to

determine how much organic C contributes to the total C nutrition of *A. anophagefferens* populations as blooms initiate, develop and persist.

### **Materials and Methods**

I examined interannual and intersite variability in bloom formation within Chincoteague Bay, a coastal bay along the mid-Atlantic coast of North America that experiences seasonal blooms of *A. anophagefferens*. Chincoteague Bay extends from Maryland to Virginia and has two small inlets at its southern and northern ends that allow it to exchange water with the Atlantic Ocean. This configuration causes the bay to have a fairly long residence time (about 63 days; Pritchard 1960). However, because of the lack of riverine input, the salinity in Chincoteague Bay is closer to that of seawater than freshwater and ranged between 21-33 ppt during this study. The watershed for the bay is approximately 72.6 square miles and is made up mostly of forested areas and wetlands. However, the main source of freshwater coming into Chincoteague Bay is groundwater. Thirty-three percent of the watershed is made up of agricultural lands (Maryland DNR, 2005) to which fertilizers that now contain a higher percentage of urea are commonly applied (Glibert et al. 2006). These nutrients can enter the coastal bays.

Brown tide blooms have been monitored by the Maryland Department of Natural Resources in Chincoteague Bay since 1999. For this study, I selected two sites in Chincoteague Bay, MD and VA; one site that had previously experienced brown tide blooms, Public Landing, Maryland (PL), and one site which there had been no previous reports of blooms as of 2002, Greenbackville, Virginia (GB) (Fig. 1). At each site, a Hydrolab Surveyor 4a Water Quality Multiprobe equipped with sensors for temperature, salinity, pH, dissolved oxygen, and photosynthetically active irradiance (PAR) was



deployed to record physical parameters. Water was collected from just below the surface with acid-cleaned 20 L polyethylene carboys and transported to the Marine Science Consortium laboratory located in Greenbackville, VA. Carboys, buckets, and all other materials associated with the sampling, handling, and storage of seawater during this project were soaked in 10% HCl between sampling events, and rinsed liberally with distilled-deionized water before each use. Because Chincoteague Bay is shallow (~ 4m) and well mixed, it is likely the sample water collected near the surface was representative of the entire water column.

Upon arrival at the laboratory, nutrient samples were filtered using 0.2  $\mu\text{m}$  Supor filter disk (2002 and 2003) or a 0.2  $\mu\text{m}$  Supor cartridge filter (2006 and 2007) and filtrate was frozen in acid-cleaned bottles for subsequent analyses. Nutrient samples were collected within 30 minutes of sample collection. Chlorophyll *a* (Chl *a*) samples were collected onto GF/F filters that were then frozen and analyzed within 2 weeks of their collection. Samples were preserved with glutaraldehyde (1% final concentration) in sterile polycarbonate bottles for later enumeration of bacteria and *A. anophagefferens*. Counts were performed within 72 hours of collection.

Nitrate plus nitrite (hereafter referred to as  $\text{NO}_3^-$  for simplicity), phosphate, urea, and silicate concentrations were determined colorimetrically using an Astoria Pacific Autoanalyzer (Parsons et al. 1984). Ammonium concentrations were determined using the manual phenol hypochlorite method (Solorzano 1969). Total dissolved nitrogen (TDN) and phosphorus (TDP) were measured after persulfate oxidation (Valderrama 1991). Dissolved organic N (DON) and dissolved organic P (DOP) were calculated as the difference between TDN and DIN and TDP and DIP, respectively. Dissolved free

amino acid (DFAA) concentrations were measured using high performance liquid chromatography (HPLC) (Cowie and Hedges 1992).

Chl *a* concentrations were measured using standard fluorometric methods (Welschmeyer 1994). Bacteria and *A. anophagefferens* cells were enumerated using epifluorescent microscopy. Heterotrophic bacteria were first stained with 4',6-di-amidinophenyl-indole (DAPI) as outlined by Porter and Feig (1980). *A. anophagefferens* were enumerated using the immunofluorescence (fluorescein isothiocyanate) method of Anderson et al. (1989). The protocol was modified by doubling the amount of primary and secondary antibody (Mulholland et al. 2009a). Samples were gently (<5 kPa) filtered onto 0.8  $\mu\text{m}$  black polycarbonate filters for counting (Anderson et al. 1989). A minimum of 100 cells were counted per sample in at least 10 fields to yield a relative standard deviation of 9% for replicate counts of the same sample ( $n = 6$ ) at cell densities of  $2 \times 10^5$  cells  $\text{mL}^{-1}$ , within the range of average *A. anophagefferens* cell densities during blooms. Blooms were defined as *A. anophagefferens* concentrations  $>35,000$  cells  $\text{mL}^{-1}$  (Gastrich and Wazniak 2002).

The amount of Chl *a* contributed by *A. anophagefferens* was estimated by assuming a constant Chl *a* content per cell for *A. anophagefferens* ( $0.035 \pm 0.003$  pg  $\text{cell}^{-1}$ ), as has been done previously (Gobler and Sañudo-Wilhelmy 2001a; Gobler et al. 2002; Mulholland et al. 2002). Variability in cellular Chl *a* concentrations could potentially bias these calculations.

Nutrient uptake experiments were conducted from March-October (2002) (Mulholland et al. 2009a), and from May-July (2003, 2006, and 2007) during daylight hours. Incubations for rate measurements were initiated in acid-cleaned polycarbonate

bottles by adding highly enriched (96-99%)  $^{15}\text{N}$  and  $^{13}\text{C}$ -labeled substrates ( $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , urea, bicarbonate, glucose, and leucine) to the respective incubation bottles. Triplicate incubations were performed for each substrate. Urea and amino acids were dually labeled with  $^{15}\text{N}$  and  $^{13}\text{C}$ . Additions of labeled substrate were  $0.03 \mu\text{mol L}^{-1}$ . This represented an atom % enrichment ranging from 1 to 94% but averaged  $<10\%$ . Bottles were then transported to an incubator where temperatures were maintained within  $2^\circ\text{C}$  of ambient levels in Chincoteague Bay under ambient light conditions. The average incoming solar radiation during light incubations ranged from  $49\text{--}2234 \mu\text{E m}^{-2} \text{ sec}^{-1}$  (measured using the Hydrolab dual-PAR sensor).

After 15-30 minutes, incubation experiments were terminated by filtering the sample onto a precombusted ( $450^\circ\text{C}$  for 2 hours) GF/C filter (nominal pore size of  $1.2 \mu\text{m}$ ), rinsed with filtered seawater, and stored frozen until analysis. Light and dark bicarbonate incubations lasted for 2-3 hours. The frozen filters were then dried at  $50^\circ\text{C}$  for 48 hours in a drying oven and pelletized in tin disks. The isotopic composition of samples was determined using a Europa Scientific isotope ratio mass spectrometer (IRMS), equipped with an automated nitrogen and carbon analyzer (ANCA). Uptake rates were calculated using the equations from Mulholland et al. (2006).

N and C content of the DFAA pool was calculated based on the C:N ratio of the ambient DFAA pool from individual HPLC runs during 2002, as described by Mulholland et al. (2002). I established that on average there was  $1.18 (\pm 0.21) \mu\text{mol L}^{-1}$  DFAA-N and  $4.41 (\pm 0.47) \mu\text{mol L}^{-1}$  DFAA-C per  $1 \mu\text{mol L}^{-1}$  DFAA. The ambient dissolved inorganic C (DIC) concentrations were calculated based on salinity assuming that  $\text{CO}_2$  concentrations were saturating in collected samples. The initial glucose

concentration was estimated as 2% of the ambient DOC pool, the lower end of the range estimated by Benner (2-6%; 2002) for marine surface waters.

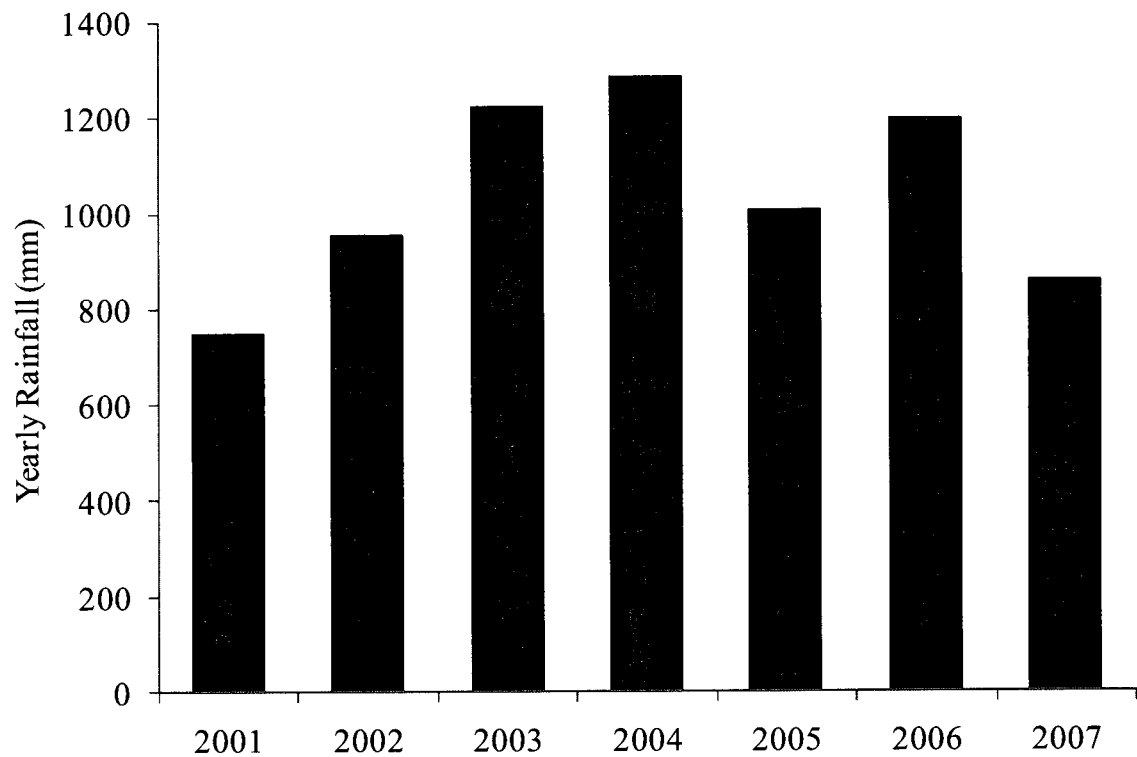
## Results

### *Physical parameters*

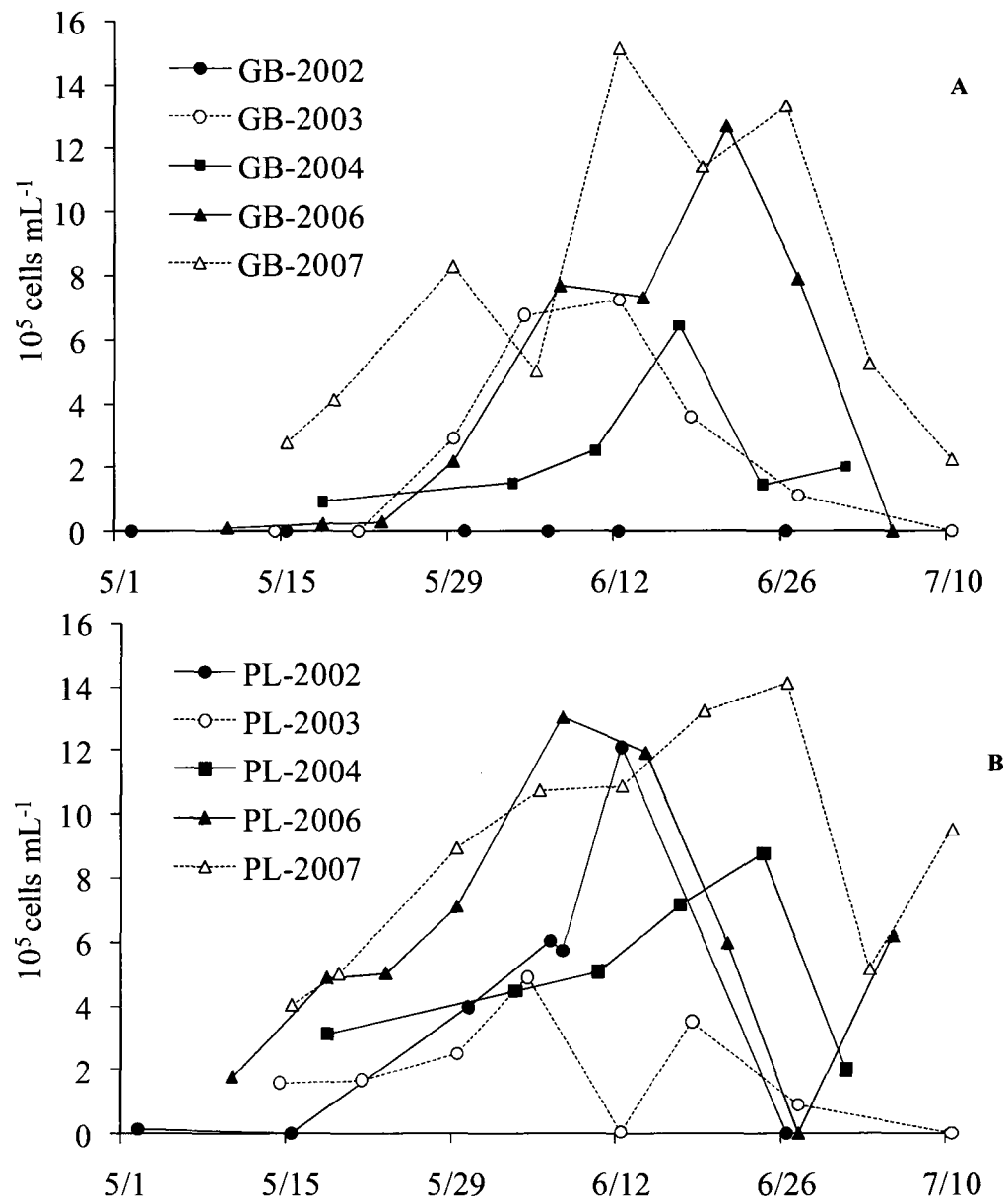
Seasonal temperature patterns did not show much variation from year to year (Table 1, 2). Typically, there was a period of rapid warming between April and May with maximum temperatures at the end of July. Salinity, however, did show a high degree of interannual variation. In 2002, salinities were  $> 30$  throughout the sampling period (Simjouw et al. 2004, Mulholland et al. 2009a). During 2003, a particularly wet year (Fig. 5), salinity ranged from 21.5-27.1 between April and June. In 2006 and 2007, salinities were generally lower than in 2002, ranging from 25.0 – 32.8 (Table 1, 2) but were higher on average than during 2003. Typically, salinities were higher at Greenbackville than Public Landing, likely because GB is closer to Chincoteague Inlet where water is exchanged with the ocean. After large rain events however, the salinity at Greenbackville was lower than that at Public Landing due to its proximity to Swans Gut Creek, which drains into Chincoteague Bay just south of that sampling site (Table 1 and 2).

### *Microbial biomass*

In contrast to 2002 when there was no brown tide bloom and only low brown tide cell density at GB (Mulholland et al. 2009a), there were blooms at both sites in 2003 (Minor et al. 2006), 2004 (Fig. 6), 2006, and 2007 (Fig. 6, Tables 1, 2). During 2003, *A. anophagefferens* abundance at PL increased during the spring and reached a peak concentration of  $4.9 \times 10^5$  cells  $\text{mL}^{-1}$  in June (Fig. 6A, Table 1). Despite there being no



**Fig. 5** Total rainfall for Chincoteague Bay during the study period (2002-2007; 2001 data were added for comparison). Data were collected from Wallops Flight FAC Airport, VA, United States (KWAL). <http://www.wunderground.com>



**Fig. 6** *A. anophagefferens* abundance during 2002, 2003, 2004, 2006, and 2007 blooms in Chincoteague Bay at (A) Greenbackville, VA and (B) Public Landing, MD. Figure includes data reported in Simjouw et al. (2004), Minor et al. (2006), and Mulholland et al. (2009)

**Table 1** Physical, biological and chemical parameters at Public Landing in Chincoteague Bay, MD and VA, during 2003, 2006, and 2007

Date	Sal.	Temp (°C)	pH	Chl <i>a</i> (µg Chl L <sup>-1</sup> )	<i>A. anophagefferens</i> (cells mL <sup>-1</sup> )x10 <sup>5</sup>	Bacteria (cells mL <sup>-1</sup> )x10 <sup>5</sup>	PC (µmol C L <sup>-1</sup> )	PN (µmol N L <sup>-1</sup> )	NH <sub>4</sub> <sup>+</sup> (µmol L <sup>-1</sup> )	NO <sub>3</sub> <sup>-</sup> + NO <sub>2</sub> <sup>-</sup> (µmol L <sup>-1</sup> )	DIP (µmol L <sup>-1</sup> )
<b>2003:</b>											
07 Mar	24.3	4.9	7.9	5.10 (0.1)	BDL	3.86 (0.02)			0.23 (0.01)	0.77 (0.01)	0.33 (0.00)
14 Apr	24.9	12.8	7.9	4.35 (0.3)	BDL	5.15 (0.02)			0.18 (0.02)	1.40 (0.02)	0.20 (0.00)
14 May	26.0	18.8	7.9	0.83 (0.5)	1.59 (0.17)	5.61 (0.04)			0.25 (0.02)	0.93 (0.02)	0.72 (0.00)
29 May	26.5	16.9	8.0	7.15 (0.7)	2.50 (0.31)	13.34 (0.08)			0.17 (0.03)	0.89 (0.00)	0.59 (0.00)
04 Jun	25.2	18.4	8.0	6.87 (1.3)	4.91 (0.53)	12.14 (1.06)			0.28 (0.03)	0.65 (0.01)	0.67 (0.09)
12 Jun	25.9	19.1	8.0	9.66 (0.7)	0.06 (0.01)	11.13 (0.14)			0.36 (0.08)	0.54 (0.02)	0.58 (0.04)
18 Jun	25.7	24.6	7.9	4.52 (0.2)	3.51 (0.56)	12.76 (0.08)			0.15 (0.01)	0.64 (0.00)	0.50 (0.35)
27 Jun	24.5	21.9	7.9	21.8 (0.5)	0.89 (0.16)	15.67 (0.15)			0.14 (0.07)	0.73 (0.00)	0.56 (0.21)
10 Jul	26.0	26.5	8.0	11.2 (0.5)	BDL	10.98 (0.03)			0.29 (0.06)	0.84 (0.02)	0.45 (0.02)
07 Aug	25.8	29.3	7.8	8.04 (0.2)	BDL				0.49 (0.04)	1.03 (0.10)	0.43 (0.04)
<b>2006:</b>											
10 May	30.4	17.1	8.8	6.4 (0.3)	0.17 (0.09)	7.97 (0.59)	109 (5)	14 (0.8)	0.61 (0.03)	0.08 (0.01)	0.06 (0.05)
18 May	30.3	21.0	8.8	8.6 (0.1)	4.88 (0.69)	9.67 (0.38)	164 (9)	15 (1.1)	1.08 (0.02)	0.13 (0.01)	0.11 (0.01)
23 May	31.1	18.3	8.8	8.7 (0.5)	5.03 (0.79)	12.07 (0.88)	225 (11)	20 (0.8)	0.93 (0.05)	0.17 (0.02)	0.03 (0.01)
31 May	31.4	19.3	8.9	12.9 (0.5)	7.11 (0.95)	16.43 (0.80)	251 (13)	22 (0.8)	0.55 (0.01)	0.19 (0.08)	0.07 (0.04)
07 Jun	31.2	21.8	8.7	25.7 (0.4)	13.1 (1.19)	16.19 (0.66)	327 (11)	32 (1.2)	0.64 (0.01)	0.11 (0.01)	0.66 (0.01)
14 Jun	31.8	21.7	8.8	17.6 (0.6)	12.0 (0.58)	17.74 (1.50)	299 (14)	31 (1.5)	0.42 (0.00)	0.21 (0.09)	0.55 (0.08)
21 Jun	30.7	26.5	8.9	28.5 (0.4)	5.96 (0.45)	22.13 (1.81)	342 (15)	33 (1.1)	0.46 (0.08)	0.11 (0.05)	0.46 (0.07)
28 Jun	30.3	25.4	8.6	26.8 (1.6)		25.38 (0.99)	109 (5)	14 (0.8)	0.51 (0.05)	0.15 (0.05)	2.32 (0.00)
05 Jul	30.8	27.8	8.7	11.2 (0.3)	6.19 (0.51)				0.65 (0.02)	0.11 (0.00)	1.58 (0.12)
<b>2007:</b>											
15 May	25.6	18.4	8.8	10.8 (0.1)	4.04 (0.54)	12.80 (0.74)	232 (21)	20 (2.1)	0.25 (0.02)	1.33 (0.01)	0.04 (0.00)
19 May	26.7	19.3	8.9	20.7 (0.3)	5.02 (0.29)	11.54 (0.82)	210 (11)	25 (1.3)	1.07 (0.01)	1.64 (0.01)	0.25 (0.00)
29 May	26.9	24.0	8.8	19.5 (0.2)	8.94 (1.28)	20.13 (1.42)	320 (22)	31 (2.2)	0.33 (0.02)	2.00 (0.08)	0.10 (0.00)
05 Jun	26.9	25.3	8.9	27.7 (0.4)	10.7 (0.87)	22.30 (1.07)	362 (35)	35 (2.5)	0.25 (0.02)	1.31 (0.01)	0.13 (0.00)
12 Jun	27.9	24.3	8.9	34.4 (0.7)	10.9 (1.10)	26.25 (0.78)	429 (23)	38 (2.7)	0.23 (0.01)	1.03 (0.01)	0.36 (0.00)
19 Jun	28.3	25.0	8.9	28.7 (1.0)	13.2 (1.10)	28.07 (1.20)	368 (32)	34 (1.6)	0.18 (0.02)	0.80 (0.01)	0.29 (0.01)
26 Jun	28.7	25.2	8.9	33.6 (0.3)	14.1 (1.59)	28.73 (1.32)	387 (29)	38 (4.0)	0.51 (0.01)	0.02 (0.07)	0.29 (0.01)
03 Jul	28.3	24.3	8.9	21.1 (0.7)	5.15 (0.43)	31.34 (0.98)	191 (20)	27 (1.7)	1.07 (0.01)	0.94 (0.01)	0.78 (0.00)
10 Jul	28.6	28.5	8.8	15.6 (0.9)	9.51 (0.54)	28.79 (1.51)	233 (14)	27 (4.8)	0.82 (0.06)	1.13 (0.00)	0.30 (0.01)

Standard deviations are in parentheses and BDL indicates that analytes were below the limits of analytical detection (about 0.03 µmol L<sup>-1</sup> for nutrients and 0.2 x 10<sup>3</sup> cell mL<sup>-1</sup> for *A. anophagefferens* abundance). Dissolved inorganic N was calculated as the sum of the average NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> concentrations and ratios were calculated from average values. Salinity, temperature, Chl *a* and *A. anophagefferens* abundance from 2003 are also reported in Minor et al. (2006). Empty fields indicate that there were no data

**Table 2** Physical, biological and chemical parameters at Greenbackville in Chincoteague Bay, MD and VA, during 2003, 2006, and 2007

Date	Sal.	Temp (°C)	pH	Chl <i>a</i> ( $\mu\text{g Chl L}^{-1}$ )	<i>A. anophagefferens</i> (cells $\text{mL}^{-1}$ ) $\times 10^5$	Bacteria (cells $\text{mL}^{-1}$ ) $\times 10^5$	PC ( $\mu\text{mol C L}^{-1}$ )	PN ( $\mu\text{mol N L}^{-1}$ )	$\text{NH}_4^+$ ( $\mu\text{mol L}^{-1}$ )	$\text{NO}_3^- + \text{NO}_2^-$ ( $\mu\text{mol L}^{-1}$ )	DIP ( $\mu\text{mol L}^{-1}$ )
<b>2003:</b>											
07 Mar	22.5	6.0	7.8	5.11 (0.0)	BDL	2.67 (0.15)			0.27 (0.00)	1.19 (0.01)	0.42 (0.04)
14 Apr	21.5	12.0	8.1	6.90 (0.0)	BDL	10.78 (0.32)			1.34 (0.04)	0.79 (0.00)	0.26 (0.00)
14 May	26.7	19.0	7.8	0.57 (0.0)	BDL	2.42 (0.21)			0.20 (0.01)	1.48 (0.07)	0.20 (0.17)
29 May	23.6	17.9	7.9	8.25 (0.6)	2.90 (0.33)	8.09 (1.12)			1.01 (0.03)	0.70 (0.00)	0.45 (0.01)
04 Jun	24.2	18.8	8.0	10.89 (0.4)	6.77 (0.40)	13.30 (0.77)			0.21 (0.02)	0.64 (0.02)	0.39 (0.03)
12 Jun	25.1	24.3	8.0	14.88 (2.0)	7.24 (0.51)	9.86 (0.47)			1.31 (0.01)	0.78 (0.04)	0.41 (0.02)
18 Jun	25.7	22.4	8.0	9.51 (0.3)	3.58 (0.43)	9.22 (0.25)			0.27 (0.01)	1.19 (0.03)	0.46 (0.05)
27 Jun	27.1	27.5	8.1	13.73 (1.1)	1.11 (0.06)				0.25 (0.02)	1.47 (0.20)	0.52 (0.22)
10 Jul	27.5	29.3	7.8	15.61 (0.2)	BDL				1.07 (0.01)	1.81 (0.01)	1.15 (0.00)
07 Aug	29.7	25.9	7.9	11.17 (0.9)	BDL				0.33 (0.02)	1.65 (0.00)	0.67 (0.00)
<b>2006:</b>											
10 May	31.8	18.3	8.7	7.1 (0.23)	0.10 (0.02)	10.37 (0.77)	71 (7.0)	7 (0.7)	0.40 (0.01)	0.10 (0.06)	0.69 (0.10)
18 May	31.8	21.9	8.6	7.9 (0.5)	0.22 (0.04)	12.06 (0.50)	171 (11)	10 (1.2)	0.47 (0.05)	0.09 (0.01)	1.23 (0.21)
23 May	32.4	19.2	8.5	2.7 (0.1)	0.29 (0.04)	9.56 (1.30)	44 (4.5)	6 (0.6)	0.48 (0.03)	0.34 (0.02)	0.83 (0.10)
31 May	32.8	20.3	8.6	12.5 (0.2)	2.19 (0.22)	12.68 (1.78)	168 (9.1)	20 (1.1)	0.73 (0.02)	0.31 (0.09)	1.46 (0.08)
07 Jun	32.0	21.4	8.7	24.5 (0.4)	7.69 (0.60)	19.65 (1.98)	121 (7.4)	11 (0.4)	0.48 (0.00)	0.27 (0.04)	1.76 (0.16)
14 Jun	31.2	21.1	8.7	31.4 (0.3)	7.31 (0.55)	19.01 (1.41)	817 (38)	79 (3.2)	0.44 (0.02)	0.18 (0.05)	2.20 (0.20)
21 Jun	31.7	26.4	8.8	26.8 (0.8)	12.7 (0.91)	23.76 (3.75)	309 (17)	32 (0.6)	0.31 (0.00)	0.18 (0.08)	3.24 (0.24)
28 Jun	25.0	25.8	8.5	19.6 (0.2)					0.56 (0.01)	0.15 (0.13)	3.21 (0.03)
05 Jul	27.7	27.7	8.5	12.8 (0.2)	7.90 (1.22)	18.32 (2.39)	71 (7.0)	7 (0.7)	0.53 (0.06)	0.20 (0.07)	4.28 (0.79)
<b>2007:</b>											
15 May	26.6	19.2	8.7	15.3 (0.1)	2.76 (0.18)	11.44 (0.98)	237 (12)	22 (2.5)	0.27 (0.00)	1.43 (0.01)	0.37 (0.00)
19 May	28.2	18.2	8.6	10.4 (0.1)	4.12 (0.36)	12.93 (0.67)	97 (6)	12 (1.5)	1.34 (0.04)	2.13 (0.02)	0.51 (0.00)
29 May	28.4	24.1	8.7	25.3 (0.5)	8.27 (1.00)	21.65 (0.92)	371 (15)	38 (3.1)	0.20 (0.01)	1.19 (0.01)	0.66 (0.00)
05 Jun	30.0	25.4	8.6	12.2 (0.2)	5.02 (0.42)	24.93 (0.88)	177 (11)	17 (2.6)	1.01 (0.03)	2.10 (0.05)	0.56 (0.00)
12 Jun	30.2	24.9	8.7	30.5 (0.2)	15.1 (0.67)	25.97 (1.33)	746 (17)	37 (1.9)	0.21 (0.02)	1.06 (0.01)	0.50 (0.01)
19 Jun	30.2	25.9	8.8	29.9 (1.0)	11.4 (1.50)	22.72 (1.33)	412 (12)	40 (3.1)	3.64 (0.25)	0.81 (0.03)	1.18 (0.00)
26 Jun	30.5	25.7	8.8	45.3 (1.0)	13.3 (1.42)	32.15 (1.34)	345 (22)	40 (3.6)	0.51 (0.02)	0.86 (0.01)	1.53 (0.01)
03 Jul	30.2	24.8	8.7	33.2 (0.3)	5.23 (0.37)	33.43 (1.83)	145 (19)	26 (2.6)	0.94 (0.11)	1.04 (0.02)	1.88 (0.01)
10 Jul	29.4	29.2	8.7	22.0 (1.0)	2.23 (0.28)	38.67 (1.93)	123 (11)	19 (1.8)	0.74 (0.03)	0.70 (0.01)	1.37 (0.01)

Standard deviations are in parentheses and DL indicates that analytes were below the limit of analytical detection (about  $0.03 \mu\text{mol L}^{-1}$  for nutrients and  $0.2 \times 10^3 \text{ cell mL}^{-1}$  for *A. anophagefferens* abundance). Dissolved inorganic N was calculated as the sum of the average  $\text{NH}_4^+$  and  $\text{NO}_3^-$  concentrations and ratios were calculated from average values. Salinity, temperature, Chl *a* and *A. anophagefferens* abundance from 2003 are also reported in Minor et al. (2006). Empty fields indicate that there were no data



previous reports of brown tide blooms at GB, there were higher overall *A.*

*anophagefferens* cell concentrations during the bloom there than at PL (up to  $7.2 \times 10^5$  cells  $\text{mL}^{-1}$ ) during 2003, and cell concentrations remained high at GB through the end of June (Fig. 6B, Table 2).

In 2005, there were no brown tide blooms at either site (data not shown). In 2006 and 2007, both sites again experienced intense brown tide blooms with *A. anophagefferens* cell numbers in excess of  $10^5$  cells  $\text{mL}^{-1}$  (Fig. 6). As in previous years, blooms in 2006 and 2007 initiated in May and persisted through early July (Tables 1, 2).

#### *Nutrient dynamics*

Unlike 2002, when  $\text{NH}_4^+$  concentrations were below detection limits on several occasions at both sites most of the year (Mulholland et al. 2009a),  $\text{NH}_4^+$  was always measureable during subsequent blooms in 2003, 2006, and 2007 (Tables 1, 2; no nutrient data were collected in 2004). In 2003,  $\text{NH}_4^+$  concentrations at GB were higher than those at PL on seven out of the ten sampling days and concentrations ranged from 0.20-1.34  $\mu\text{mol L}^{-1}$ . In 2006,  $\text{NH}_4^+$  concentrations were always detectable at both sites but  $< 1.00$   $\mu\text{mol L}^{-1}$  on all but one sampling date. In 2007,  $\text{NH}_4^+$  concentrations ranged from 0.18 to 3.64  $\mu\text{mol L}^{-1}$  but were usually  $< 1.00$   $\mu\text{mol L}^{-1}$ .

During 2002,  $\text{NO}_3^-$  concentrations at PL were below analytical detection during and after the bloom, consistent with previous studies; however,  $\text{NO}_3^-$  concentrations at GB, where there was no bloom, were also below the detection limit or  $< 0.30$   $\mu\text{mol L}^{-1}$  during the same period (Mulholland et al. 2009a). In contrast, during 2003, 2006, and 2007,  $\text{NO}_3^-$  concentrations were detectable throughout the bloom period at both sites in Chincoteague Bay (Tables 1, 2). In 2003,  $\text{NO}_3^-$  concentrations ranged from 0.54-1.81

$\mu\text{mol L}^{-1}$ . However, in 2006,  $\text{NO}_3^-$  concentrations were lower, ranging from 0.09-0.48  $\mu\text{mol L}^{-1}$  and in 2007,  $\text{NO}_3^-$  concentrations were the highest of all sampling years, reaching as high as 2.13  $\mu\text{mol L}^{-1}$ . During 2007,  $\text{NO}_3^-$  concentrations were in excess of 2.00  $\mu\text{mol L}^{-1}$  even during the initiation of brown tide blooms in May.

During 2002, urea concentrations were generally higher (0.24-2.30  $\mu\text{mol L}^{-1}$ ; Mulholland et al. 2009a) than during subsequent sampling years when concentrations were consistently  $< 1.0 \mu\text{mol L}^{-1}$  (Tables 3, 4). During 2003, urea concentrations ranged from 0.10-0.89  $\mu\text{mol L}^{-1}$ , higher than those observed during 2006 and 2007 (0.02-0.36  $\mu\text{mol L}^{-1}$ ). During 2007, urea concentrations were low as well (0.07-0.63  $\mu\text{mol L}^{-1}$ ) except during July when concentrations were 3.61  $\mu\text{mol L}^{-1}$  and 3.65  $\mu\text{mol L}^{-1}$  at GB and PL, respectively. DFAA concentrations were fairly consistent throughout the sampling period. Typically, DFAA concentrations were  $< 1.0 \mu\text{mol L}^{-1}$  with the only exception being in July, 2003 (Table 4). The average C and N concentration for the DFAA pool during 2002 was 1.18  $\mu\text{mol L}^{-1}$  DFAA-N and 4.41  $\mu\text{mol L}^{-1}$  DFAA-C for each 1  $\mu\text{mol L}^{-1}$  DFAA (data not shown). The most abundant amino acids were serine, glycine, and histidine.

DIP concentrations were always detectable and ranged from 0.03 – 4.28  $\mu\text{mol L}^{-1}$  over the study period. As during 2002 (Mulholland et al. 2009a), DIN:DIP ratios were less than 16, the Redfield ratio, at both sites (Tables 1, 2), suggesting N limitation, with only three exceptions at PL; May in 2006 (once) and 2007 (twice) (Table 1). Low concentrations of DIP (0.03-0.10  $\mu\text{mol L}^{-1}$ ) were observed at these times. The lowest DIN:DIP ratio (0.2 – 1.1) were observed at GB during the 2006 bloom. These low ratios resulted from high DIP concentrations at GB during the bloom period that year. Some

**Table 3** Concentrations and ratios of organic nutrients at Public Landing in Chincoteague Bay, MD during 2003, 2006, and 2007

Date	DOP ( $\mu\text{mol L}^{-1}$ )	Urea ( $\mu\text{mol L}^{-1}$ )	DFAA ( $\mu\text{mol L}^{-1}$ )	DON ( $\mu\text{mol L}^{-1}$ )	DOC ( $\mu\text{mol L}^{-1}$ )	DOC:DON	DOC:DOP	DON:DOP	TDN:DOP
<b>2003</b>									
07 Mar	0.32	0.33 (0.00)	0.27 (0.05)	24.1	276 (1.3)	12	873	76	39.4
14 Apr	0.26	0.20 (0.00)	0.38 (0.13)	28.0	268 (9.2)	10	1,021	107	66.8
14 May	BDL	0.72 (0.00)	0.71 (0.04)	26.8	260 (1.4)	10			152
29 May	0.07	0.67 (0.09)	0.58 (0.02)	36.4	313 (4.0)	9	4,611	536	78.4
04 Jun	0.15	0.58 (0.04)	0.63 (0.38)	29.0	294 (1.3)	10	1,954	193	59.8
12 Jun	0.25	0.50 (0.35)	0.27 (0.03)	34.3	286 (6.7)	8	1,154	138	93.1
18 Jun	0.19	0.56 (0.21)	0.53 (0.03)	36.5	382 (11.5)	11	2,011	192	88.9
27 Jun	0.14	0.45 (0.02)	0.94 (0.06)	40.1	353 (2.5)	9	2,562	291	108
10 Jul	0.28	0.43 (0.04)	0.66 (0.01)	45.7	397 (2.7)	9	1,418	163	86.7
07 Aug	0.16	0.67 (0.00)	0.40 (0.04)	47.7	376 (1.2)	8	2,350	298	100
<b>2006</b>									
10 May	0.41	BLD	0.25 (0.00)	33.9	352	10	859	82	73.4
18 May	0.26	0.02 (0.02)	0.13 (0.02)	29.6	529	18	2,035	114	84.7
23 May	0.49	0.36 (0.12)	0.43 (0.01)	36.2	466	13	951	73	70.9
31 May	0.76	0.07 (0.01)	0.49 (0.02)	41.1	577	14	759	54	50.6
07 Jun	0.29	0.12 (0.03)	0.40 (0.02)	35.7	1188	33	4,097	124	38.8
14 Jun	0.37	0.22 (0.13)	0.35 (0.01)	38.0	580	15	1,568	102	42.3
21 Jun	0.42	0.10 (0.05)	0.31 (0.01)	34.9	652	19	1,552	83	40.7
28 Jun	BDL	0.14 (0.01)	0.31 (0.03)	41.9	710	17			19.5
05 Jul	0.38	0.20 (0.03)	0.56 (0.05)	45.4	751	17	1,976	118	19.2
<b>2007</b>									
15 May	0.36	0.63 (0.01)	0.44 (0.05)	30.0	473	16	1,314	82	78.5
19 May	0.32	0.21 (0.01)	0.83 (0.00)	27.8	378	14	1,181	88	54.2
29 May	0.29	0.07 (0.01)	0.25 (0.02)	31.3	459	15	1,583	108	86.0
05 Jun	0.34	0.15 (0.01)	0.24 (0.03)	36.1	630	17	1,853	105	79.4
12 Jun	0.39	0.41 (0.01)	0.63 (0.01)	34.1	500	15	1,282	89	47.6
19 Jun	0.42	0.09 (0.02)	0.41 (0.00)	32.1	476	15	1,133	76	46.8
26 Jun	0.31	0.20 (0.02)	0.26 (0.01)	35.3	524	15	1,690	113	59.6
03 Jul	0.37	3.65 (0.00)	0.24 (0.01)	43.7	561	13	1,516	119	40.0
10 Jul	0.51	0.16 (0.03)	0.20 (0.03)	35.3	1,638	46	3,212	69	45.9

Standard deviations are in parentheses. DOC concentrations from 2003 are also reported in Minor et al. (2006). BDL indicates concentrations below the limit of analytical detection ( $< 0.03 \mu\text{mol L}^{-1}$ ). Empty fields indicate that there were no data

**Table 4** Concentrations and ratios of organic nutrients at Greenbackville in Chincoteague Bay VA, during 2003, 2006, and 2007

Date	DOP ( $\mu\text{mol L}^{-1}$ )	Urea ( $\mu\text{mol L}^{-1}$ )	DFAA ( $\mu\text{mol L}^{-1}$ )	DON ( $\mu\text{mol L}^{-1}$ )	DOC ( $\mu\text{mol L}^{-1}$ )	DOC:DON	DOC:DOP	DON:DOP	TDN:TDP
<b>2003</b>									
07 Mar	0.38	0.36 (0.01)	0.56 (0.00)	37.3	344 (2.0)	9	911	99	48.6
14 Apr	0.15	0.10 (0.04)	0.18 (0.03)	28.9	310 (4.4)	11	2,066	193	75.7
14 May	0.74	0.88 (0.17)	0.74 (0.01)	33.7	242 (2.2)	7	326	45	37.5
29 May	0.30	0.88 (0.02)	0.25 (0.00)	34.6	350 (2.7)	10	1,167	115	48.4
04 Jun	0.22	0.67 (0.03)	0.74 (0.03)	36.7	315 (2.5)	9	1,430	167	61.5
12 Jun	0.15	0.54 (0.02)	0.23 (0.01)	26.8	295 (5.0)	11	1,985	180	51.6
18 Jun	0.06	0.22 (0.10)	1.53 (0.53)	34.8	336 (3.9)	10	5,600	580	69.7
27 Jun	0.32	0.66 (0.04)	0.38 (0.11)	37.9	330 (1.1)	9	1,031	118	47.1
10 Jul	BDL	0.82 (0.05)	0.42 (0.02)	65.6	338 (11.1)	5			59.1
07 Aug	0.25	0.89 (0.12)	0.47 (0.03)	64.2	264 (0.5)	4	1,056	257	72.0
<b>2006</b>									
10 May	0.29	0.13 (0.03)	0.40 (0.01)	31.6	304	10	1,048	109	32.9
18 May	BDL	0.29 (0.15)	0.29 (0.00)	33.2	324	10			31.5
23 May	0.23	0.08 (0.04)	0.18 (0.03)	28.9	325	11	1,413	123	28.0
31 May	BDL	0.11 (0.02)	0.27 (0.01)	26.5	374	14			24.5
07 Jun	BDL	0.10 (0.07)	0.31 (0.01)	27.6	362	13			17.0
14 Jun	BDL	0.03 (0.01)	0.60 (0.01)	36.4	442	12	11,050	845	16.5
21 Jun	BDL	0.05 (0.02)	0.65 (0.03)	34.9	492	14			15.7
28 Jun	BDL	0.22 (0.00)	0.33 (0.02)	41.3	597	14			14.4
05 Jul	BDL	0.28 (0.18)	0.32 (0.04)	28.6	402	14			10.5
<b>2007</b>									
15 May	0.51	0.20 (0.04)	0.32 (0.13)	32.2	356	11	698	63	38.8
19 May	0.25	0.27 (0.02)	0.40 (0.01)	31.3	257	8	1,028	126	46.3
29 May	0.49	0.32 (0.02)	0.43 (0.03)	31.1	437	14	892	63	28.2
05 Jun	0.26	0.31 (0.00)		23.3	254	11	977	88	32.6
12 Jun	0.42	0.09 (0.01)	0.18 (0.01)	30.0	499	17	1,188	71	33.9
19 Jun	0.53	0.25 (0.06)	0.37 (0.01)	29.6	513	17	968	56	20.0
26 Jun	1.47	0.19 (0.01)	0.70 (0.01)	39.1	678	17	461	27	13.5
03 Jul	0.53	3.61 (0.07)	0.27 (0.03)	40.3	484	12	913	76	17.6
10 Jul	0.83	0.44 (0.02)	0.38 (0.05)	31.4	841	27	1,013	38	15.0

Standard deviations are in parentheses. DOC concentrations from 2003 are also reported in Minor et al. (2006). BDL indicates concentrations below the limit of analytical detection ( $< 0.03 \mu\text{mol L}^{-1}$ ). Empty fields indicate that there were no data

low DIN:DIP ratios were also observed in 2007 at GB at the end of the bloom when DIP concentrations were again high (Table 2).

In 2002, bulk DON concentrations in Chincoteague Bay ranged from about 5.5 in March to 49.9  $\mu\text{mol N L}^{-1}$  during the fall at PL, and DON concentrations were up to 2.5 times higher at PL, where there was a bloom, than at GB where there was no bloom (Mulholland et al. 2009a). During subsequent bloom years (2003, 2006, and 2007), bulk DON concentrations were much more similar between sites, ranging from 23.3-65.6  $\mu\text{mol N L}^{-1}$  (Tables 3, 4). In 2003, DON concentrations at PL ranged from 24.1-47.7  $\mu\text{mol N L}^{-1}$  with a mean concentration of 34.9  $\mu\text{mol N L}^{-1}$  (Table 3). GB had a larger range (26.8-65.8  $\mu\text{mol N L}^{-1}$ ) and a higher mean (40.1  $\mu\text{mol N L}^{-1}$ ). In 2006, DON concentrations at PL ranged from 29.6-45.4  $\mu\text{mol N L}^{-1}$  with a mean concentration of 37.5  $\mu\text{mol N L}^{-1}$ , similar to 2003 (Table 3). In contrast, at GB, DON concentrations were lower in 2006, ranging from 26.5 - 41.3  $\mu\text{mol N L}^{-1}$  (mean of 31.8  $\mu\text{mol N L}^{-1}$ ) (Table 4). In 2007, DON concentrations at PL had a range of 27.8 - 43.7  $\mu\text{mol N L}^{-1}$  and a mean of 34.0  $\mu\text{mol N L}^{-1}$  (Table 3) while GB had a range of 23.3 - 40.3  $\mu\text{mol N L}^{-1}$  and a mean of 32.0  $\mu\text{mol N L}^{-1}$  (Table 4).

During 2002, DOC concentrations were higher at PL than at GB and concentrations were also always less than 400  $\mu\text{mol C L}^{-1}$  (Simjouw et al. 2004, Mulholland et al. 2009a). In the 2002 study, large differences in the DOC concentrations and characteristics were attributed to the blooms at PL versus the lack of bloom at GB (Simjouw et al. 2004). However, during 2003, both GB and PL experienced brown tide blooms and DOC concentrations were similar at both sites (within 25% of each other; Tables 3, 4). During 2006, both sites experienced blooms, but DOC concentrations were

always greater at PL (mean 661  $\mu\text{mol C L}^{-1}$ ) than at GB (mean 393  $\mu\text{mol C L}^{-1}$ ) (Tables 3, 4). Finally, during 2007, DOC concentrations were highest and showed more variation over the sampling period than in any other year, ranging from 254-841  $\mu\text{mol C L}^{-1}$  at GB and 378-1,638  $\mu\text{mol C L}^{-1}$  at PL. Overall, an increase in DOC concentrations in Chincoteague Bay was observed over the 6-year sampling period from a mean of 296  $\mu\text{mol C L}^{-1}$  and 461  $\mu\text{mol C L}^{-1}$  at GB and PL, respectively, during 2002 (Simjouw et al. 2004), to a mean of 480  $\mu\text{mol C L}^{-1}$  and 627  $\mu\text{mol C L}^{-1}$  at GB and PL, respectively, during 2007. Throughout the study period, DOC:DON ratios were greater than 6.6, the Redfield ratio, with the exception of the post-bloom period (Jul-Aug) at GB during 2003.

In 2002, DOP concentrations were similar between sites (Mulholland et al. 2009a). In 2003, DOP concentrations ranged from below the detection limit (BDL) - 0.74  $\mu\text{mol L}^{-1}$  at GB, and BDL - 0.32  $\mu\text{mol L}^{-1}$  at PL. During 2006, DOP concentrations were much lower at GB than PL; and on eight of the ten sampling days, DOP concentrations were below detection at GB with a maximum concentration of only 0.29  $\mu\text{mol L}^{-1}$ . PL had a greater range of DOP concentrations, BDL - 0.76  $\mu\text{mol L}^{-1}$ , and DOP was detectable on all but one occasion. During 2007, DOP concentrations were always measurable and higher than during 2006, ranging from 0.25-1.47  $\mu\text{mol L}^{-1}$  at GB and 0.29-0.51  $\mu\text{mol L}^{-1}$  at PL.

During 2002, TDN:TDP ratios ranged from 16.1 – 23.7 between May and July at GB, where there was no bloom, and from 16.3 – 48.5 at PL during the same period (28.9-42.2 during the bloom period) (Mulholland et al. 2009a). In contrast, during 2003, TDN:TDP ratios ranged from 37.5-69.7 at GB and 59.8-152.0 at PL between May and July in 2003. High TDN:TDP ratios persisted during 2006 and 2007 at PL (range of

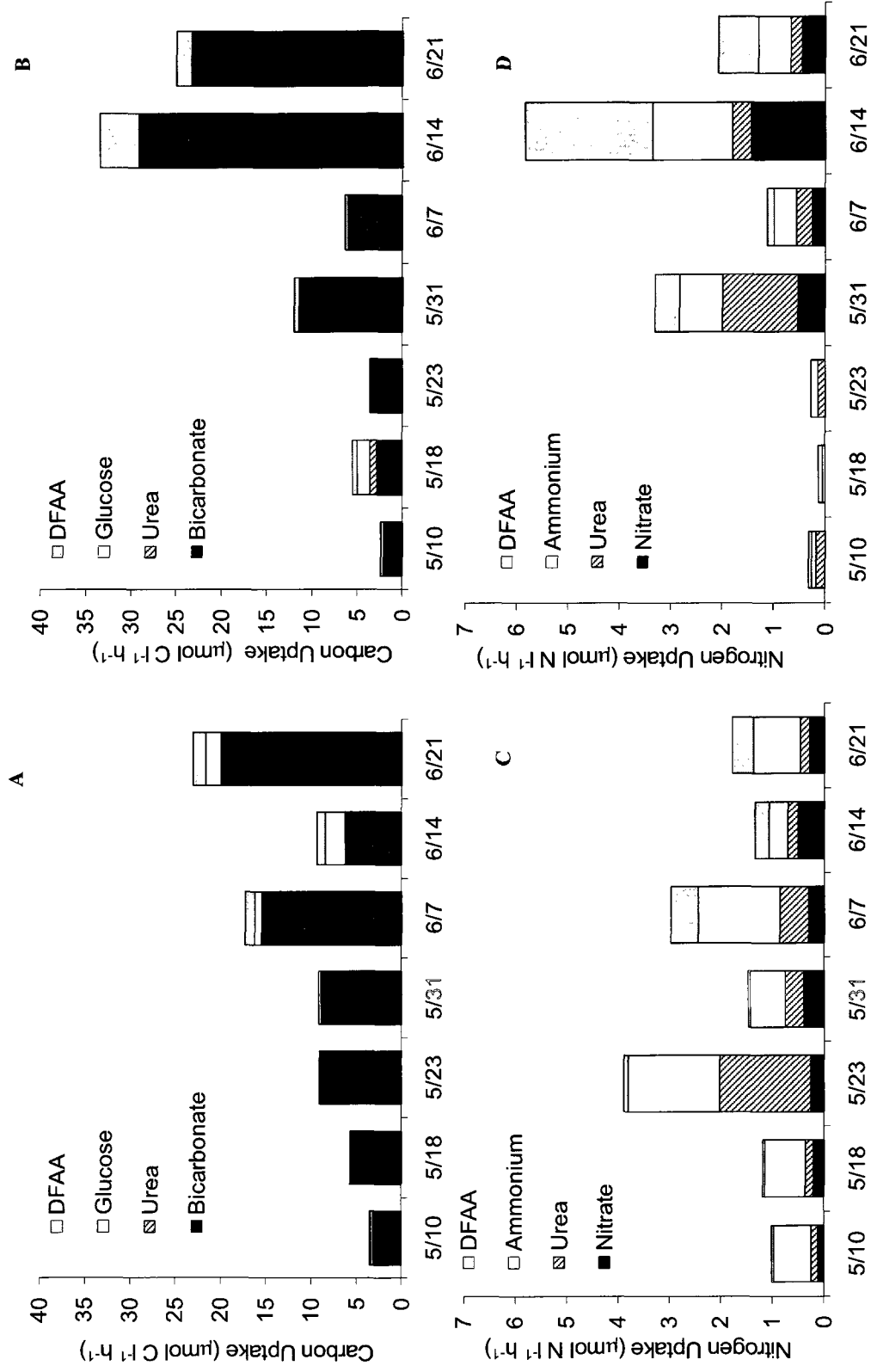
38.8–86.0, excluding 2 post bloom dates in 2006), but decreased at GB during the subsequent bloom years (range of 10.5 – 46.3).

#### *Nitrogen and carbon uptake*

In 2002, urea was the dominant form of N taken up by cells during most of the year at both bloom and non-bloom sites in Chincoteague Bay (Mulholland et al. 2009a). During most of the year, total N uptake was much higher (almost an order of magnitude) at the PL site than at GB, consistent with the higher biomass during and after the bloom at that site. While photosynthetic uptake of  $\text{HCO}_3^-$  provided the bulk of the measured C uptake during the 2002 bloom, urea, DFAA and glucose contributed to the microbial C demand, particularly at the end of and subsequent to the bloom (Mulholland et al. 2009a).

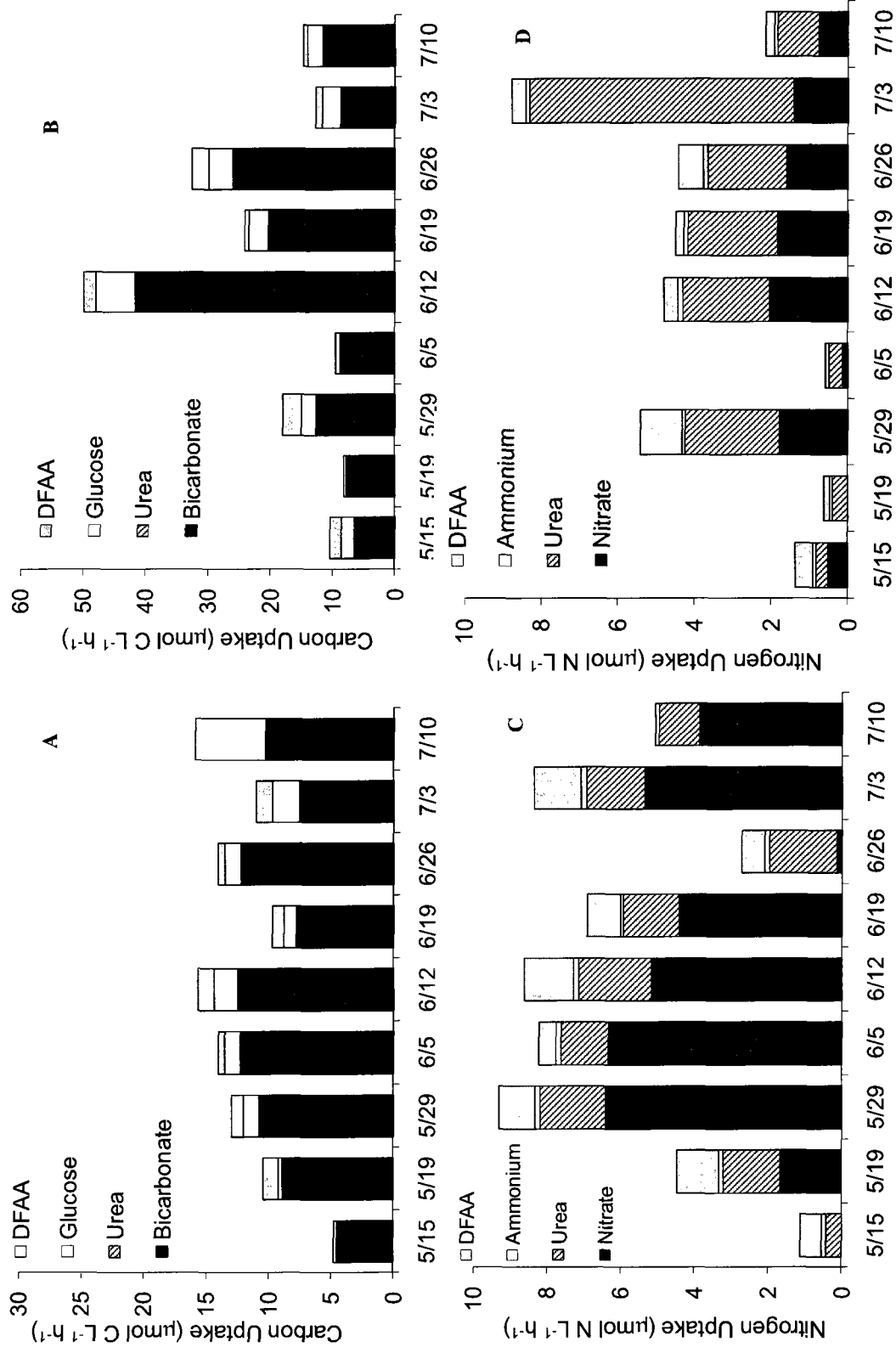
In contrast, N uptake at PL was dominated by  $\text{NH}_4^+$  in 2006 (Fig. 7, Table 5) and  $\text{NO}_3^-$  in 2007 (Fig. 8, Table 6). In 2006,  $\text{NH}_4^+$  uptake averaged 52% ( $\pm 15\%$ ) of the total N uptake at PL and urea,  $\text{NO}_3^-$ , and DFAA accounted for 20% ( $\pm 12\%$ ), 18% ( $\pm 11\%$ ), and 10% ( $\pm 9\%$ ) of the N uptake, respectively, and DIN uptake was higher than DON uptake (Fig. 3). At GB, on average, DON compounds (urea plus DFAA) accounted for 56% ( $\pm 15\%$ ) of the total nitrogen uptake. Urea,  $\text{NH}_4^+$ , DFAA and  $\text{NO}_3^-$  contributed 29% ( $\pm 18\%$ ), 30% ( $\pm 9\%$ ), 27% ( $\pm 21\%$ ), and 15% ( $\pm 9\%$ ), of the total N uptake, respectively.

In contrast to previous years, during 2007,  $\text{NO}_3^-$  was the dominant form of N taken up during the bloom representing 50% ( $\pm 29\%$ ) of the total N uptake at PL (Fig. 8). During the 2007 bloom,  $\text{NO}_3^-$  concentrations were also the highest observed over the 6-year study period (Table 1), reaching as high as  $2.13 \mu\text{mol L}^{-1}$ . Higher  $\text{NO}_3^-$  concentrations were also observed at GB in 2007 (Table 2), but  $\text{NO}_3^-$  uptake only comprised 30% ( $\pm 13\%$ ) of the total N uptake at that site (Fig. 8). At GB, urea uptake was



**Fig. 7** Carbon uptake at (A) Public Landing, MD and (B) Greenbackville, VA and nitrogen uptake at (C) Public Landing, MD and (D) Greenbackville, VA during 2006





**Fig. 8** Carbon uptake at (A) Public Landing, MD and (B) Greenbackville, VA and nitrogen uptake at (C) Public Landing, MD and (D) Greenbackville, VA, during 2007

**Table 5** Carbon uptake rates during the 2006, and 2007 blooms

Date	Site	Bicarbonate $\mu\text{mol C L}^{-1} \text{ h}^{-1}$	Urea $\mu\text{mol C L}^{-1} \text{ h}^{-1}$	Glucose $\mu\text{mol C L}^{-1} \text{ h}^{-1} \text{ L}$	DFAA $\mu\text{mol C L}^{-1} \text{ h}^{-1}$	Total $\mu\text{mol C L}^{-1} \text{ h}^{-1}$
<b>2006:</b>						
18 May	GB	2.04 (0.10)	0.06 (0.00)	0.21 (0.00)	0.12 (0.01)	2.43
23 May	GB	2.78 (0.19)	0.79 (0.01)	1.42 (0.06)	0.49 (0.06)	5.49
31 May	GB	3.48 (0.03)	0.01 (0.00)	0.05 (0.01)	0.00 (0.00)	3.55
07 Jun	GB	11.35 (0.29)	0.00 (0.00)	0.09 (0.02)	0.54 (0.07)	11.97
14 Jun	GB	6.07 (0.14)	0.00 (0.00)	0.00 (0.00)	0.27 (0.01)	6.34
21 Jun	GB	29.14 (0.46)	0.00 (0.00)	0.00 (0.00)	4.30 (0.39)	33.44
<b>2006:</b>						
18 May	PL	3.11 (0.09)	0.00 (0.00)	0.31 (0.03)	0.10 (0.01)	3.52
23 May	PL	5.68 (0.22)	0.00 (0.00)	0.00 (0.00)	0.03 (0.01)	5.71
31 May	PL	9.03 (0.27)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	9.03
07 Jun	PL	8.92 (0.26)	0.00 (0.00)	0.25 (0.39)	0.00 (0.00)	9.17
14 Jun	PL	15.42 (0.00)	0.05 (0.01)	0.73 (0.07)	1.16 (0.14)	17.35
21 Jun	PL	6.17 (0.38)	0.10 (0.01)	2.18 (0.15)	0.88 (0.04)	9.33
<b>2007:</b>						
15 May	GB	6.52 (0.38)	0.00 (0.00)	2.06 (0.97)	1.90 (0.07)	10.48
19 May	GB	7.59 (2.60)	0.03 (0.00)	0.12 (0.02)	0.45 (0.02)	8.19
29 May	GB	12.55 (0.07)	0.07 (0.01)	2.47 (0.14)	2.91 (0.07)	18.00
05 Jun	GB	8.74 (2.03)	0.03 (0.00)	0.72 (0.05)	0.07 (0.01)	9.56
12 Jun	GB	41.63 (0.65)	0.09 (0.02)	6.22 (0.05)	2.05 (0.08)	49.99
19 Jun	GB	20.33 (0.24)	0.02 (0.00)	3.07 (0.21)	0.62 (0.93)	24.05
26 Jun	GB	25.87 (0.94)	0.06 (0.01)	3.93 (0.10)	2.66 (1.06)	32.53
03 Jul	GB	8.65 (0.29)	0.17 (0.00)	2.89 (0.08)	1.09 (0.04)	12.80
10 Jul	GB	11.52 (1.98)	0.05 (0.00)	2.45 (0.07)	0.69 (0.05)	14.70
<b>2007:</b>						
15 May	PL	4.52 (0.16)	0.00 (0.00)	0.02 (0.06)	0.59 (0.01)	5.13
19 May	PL	8.88 (0.29)	0.02 (0.00)	0.03 (0.21)	1.15 (0.95)	10.34
29 May	PL	10.78 (0.38)	0.03 (0.00)	1.24 (0.34)	0.99 (0.08)	13.03
05 Jun	PL	12.30 (0.30)	0.01 (0.00)	1.24 (0.09)	0.47 (0.01)	14.02
12 Jun	PL	12.45 (0.43)	0.03 (0.01)	1.89 (0.05)	1.35 (0.12)	15.71
19 Jun	PL	7.79 (0.22)	0.01 (0.00)	1.02 (0.13)	0.91 (0.05)	9.74
26 Jun	PL	12.19 (1.88)	0.03 (0.00)	1.30 (0.15)	0.63 (0.27)	14.15
03 Jul	PL	7.53 (0.54)	0.06 (0.00)	2.16 (0.21)	1.28 (0.23)	11.04
10 Jul	PL	10.28 (1.04)	0.04 (0.00)	5.61 (0.56)	0.00 (0.00)	15.93

Standard deviations are in parentheses

**Table 6** Nitrogen uptake rates during the 2006, and 2007 blooms

Date	Site	NO <sub>3</sub> μmol N L <sup>-1</sup> h <sup>-1</sup>	Urea μmol N L <sup>-1</sup> h <sup>-1</sup>	NH <sub>4</sub> <sup>+</sup> μmol N L <sup>-1</sup> h <sup>-1</sup>	DFAA μmol N L <sup>-1</sup> h <sup>-1</sup>	Total μmol N L <sup>-1</sup> h <sup>-1</sup>
<b>2006:</b>						
18 May	GB	0.01 (0.00)	0.17 (0.01)	0.09 (0.00)	0.06 (0.00)	0.32
23 May	GB	0.00 (0.00)	0.02 (0.00)	0.02 (0.00)	0.08 (0.01)	0.13
31 May	GB	0.03 (0.00)	0.12 (0.00)	0.13 (0.01)	0.00 (0.00)	0.27
07 Jun	GB	0.52 (0.01)	1.47 (0.00)	0.83 (0.06)	0.48 (0.06)	3.30
14 Jun	GB	0.24 (0.01)	0.31 (0.00)	0.43 (0.01)	0.14 (0.00)	1.12
21 Jun	GB	1.43 (0.10)	0.36 (0.00)	1.56 (0.03)	2.48 (0.18)	5.83
<b>2006:</b>						
18 May	PL	0.10 (0.02)	0.13 (0.00)	0.73 (0.01)	0.04 (0.00)	1.00
23 May	PL	0.21 (0.01)	0.16 (0.00)	0.78 (0.01)	0.03 (0.00)	1.18
31 May	PL	0.31 (0.04)	1.76 (0.00)	1.79 (0.07)	0.09 (0.01)	3.94
07 Jun	PL	0.37 (0.02)	0.35 (0.02)	0.69 (0.03)	0.04 (0.01)	1.44
14 Jun	PL	0.31 (0.02)	0.55 (0.00)	1.58 (0.03)	0.53 (0.07)	2.97
21 Jun	PL	0.44 (0.07)	0.20 (0.00)	0.37 (0.01)	0.27 (0.01)	1.27
<b>2007:</b>						
15 May	GB	0.51 (0.55)	0.31 (0.02)	0.08 (0.00)	0.45 (0.02)	1.36
19 May	GB	0.02 (0.00)	0.38 (0.02)	0.07 (0.00)	0.14 (0.01)	0.62
29 May	GB	1.79 (0.06)	2.45 (0.01)	0.09 (0.02)	1.09 (0.10)	5.42
05 Jun	GB	0.13 (0.00)	0.36 (0.01)	0.08 (0.00)	0.02 (0.00)	0.60
12 Jun	GB	2.06 (0.10)	2.24 (0.19)	0.14 (0.00)	0.36 (0.01)	4.80
19 Jun	GB	1.84 (0.06)	2.33 (0.06)	0.10 (0.00)	0.22 (0.34)	4.49
26 Jun	GB	1.59 (0.04)	2.06 (0.06)	0.11 (0.02)	0.66 (0.50)	4.42
03 Jul	GB	1.41 (0.05)	6.91 (0.13)	0.10 (0.00)	0.37 (0.01)	8.79
10 Jul	GB	0.76 (0.04)	1.08 (0.05)	0.09 (0.01)	0.22 (0.13)	2.15
<b>2007:</b>						
15 May	PL	0.00 (0.00)	0.42 (0.00)	0.13 (0.00)	0.31 (0.06)	0.86
19 May	PL	1.67 (0.18)	1.53 (0.03)	0.12 (0.00)	0.46 (0.28)	3.78
29 May	PL	6.42 (0.20)	1.79 (0.01)	0.13 (0.00)	0.48 (0.02)	8.82
05 Jun	PL	6.33 (0.13)	1.29 (0.02)	0.13 (0.04)	0.30 (0.02)	8.06
12 Jun	PL	5.18 (0.21)	1.98 (0.00)	0.13 (0.01)	0.82 (0.08)	8.10
19 Jun	PL	4.42 (0.26)	1.52 (0.19)	0.07 (0.02)	0.48 (0.02)	6.50
26 Jun	PL	0.13 (0.02)	1.83 (0.06)	0.13 (0.00)	0.31 (0.03)	2.41
03 Jul	PL	5.36 (0.07)	1.58 (0.04)	0.15 (0.00)	0.68 (0.29)	7.76
10 Jul	PL	3.87 (1.23)	1.11 (0.01)	0.10 (0.00)	0.00 (0.00)	5.08

Standard deviations are in parentheses

52% ( $\pm 15\%$ ) of the total measured N uptake and DON uptake was greater than 50% of the total N uptake on 9 of the sampling days.

As in 2002, photosynthetic bicarbonate uptake was the main form of carbon taken at both sites during 2006 (Fig. 7) and 2007 (Fig. 8). On average, photosynthetic uptake of  $\text{HCO}_3^-$  accounted for 89% ( $\pm 14\%$ ) and 86% ( $\pm 16\%$ ) of the total measured C uptake at PL and GB, respectively, during 2006 (Fig. 7). Similar to 2002, urea C was a small fraction of the total measured carbon at both sites during 2006 and 2007, averaging  $< 2\%$  of the total C uptake.

Carbon uptake from DFAA and glucose however, accounted for a substantial fraction of the total measured C uptake (Figs. 7, 8). In 2006, glucose uptake was important at the beginning of the bloom in GB, accounting for 9% ( $\pm 2\%$ ) of the C uptake on 10 May and 26% ( $\pm 4\%$ ) on 18 May (Fig. 7). At PL, glucose uptake averaged 15% ( $\pm 10\%$ ) during the end of the bloom, reaching as high as 23% ( $\pm 2\%$ ) on 14 June. In 2007, glucose uptake was highest during the peak and at the end of the bloom and was as high as 35% ( $\pm 3\%$ ) on 10 July (Fig. 8). On average, glucose uptake represented 12% ( $\pm 10\%$ ) of the total measured C uptake at GB.

## Discussion

### *Bloom dynamics*

Between 2002 and 2007, Chincoteague Bay experienced brown tide blooms ( $> 35,000$  cells  $\text{mL}^{-1}$ ; Gastrich and Wazniak, 2002) every spring except during 2005. In each of the bloom years, there was a gradual warming trend during which the *A. anophagefferens* bloom initiated; during 2005, the water warmed abruptly in the spring, likely preventing a large bloom development

([http://www.dnr.state.md.us/coastalbays/bt\\_results.html](http://www.dnr.state.md.us/coastalbays/bt_results.html) ). Subsequent to 2002, blooms have spread to previously unimpacted areas in Chincoteague Bay and the duration and intensity of blooms have increased over time (Fig. 6). Although the 2003 bloom at PL did not reach the peak *A. anophagefferens* concentrations observed in 2002, the 2003 bloom lasted longer than the 2002 bloom and a bloom was also observed at GB. During 2006 and 2007, blooms at both sites reached higher densities and lasted longer than in previous years (Fig. 6). For the entire 2007 sampling period (15 May – 10 July), *A. anophagefferens* concentrations were above the Category 3 threshold for brown tide blooms ( $> 200,000$  cells  $\text{mL}^{-1}$ ). This differs from the 2002 bloom at PL where *A. anophagefferens* concentrations were above the Category 3 threshold from 5/30 to 6/12 (Mulholland et al. 2009). These high concentrations have been shown to result in negative impacts for shellfish, seagrasses and planktonic organisms (Bricelj and Lonsdale 1997). In addition, growth rates of the hard clam *Mercenaria mercenaria* can be negatively impacted at *A. anophagefferens* concentrations as low as  $20,000$  cells  $\text{mL}^{-1}$  (Wazniak and Glibert 2004). Therefore, productivity within shellfish aquaculture facilities located in or on Chincoteague Bay could be negatively affected by blooms. In addition to current aquaculture facilities, ~250 acres of bay bottom have been leased for potential use in raising hard clams (Tarnowski 2008).

#### *Nitrogen dynamics*

Previous studies have shown that populations of *A. anophagefferens* bloom only after  $\text{NO}_3^-$  concentrations have been depleted (Gobler and Sañudo-

Wilhelmy 2001a) or when DIN concentrations are low (Lomas et al. 2004). In Chincoteague Bay, these criteria were met in 2002, when  $\text{NO}_3^-$  concentrations were below the limit of analytical detection from April through the end of June at PL and DIN near or at the limit of analytical detection during the bloom period (Mulholland et al. 2009a). However, during 2002,  $\text{NO}_3^-$  and DIN concentrations were low or at the analytical detection limit at the non-bloom site as well, suggesting that low DIN is not sufficient for bloom formation since *A. anophagefferens* cells were present at both sites in 2002 (Mulholland et al. 2009a). In contrast to 2002, neither  $\text{NO}_3^-$  nor DIN was depleted during brown tide blooms at GB and PL during 2003, 2006, and 2007. Although  $\text{NO}_3^-$  concentrations were higher in 2007, the DIN pool did not vary greatly between years, ranging from 0.5-4.5  $\mu\text{mol L}^{-1}$  and averaging 1.4  $\mu\text{mol L}^{-1}$  (Tables 1, 2). DIN concentrations were similar to what has been observed in other blooms (Mulholland et al. 2002). The elevated  $\text{NO}_3^-$  concentrations were also within range of what is typically seen in Chincoteague Bay, where  $\text{NO}_3^-$  concentrations are typically below 5  $\mu\text{mol N L}^{-1}$  (Glibert et al. 2007).

During this 6-year study, N uptake during blooms varied greatly and  $\text{NH}_4^+$ , urea,  $\text{NO}_3^-$  and DFAA all contributed to the total measured N uptake (Figs. 7, 8). Previously it was shown that *A. anophagefferens* has a high affinity for  $\text{NH}_4^+$  and urea (Lomas et al. 1996, Berg et al. 1997). Consistent with those observations, these two compounds accounted for the majority of the N uptake in Chincoteague Bay during 2002 (Mulholland et al. 2009) and 2006 (Figs. 7, 8). Similarly, in Long Island, NY, coastal bays,  $\text{NH}_4^+$  and DFAA were the primary N

compounds taken up during brown tide blooms there (Mulholland et al. 2002).

High  $\text{NO}_3^-$  concentrations in Chincoteague Bay during 2007 were accompanied by high uptake rates of this compound during that year (Figs. 7, 8). This was unexpected since studies have shown that the growth of *A. anophagefferens* relative to other competing phytoplankton can be suppressed with the addition of  $\text{NO}_3^-$  (Gobler and Sañudo-Wilhelmy 2001a; Taylor et al., 2006). Interestingly, cultures of *A. anophagefferens* grow equally as well on  $\text{NO}_3^-$  and urea (Pustizzi et al. 2004; MacIntyre et al. 2004). Together, these results suggest that blooms of *A. anophagefferens* can be supported by a variety of organic and inorganic N compounds.

#### *Carbon dynamics*

A major goal of this study was to determine the degree to which brown tide organisms augment autotrophic uptake of DIC with heterotrophic uptake of DOC and to determine how this changes over the course of blooms as cell densities increase, potentially self-shade, and populations draw down DIC. Overall, DIC uptake was the dominant form of C taken up (Figs. 7, 8), however, C from glucose, DFAA, and urea could be taken up during blooms and that DOC uptake could account for as much as 49% of the total measured C uptake. These results are similar to what was observed during a 2002 bloom at PL where organic C uptake in whole water accounted for 17-71% of the total carbon uptake, with the percentage increasing as the bloom progressed (Mulholland et al. 2009). C uptake from DFAA were also similar to rates observed in a 2000 bloom in

Quantuck Bay, NY (Mulholland et. al. 2002).

On average, DOC uptake accounted for 16% of the total C uptake throughout this study. However, all of the incubations in this study were done during the middle of the day, when photosynthetic uptake rates were likely at or near maximum levels. If *A. anophagefferens* are also able to take up organic C at night, this might give populations a large advantage over competing phytoplankton that can only use the inorganic C pool during the day in conjunction with photosynthesis. Nighttime uptake of C will be examined in Chapter IV.

During 2006 and 2007, pH reached 8.9 just after peak bloom density, presumably due to the drawdown of DIC (Tables 1, 2) while during 2003, pH levels never exceeded 8.1 at either site. Declines in photosynthesis and growth rates have been observed in coastal and oceanic marine diatoms and a natural assemblage of phytoplankton from Narragansett Bay at pH > 8.8 (Chen and Durbin 1994) and pH of 8.9 and higher have been shown to affect the growth rate of some heterotrophic protists (Pedersen and Hansen 2003) and dinoflagellates (Hansen et al. 2007)). While there was some uptake of organic C during the 2006 bloom at PL and GB, uptake was much higher during 2007, when pH was consistently higher and DIC may have become limiting. This suggests that *A. anophagefferens* could be switching to an organic carbon as a result of C limitation. DOC uptake increased as blooms progressed during both 2006 and 2007 (Figs. 7, 8), consistent with the idea that DOC can supplement photosynthetic C uptake when high cell densities might result in light or carbon-



limited photosynthesis. Previous studies found that glucose additions stimulated brown tide growth relative to other algae and that the DOC pool was drawn down during brown tide blooms (Gobler et al. 2004, Minor et al. 2006).

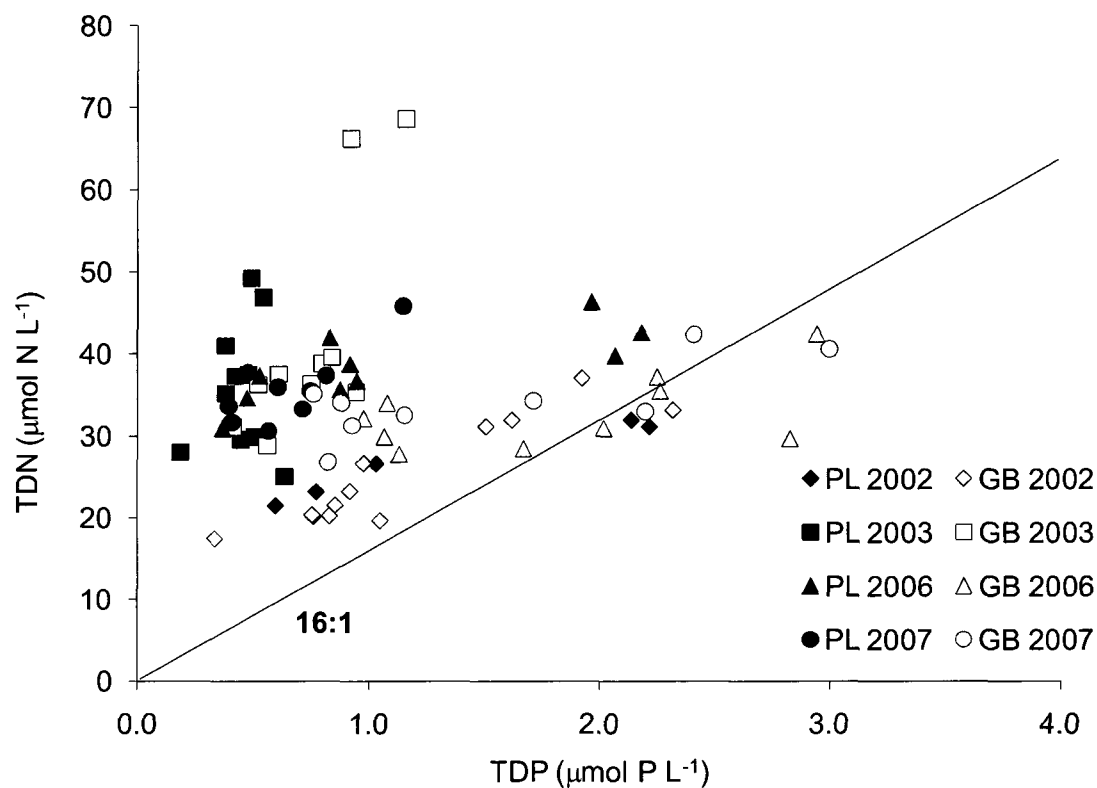
Despite their ability to take up DOC compounds, *A. anophagefferens* appears to be a net source of DOC to Chincoteague Bay (Simjouw et al. 2004; Mulholland et al. 2009a) where we observed an overall increase in DOC concentrations in response to brown tide blooms that appears to have been carried forward into subsequent years resulting in an increase in system-wide DOC concentrations over time. DOC concentrations increased in Chincoteague Bay over the study period (2002 – 2007) by approximately 50%. It is likely that the long residence time of this coastal lagoon (Pritchard 1960) has contributed to the trapping and accumulation of material in the system. If blooms continue to occur in this lagoon and organic carbon concentrations continue to accumulate, this may push Chincoteague Bay towards a heterotrophic system. Not only might this favor the growth of heterotrophic bacteria and phytoplankton mixotrophs such as *A. anophagefferens*, but this system change may also favor other mixotrophic harmful algal species (HAB's) that tend to flourish in eutrophic estuaries (Burkholder et al. 2008). Elevated bacterial production might change the trophic status of this system, provide increased prey for bacterivorous protists and may negatively impact the system by depleting dissolved oxygen.

*A. anophagefferens* blooms may also have a positive feedback on HAB's (Sunda et al. 2006). Because grazing is reduced during *A. anophagefferens* blooms (Gobler et al. 2002), there are fewer recycled nutrients available (Sunda et

al. 2006) to support the growth of other species. As *A. anophagefferens* blooms intensify, light penetration is reduced which limits the growth of benthic algae (MacIntyre et al. 2004). Without benthic algae intercepting nutrients coming from the sediments, there may be a greater flux of nutrients coming out of the sediments and into the water column (MacIntyre et al. 2004; Sunda et al. 2006) and this may further stimulate the growth of *A. anophagefferens*. Wazniak (2004) found that Chincoteague Bay has a considerable benthic microalgae population, with summertime benthic chlorophyll concentrations averaging  $38.69 \text{ mg m}^{-2}$  in 2002 and  $28.6 \text{ mg m}^{-2}$  in 2003. If these blooms block light reaching the sediments on a regular basis, the benthic algal community could also be impacted by dense blooms. These positive feedbacks could result in increases in brown tide bloom intensity in Chincoteague Bay in the future.

#### *Nutrient ratios*

During this study, DIN:DIP ratios were consistently below 16. At both sites, the DIN:DIP ratio ranged from 0.2 to 43 and averaged  $5.4 (\pm 7.7)$ . The low DIN:DIP ratios suggest that the system was depleted in N relative to P, as has been shown previously for coastal and estuarine systems (Fisher et al. 1992; Kemp et al. 1992). This was also observed during 2002 (Mulholland et al. 2009a) at both the bloom and non-bloom sites. However, TDN:TDP ratios were usually in excess of the Redfield ratio, suggesting P limitation (Fig. 9). This presumes that the DON and DOP pools are bioavailable. DOP concentrations were generally low, resulting in high DOC:DOP and DON:DOP ratios. DON concentrations were similar to what has been observed in other brown tide prone estuaries,



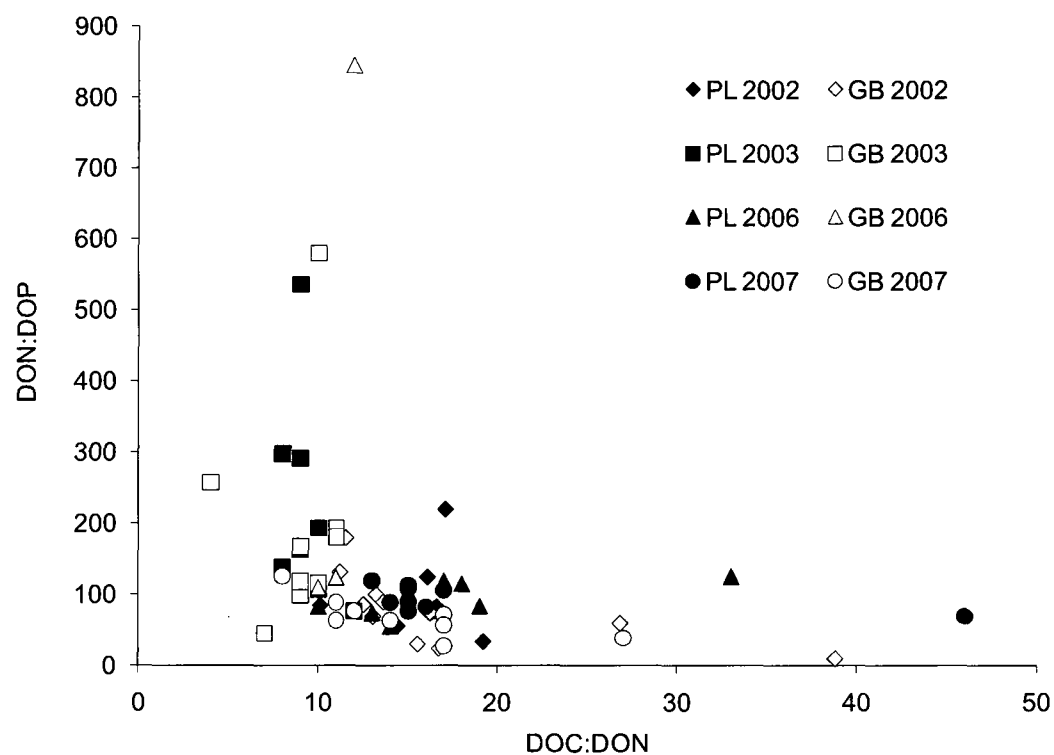
**Fig. 9** Total dissolved nitrogen (TDN) versus total dissolved (TDP) phosphorus for all the blooms (2002, 2003, 2006, and 2007) sampled as part of this project (including data reported in Simjouw et al. 2004; Minor et al. 2006; and Mulholland et al. 2009). The black line is the 16:1 line (Redfield)

including those on Long Island (Lomas et al. 2001; Lomas et al. 2004).

It has also been suggested that brown tide blooms are associated with high DOC:DON and low DON:DOP (Lomas et al. 2001). However, this was not observed in not observed in Chincoteague Bay in 2002 (Mulholland et al. 2009a) or in subsequent bloom years reported here (Fig. 10). So, while it has been suggested that organic nutrient ratios and DIN depletion are causative agents promoting brown tide bloom formation, my results suggest that relating blooms to nutrient concentrations and ratios may be more complicated than previously thought.

The total measured C:N uptake ratio estimated from the short term incubations averaged 7.6 but the range was quite high (1-44), suggesting that there may be short term uncoupling between C and N uptake during blooms. The short-term C:N uptake ratios at PL were below Redfield for most of the study, suggesting short-term imbalances in C and N uptake or unquantified C sources supporting the growth of bloom organisms. I estimated uptake of C from glucose, urea, and amino acids during mid-day incubations. Combined these are only a very small fraction of the DOC pool (Benner 2002). Uptake of DOC during dark periods or of compounds not measured here may also have contributed to *A. anophagefferens* growth.

It is also interesting to note that although cells were taking up urea as a nitrogen source, they were usually not incorporating the carbon associated with the urea. This has also been observed during brown tide blooms in Quantuck Bay, NY (Lomas 2003). Urea carbon was taken up at a higher rate than urea



**Fig. 10** Comparison of DOC:DON and DON:DOP ratios during brown tide blooms in 2003, 2006, and 2007 at both locations, GB and PL. The 2002 PL and GB data is added from Mulholland et al. 2009)

nitrogen at only timepoint during the 2006 GB bloom (Fig. 7). Excluding this one exception, the C:N uptake ratio for urea averaged just 0.06. In contrast the C:N ratio for DFAA averaged 2.6 throughout the study. This is similar to what was observed in a 2000 bloom in Quantuck Bay, NY and the 2002 bloom at PL when C:N uptake for DFAA was about 2 (Mulholland et al. 2002; Mulholland et al. 2009).

It is unclear why urea C was not assimilated but DFAA C was. Previous studies suggest that urea may be degraded to produce ammonium in the environment (Kamennaya et al. 2008). Alternatively urease catalyzes the degradation of urea to 2 ammonium ions and CO<sub>2</sub> within the cell. Amino acids on the other hand may be assimilated directly into the cell where C is conserved in intermediate metabolites in the cell.

### *Conclusions*

During this six-year study of brown tide blooms in Chincoteague Bay MD and VA, I found an increase in bloom intensity and duration over time and an overall accumulation of DOC in this lagoonal system. This has important implications for the overall health of the bay and may lead to changes in ecosystem structure and metabolism, trophic status, and food web interactions.

Further, *A. anophagefferens* is nutritionally versatile and are able to use a wide range of nitrogen and carbon sources to meet their nutritional demands. Consequently, any strategy for managing nutrient loads to prevent blooms should also take into account the ability of both inorganic and organic C and N to be used by bloom organisms. Because no single N compound was responsible for fueling

brown tide growth, the total N load and retention of that load within the system may be key factors contributing to brown tides rather than inputs of any particular form of N.

During blooms, organic C uptake subsidized C acquisition from photosynthesis. Although bicarbonate uptake was higher than organic carbon uptake, sampling and rate measurements were made at mid-day when PAR was at its peak. *Further investigations are needed to determine the contribution of DOC to daily cellular C demand, particularly when light or C may be limiting.*

**CHAPTER III**

**NITROGEN AND CARBON UPTAKE BY *AUREOCOCCUS***

***ANOPHAGEFFERENS* VERSUS CO-OCCURRING BACTERIA DURING**

**A BLOOM: A FLOW CYTOMETRY APPROACH**

**Introduction**

Blooms of *Aureococcus anophagefferens* have been recorded in coastal bays along the Eastern United States since 1985. Numerous studies have examined the utilization of nutrients during these blooms. The uptake of dissolved organic matter (DOM) could give *A. anophagefferens* a competitive advantage over strictly autotrophic phytoplankton (Chapter II) since it can supply nitrogen, phosphorus, and carbon even during the dark when the light-dependent reactions of photosynthesis cannot occur (Chapter IV). This can, however, put *A. anophagefferens* in direct competition with bacteria for DOM.

Studies have shown that *A. anophagefferens* has the ability to take up a wide range of compounds to meet its nitrogen (N) demand (Dzurica et al. 1989; Lomas et al. 1996; Berg et al. 1997; Glibert et al. 2001; Berg et al. 2002; Mulholland et al. 2002; Mulholland et al. 2004; Mulholland et al. 2009a). In Chincoteague Bay,  $\text{NH}_4^+$ , nitrate ( $\text{NO}_3^-$ ), urea, and amino acids are all taken up during blooms of *A. anophagefferens* (Chapter II; Mulholland et al. 2009a). Further, a recent examination of its genome has determined that *A. anophagefferens* has the capacity to take up eight different forms of N including those mentioned above, as well as amides, cyanate,  $\text{NO}_2^-$ , and guanine (Berg et al.



2008). In culture and field studies, *A. anophagefferens* has been shown to take up organic nitrogen including urea and amino acids (Lomas et al. 1996; Berg et al. 1997; Berg et al. 2002; Mulholland et al. 2002; Mulholland et al. 2004; Mulholland et al. 2009a). In addition, it has a high affinity for ammonium ( $\text{NH}_4^+$ ) and urea (Lomas et al. 1996; Berg et al. 1997).

Bacteria can also take up a wide range of nitrogen compounds. Studies have found that bacteria take up  $\text{NH}_4^+$  (Wheeler and Kirchman 1986; Keil and Kirchman 1991; Lipschultz 1995; Hoch and Kirchman 1995; Tungaraza et al. 2003; Fouilland et al. 2007) and amino acids (Billen 1984; Fuhrman 1987; Jørgensen et al. 1993) to meet their nitrogen demand. Although  $\text{NH}_4^+$  and amino acids are thought to be the primary sources of N for bacteria (Hoch and Kirchman 1995; Kirchman 2000), numerous studies have also found that bacteria can also take up  $\text{NO}_3^-$  (Horrigan et al. 1988; Harrison and Wood 1998; Kirchman and Wheeler 1998; Middleburg and Nieuwenhuize 2000; Allen et al. 2002, Fouilland et al. 2007) and urea (Tungaraza et al. 2003; Veuger et al. 2004; Jørgensen 2006; Fouilland et al. 2007; Sanderson et al. 2008; Bradley et al. 2010).  $\text{NO}_3^-$  uptake rates by bacteria are especially high when  $\text{NO}_3^-$  concentrations are elevated (Middleburg and Nieuwenhuize 2000; Allen et al. 2002).

Because *A. anophagefferens* and heterotrophic bacteria both appear to be capable of taking up many of the same organic and inorganic compounds, the two groups may be competing for these nutrients in the environment. Such competition has been observed in cultures (Berg et al. 2002), where it was shown that three strains of bacteria isolated from an *A. anophagefferens* culture had

higher mean hydrolysis rates for urea and acetamide than *A. anophagefferens*.

However, *A. anophagefferens* was able to hydrolyze aminopeptide and chitobiose at higher rates than the bacterial strains. Together, these observations suggest that not only do we need to understand the interactions between *A. anophagefferens* and co-occurring phytoplankton, but that interactions between heterotrophic bacteria and *A. anophagefferens* may also be important in bloom development.

There is evidence of interactions between *A. anophagefferens* and bacteria from previous field studies (Gobler and Sanudo-Wihelmy 2001a; Mulholland et al. 2002; Mulholland et al. 2009a). During a 2000 bloom in Quantuck Bay, New York (NY), bacterial cell densities increased in tandem with increases in *A. anophagefferens* abundance (Mulholland et al. 2002; Mulholland et al. 2004). This pattern was also observed during the 2003, 2006, and 2007 blooms in Chincoteague Bay, MD (Chapter II). During a 1998 bloom in West Neck Bay, NY, bacterial populations reached their maximum cell densities immediately after *A. anophagefferens* began to decrease (Gobler and Sanudo-Wihelmy 2001a). This may be because viral lysis and degradation of the *A. anophagefferens* cells released a significant amount of DOM and remineralized nutrients into the water column (Gobler et al. 1997; Gobler and Sanudo-Wihelmy 2001a). Similar dynamics were observed during a 2002 bloom in Chincoteague Bay, when bacterial cell density and DOC concentrations peaked only after the *A. anophagefferens* bloom began to decline (Simjouw et al. 2004; Mulholland et al. 2009).

This study employed flow cytometry to examine taxon-specific uptake of

commonly available inorganic and organic N compounds ( $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , urea, and DFAA) by *A. anophagefferens* and heterotrophic bacteria in incubations of natural populations. The goal of this study was to ascertain whether these two groups directly compete for the same N resources or whether they each use different, specific components of the dissolved N pool, thereby avoiding direct competition. At the same time, uptake of organic C by both *A. anophagefferens* and heterotrophic bacteria was also examined. Stable isotopes were used as tracers to measure N and C uptake and flow cytometry was used to isolate *A. anophagefferens* and bacteria from natural populations.

There are major challenges involved in determining taxon-specific N and C uptake rates. A variety of methods have been used in past studies to compare uptake of C and N by phytoplankton and heterotrophic bacteria. Size fractionation is probably the most widely employed method to distinguish N and C uptake by different phytoplankton size fractions (Tamminen and Irmisch 1996; Allen et al. 2002; Fouilland et al. 2007; Sanderson et al. 2008). However, because *A. anophagefferens* and bacteria are similar in size, size fractionation techniques fail to adequately separate the two groups. Another issue with size-fractionation techniques is that filters can easily clog, resulting in retention of smaller sized cells on filters, and leading to spuriously high or low calculation of uptake rates by different planktonic groups.

Antibiotics have also been used to inhibit bacterial N and C uptake rates (Wheeler and Kirchman 1986; Middleburg and Nieuwenhuize 2000; Tungaraza et al. 2003; Veuger et al. 2004) and thereby assess uptake of N and C by

phytoplankton, but this method may not be 100% effective at inhibiting bacterial N and C uptake (Hamdan and Jonas 2007). Tungaraza et al. (2003) suggested that uptake rates measured using antibiotics should be interpreted carefully because, while phytoplankton growth rates were not affected by the addition of antibiotics, other physiological effects of antibiotic treatments on phytoplankton are unknown. In addition, antibiotics stop bacteria not only from taking up, but also regenerating nutrients, which could also bias interpretation of incubation experiments.

In order to distinguish and quantify N and C uptake by bacteria versus *A. anophagefferens*, I coupled isotopic tracer techniques with flow cytometry. Tracer incubations using  $^{15}\text{N}$  and  $^{13}\text{C}$  labeled substrates were conducted and then cells were sorted using flow cytometry. Uptake rates of N and C compounds by *A. anophagefferens* versus heterotrophic bacteria were then calculated to evaluate the relative uptake of N and C substrates by bacteria versus *A. anophagefferens*, the dominant phytoplankton during this study.

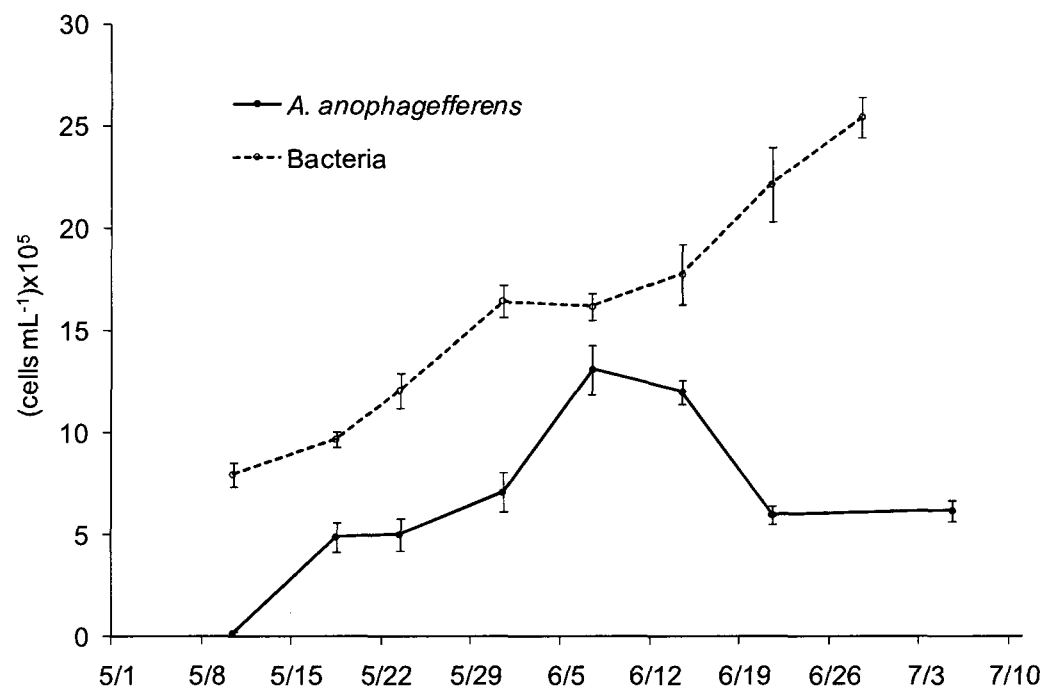
### **Materials and Methods**

Water samples were collected during the beginning, peak, and demise of an intense brown tide bloom during 2006 in Chincoteague Bay. Uptake experiments were conducted on three dates during three phases of bloom development: May 23 (early bloom), June 7 (peak bloom), and June 21 (late bloom), when *A. anophagefferens* concentrations were at  $5.0 \times 10^5$ ,  $13.1 \times 10^5$ , and  $6.0 \times 10^5$  cells  $\text{mL}^{-1}$ , respectively (Table 7 and Fig. 11). For this study, blooms were defined as cellular concentrations of *A. anophagefferens*  $>20,000$  cells  $\text{mL}^{-1}$ .

**Table 7** Physical, biological and chemical parameters at Public Landing in Chincoteague Bay, MD and VA

Date	Sal.	Temp (°C)	pH	Chl a (µg Chl L <sup>-1</sup> )	AA (cells mL <sup>-1</sup> )x10 <sup>5</sup>	Bacteria (cells mL <sup>-1</sup> )x10 <sup>5</sup>	NH <sub>4</sub> <sup>+</sup> (µmol L <sup>-1</sup> )	NO <sub>3</sub> <sup>-</sup> + NO <sub>2</sub> <sup>-</sup> (µmol L <sup>-1</sup> )	DIN (µmol L <sup>-1</sup> )	DIP (µmol L <sup>-1</sup> )	DIN:DIP
23 May	31.1	18.3	8.8	8.7 (0.5)	5.03 (0.79)	12.07 (0.88)	0.93 (0.05)	0.17 (0.02)	1.2	0.03 (0.01)	34.4
07 Jun	31.2	21.8	8.7	25.7 (0.4)	13.1 (1.19)	16.19 (0.66)	0.64 (0.01)	0.11 (0.01)	1.0	0.66 (0.01)	1.5
21 Jun	30.7	26.5	8.9	28.5 (0.4)	5.96 (0.45)	22.13 (1.81)	0.46 (0.08)	0.11 (0.05)	0.7	0.46 (0.07)	1.5

Standard deviations are in parentheses. Dissolved inorganic N was calculated as the sum of the average NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup> concentrations and ratios were calculated from average values. AA represents *A. anophagefferens*



**Fig. 11** Bacterial (dashed line) and *A. anophagefferens* (solid line) concentrations during the 2006 PL bloom. Vertical bars indicate standard deviation

This is the concentration at which *A. anophagefferens* can have a negative impact on *Mercenaria mercenaria* growth rates in Chincoteague Bay (Wazniak and Glibert 2004).

Water was collected from Public Landing, Maryland (PL) in the same manner as described in Chapter II. Whole water samples were filtered through a 0.2  $\mu\text{m}$  Supor cartridge filters in triplicate and filtrate was frozen in acid-cleaned bottles for subsequent analyses of dissolved constituents. An Astoria Pacific Autoanalyzer was used to colorimetrically determine concentrations of: nitrate plus nitrite (hereafter referred to as  $\text{NO}_3^-$  for simplicity), phosphate, and urea, (Parsons et al. 1984).  $\text{NH}_4^+$  concentrations were determined using the manual phenol hypochlorite method (Solorzano 1969). Dissolved free amino acid (DFAA) concentrations were measured using high performance liquid chromatography (HPLC) (Cowie and Hedges 1992).

For chlorophyll *a* (Chl *a*) analyses, samples were collected in triplicate and were gently filtered (< 5 kPa) onto GF/F filters. Chl *a* was measured using standard fluorometric methods (Welschmeyer 1994) on a Turner fluorometer. Bacteria and *A. anophagefferens* cell abundances were enumerated using epifluorescent microscopy; heterotrophic bacteria were stained with 4',6-di-amidinophenyl-indole (DAPI) as outlined by Porter and Feig (1980) and *A. anophagefferens* concentrations were enumerated using epifluorescent microscopy described by Anderson et al. (1989).

Whole water was transferred into acid-cleaned 1 L polycarbonate bottles for uptake experiments. Duplicate incubations to measure the uptake of  $^{15}\text{NO}_3^-$ ,

$^{15}\text{NH}_4^+$ ,  $^{13}\text{C}$ -labeled glucose, and  $^{15}\text{N}$  and  $^{13}\text{C}$  dually labeled urea and leucine were conducted. Leucine was chosen for these experiments because, while *A. anophagefferens* has been shown to utilize amino acids (Mulholland et al. 2002), leucine is also used to estimate bacterial production (Bell 1993; Kirchman 1993) and so uptake of this amino acid by *A. anophagefferens* could compromise the use of this technique to estimate bacterial productivity in systems where they are abundant. Incubations were initiated by adding  $0.03\ \mu\text{mol L}^{-1}$  of highly enriched (96-99%)  $^{15}\text{N}$  and  $^{13}\text{C}$ -labeled substrates to the respective incubation bottles. Additions resulted in atom % enrichments ranging from 1 to 94%. Enrichments of  $\geq 1\%$  have been shown previously to produce reliable uptake estimates (Mulholland et al. 2009a), although enrichments of  $>10\%$  may artificially stimulate uptake (Glibert and Capone 1993). After 30 minutes, incubation experiments were terminated by the addition of 10% glutaraldehyde (1% final concentration).

After isotope additions, incubation bottles were transported to an incubator where temperatures were maintained within  $2^\circ\text{C}$  of ambient levels in Chincoteague Bay and under ambient light conditions using neutral density screens. The average incoming solar radiation during light incubations, measured using Hydrolab dual-PAR sensor, ranged from 226 to  $1517\ \mu\text{E m}^{-2}\ \text{sec}^{-1}$ . Control samples receiving no isotope additions were preserved in the same manner as the tracer experiments with a final concentration of 1% glutaraldehyde. This was done so that the effect of the glutaraldehyde on both the C mass and the atom % enrichment of the isotope could be accounted for in the final uptake calculations



for C compounds. Natural abundance samples were also collected by filtering 100-300 mL of whole water onto combusted GF/F filters (450°C for 2 hour). Initial studies to examine the effects of using glutaraldehyde on the C mass and atom % enrichment of particulate C on the filter were conducted using whole water from the Elizabeth River, Virginia. Results showed that while glutaraldehyde did affect the carbon mass (it added mass) and C isotopic signature (it resulted in isotopically lighter particulate samples), these effects were consistent and could be accounted for in the final uptake calculations with the appropriate controls (Table 8). Preservation with glutaraldehyde added about 24% C biomass that was isotopically lighter than the non-preserved samples.

Samples from uptake experiments were concentrated and then sorted using a flow cytometer equipped with a high speed sorter at the Bermuda Institute of Ocean Sciences Marine Particle Imaging Facility. First, 50-100 mL of preserved sample from the uptake experiments were gently concentrated to a volume of 5mL onto silver filters (pore size of 0.2  $\mu$ m). Concentrated samples were then sorted using a flow cytometer equipped with a Cytopenia Influx Cell Sorter using 0.2  $\mu$ m filtered 3.6% NaCl solution as sheath fluid and a 100  $\mu$ m tip. Sort logic for autotrophic populations was based upon characteristic red fluorescence/orange fluorescence/forward scatter patterns. Sort logic for heterotrophic bacteria, was based on positive HOECHST stain and absence of red chlorophyll fluorescence. This gating scheme made it possible to separate detrital particles from DNA containing particles and separate DNA containing particles into heterotrophic bacteria (absence of red fluorescence) and autotrophs (presence of red

**Table 8** Measurements of carbon mass ( $\mu\text{mol C L}^{-1}$ ), Atom % C, isotopic signature and carbon uptake rates ( $\mu\text{mol C L}^{-1} \text{ h}^{-1}$ ) for natural water samples before (initial) and after (final) incubations with  $^{13}\text{C}$ -labeled bicarbonate

Time	Preservation	Mass C ( $\mu\text{M}$ )	% C	Atm % C	$\delta^{13}\text{C}$	Carbon Uptake
		Avg	From GLUT	Avg		( $\mu\text{mol C L}^{-1} \text{ h}^{-1}$ )
Initial	None	1084 (117)		1.08630 (0.00009)	-22.69 (0.08)	
Initial	Glut	1389 (31.6)	28.1	1.08591 (0.00017)	-23.04 (0.16)	
Final	None	1158 (137)		1.43185 (0.06483)		10.95 (2.05)
Final	Glut	1384 (198)	19.5	1.41531 (0.06401)		10.44 (2.03)

Parameters are shown with and without glutaraldehyde (GLUT) preservation. Standard deviations from 5 replicate incubations are in parentheses.

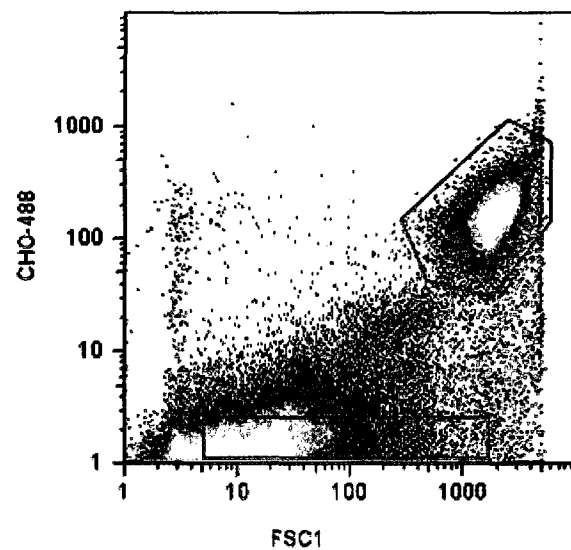
fluorescence). All data was acquired by Spigot™ (Cytospeia Inc., Seattle, WA) and analyzed with FCS Express 2™ software (De Novo Software, Thornhill, Ontario). The gates used for sorting are shown in Fig. 12.

Once sorted, the *A. anophagefferens* fraction was filtered onto a combusted (450°C for 2 hours) GF/C filter (nominal pore size of ~1.2 µm). Bacteria samples were filtered onto silver filters (pore size of 0.2 µm). Filters were rinsed with 0.2 µm filtered seawater and stored frozen until analysis. Filters were dried at 50°C for up to 48 hours in a drying oven and then pelletized in tin disks. The isotopic composition of samples was determined using a Europa Scientific GEO 20/20 isotope ratio mass spectrometer (IRMS), equipped with an automated nitrogen and carbon analyzer (ANCA). Uptake rates were calculated using the following equations:

***A. anophagefferens*:** PN<sub>AA</sub> is particulate nitrogen due to *A. anophagefferens*, in the final sorted incubation or in the initial sample, and PC<sub>AA</sub> is the particulate carbon of *A. anophagefferens*, in the final sorted incubation or in the initial sample.

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

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



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Count: 200000  
Ungated  
Pseudocolor Plot of FSC1 vs. CHO-488

**Fig. 12** Gates showing where *A. anophagefferens* and bacteria populations were sorted

**Bacteria:**  $PN_B$  is particulate nitrogen due to bacteria, in the final sorted incubation or in the initial sample, and  $PC_B$  is the particulate carbon of bacteria, in the final sorted incubation or in the initial sample.

$$\frac{^{15}\text{N}}{\text{Uptake}} = \frac{(\text{atom \% } PN_B)_{\text{final}} - (\text{atom \% } PN_B)_{\text{initial}}}{(\text{atom \% N source pool} - \text{atom \% } PN_B)_{\text{initial}} * \text{time}} \times [PN_B], \quad (3)$$

$$\frac{^{13}\text{C}}{\text{Uptake}} = \frac{(\text{atom \% } PC_B)_{\text{final}} - (\text{atom \% } PC_B)_{\text{initial}}}{(\text{atom \% C source pool} - \text{atom \% } PC_B)_{\text{initial}} * \text{time}} \times [PC_B], \quad (4)$$

Initial atom% values were determined by measuring the atom% of *A.*

*anophagefferens* and bacteria in the preserved controls. The controls had no added isotope but were preserved, consistent with treatment incubations, in glutaraldehyde, and flow cytometrically sorted the same way as the other treatments. This was done to correct for the isotopic signature of the carbon in the glutaraldehyde used to preserve samples. Glutaraldehyde added C mass to samples and resulted in significantly lighter C signatures in the samples (Table 8).

To calculate specific uptake rates by *A. anophagefferens*, it was necessary to determine the N or C mass associated with *A. anophagefferens*. *A. anophagefferens* concentrations present in natural water samples based on cell counts were multiplied by cellular C or N concentrations for *A. anophagefferens*. For *A. anophagefferens*, 0.35 pg N cell<sup>-1</sup> (Gobler, 1995) and 2.33 pg C cell<sup>-1</sup> (Milligan and Cosper, 1997) were used to calculate the PN and PC in natural samples due to *A. anophagefferens*. Cellular N and C were multiplied by the concentration of *A. anophagefferens* (per liter) to calculate the PN and PC per liter for the *A. anophagefferens* fraction in natural samples. Similarly, to

determine the PC and PN per unit volume due to bacteria, cellular concentrations of  $0.20 \text{ fg C cell}^{-1}$  and  $0.05 \text{ fg N cell}^{-1}$  were used (Lee and Fuhrman 1987). Cellular N and C were then multiplied by the concentrations of bacteria (per liter) in the initial water samples to calculate the PN and PC per liter due to bacterial biomass.

As described in Chapter II, N and C content of the DFAA pool was calculated based on the C:N ratio of the ambient DFAA pool from individual HPLC runs during 2002 (Mulholland et al. 2009). I established that on average there was  $1.18 \mu\text{mol L}^{-1}$  DFAA-N and  $4.41 \mu\text{mol L}^{-1}$  DFAA-C per  $1 \mu\text{mol L}^{-1}$  DFAA. I assumed that leucine uptake was representative of the uptake of all amino acids. As such, atom % enrichment of the amino acid pool was calculated using the total DFAA concentration as the substrate pool. The ambient dissolved inorganic C (DIC) concentrations were calculated based on salinity and assumed that  $\text{CO}_2$  concentrations were saturated in collected samples. The initial glucose concentration was estimated as 2% of the ambient DOC pool, the lower end of the range estimated by Benner 2002 (2-6%) for marine surface waters.

## Results

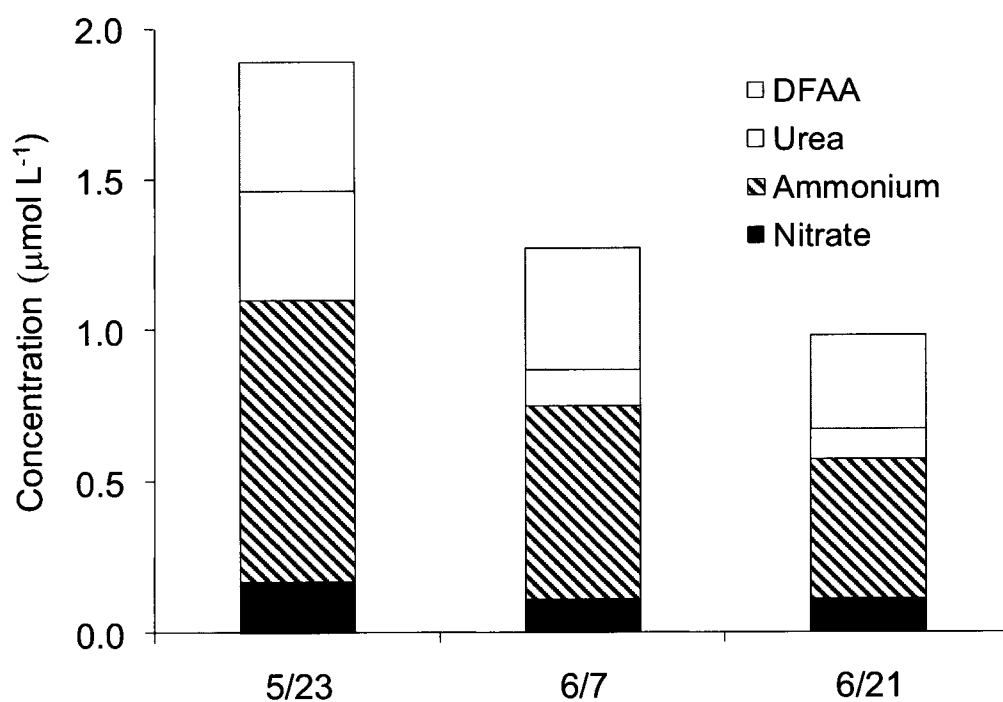
### *Chemical and biological parameters*

*A. anophagefferens* cells were detected in Chincoteague Bay during 2006 in early May (Fig. 11) and their concentrations quickly increased to harmful levels (concentrations  $>35,000 \text{ cells mL}^{-1}$ ; Gastrich and Wazniak, 2002) reaching a maximum density of  $13.1 \times 10^5 \text{ cells mL}^{-1}$  on June 7 (Fig. 11). Bacterial concentrations increased in tandem with *A. anophagefferens* cell densities but then continued to rise even as *A.*

*anophagefferens* cell abundances declined in late June and early July (Fig. 11), similar to the pattern during the 2002 bloom in Chincoteague Bay (Mulholland et al. 2009a).

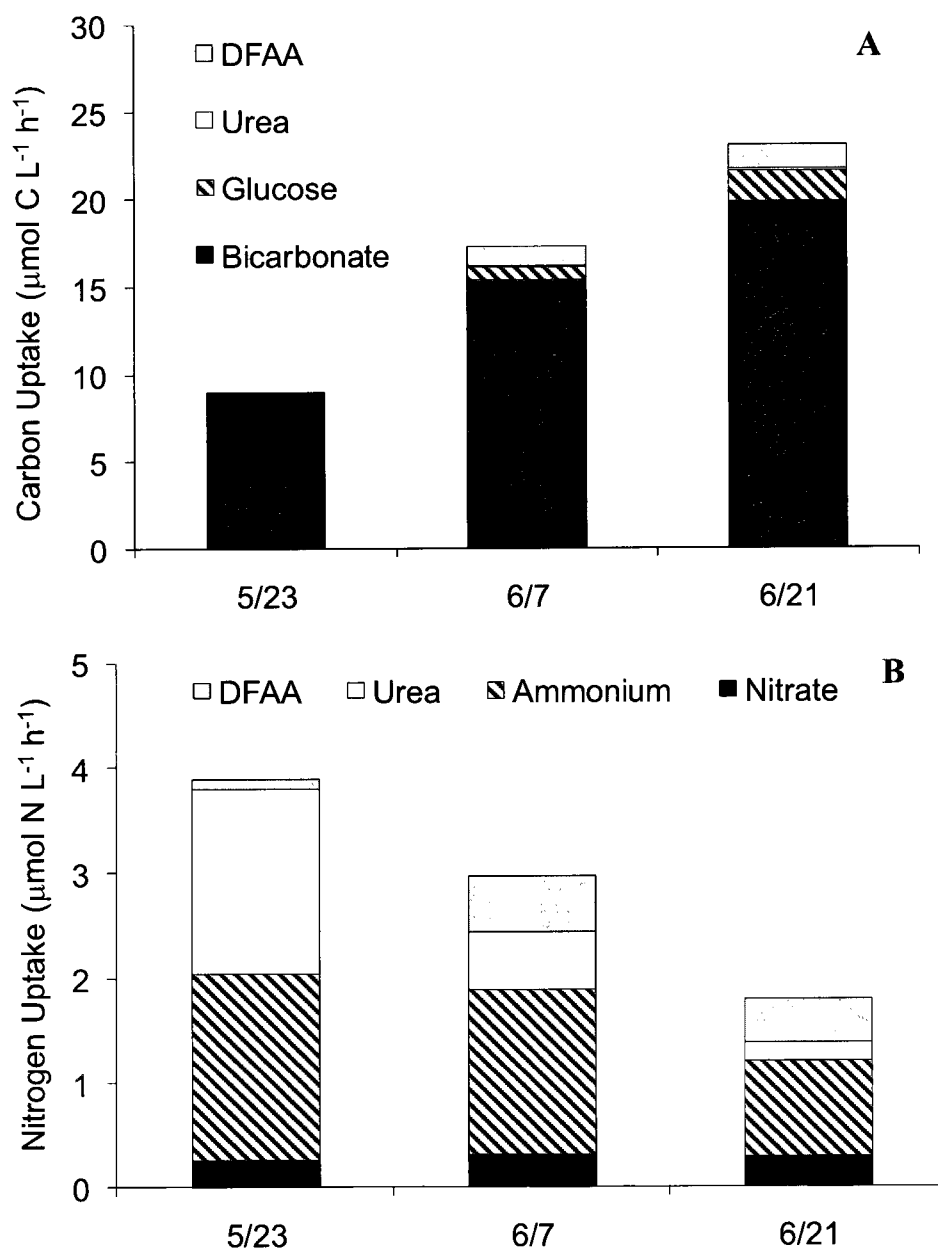
During the 2006 bloom at Public Landing, temperatures ranged from 18-26.5°C during May and June (Table 11, Chapter II), which was within range of optimal growth temperatures for *A. anophagefferens* (Casper et al. 1989). Salinities ranged from 30.7-31.2 (Table 7, Chapter II), which is also ideal for *A. anophagefferens* growth (Casper et al. 1989) and characteristic of Chincoteague Bay at this time of year (see Chapter II). pH showed little variation, ranging from 8.7-8.9 (Table 7 and Chapter II). Chl *a* concentrations increased from 8.7 µg Chl L<sup>-1</sup> on 5/23 to 25.7 µg Chl L<sup>-1</sup> on 6/7 and 28.5 µg Chl L<sup>-1</sup> on 6/21 (Table 7 and Chapter II). Assuming an average Chl *a* per cell for *A. anophagefferens* of 0.035 pg cell<sup>-1</sup> (Gobler and Sañudo-Wilhelmy 2001a, Gobler et al. 2002), 100% of the Chl *a* in the greater than 1.2 µm fraction could be attributed to *A. anophagefferens* on 5/23 and 6/7. On 6/21, as the bloom was declining, only 75% of Chl *a* could be attributed to *A. anophagefferens*. While *A. anophagefferens* concentrations were lower on this date, 5.96x10<sup>5</sup> cells mL<sup>-1</sup>, Chl *a* concentrations did not decrease, suggesting that another population of phytoplankton was present or that cellular Chl *a* concentrations changed over the course of the bloom (Table 7).

During this study, DIN concentrations did not vary much and ranged from 0.7 µmol L<sup>-1</sup> to 1.2 µmol L<sup>-1</sup> (Table 7). On all three dates, NH<sub>4</sub><sup>+</sup> was the most abundant form of N measured (Fig. 13) and the dominant source of N taken up in whole water incubations (Fig. 14). DIP concentrations were near the detection limit at the beginning of the bloom but higher at the peak of the bloom (0.66 µmol L<sup>-1</sup>) (Table 7). DOP and DON concentrations were relatively constant and ranged from 0.29 to 0.49 µmol L<sup>-1</sup> and



**Fig. 13** Dissolved nutrient concentrations during a 2006 brown tide bloom at PL





**Fig. 14** Carbon (A) and nitrogen (B) uptake in whole water on 5/23, 6/7, and 6/21

34.9 to 36.2  $\mu\text{mol L}^{-1}$ , respectively (Table 9). DFAA concentrations did not vary much (0.31 to 0.43  $\mu\text{mol L}^{-1}$ ) and on average were less than 2% of the DON pool (Table 9). Urea concentrations were higher at the beginning of the bloom (0.36  $\mu\text{mol L}^{-1}$ ) but lower by the end of the bloom (0.10  $\mu\text{mol L}^{-1}$ ) and, like DFAA concentrations, were on average less than 2% of the DON pool (Table 9). DOC concentrations were the highest on 6/7 at 1,188  $\mu\text{mol L}^{-1}$  (Table 9), corresponding with peak in *A. anophagefferens* cell densities (Table 7 and Chapter II). On the other two sampling days, DOC concentrations were 466  $\mu\text{mol L}^{-1}$  on 5/23 and 652  $\mu\text{mol L}^{-1}$  on 6/21 (Table 9).

#### *Whole water C and N uptake*

As seen in previous blooms (Mulholland et al. 2009a, Chapter II), whole water C uptake was dominated by bicarbonate (Fig. 14A). On 5/23, almost 100% of the measured C uptake was from bicarbonate, however, as the bloom progressed, more carbon uptake was from organic compounds (Fig. 14A). On 6/7, 11% of the carbon uptake was from two organic compounds (glucose and DFAA), and on 6/21 organic carbon, uptake of these compounds was 14% of the total measured C uptake (Fig. 14A). At the same time, total C uptake more than doubled between 5/23 and 6/21. While total measured C uptake increased over the course of the bloom, total measured N uptake decreased.

A diverse group of N compounds was taken up during 2006, as seen in previous blooms (Mulholland et al. 2009a, Chapter II). On 5/23, DIN and DON N uptake rates in whole water samples were almost equal, and together  $\text{NH}_4^+$  and urea accounted for 91% of the total measured N uptake (Fig. 14B). As the bloom progressed, the total measured N uptake decreased. While DIN uptake increased to 67% of the total N uptake, mainly due to a decrease in urea uptake (Fig. 14B), urea uptake rates decreased from 45% of the

**Table 9** Nutrients at Public Landing in Chincoteague Bay, MD and VA

Date	DOP ( $\mu\text{mol L}^{-1}$ )	Urea ( $\mu\text{mol L}^{-1}$ )	DFAA ( $\mu\text{mol L}^{-1}$ )	DON ( $\mu\text{mol L}^{-1}$ )	DOC ( $\mu\text{mol L}^{-1}$ )	DOC/DON	DOC/DOP	DON/DOP	TDN/TDP
23 May	0.49	0.36 (0.12)	0.43 (0.01)	36.2	466	13	951	73	70.9
07 Jun	0.29	0.12 (0.03)	0.40 (0.02)	35.7	1,188	33	4,097	124	38.8
21 Jun	0.42	0.10 (0.05)	0.31 (0.01)	34.9	652	19	1,552	83	40.7

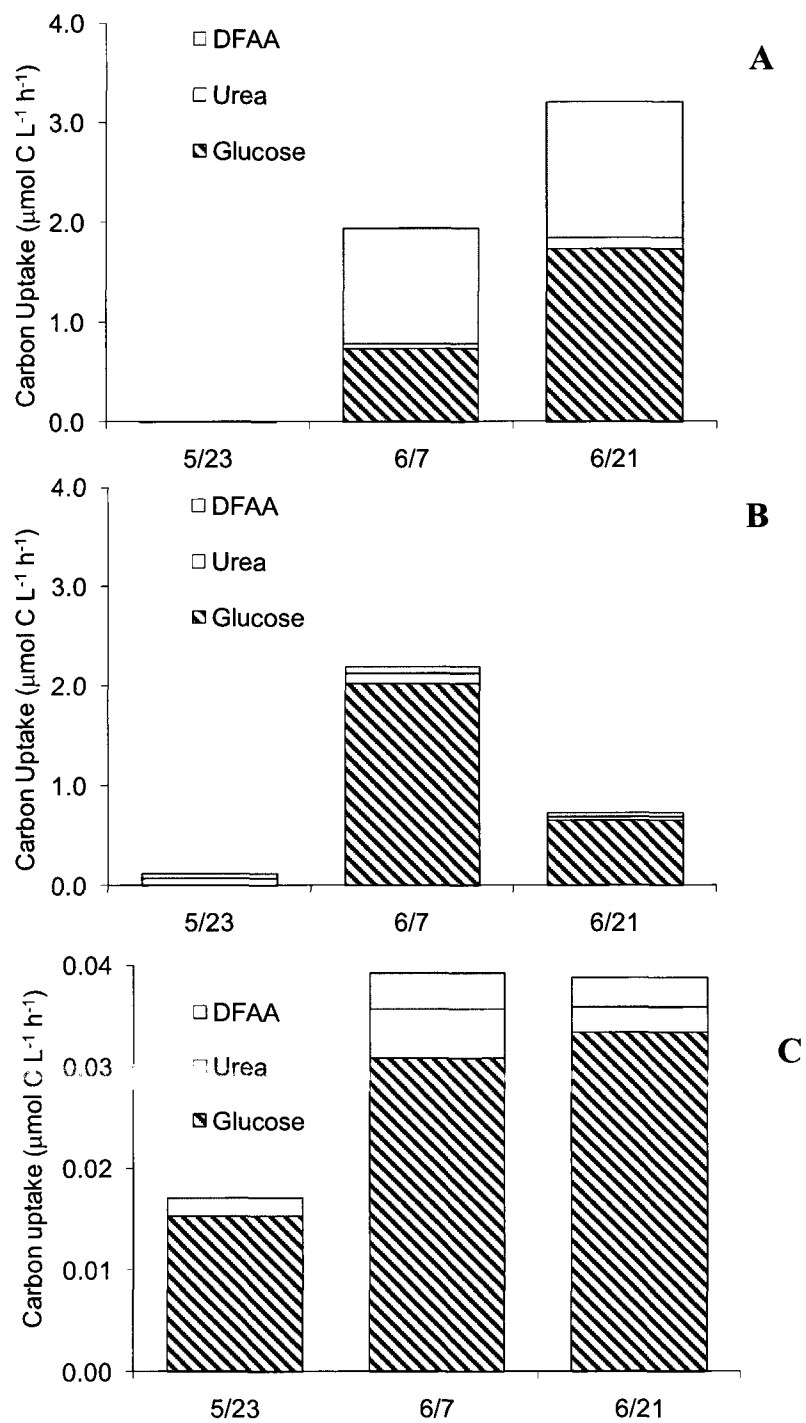
Standard deviations are in parentheses

total N uptake on 5/23 to 10% of the total N uptake on 6/21. Over the same time period, DFAA uptake increased as a fraction of the total N uptake, increasing from 3% to 23% on 6/21 (Fig. 14B).

As seen in previous brown tide blooms, urea nitrogen was taken up at high rates (Fig. 14). On 6/7, little of the urea C was taken up and the C:N uptake ratio for urea was 0.1. By 6/21, although total urea uptake was lower than at the beginning of the bloom, the urea C:N uptake ratio was 0.6. These results indicate that urea was taken up in stoichiometric proportion later in the bloom since a C:N ratio of 0.5 indicates balanced stoichiometric uptake. A decrease in urea N uptake during this time may be the cause of the increase in the C:N uptake ratio at the end of the bloom.

#### *A. anophagefferens* C and N uptake

Although uptake of all three DOC compounds measured was detected during this study, glucose was the quantitatively most important source of the three being utilized by *A. anophagefferens*. *A. anophagefferens* accounted for a majority of the glucose uptake on 6/7 and on 6/21 when *A. anophagefferens* accounted for 100 and 74% of the algal biomass, respectively (Figs. 15A, 15B). Organic C uptake was very low on 5/23 for both whole water and sorted *A. anophagefferens* cells (Fig. 15). Urea C uptake by *A. anophagefferens* was low on all three dates. There was no detectable DFAA C uptake on 5/23 but DFAA C uptake by *A. anophagefferens* accounted for >5% of the total C uptake by *A. anophagefferens* on 6/7 and 6/21. DFAA C uptake rates calculated for *A. anophagefferens* were an order of magnitude less than what was observed in whole water incubations (Fig. 15A), but an order of magnitude greater than what was observed for heterotrophic bacteria (Fig. 15C). When comparing volumetric C uptake rates by *A.*



**Fig. 15** Organic carbon uptake in whole water (A), *A. anophagefferens* carbon uptake rates (B), and bacteria carbon uptake rates (C). Please note the different scale for (C)

*anophagefferens* and heterotrophic bacteria, *A. anophagefferens* accounted for more organic C uptake than bacteria (Figs 15B, 15C). Together, uptake of the 3 organic C compounds by *A. anophagefferens* averaged  $1.01 \mu\text{mol C L}^{-1} \text{ h}^{-1}$  over the sampling period while bacterial uptake only accounted for  $0.03 \mu\text{mol C L}^{-1} \text{ h}^{-1}$ . When organic carbon uptake rates were compared on a per cell basis, the difference was even greater. Organic C uptake per *A. anophagefferens* cell ranged from  $0.22\text{--}3.22 \text{ fmol C cell}^{-1} \text{ h}^{-1}$  (Table 10) while cell-specific uptake rates by bacteria only ranged from  $17.17\text{--}24.24 \text{ amol C cell}^{-1} \text{ h}^{-1}$  (Table 11). In both cases, the highest uptake rates per cell were measured on 6/7, when *A. anophagefferens* concentrations were near their maximum and they accounted for 100% of the Chl *a* biomass (Table 10 and 11).

As with whole water samples, *A. anophagefferens* cell-specific urea N uptake rates were lower on the latter sampling dates than on 5/23,  $\text{NO}_3^-$  uptake remained constant over all 3 sampling dates, but DFAA uptake was higher as the bloom progressed (Figs. 16A, 16B). In contrast, while  $\text{NH}_4^+$  uptake dominated in the whole water fraction,  $\text{NH}_4^+$  uptake by *A. anophagefferens* was not detectable on 5/23 and 6/7 in sorted samples (Figs. 16A, 16B). On 6/21,  $\text{NH}_4^+$  uptake by *A. anophagefferens* was greater than urea and  $\text{NO}_3^-$  uptake but less than DFAA uptake (Fig. 16B).

As observed for the whole water uptake incubations, the C:N uptake ratio for urea in the sorted *A. anophagefferens* cells was higher as the bloom progressed. The ratio was 0.6 on 5/23, 2.32 on 6/7, and 4.45 on 6/21. In contrast, *A. anophagefferens* C:N uptake ratio for DFAA was lower as the bloom progressed. The C:N uptake ratio was 7.1 on 5/23, suggesting that DFAA C was taken up at near stoichiometric proportions as DFAA N (leucine has a 6:1 C:N ratio). The ratio was  $<1$  on the following two dates, indicating

**Table 10** Cell-specific organic carbon uptake rates for *Aureococcus anophagefferens* and calculated C turnover times

	Urea C Uptake (fmol C cell <sup>-1</sup> h <sup>-1</sup> )	Glucose Uptake (fmol C cell <sup>-1</sup> h <sup>-1</sup> )	DFAA C Uptake (fmol C cell <sup>-1</sup> h <sup>-1</sup> )	Total C Uptake (fmol C cell <sup>-1</sup> h <sup>-1</sup> )	C Turnover Time (days)
23 May	0.09	0.00 (0.00)	0.13	0.22	37
07 Jun	0.06	3.09	0.07 (0.05)	3.22	3
21 Jun	0.07 (0.01)	1.08 (0.36)	0.06 (0.01)	1.21	7

**Table 11** Cell-specific organic carbon uptake rates for bacteria and calculated C turnover times for bacterial biomass

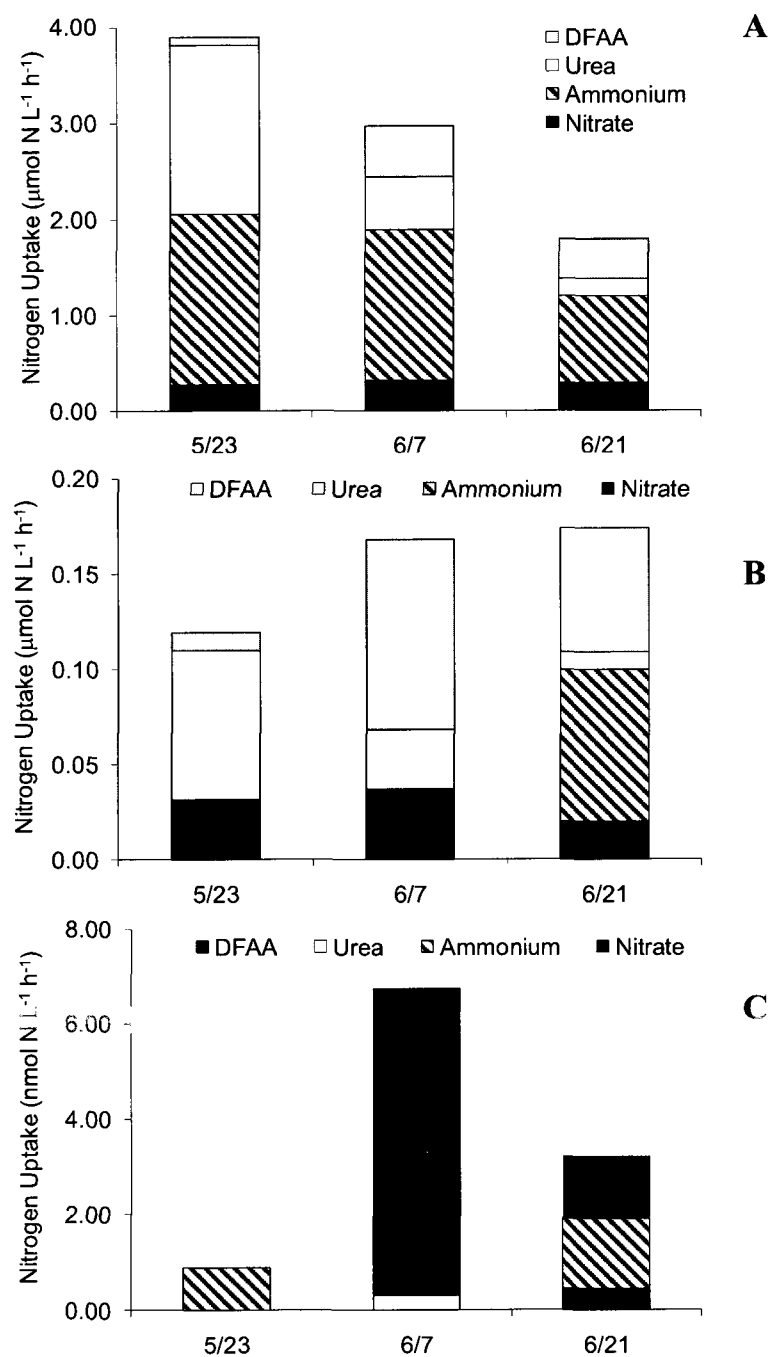
	Urea C Uptake ( $\text{amol C cell}^{-1} \text{h}^{-1}$ )	Glucose Uptake ( $\text{amol C cell}^{-1} \text{h}^{-1}$ )	DFAA C Uptake ( $\text{amol C cell}^{-1} \text{h}^{-1}$ )	Total C Uptake ( $\text{amol C cell}^{-1} \text{h}^{-1}$ )	C Turnover Time (days)
23 May	0.00 (0.00)	12.64	1.53 (0.12)	14.17	5
07 Jun	2.97	19.07 (0.34)	2.20 (1.68)	24.24	3
21 Jun	1.13	15.11	1.28 (0.38)	17.52	4



that DFAA N was taken up at a greater rate than DFAA C.

#### *Bacterial C and N uptake*

C uptake rates by bacteria were higher later in the bloom when bacteria cell numbers were also higher (Fig. 15C). As the bloom was beginning to develop on 5/23, bacteria took up  $17 \text{ nmol C L}^{-1} \text{ h}^{-1}$  with most of this uptake coming from glucose (Fig. 15C). On 6/7, when bacterial cell numbers were higher and *A. anophagefferens* cell numbers were at their peak (Fig. 11), bacterial abundances increased 34% but bacterial C uptake rates doubled to  $38 \text{ nmol C L}^{-1} \text{ h}^{-1}$  with most (84%) of that uptake coming from glucose (Fig. 15C). Although *A. anophagefferens* cell concentrations declined on 6/21 (Fig. 11), bacteria cell numbers again increased by 37% but bacterial C uptake rates remained constant at  $38 \text{ nmol C L}^{-1} \text{ h}^{-1}$  (Fig. 15C) with most of the of the carbon uptake coming from glucose (Fig. 15C). Bacterial N uptake rates were two orders of magnitude lower than *A. anophagefferens* N uptake rates (Figs. 16B, 16C). On 5/23, both N and C uptakes rates were low compared to other dates. As the bloom progressed and bacterial numbers increased (Fig. 15), bacterial nitrogen uptake was higher. On 6/7, nitrogen uptake was  $6.76 \text{ nmol N L}^{-1} \text{ h}^{-1}$  and was dominated by DFAA (93% of the total nitrogen uptake) with some urea uptake (7%) (Fig. 16C). On 6/21, nitrogen uptake was lower ( $3.20 \text{ nmol N L}^{-1} \text{ h}^{-1}$ ) and was dominated by  $\text{NH}_4^+$  and DFAA uptake (Fig. 16C). DFAA uptake by bacteria was highest on 6/7, with rate of  $6.5 \text{ nmol N L}^{-1} \text{ h}^{-1}$  (Fig. 16C). However, this was still two orders of magnitude less than the DFAA N uptake rate for *A. anophagefferens* ( $100 \text{ nmol N L}^{-1} \text{ h}^{-1}$ ; Fig. 16C). *A. anophagefferens* was also able to take up N at higher rates than bacteria on a per cell basis. Total N uptake per *A.*



**Fig. 16** Nitrogen uptake in whole water (A), *A. anophagefferens* N uptake rates (B), and bacteria N uptake rates (C). Please note the different scale for bacteria uptake

*anophagefferens* cell ranged from 0.13-0.30 fmol N cell<sup>-1</sup> h<sup>-1</sup> (Table 12) while cell-specific N uptake rates for bacteria only ranged from 0.46-4.17 amol N cell<sup>-1</sup> h<sup>-1</sup> (Table 13).

### Discussion

During this study, both *A. anophagefferens* and bacteria took up organic and inorganic N and organic C. When uptake rates were normalized per cell, *A. anophagefferens* DOC uptake rates were several orders of magnitude higher than bacterial cell-specific uptake rates (Tables 10 and 11), despite the commonly held view that bacteria are the primary consumers of DOC and phytoplankton do not take up DOC in the environment.

Similarly, when comparing cell-specific N uptake rates, *A. anophagefferens* took up organic and inorganic N at much higher rates than bacteria (Table 12 and 13). *A. anophagefferens* and bacteria had similar cellular DOC turnover times (Tables 10 and 11). The only exception to this was on 5/23 when *A. anophagefferens* organic C uptake was low. Since DIC uptake in the whole water incubations was measured, it is possible that *A. anophagefferens* was meeting its C needs through photosynthetic C uptake. The similar cellular DOC turnover times on 6/7 and 6/21 suggest that although *A. anophagefferens* is taking up DOC at higher rates, both groups may be meeting their cellular C requirements for growth. The average cellular N turnover time for bacteria was (1.9 days), less than the average cellular N turnover time for *A. anophagefferens* (5 days) (Tables 12 and 13).

While total measured organic C uptake was higher later during the *A. anophagefferens* bloom, total N uptake was lower which resulted in higher C:N uptake ratios. If inorganic carbon uptake is also considered, then the C:N uptake ratio is even

**Table 12** Cell-specific N uptake rates for *Aureococcus anophagefferens* and calculated N turnover times for *A. anophagefferens* N biomass

	$\text{NO}_3^-$ Uptake (fmol N cell <sup>-1</sup> h <sup>-1</sup> )	$\text{NH}_4^+$ Uptake (fmol N cell <sup>-1</sup> h <sup>-1</sup> )	DFAA N Uptake (fmol N cell <sup>-1</sup> h <sup>-1</sup> )	Urea N Uptake (fmol N cell <sup>-1</sup> h <sup>-1</sup> )	Total N Uptake (fmol N cell <sup>-1</sup> h <sup>-1</sup> )	N Turnover Time (days)
23 May	0.06 (0.01)	0.00 (0.00)	0.04	0.16 (0.03)	0.26	4
07 Jun	0.03 (0.01)	0.00 (0.00)	0.08 (0.05)	0.02 (0.02)	0.13	8
21 Jun	0.03 (0.01)	0.13	0.11 (0.08)	0.03	0.30	3

**Table 13** Cell-specific N uptake rates for bacteria and calculated turnover times of bacterial N biomass

	<b>NO<sub>3</sub><sup>-</sup> Uptake (amol N cell<sup>-1</sup> h<sup>-1</sup>)</b>	<b>NH<sub>4</sub><sup>+</sup> Uptake (amol N cell<sup>-1</sup> h<sup>-1</sup>)</b>	<b>DFAA N Uptake (amol N cell<sup>-1</sup> h<sup>-1</sup>)</b>	<b>Urea N Uptake (amol N cell<sup>-1</sup> h<sup>-1</sup>)</b>	<b>Total N Uptake (amol N cell<sup>-1</sup> h<sup>-1</sup>)</b>	<b>Turnover (days)</b>
23 May	0.00 (0.00)	0.74 (0.48)	0.00 (0.00)	0.00 (0.00)	0.74	2.0
07 Jun	0.00 (0.00)	0.00 (0.00)	3.99	0.19 (0.15)	4.17	0.4
21 Jun	0.20 (0.11)	0.13 (0.41)	0.13	0.00 (0.00)	0.46	3.2

higher. One possible explanation for this discrepancy may be unbalanced growth. It is also possible that there were unidentified N sources that were supplying additional N later in the bloom. This seems likely because *A. anophagefferens* has been shown to use a variety of organic N sources other than those tested here (Berg et al. 2002; Mulholland et al. 2002; Mulholland and Lee 2009). *A. anophagefferens* has been shown to hydrolyze aminopeptide and chitobiose at higher rates than several co-occurring bacteria strains (Berg et al. 2002) as well as perform peptide hydrolysis (Mulholland et al. 2002; Mulholland and Lee 2009). In addition, genomic analysis of *A. anophagefferens* suggest that a variety of other N compounds may be used by *A. anophagefferens*, including purines and cyanate (Berg et al. 2008).

#### *C and N interactions*

One important factor that may influence the relative uptake of DOM by *A. anophagefferens* and bacteria may be the C:N ratio of DOM (Gobler et al. 2005). This is because the C:N ratio of DOM may determine if bacteria are net producers or consumers of DIN (Goldman and Dennett 2000). Using a C:N mass balance model (Goldman et al. 1987), Gobler et al. (2005) suggest that when C:N ratios are low ( $<10$ ), bacteria tend to remineralize nitrogen. This is because at low C:N ratios there is a surplus of N relative to C for the bacterial cell growth which results in bacteria releasing excess N back into the environment. High DOM C:N ratios ( $>10$ ), however, result in a N deficit for bacteria and bacteria may take up DIN to balance internal C and N pools (Goldman et al. 1987; Kirchman et al. 1990). Gobler et al. (2005) suggest that if the C:N ratios of DOM are high ( $>10$ ), bacteria will use DIN rather than DON as an N source. This has the potential to be beneficial to *A. anophagefferens* in two ways. First, if bacteria take up DIN instead

of organic substrates, *A. anophagefferens* would no longer be competing with bacteria for organic N sources. The second benefit would be that because bacteria are taking up DIN, they would be competing for the same N pool as non-*A. anophagefferens* phytoplankton, thereby giving *A. anophagefferens* a competitive advantage over other phytoplankton. In support of this idea, Hasegawa et al. (2005) found that when glucose was added to water from Sagami Bay, Japan, the elevated DOC:DON ratios resulted in bacteria out-competing the existing phytoplankton for DIN. If this happened during a brown tide bloom, it could help *A. anophagefferens* outcompete other phytoplankton species and form blooms.

These findings could be important in Chincoteague Bay since the mean DOC:DON ratio at PL and GB has been increasing over the past several years largely due to an increase in DOC concentrations; DON concentrations have not changed much (Chapter II). At PL in 2003, the mean DOC:DON ratio was 9 but had increased to 18 by 2007 (Table 9 and Chapter II). During this study in 2006, DOC:DON ratios were > 10, the mean DOC:DON ratio was 17 (Chapter II), which according to the model above would promote bacterial uptake of DIN. Indeed, bacteria took up  $\text{NH}_4^+$  but *A. anophagefferens* did not during the first sampling date. On 6/7 the DOC:DON ratio was 33 (Table 9). However, on this date, most of the N taken up by bacteria was from DFAA rather than DIN (Fig. 16C). *A. anophagefferens* was also taking up DFAA at this time (Fig. 16B) at rates that were two orders of magnitude higher than those observed for bacteria (Figs. 16B, 16C). Two weeks later, when the DOC:DON ratio was 19, bacterial DIN uptake (both  $\text{NH}_4^+$  and  $\text{NO}_3^-$ ) exceeded measured DON uptake (Fig. 16C).

#### *Glucose uptake*

Surprisingly, during this study, glucose was an important source of C for both *A. anophagefferens* and bacteria. While glucose was the main source of DOC measured for bacteria on all 3 sampling dates, glucose uptake by *A. anophagefferens* was high on both 6/7 and 6/21. In fact, glucose uptake by *A. anophagefferens* always exceeded that measured for bacteria, suggesting that *A. anophagefferens* can compete with bacteria for glucose during blooms. This differs from another study in which bacteria had higher glucose uptake rates than algae (Kamjunke et al. 2008). In contrast, results presented here indicate that *A. anophagefferens* is capable of taking up glucose at higher rates than bacteria.

#### *Urea uptake*

*A. anophagefferens* took up urea N during all experiments and did so at higher rates than bacteria (Figs. 16B, 16C). This was expected since *A. anophagefferens* has a high affinity for urea (Lomas et al. 1996; Berg et al. 1997) and *A. anophagefferens* has several urea transporters (Berg et al. 2008). While the N from urea was taken up by *A. anophagefferens*, urea C was also taken up. This differs from what has been observed in previous brown tide blooms (Lomas 2004, Mulholland et al. 2009, Chapter II). When looking at *A. anophagefferens* cell-specific urea uptake rates, the C:N uptake ratio of urea was 0.6 on 5/23, 2.3 on 6/7, and 4.5 on 6/21. These results suggest that urea was being used more as a C than N source later during the bloom. This has been observed during other harmful blooms. Fan and Glibert (2005) found that during a *Prorocentrum minimum* bloom, the amount of C being used from urea doubled over the course of the bloom. The authors suggested that a possible cause of this was that as the bloom progressed, pH levels increased to 9-9.5, and dissolved inorganic carbon (DIC) became



limiting. Although such elevated pH levels were not observed during this bloom, cell densities were high and DIC uptake by whole water samples was high (Chapter II).

Bacteria are generally thought to be net producers of urea (Cho et al. 1996) since bacteria can release urea when breaking down purines and other organic compounds (Vogels and Van Der Drift 1976). Urea is generally not thought of as a significant N source for bacteria (Price and Harrison 1988; Tamminen and Irmisch 1996; Kirchman 2000; Middelburg and Nieuwenhuize 2000). Wheeler and Kirchman (1986) measured bacterial urea N uptake of 0-2 nmol N L<sup>-1</sup> h<sup>-1</sup> near Sapelo Island, GA and Middelburg and Nieuwenhuize (2000) measured urea uptake rates of <0.1 to 7 nmol N L<sup>-1</sup> h<sup>-1</sup> in the Thames estuary and North Sea. As reported previously, during this study, urea was not an important source of N for bacteria with rates ranging from 0-0.03 nmol N L<sup>-1</sup> h<sup>-1</sup>. Although bacteria did not use urea as a main N source, bacteria did take up carbon from urea, but rates were low (0-4.5 nmol C L<sup>-1</sup> h<sup>-1</sup>).

One reason for such low urea uptake by bacterial populations during this and other studies may be that only a small percent of bacteria have urease, the enzyme that breaks down urea intracellularly (Jørgensen et al. 2006). Another possible explanation of the low urea N uptake rates in this study may be that bacteria were already meeting their N needs with DIN, DFAA, and other DON present in the environment. Both DIN (0.7-1.2 μmol L<sup>-1</sup>) and DFAA (0.31-0.43 μmol L<sup>-1</sup>) were available and comprised over 95% of the total measured N uptake by bacteria. Since the bacteria in this study were actively taking up both NH<sub>4</sub><sup>+</sup> and DFAA's, it is possible that bacteria were N replete and urea N uptake was unnecessary or that they were taking up other N compounds not measured during this study. Urea uptake is also metabolically more costly than NH<sub>4</sub><sup>+</sup> and DFAA

uptake and so bacteria may prefer the latter N compounds when these are available. The metabolic cost of producing urease has been suggested as a limiting factor in urea uptake by bacteria (Jørgensen et al. 2006).

Gobler and Sanudo-Wihelmy (2001a) found that during brown tide blooms in West Neck Bay, NY, urea additions stimulated bacterial growth rates, however, they suggested that the increased bacterial growth rates were more likely related to elevated levels of phytoplankton exudation since the urea additions also stimulated phytoplankton growth. Results from this study demonstrate that bacteria were actively taking up C from urea during a brown tide in Chincoteague Bay and therefore may benefit directly from urea additions.

#### *DFAA uptake*

During this study, DFAA was used as both a C and N source in whole water incubations and in sorted *A. anophagefferens* and bacterial fractions of the population. On 6/7 and 6/21, DFAA were the dominant form of N taken up by *A. anophagefferens*, however DFAA C uptake only represented a fraction of the total measured C uptake. DFAA C and N uptake rates by *A. anophagefferens* were higher than bacterial DFAA uptake rates. Previous studies concluded that uptake of alanine and glutamate were good proxies for DFAA uptake (Mulholland et al. 2002). In 2007, glutamic acid and leucine uptake yielded similar results (data not shown) and so leucine was used as a proxy to assess DFAA uptake. While the high leucine N and C uptake by *A. anophagefferens* relative to bacteria in these short, mixed population incubations is important for determining potential competitive interactions between these two groups, it also has important implications for bacterial productivity rate estimates made using the leucine

incorporation method. One of the most common methods currently employed to determine bacterial productivity measures leucine incorporation in incubation experiments of natural water samples. It has been assumed that bacteria are the primary organisms incorporating leucine and that they do so at a much higher rates than phytoplankton during short incubations (Fuhrman and Azam 1980; Fuhrman and Azam 1982; Kirchman et al. 1985; Kirchman and Hoch 1988; Kirchamn 1992). In a recent mesocosm experiment during an *Emiliania huxleyi* bloom, Løvdal et al. (2008) found that bacteria outcompeted phytoplankton for organic N but that phytoplankton were able to utilize inorganic N more efficiently. The results presented here suggest that bacteria may not be able to outcompete phytoplankton for organic N in all systems.

Although bacteria can incorporate both DFAA carbon and nitrogen, the rate at which *A. anophagefferens* took up DFAA was orders of magnitude higher than for bacteria even after taking into account the 2 order of magnitude difference in cellular C and N concentrations between the two groups. In contrast, Kamjunke and Tittel (2008) found that although several phytoplankton species in cultures were capable of taking up leucine volumetrically, bacterial leucine uptake rates were always higher. It is possible however that the species used in their study (cyanobacteria, chorophytes, a diatom and a euglenophyte) were not as efficient as *A. anophagefferens* at taking up leucine.

Recently, Hartmann et al. (2009) used flow cytometry to determine whether phytoplankton and bacteria were competing for leucine. Results indicated that while bacteria actively took up leucine, nanoflagellates did not. However, the authors did not rule out that the nanoflagellates may take up leucine in nature because the cultures used in the study were conditioned to growing on DIN. It is also possible that since the

cultures were N replete, additional N uptake from leucine was not needed. A recent study found that more than 50% of leucine and thymidine uptake could be attributed to phytoplankton during blooms (Mulholland et al. accepted). Consistent with this observation, Kamjunke and Tittel (2008) recently determined that 13 of the 26 phytoplankton cultures they tested were capable of taking up leucine. Significant uptake of leucine by phytoplankton during bacterial productivity bioassays would lead to an overestimate of bacterial production. During this study, DFAA uptake (estimated using leucine) by *A. anophagefferens* was orders of magnitude higher than bacterial uptake of this compound, suggesting that any assessment of bacterial productivity in this system using leucine incorporation would seriously overestimate bacterial productivity.

#### *DIN uptake*

While studies have found that bacteria are the primary users of amino acids and organic N (Billen 1984; Fuhrman 1987; Jørgensen et al. 1993; Kroer et al. 1994; Middleboe et al. 1995) and phytoplankton primarily use DIN (Mulholland and Lomas 2008), numerous studies have found that bacteria can also take up inorganic N nitrogen (Wheeler and Kirchman 1986; Horrigan et al. 1988; Keil and Kirchman 1991; Lipschultz 1995; Hoch and Kirchman 1995; Lipschutz 1995; Middleburg and Nieuwenhuize 2000; Allen et al. 2002; Tungaraza et al. 2003; Fouilland et al. 2007). Using metabolic inhibitors, Wheeler and Kirchman (1986) reported that in addition to taking up amino acids, heterotrophic bacteria utilized a large portion of the  $\text{NH}_4^+$  pool. However it was also noted that completely separating the bacteria and phytoplankton fractions was difficult. Similarly, using size fractionation, Hoch and Kirchman (1995) found that  $\text{NH}_4^+$  uptake by the bacteria fraction could be as high as 50% of the total N demand in the

Delaware estuary. This was true especially in the summer when amino acid concentrations were low (Hoch and Kirchman 1995).

During this study, DFAA and  $\text{NH}_4^+$  were the dominant forms of N taken up by bacteria during the *A. anophagefferens* bloom (Fig. 16C). This was expected since bacteria have been shown to take up amino acids as an N source (Billen 1984; Fuhrman 1987; Jørgensen et al. 1993; Kroer et al. 1994; Middleboe et al. 1995). What was surprising was the uptake of nitrate by bacteria at the end of the bloom. Bacteria are generally not thought to utilize  $\text{NO}_3^-$  at significant rates due to the high metabolic cost of its uptake and intracellular reduction (Vallino et al. 1996). Some studies, however, have found that when ambient  $\text{NO}_3^-$  concentrations are high, bacterial nitrate uptake can be significant, as observed in the  $\text{NO}_3^-$ -rich sub-Arctic Pacific (Kirchman and Wheeler, 1998) and in estuaries that have been impacted with high nutrients (Middleburg and Nieuwenhuize 2000). Middleburg and Nieuwenhuize (2000) found that in the Thames estuary, amino acids were the main source of nitrogen for bacteria offshore but  $\text{NO}_3^-$  was the dominant N source for bacteria within the estuary. These authors attributed high  $\text{NO}_3^-$  uptake to the high  $\text{NO}_3^-$  concentrations (up to 650  $\mu\text{M}$ ) in the estuary (Middleburg and Nieuwenhuize 2000).

Such high concentrations of  $\text{NO}_3^-$  were not observed in Chincoteague Bay during this study, and  $\text{NO}_3^-$  concentrations were not higher on 6/21 than the other 2 dates (Table 7 and Fig. 13). In Chincoteague Bay,  $\text{NO}_3^-$  concentrations are typically less than 5  $\mu\text{M}$  throughout the year (Glibert et al. 2007) and during *A. anophagefferens* blooms in 2002, 2003, 2006, and 2007,  $\text{NO}_3^-$  concentrations were well below 5  $\mu\text{M}$ , and usually < 1  $\mu\text{M}$  (Chapter II). The highest  $\text{NO}_3^-$  concentrations were observed in 2007, when

concentrations were above 2  $\mu\text{M}$  for the first time on 5/29 at PL and 5/19 and 6/5 at GB (Chapter II). During 2006,  $\text{NO}_3^-$  concentrations ranged from 0.11-0.17  $\mu\text{M}$  (Table 7) on our 3 sampling dates and although  $\text{NO}_3^-$  was the dominant form of N taken up by the bacterial fraction on 6/21,  $\text{NO}_3^-$  uptake by bacteria was only 1  $\text{nmol N L}^{-1} \text{ h}^{-1}$ , the lower end of the range Middelburg and Nieuwenhuize (2000) reported in the Thames estuary when  $\text{NO}_3^-$  concentrations were low. These authors observed bacterial  $\text{NO}_3^-$  uptake rates as low as 1  $\text{nmol N L}^{-1} \text{ h}^{-1}$  when nitrate was depleted and up to 1.44  $\mu\text{mol N L}^{-1} \text{ h}^{-1}$  when  $\text{NO}_3^-$  concentrations were higher.

#### *Taxon-specific uptake versus whole water uptake*

Uptake of C and N by *A. anophagefferens* and bacteria combined were lower than uptake rates measured in whole water samples. One of the reasons for this discrepancy may be the presence of detritus, the narrow gating for flow cytometric sorting, or the presence of other picophytoplankton that were not quantified. A large amount of the C and N biomass during the bloom was likely detritus. GF/C filters used to collect samples from whole water incubations would undoubtedly contain a large amount of detrital N and C as well as living cells. Consequently, PN and PC concentrations measured in the environment are likely to overestimate living cellular material and thus would result in an overestimation of uptake rates (see equations). For example, the calculated PC concentrations due to *A. anophagefferens* on 6/7 when *A. anophagefferens* was estimated to be 100% of the chlorophyll biomass (Gobler and Sañudo-Wilhelmy 2001a, Gobler et al. 2002) was 3,052  $\mu\text{g C L}^{-1}$ . The total amount of PC measured in the  $>1.2\mu\text{m}$  fraction (which should exclude most bacterial C) was 3,926  $\mu\text{g C L}^{-1}$ . This suggests that nearly one quarter of the C was detrital. This was also observed on 6/21, when the bloom was

beginning to decrease. At this point, *A. anophagefferens* represented about 75% of the total Chl *a*, but only 34% of the PC concentration ( $4,104 \mu\text{g L}^{-1}$ ). Since absolute uptake rates are calculated by multiplying specific rates by the PC and PN concentrations, a 50% decrease in PC or PN concentrations would result in a 50% decrease in absolute uptake rates. While this may explain some of the discrepancies between whole water and *A. anophagefferens* cell-specific uptake rates, it cannot explain all of them.

Uptake of N and C by groups other than *A. anophagefferens* and bacteria may be another reason for the differences in whole water versus *A. anophagefferens* and bacteria cell-specific N and C uptake. From flow cytometry data (Fig. 12), there was evidence of other cells and detritus being present. Based on the gates used in this study, *Synechococcus* and other picoplankton were excluded and not sorted. Since *Synechococcus* and *A. anophagefferens* are similar in size, it is possible that both groups may be competing for the same niche (Sieracki et al. 1999; Sieracki et al. 2004). This has been observed in field studies on Long Island, NY. When a brown tide bloom in Great South Bay began to decline in 2002, the dominant species shifted from *A. anophagefferens* to picocyanobacteria (Gobler et al. 2002; Gobler et al. 2004). During a 2000 bloom in Quantuck Bay, the opposite happened, *Synechococcus* concentrations peaked before the brown tide bloom began (Sieracki et al. 2004). In both studies, only one group dominated the niche at a time suggesting competition for the niche. *Synechococcus* counts were not performed in 2006 at PL but flow cytometry analysis shows another group of picoplankton other than *A. anophagefferens* (Fig. 12). *Synechococcus* counts were done at PL in 2007 (data not shown) but peak concentrations ( $9.7 \times 10^4 \text{ cells mL}^{-1}$ ) were less than what was observed on Long Island during brown tide

blooms (Gobler et al. 2004).

*A. anophagefferens* and *Synechococcus* might compete for similar resources since *Synechococcus* is also capable of taking up and growing on organic N (Chen et al. 1991; Berman and Chava 1999; Paerl 1991; Collier et al. 1999; Sakamoto and Bryant 2001; Moore et al. 2002; Wawrik et al. 2009). Wawrik et al. (2009) used DNA stable isotope probing to measure N uptake by *Synechococcus*. The authors discovered that besides taking up DIN, *Synechococcus* also took up urea and amino acids. Palenik et al. (2003) examined the genome of *Synechococcus* and determined that *Synechococcus* has the potential to utilize organic N sources such as amino acids and cyanate and Kamjunke and Tittel (2008) determined that *Synechococcus* actively takes up leucine. These findings suggest that besides competing for the same niche, *Synechococcus* and *A. anophagefferens* may be competing for the same nutrient resources. Since *Synechococcus* uptake was not measured during this study, it is possible that some of the difference between whole water and *A. anophagefferens* cell-specific uptake rates may be due to *Synechococcus* or other picoplankton uptake.

Other studies have used flow cytometry to calculate phytoplankton-specific uptake rates. For example, Lipschultz (1995) determined N uptake rates for phytoplankton (chlorophyll-containing particles in the 3-53  $\mu\text{m}$  size fraction) in Boothbay Harbor, ME. The study found absolute  $\text{NO}_3^-$  uptake rates for phytoplankton ranged from 4.4-9.5  $\text{nmol N L}^{-1} \text{ h}^{-1}$  during light periods and 0.1-21.0  $\text{nmol N L}^{-1} \text{ h}^{-1}$  during dark periods.  $\text{NH}_4^+$  uptake rates were found to be higher and ranged from 24.8-34.6  $\text{nmol N L}^{-1} \text{ h}^{-1}$  during light periods and 3.6-5.3  $\text{nmol N L}^{-1} \text{ h}^{-1}$  during dark periods. The author was able to “crudely estimate” bacterial uptake by comparing uptake rates



from the different fractions and determined that bacteria were responsible for 34% of the total ammonium uptake. The phytoplankton N uptake rates were lower than what was observed for *A. anophagefferens* during brown tide blooms in Chincoteague Bay where N uptake ranged from 118-174 nmol N L<sup>-1</sup> h<sup>-1</sup>. However, in Chincoteague Bay, absolute nitrogen uptake rates for bacteria ranged from 0.1-3.1 nmol N L<sup>-1</sup> h<sup>-1</sup>, comparable to the estimates made by Lipschultz (1995) but representing a much smaller (<1 to 2.6%) fraction of that measured for *A. anophagefferens*, the dominant phytoplankton.

Flow cytometry has also been used to compare phytoplankton and heterotrophic bacterial N uptake in the Mid-Atlantic Bight (Bradley et al. 2010). These authors used flow cytometry to separate autotrophic cells from heterotrophic bacteria. They found that the bacteria were responsible for 20-93% of the total DIN uptake (NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>), which was a much greater percentage than was measured in this study during an *A. anophagefferens* bloom. They do point out, however, that since size fractionation was used to determine bacterial uptake rates (0.2-0.8µm), it was possible that autotrophic cells could have been present in the 0.2-0.8µm fraction. When combining the results from this study with results from the Bradley et al. (2010) study, an open ocean to eutrophic lagoon gradient emerges. The percent that bacteria contributed to total DIN uptake was highest in the oligotrophic ocean, lower in the highly productive coastal ocean, and lowest in an extreme bloom.

### *Conclusions*

This study demonstrated that it is possible to measure taxon-specific N and C uptake during brown tide blooms for *A. anophagefferens* and heterotrophic bacteria by sorting cells with flow cytometry. *A. anophagefferens* cell-specific uptake rates of N

uptake confirm that *A. anophagefferens* uses a wide range of N compounds in the environment during blooms, including  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea, and DFAA N. Results also confirm that *A. anophagefferens* supplements photosynthetic C uptake with the uptake of organic compounds. This study also demonstrated that although bacteria are thought to be the primary consumers of amino acids such as leucine, *A. anophagefferens* can take up both C and N from amino acids at a much higher rates than bacteria. This finding has important implications for bacteria productivity studies that assume bacteria are the primary consumers of leucine (see also Mulholland et al. accepted).

## CHAPTER IV

### DIURNAL CARBON AND NITROGEN UPTAKE DURING *AUREOCOCCUS ANOPHAGEFFERENS* BLOOMS (BROWN TIDE)

#### Introduction

*A. anophagefferens* can acquire carbon (C) and nitrogen (N) from numerous sources including dissolved organic matter (DOM). Studies have shown that *A. anophagefferens* can take up N from urea (Lomas et al. 1996; Berg et al. 1997; Lomas et al. 2001; Berg et al. 2002; Mulholland et al. 2002; Mulholland et al. 2009a), N and C from amino acids (Berg et al. 1997; Mulholland et al. 2002; Berg et al. 2003; Mulholland et al. 2009a), and N from other organic compounds such as peptides, proteins, chitobiose, and acetamide (Berg et al. 2002; Mulholland and Lee 2009). In cultures, *A. anophagefferens* can grow at comparable rates on media containing N as DIN or urea (MacIntyre et al. 2004 and Pustizzi et al. 2004) and additions of DON in field studies stimulated *A. anophagefferens* growth in natural populations (Kana et al. 2004).

Numerous studies have examined how light affects N uptake by phytoplankton. Studies have shown that there is generally diel periodicity in  $\text{NO}_3^-$  uptake in the Subarctic Pacific (Koike et al. 1986; Cochlan et al. 1991), the Chesapeake Bay plume (Glibert and Garside 1992), and during blooms of *Gonyaulax polyedra* off the coast of Baja, California (MacIssac 1978). In these studies  $\text{NO}_3^-$  uptake was highest during the day and decreased or ceased at night. Other studies have found that  $\text{NO}_3^-$  can be taken up during the dark period at rates comparable to daytime uptake rates (Dortch and Maske 1982; Petterson and Salhsten 1990; Kudela and Cochlan 2000). Paasche (1984) noted that dark

uptake of  $\text{NO}_3^-$  varied by species.

Studies examining the diel uptake of N from urea present conflicting results. Urea N uptake rates were higher during the daytime in the Chesapeake Bay (Bronk et al. 1998) and during a *Prorocentrum minimum* bloom in the Choptank River, a tributary of the Chesapeake Bay (Fan and Glibert 2005). However, the opposite was observed in the Chesapeake Bay plume in August when urea uptake rates were higher at night (Glibert et al. 1991). Similar daytime and nighttime urea N uptake rates were also observed in some *Karenia brevis* cultures (Sinclair et al. 2009).

In addition to photosynthesis, *A. anophagefferens* can also take up the C from DOM (Dzurica 1989). Field studies using dually labeled  $^{15}\text{N}$  and  $^{13}\text{C}$  organic tracers have shown that *A. anophagefferens* can take up both C and N from amino acids (Mulholland et al. 2002; Mulholland et al. 2009a). Other field studies have shown that the addition of dissolved organic carbon (DOC) stimulates the growth of *A. anophagefferens* (Gobler and Sanudo-Wihelmy 2001a), and during intense monospecific blooms, a significant drawdown the DOC pool has been documented (Gobler et al. 2004), suggesting that *A. anophagefferens* is directly utilizing or indirectly benefiting from that pool of carbon. The ability to take up both organic and inorganic carbon could give *A. anophagefferens* an advantage over species that can only acquire C via photosynthesis.

The ability of phytoplankton to take up DOC has been documented in several marine environments. Using isotopic tracers, phytoplankton have been shown to take up carbon from glucose (Rivkin and Putt 1987; Paerl et al. 1991; Gómez-Baena et al. 2008; Kamjunke et al. 2008), glycine (Wheeler et al. 1977), methionine (Zubkov et al. 2003) other amino acids (Paerl et al. 1991; Mulholland et al. 2009b), and urea (Mulholland et

al. 2009b; Chapter III). The ability to use DOC as a carbon source might be especially beneficial to *A. anophagefferens* when DIC concentrations are low or light limits photosynthetic C uptake. Because blooms of *A. anophagefferens* can reach concentrations in excess of  $1.0 \times 10^5$  cells mL<sup>-1</sup> (Lomas et al. 2001; Mulholland et al. 2002; Gobler and Sanudo-Wihelmy 2001a; Lomas et al. 2004; Mulholland et al. 2009a), self-shading can decrease light available for cellular photosynthesis. In low light environments the ability to supplement photosynthesis with organic C uptake would give *A. anophagefferens* access to alternative carbon sources unavailable to co-occurring phytoplankton that are strictly photoautotrophic. Studies have demonstrated that *A. anophagefferens* can grow at low light levels (Milligan 1992; Lomas et al. 1996; Milligan and Cosper; 1997). Further, *A. anophagefferens* is prone to photoinhibition at high light levels and appear better adapted to low light conditions such as those typical during brown tide blooms (Yentsch et al. 1989; MacIntyre et al. 2004).

In this study, I examined C and N uptake by natural populations dominated by *A. anophagefferens* over light-dark cycles during blooms. I hypothesized that organic carbon uptake would be higher at night when photosynthesis is not possible. I also hypothesized that since light penetration becomes limited as blooms progress and cell densities increase, the percent of organic carbon taken up would increase over the course of blooms. The ability to utilize organic C and N over the entire diurnal light cycle would provide a competitive advantage for *A. anophagefferens* over strict photoautotrophs that acquire C via photosynthesis only during daylight hours, and may help explain why *A. anophagefferens* can outcompete co-occurring phytoplankton and form dense monospecific blooms when environmental conditions are conducive. To test

these hypotheses, I performed nutrient uptake experiments over several diurnal cycles during brown tide blooms in Chincoteague Bay. Both C and N uptake were measured during the day and at night. Diel uptake experiments were performed during multiple years and during different phases of the blooms (including bloom initiation, peak bloom, and as the bloom was waning) to determine how C uptake dynamics changes as blooms mature and then decline.

I further hypothesized that when cell densities are high and light is limiting photosynthesis, or DIC becomes limiting, C uptake from urea will increase. Although studies have found that *A. anophagefferens* can use both the C and N from amino acids (Mulholland et al. 2002; Chapter II), urea C was not an important source of C during previous brown tide blooms (Lomas 2004; Mulholland 2009; Chapter II). However, Lomas (2004) observed that when light levels were low, C uptake from urea could be as much as 40% of the bicarbonate uptake. Other studies have reported a 50% increase in urea C uptake as a *Prorocentrum minimum* bloom progressed (Fan and Glibert 2005). These authors suggested that bicarbonate limitation may have caused the increase in urea C uptake. Further in Chapter III, when looking at *A. anophagefferens* cell-specific urea C uptake rates, urea was not a major source of C for *A. anophagefferens* relative to its C demand, but C:N uptake ratios indicated that urea C was taken up in stoichiometric proportion to urea N.

## Methods

Water was collected in the same manner as described in previous chapters. Prior to sampling, a Hydrolab Surveyor 4a Water Quality Multiprobe equipped with sensors for temperature, salinity, pH, dissolved oxygen, and photosynthetically active irradiance

(PAR) was deployed. Water was collected from just below the surface with acid-cleaned 20 L polyethylene carboys and transported to the Marine Science Consortium laboratory located in Greenbackville, VA. Samples collected during dark periods were transported in opaque carboys to ensure samples were not exposed to light during transport.

Upon arrival at the laboratory, nutrient samples were filtered using 0.2  $\mu\text{m}$  Supor filter disks (2003 and 2004) or a 0.2  $\mu\text{m}$  Supor cartridge filter (2006) and stored frozen. Chlorophyll *a* samples were collected onto GF/C filters and placed into sterile centrifuge tubes and stored frozen. Samples for enumerating *A. anophagefferens* were preserved with glutaraldehyde (1% final concentration) in sterile polycarbonate bottles for later enumeration.

$\text{NO}_3^-$ ,  $\text{NH}_4^+$ , and urea concentrations were analyzed using an Astoria Pacific nutrient autoanalyzer or manually using colorimetric methods (Parsons et al. 1984; Price and Harrison 1987). DFAA concentrations were measured using high performance liquid chromatography (HPLC) (Cowie and Hedges 1992). Dissolved organic carbon concentrations were measured by high temperature combustion using a Shimadzu TOC-5000 (Burdige and Homstead 1994). Chlorophyll *a* samples were extracted with 90% acetone, and analyzed using a Turner fluorometer within 2 weeks of sample collection (Welschmeyer 1994). *A. anophagefferens* concentrations were enumerated using the immunofluorescence method of Anderson et al. (1989).

Nutrient uptake experiments were conducted in the same manner as described in previous chapters. Incubations for rate measurements were initiated in acid-cleaned polycarbonate bottles by adding highly enriched (96-99%)  $^{15}\text{N}$  and/or  $^{13}\text{C}$ -labeled substrates that included  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , urea, bicarbonate, glucose, alanine and leucine.

Once the enriched substrate was added, incubation bottles were placed in incubators where temperatures were maintained within 2°C of ambient levels in Chincoteague Bay by pumping bay water into the incubator. During daytime incubations, a layer of neutral density screening was placed over the bottles to simulate ambient in-water light levels. For nighttime incubations, a cover was placed over the incubator to block all light. Experiments were terminated after 15-30 minutes by filtering the entire contents of incubation bottles onto a precombusted (450°C for 2 hours) GF/C filters (nominal pore size of 1.2 µm). Bicarbonate incubations were terminated after 2-3 hours. Samples were stored frozen after filtration. During the filtration of dark samples, a 60 watt red light bulb was used for visibility.

N and C uptake was measured during mid-day and at midnight on several dates during *A. anophagefferens* blooms in Chincoteague Bay in 2003, 2004, and 2006. Additionally, on several dates, N and C uptake was measured at dusk (1800) and dawn (0600). Results were divided into three categories based on *A. anophagefferens* abundances: 1) early bloom, when *A. anophagefferens* concentrations were below  $2.0 \times 10^5$  cells mL<sup>-1</sup> (Category 2 brown tide bloom: Gastrich and Wazniak 2002) and had not yet reached peak concentrations, 2) peak bloom, when concentrations were approaching or at peak levels, and 3) late bloom, when cell concentrations were past peak levels and declining. Because the 3 blooms differed in timing and magnitude, cell densities for each bloom stage varied between years.

During the 2003 bloom at GB, *A. anophagefferens* concentrations peaked at  $7.2 \times 10^5$  cells mL<sup>-1</sup> on 6/12 (Fig. 6) and by 6/18, *A. anophagefferens* concentrations had decreased by half to  $3.6 \times 10^5$  cells mL<sup>-1</sup>. During 2004, *A. anophagefferens* concentrations



peaked at  $6.4 \times 10^5$  cells  $\text{mL}^{-1}$  on 6/17 at GB (Fig. 6). The peak *A. anophagefferens* concentration at PL during 2004 was  $8.6 \times 10^5$  cells  $\text{mL}^{-1}$  on 6/24 (Fig. 6). During the 2006 bloom at PL, *A. anophagefferens* concentrations peaked at  $13.1 \times 10^5$  cells  $\text{mL}^{-1}$  on 6/7 (Fig. 6). On 6/14, *A. anophagefferens* concentrations were still high,  $11.6 \times 10^5$  cells  $\text{mL}^{-1}$ , but decreased to  $0.6 \times 10^5$  cells  $\text{mL}^{-1}$  the following week (6/21) (Fig. 6). During this study, early bloom conditions were sampled: 1) during 2004 (6/3-6/4) at GB, and 2) during 2006 at GB (5/18-5/19). *A. anophagefferens* concentrations on these dates averaged  $1.55 \times 10^5$  cells  $\text{mL}^{-1}$  and  $0.23 \times 10^5$  cells  $\text{mL}^{-1}$ , respectively (Table 14). Peak bloom conditions were sampled: 1) during 2003 on 6/4-6/5 at PL (average of  $4.65 \times 10^5$  cells  $\text{mL}^{-1}$ ), 2) during 2004 on 6/10-6/11 at PL (average of  $2.34 \times 10^5$  cells  $\text{mL}^{-1}$ ) and PL (average of  $5.04 \times 10^5$  cells  $\text{mL}^{-1}$ ), and 3) during 2006 on 5/18-5/19 at PL (average of  $4.8 \times 10^5$  cells  $\text{mL}^{-1}$ ) and 6/14-6/15 at GB (average of  $7.03 \times 10^5$  cells  $\text{mL}^{-1}$ ). Late bloom conditions were sampled during 2003 at GB on 6/18-6/19 (average of  $3.77 \times 10^5$  cells  $\text{mL}^{-1}$ ) and during 2006 at PL on 6/14-15 (average of  $11.6 \times 10^5$  cells  $\text{mL}^{-1}$ ).

Isotopic composition of the samples was determined using a Europa Scientific isotope ratio mass spectrometer (IRMS), equipped with an automated nitrogen and carbon analyzer (ANCA). Uptake rates were calculated as described in previous chapters. The N and C content of the DFAA pool were calculated based on the C:N ratio of the ambient DFAA pool from individual HPLC runs (average  $1.18 \mu\text{mol L}^{-1}$  DFAA-N and  $4.41 \mu\text{mol L}^{-1}$  DFAA-C per  $1 \mu\text{mol L}^{-1}$  DFAA). The ambient dissolved inorganic C (DIC) concentrations were calculated based on salinity and assumed that  $\text{CO}_2$  concentrations were saturated. The initial glucose concentration was estimated as 2% of

**Table 14** Physical, biological and chemical parameters in Chincoteague Bay, MD and VA, during the 2003, 2004, and 2006 blooms

Date	Site	Time	Sal.	Temp (°C)	PAR ( $\mu\text{E m}^{-2} \text{sec}^{-1}$ )	Chl <i>a</i> ( $\mu\text{g Chl L}^{-1}$ )	<i>A. anophagefferens</i> (cells $\text{mL}^{-1}$ ) $\times 10^5$	PC ( $\mu\text{mol C L}^{-1}$ )	PN ( $\mu\text{mol N L}^{-1}$ )	C:N
<b>2003:</b>										
04 June	PL	1200	25.9	19.1	894	9.7 (0.7)	4.91 (0.53)	189 (28)	22 (2.6)	8.4
04 June	PL	1800	25.6	19.4	258	10.6 (0.2)	4.61 (0.33)	175 (21)	22 (3.1)	8.0
05 June	PL	0000	25.4	19.3	0	8.4 (0.1)	4.44 (0.61)	153 (3.9)	19 (0.7)	8.1
18 June	GB	1200	25.7	22.4	520	9.5 (0.3)	3.58 (0.43)	250 (11)	31 (1.6)	8.2
19 June	GB	0000	25.5	22.7	1	13.5 (2.6)	3.95 (0.47)	215 (6.3)	26 (4.4)	8.3
<b>2004:</b>										
03 June	GB	1200	29.6	25.0	2195	6.2 (0.0)	1.52 (0.20)	95 (5.3)	11 (2.5)	8.8
03 June	GB	1800	29.9	25.4	907	5.5 (0.4)	1.66 (0.21)	115 (6.7)	10 (2.4)	11.1
04 June	GB	0000	29.9	24.5	0	4.0 (0.2)	1.48 (0.14)	101 (9.3)	12 (5.6)	8.5
04 June	GB	0600	29.9	24.0	106	4.3 (0.0)	1.53 (0.05)	96 (6.1)	10 (2.4)	9.9
10 June	GB	1200	29.5	28.3	1850	12.0 (0.1)	2.54 (0.16)	145 (7.3)	19 (1.5)	7.5
11 June	GB	0000	29.7	26.7	0	6.5 (0.2)	2.14 (0.11)	107 (4.4)	13 (3.7)	8.3
10 June	PL	1200	24.4	25.9	903	17.4 (0.4)	5.07 (0.41)	336 (10)	41 (1.5)	8.2
11 June	PL	0000	24.3	25.8	0	17.0 (0.2)	5.01 (0.20)	310 (35)	39 (4.7)	7.9
<b>2006:</b>										
18 May	GB	1200	31.8	21.9	251	7.9 (0.5)	0.22 (0.04)	167 (21)	10 (1.0)	15.6
19 May	GB	0000	31.6	21.5	0	7.2 (0.2)	0.24 (0.01)	93 (25)	8.9 (4.9)	10.5
18 May	PL	1200	30.3	21.0	1977	8.6 (0.1)	4.88 (0.69)	165 (9.2)	15 (1.1)	11.0
18 May	PL	1800	30.6	22.4	394	6.4 (0.3)	4.37 (0.71)	185 (16)	18 (1.5)	10.3
19 May	PL	0000	30.4	21.6	0	6.3 (0.2)	5.14 (0.44)	169 (16)	18 (2.0)	9.2
14 June	GB	1200	31.2	21.1	49	31.4 (0.3)	7.30 (0.55)	816 (42)	79 (2.9)	10.4
15 June	GB	0000	31.1	20.1	0	25.4 (0.4)	6.75 (0.91)	188 (17)	27 (1.8)	7.0
14 June	PL	1200	31.8	21.7	87	17.6 (0.6)	12.0 (0.58)	294 (16)	31 (1.5)	9.6
15 June	PL	0000	31.0	20.6	0	18.0 (0.6)	11.1 (1.78)	269 (5.4)	29 (1.1)	9.2

Standard deviations are in parentheses. Salinity, temperature, Chl *a* and *A. anophagefferens* abundance for 2003 are also reported in Minor et al. (2006). Empty fields indicate that there were no data. Shaded area indicates a dark sampling period

the ambient DOC pool, the lower end of the range estimated by Benner (2-6%; 2002) for marine surface waters.

During the 2004 bloom, nutrient concentrations were only measured during the daytime and DFAA and DOC concentrations were not measured. To calculate uptake rates during 2004, the mean DFAA and DOC concentration during the 2003 bloom were used. During 2003, the average DFAA concentration at GB was  $0.55 \mu\text{mol L}^{-1}$  ( $\pm 0.40$ ) and  $0.54 \mu\text{mol L}^{-1}$  ( $\pm 0.21$ ) at PL. These concentrations are similar to what has been reported for Chesapeake Bay (Bronk et al. 1998), the Delaware Estuary (Middelboe et al. 1995), and during a brown tide in Quantuck Bay, NY (Mulholland et al. 2002). The average DOC concentration in 2003 was  $312 \mu\text{mol L}^{-1}$  ( $\pm 36$ ) at GB and  $321 \mu\text{mol L}^{-1}$  ( $\pm 52$ ) at PL, which is within the range reported for similar brown tide prone systems (Lomas et al. 2001; Gobler and Sañudo-Wilhelmy 2001a; Gobler and Sañudo-Wilhelmy 2001b; Gobler et al. 2002).

## Results

### *Microbial and nutrient dynamics*

In 2003, PL experienced a brown tide bloom but it was less intense than during other years (Fig. 6). At the same time, the first brown tide blooms were reported at GB, the VA site where no blooms had been previously reported (but the site had not been routinely monitored as had PL). This was the first brown tide reported in Virginian waters (see Chapter II).

During the first diel uptake experiments conducted in 2003 (6/4 at PL), *A. anophagefferens* concentrations were  $4.91 \times 10^5$  cells  $\text{mL}^{-1}$  (Table 14), which was the peak concentration observed during the 2003 bloom (Table 1). This was less than the peak

concentration observed the previous year at PL ( $12.1 \times 10^5$  cells  $\text{mL}^{-1}$ ) (Mulholland et al. 2009). The GB bloom reached a peak concentration of  $7.24 \times 10^5$  cells  $\text{mL}^{-1}$  on 6/12 (Table 2). On 6/18, when the 2003 GB diel experiment was conducted, *A. anophagefferens* concentrations were  $3.58 \times 10^5$  cells  $\text{mL}^{-1}$  (Table 14), indicating that the bloom was beginning to decline (Table 2).

Nutrient concentrations during the 2003 bloom followed a trend similar to what was observed in previous years (Mulholland et al. 2009). A notable exception was that  $\text{NO}_2^- + \text{NO}_3^-$  concentrations were higher than what was observed during 2002 (Mulholland et al. 2009); however, concentrations were not as high as what was observed in 2007.

During the 2004 bloom, diel experiments were conducted at GB on 6/3-6/4 and 6/10-6/11 when daytime *A. anophagefferens* concentrations were  $1.52 \times 10^5$  cells  $\text{mL}^{-1}$  and  $2.54 \times 10^5$  cells  $\text{mL}^{-1}$ , respectively (Table 14). A diel experiment was also conducted on 6/10-6/11 at PL. The bloom at PL was more intense, reaching a peak *A. anophagefferens* concentration of  $8.76 \times 10^5$  cells  $\text{mL}^{-1}$  on 6/24, and lasted longer than the bloom at GB (Fig. 2). On 6/10 at PL, *A. anophagefferens* concentrations were  $5.07 \times 10^5$  cells  $\text{mL}^{-1}$  (Table 14).

Nutrient concentrations during the 2004 bloom varied over time and between sites but differences were small and the variability was low. At both GB and PL, urea concentrations were higher on 6/10 than on 6/3; in contrast  $\text{NH}_4^+$  concentrations were lower on 6/10 than on 6/3 (Table 15). At GB, urea increased from  $0.20 \mu\text{mol L}^{-1}$  to  $0.94 \mu\text{mol L}^{-1}$  and  $\text{NH}_4^+$  concentrations decreased from  $1.18 \mu\text{mol L}^{-1}$  to  $0.54 \mu\text{mol L}^{-1}$ .  $\text{NO}_3^-$  concentrations at GB increased from  $0.57 \mu\text{mol L}^{-1}$  on 6/3 to  $0.86 \mu\text{mol L}^{-1}$  on 6/10.

**Table 15** Nutrients in Chincoteague Bay, MD and VA, during the 2003, 2004, and 2006 blooms

Date	Site	Time	NH <sub>4</sub> <sup>+</sup> ( $\mu\text{mol L}^{-1}$ )	NO <sub>3</sub> <sup>-</sup> + NO <sub>2</sub> <sup>-</sup> ( $\mu\text{mol L}^{-1}$ )	Urea ( $\mu\text{mol L}^{-1}$ )	DFAA ( $\mu\text{mol L}^{-1}$ )	DOC ( $\mu\text{mol L}^{-1}$ )	TDN ( $\mu\text{mol L}^{-1}$ )	DIN ( $\mu\text{mol L}^{-1}$ )
<b>2003:</b>									
04 June	PL	1200	0.28 (0.03)	0.65 (0.01)	0.58 (0.04)	0.63 (0.38)	294	29.0	0.93
04 June	PL	1800	0.47 (0.05)	0.25 (0.03)	0.54 (0.03)		290	33.1	0.72
05 June	PL	0000	0.48 (0.04)	0.18 (0.07)	0.76 (0.03)		286	30.1	0.66
18 June	GB	1200	0.27 (0.01)	1.19 (0.03)	0.22 (0.10)	1.53 (0.53)	336	35.2	1.46
19 June	GB	0000	0.66 (0.09)	0.27 (0.04)	0.77 (0.11)		316	42.3	0.93
<b>2004:</b>									
03 June	GB	1200	1.18 (0.04)	0.57 (0.07)	0.20 (0.05)				1.75
03 June	GB	1800							
04 June	GB	0000							
04 June	GB	0600							
10 June	GB	1200	0.54 (0.03)	0.86 (0.17)	0.94 (0.04)				1.40
11 June	GB	0000							
10 June	PL	1200	0.40 (0.05)	0.33 (0.08)	1.05 (0.05)				0.73
11 June	PL	0000							
<b>2006:</b>									
18 May	GB	1200	0.47 (0.05)	0.09 (0.01)	0.29 (0.15)	0.29 (0.00)	324	34.0	0.56
19 May	GB	0000	0.46 (0.01)	0.16 (0.09)	0.27 (0.11)		307	35.9	0.62
18 May	PL	1200	1.08 (0.02)	0.13 (0.01)	0.02 (0.01)	0.13 (0.02)	529	30.9	1.21
18 May	PL	1800	0.52 (0.05)	0.10 (0.00)	0.02 (0.01)	0.38 (0.01)	379	30.3	0.62
19 May	PL	0000	2.27 (0.05)	0.08 (0.00)	0.07 (0.01)	0.51 (0.00)	375	34.1	2.35
14 June	GB	1200	0.44 (0.02)	0.18 (0.05)	0.03 (0.01)	0.60 (0.14)	442	37.2	0.62
15 June	GB	0000	0.39 (0.04)	0.33 (0.05)	0.05 (0.04)		539	36.9	0.72
14 June	PL	1200	0.42 (0.00)	0.21 (0.09)	0.23 (0.13)	0.35 (0.01)	580	38.8	0.63
15 June	PL	0000	0.40 (0.01)	0.32 (0.01)	0.05 (0.01)	0.64 (0.03)	513	39.3	0.72

Standard deviations are in parentheses. Dissolved inorganic N was calculated as the sum of the average NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> concentrations and ratios were calculated from average values. DOC values for 2003 are also reported in Minor et al. (2006). Empty fields indicate that there were no data. Shaded area indicates a dark sampling period

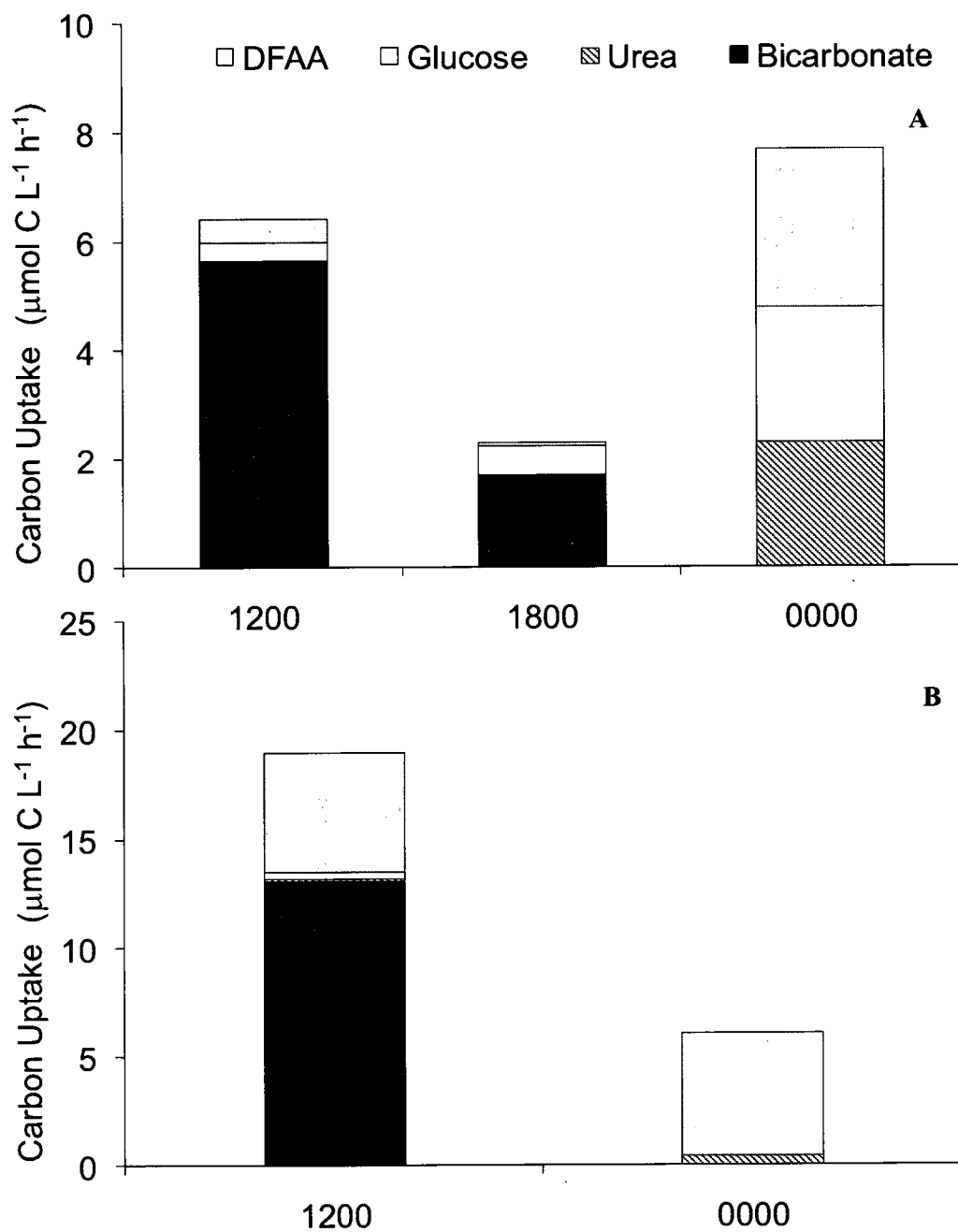
At PL on 6/10, concentrations of urea,  $\text{NH}_4^+$ , and  $\text{NO}_3^-$  concentrations were  $1.05 \mu\text{mol L}^{-1}$ ,  $0.40 \mu\text{mol L}^{-1}$ , and  $0.33 \mu\text{mol L}^{-1}$ , respectively.

In 2006, both sites experienced intense brown tide blooms (Fig. 6). The bloom in 2006 reached a peak concentration of  $12.7 \times 10^5 \text{ cells mL}^{-1}$  at GB and  $12.0$  at PL. These concentrations were higher than what was observed during the 2003 and 2004 blooms (Fig. 6) and similar to peak concentrations in 2002 (Mulholland et al. 2009). The duration of the 2006 bloom, however, was greater than what had been observed in previous years.

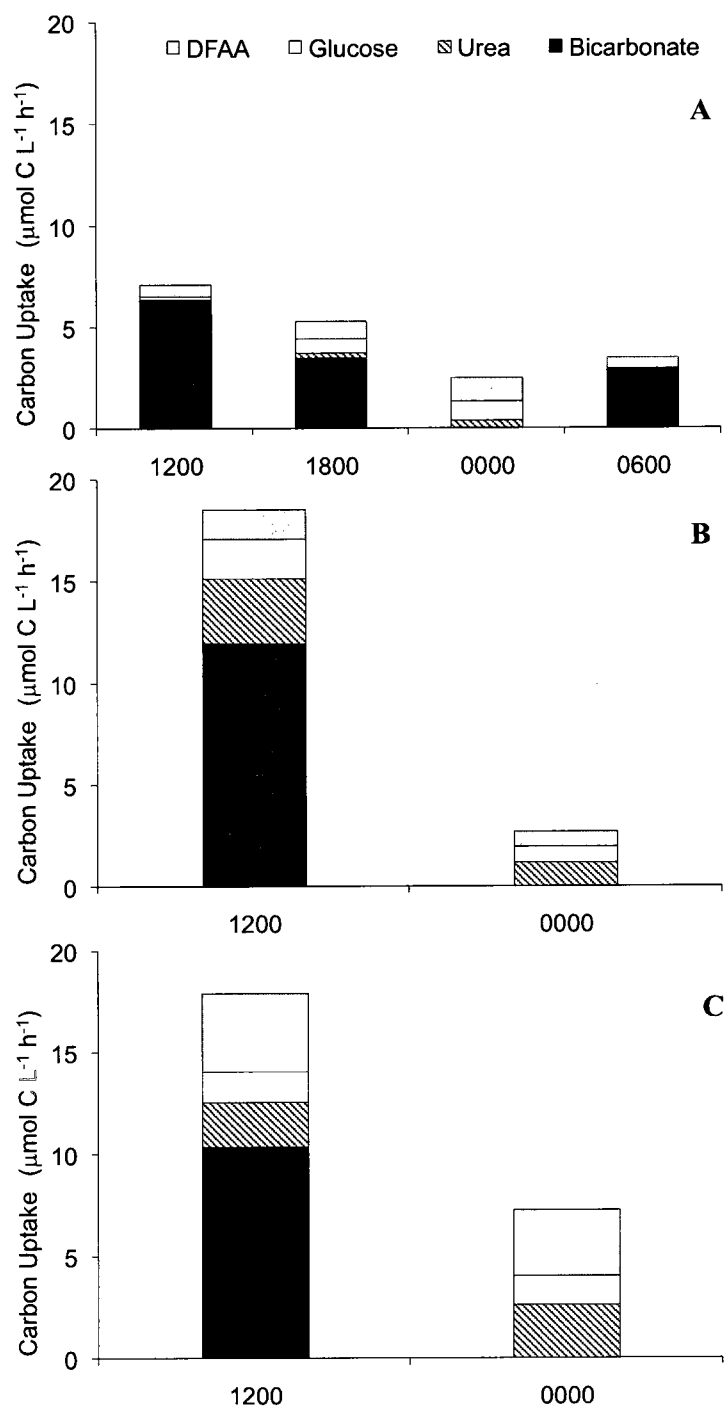
During the 2006 brown tide bloom,  $\text{NH}_4^+$  and  $\text{NO}_3^-$  were always detectable in the water column at both sites.  $\text{NO}_3^-$  was always  $< 0.4 \mu\text{mol L}^{-1}$  and  $\text{NH}_4^+$  was always  $< 1.0 \mu\text{mol L}^{-1}$ , except on 5/18 at PL where  $\text{NH}_4^+$  concentrations reached  $1.08 \mu\text{mol L}^{-1}$  (Tables 1, 2). Urea concentrations ranged from below the detection limit to  $0.36 \mu\text{mol L}^{-1}$  at PL and  $0.03\text{-}0.29 \mu\text{mol L}^{-1}$  at GB (Tables 1, 2). DFAA concentrations were consistent with other years (Tables 1, 2).

#### *Carbon uptake during early bloom conditions*

As expected, bicarbonate uptake was the dominant form of C taken up during mid-day during all phases of the bloom even though DOC uptake was always observed on all sampling dates (Figs. 17, 18, 19) with one exception. During 2006, on 5/18 at GB when the bloom was just beginning to form, total C uptake was  $7.26 \mu\text{mol C L}^{-1} \text{ h}^{-1}$  at 1200 (Table 16) and 62% of this C came from DOC (urea:  $1.31 \mu\text{mol C L}^{-1} \text{ h}^{-1}$ , glucose:  $2.26 \mu\text{mol C L}^{-1} \text{ h}^{-1}$ , DFAA  $0.91 \mu\text{mol C L}^{-1} \text{ h}^{-1}$ ) (Fig. 19A). However, during the other experiment completed during early bloom conditions (6/3-6/4 during the 2004

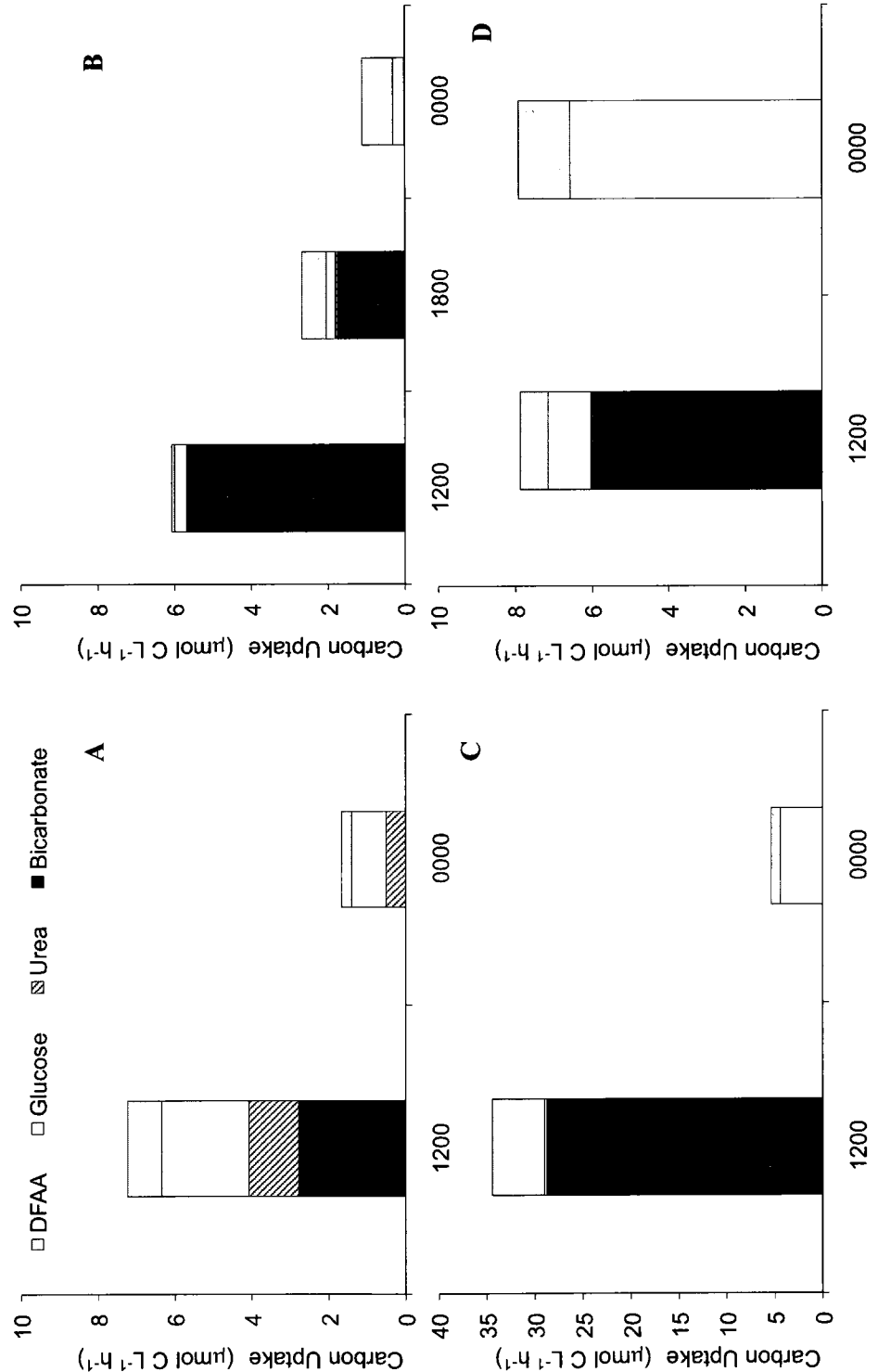


**Fig. 17** 2003 carbon uptake for (A) June 4 and 5 (peak bloom conditions) at PL and (B) June 18 and 19 (late bloom conditions) at GB



**Fig. 18** 2004 carbon uptake for (A) June 3 and 4 (early bloom conditions) at GB, (B) June 10 and 11 (peak bloom conditions) at GB and (C) June 10 and 11 (peak bloom conditions) at PL





**Fig. 19** 2006 carbon uptake for (A) May 18 and 19 (early bloom conditions) at GB, (B) May 18 and 19 (peak bloom conditions) at PL, (C) June 18 and 19 (peak bloom conditions) at GB, and (D) June 18 and 19 (late bloom conditions) at PL

bloom at GB), photosynthesis was the dominant form of C uptake at 1200 (Fig. 18A), accounting for 89% of the total C uptake at noon (Table 16). As PAR levels dropped from  $2,195 \mu\text{E m}^{-2} \text{sec}^{-1}$  at 1200 to  $907 \mu\text{E m}^{-2} \text{sec}^{-1}$  at 1800 (Table 14), bicarbonate uptake dropped from  $6.32 \mu\text{mol C L}^{-1} \text{h}^{-1}$  to  $3.49 \mu\text{mol C L}^{-1} \text{h}^{-1}$  (Table 16) and organic carbon uptake increased (Fig. 18A) from  $0.79 \mu\text{mol C L}^{-1} \text{h}^{-1}$  at 1200 to  $2.93 \mu\text{mol C L}^{-1} \text{h}^{-1}$  at 0000 (Table 16).

During all nighttime uptake experiments, there was always dark uptake of DOC (Table 16). These rates were always greater than  $2 \mu\text{mol C L}^{-1} \text{h}^{-1}$  except on 5/18 at GB during 2006 when nighttime C uptake was only  $1.69 \mu\text{mol C L}^{-1} \text{h}^{-1}$  (Fig. 19, Table 16). During 2004, of the compounds measured, DFAA-C was the dominant form of DOC taken up at all time points (>50%) and the only form of DOC taken up at 0600 (Fig. 18A). During 2006, glucose was about 50% of the measured DOC uptake with DFAA and urea providing the other 50%. Uptake of all the DOC compounds tested was higher during the day during the early part of the bloom during 2006 and higher at night during this same stage of the bloom in 2004 (Fig. 20).

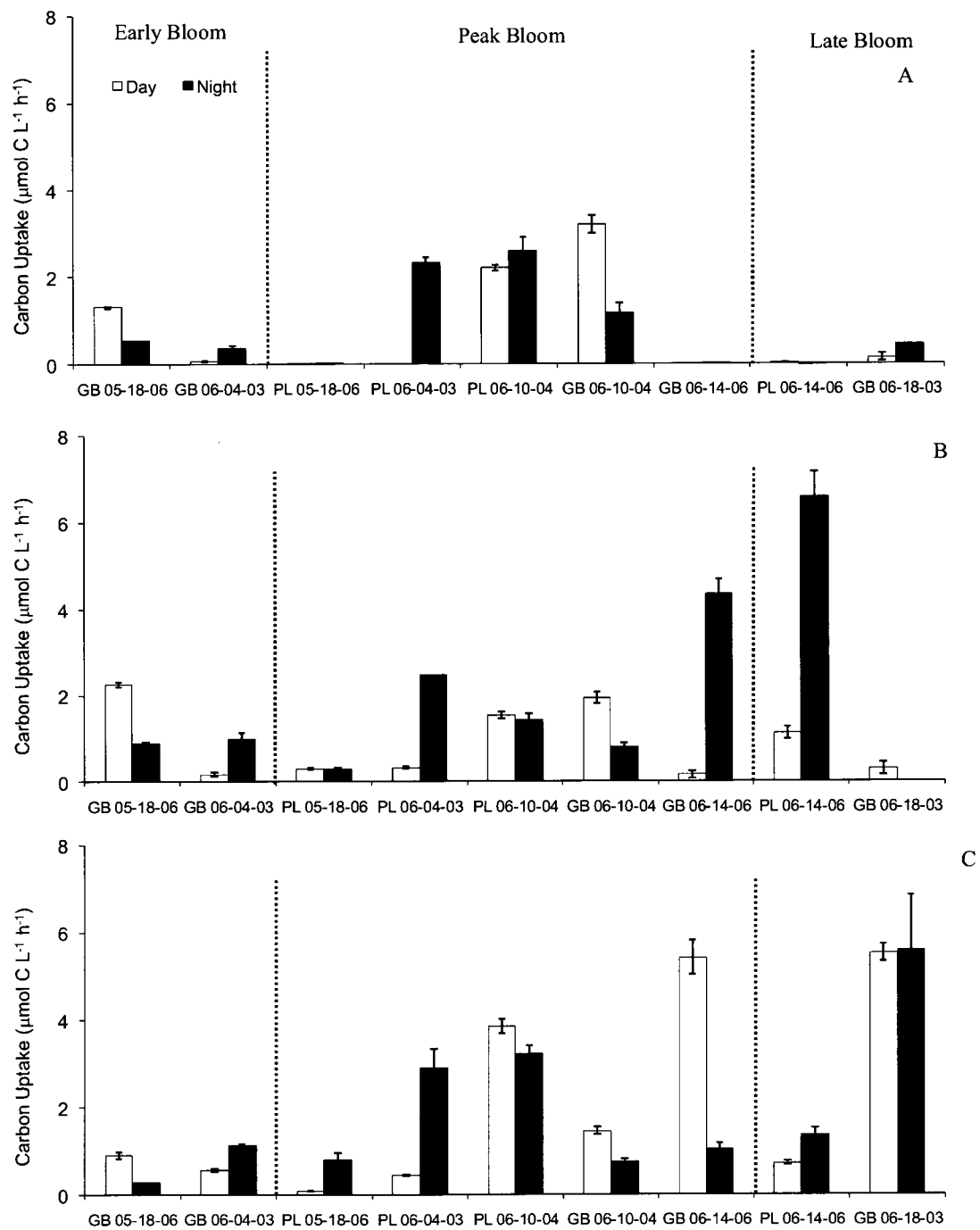
#### *Carbon uptake during peak bloom conditions*

As was observed during the early bloom conditions, all C and N compounds tested were taken up (Figs. 17, 18, 19). Bicarbonate uptake was the dominant form of C uptake during the day but DOC was taken up during the day and at night during most experiments (Fig. 20). The highest urea C uptake rates were measured during experiments at the peak of the bloom ( $0.00$  to  $3.19 \mu\text{mol C L}^{-1} \text{h}^{-1}$ ) (Fig. 20A, Table 16). On 6/4 and 6/5 during the 2003 bloom at PL, there was no detectable urea C uptake during the day (Fig. 17A). At night however, urea C uptake increased to  $2.31$

**Table 16** Carbon uptake rates during the 2003, 2004, and 2006 blooms

Date	Site	Time	Bicarbonate $\mu\text{mol C L}^{-1} \text{ h}^{-1}$	Urea $\mu\text{mol C L}^{-1} \text{ h}^{-1}$	Glucose $\mu\text{mol C L}^{-1} \text{ h}^{-1}$	DFAA $\mu\text{mol C L}^{-1} \text{ h}^{-1}$	Total $\mu\text{mol C L}^{-1} \text{ h}^{-1}$	DOC $\mu\text{mol C L}^{-1} \text{ h}^{-1}$	% DOC uptake
<b>2003:</b>									
04 June	PL	1200	5.66 (0.35)	0.00 (0.00)	0.32 (0.02)	0.44 (0.01)	6.42	0.76	12%
04 June	PL	1800	1.69 (0.17)	0.00 (0.00)	0.55 (0.39)	0.06 (0.01)	2.31	0.61	26%
05 June	PL	0000	0.00 (0.00)	2.31 (0.12)	2.47 (0.00)	2.89 (0.45)	7.67	7.67	100%
18 June	GB	1200	13.07 (0.77)	0.14 (0.09)	0.29 (0.14)	5.52 (0.19)	19.01	5.94	31%
19 June	GB	0000	0.00 (0.00)	0.45 (0.00)	0.00 (0.00)	5.59 (1.25)	6.04	6.04	100%
<b>2004:</b>									
03 June	GB	1200	6.32 (1.10)	0.07 (0.01)	0.17 (0.04)	0.56 (0.03)	7.12	0.80	11%
03 June	GB	1800	3.49 (0.19)	0.26 (0.02)	0.70 (0.05)	0.85 (0.32)	5.30	1.81	34%
04 June	GB	0000	0.00 (0.00)	0.37 (0.05)	0.98 (0.15)	1.13 (0.03)	2.48	2.48	100%
04 June	GB	0600	2.93 (0.82)	0.00 (0.00)	0.00 (0.00)	0.55 (0.46)	3.48	0.55	16%
10 June	GB	1200	11.98 (0.34)	3.19 (0.21)	1.94 (0.14)	1.45 (0.09)	18.56	6.58	35%
11 June	GB	0000	0.00 (0.00)	1.17 (0.23)	0.08 (0.09)	0.75 (0.07)	2.72	2.72	100%
10 June	PL	1200	10.34 (0.50)	2.20 (0.06)	1.54 (0.08)	3.85 (0.16)	17.92	7.58	42%
11 June	PL	0000	0.00 (0.00)	2.59 (0.31)	1.43 (0.13)	3.22 (0.19)	7.24	7.24	100%
<b>2006:</b>									
18 May	GB	1200	2.79 (0.19)	1.31 (0.02)	2.26 (0.06)	0.91 (0.07)	7.26	4.47	62%
19 May	GB	0000	0.00 (0.00)	0.53 (0.01)	0.87 (0.03)	0.29 (0.00)	1.69	1.69	100%
18 May	PL	1200	5.70 (0.22)	0.00 (0.00)	0.30 (0.03)	0.09 (0.01)	6.09	0.39	6%
18 May	PL	1800	1.82 (0.04)	0.00 (0.00)	0.24 (0.05)	0.64 (0.08)	2.69	0.88	33%
19 May	PL	0000	0.00 (0.00)	0.01 (0.00)	0.30 (0.03)	0.80 (0.16)	1.11	1.11	100%
14 June	GB	1200	28.78 (0.46)	0.00 (0.00)	0.30 (0.09)	5.41 (0.39)	34.34	5.56	16%
15 June	GB	0000	0.00 (0.00)	0.02 (0.00)	4.34 (0.34)	1.04 (0.14)	5.40	5.40	100%
14 June	PL	1200	6.03 (0.37)	0.02 (0.01)	1.12 (0.15)	0.72 (0.04)	7.88	1.85	23%
15 June	PL	0000	0.00 (0.00)	0.00 (0.00)	6.58 (0.59)	1.35 (0.16)	7.93	7.93	100%

DOC uptake was calculated by summing the urea, glucose and DFAA uptake rates. Standard deviations are in parentheses. Shaded area indicates a dark sampling period



**Fig. 20** Day (white bars) and night (dark bars) carbon uptake from urea (A) glucose (B) and DFAA (C)

$\mu\text{mol C L}^{-1} \text{ h}^{-1}$  (Fig. 17A). During the 2004 bloom on 6/10 and 6/11, DOC uptake was lower at night than during the day at GB (Fig. 4. 2B) but at PL, DOC uptake was about the same during the day and night (Fig. 18C). The relative contributions of the 3 compounds tested to the total measured DOC uptake were about the same during the daytime and nighttime at both sites. During 2 of the 4 peak bloom experiments there was an increase in absolute rates of DOC uptake at night (Figs. 17A, 19B). On both these dates, bicarbonate uptake decreased by more than half from mid-day to 1800. During the 6/4 and 6/5 experiments at PL in 2003, the greatest increase in nighttime DOC uptake was observed, from  $0.76 \mu\text{mol C L}^{-1}$  during the day to  $7.67 \mu\text{mol C L}^{-1} \text{ h}^{-1}$  at night (Fig. 17A and Table 16). Urea, glucose and DFAA contributed nearly equally to this C uptake ( $2.31 \mu\text{mol urea C L}^{-1} \text{ h}^{-1}$ ,  $2.47 \mu\text{mol glucose C L}^{-1}$ , and  $2.89 \mu\text{mol DFAA C L}^{-1}$ ) (Table 16 and Fig. 17A).

During 2 of the 4 experiments performed during peak bloom conditions, total DOC uptake was higher at night (Table 16). Only once during the peak bloom experiments were daytime total DOC uptakes rates higher than nighttime rates (Fig. 18B). This occurred during the 2004 bloom at GB on 6/10 and 6/11, total C uptake was  $18.56 \mu\text{mol C L}^{-1} \text{ h}^{-1}$  (Table 16) at 1200 and photosynthetic C uptake at 1200 made up 65% of the total carbon uptake (Table 16). Total DOC uptake decreased from  $6.58 \mu\text{mol C L}^{-1} \text{ h}^{-1}$  at noon to  $2.72 \mu\text{mol C L}^{-1} \text{ h}^{-1}$  at midnight (Fig. 18B and Table 16). At PL on the same date, DOC uptake was about equal during the day and night (Fig. 18C). Although daytime total C uptake was similar at PL ( $17.09 \mu\text{mol C L}^{-1} \text{ h}^{-1}$ ), there was no decrease in DOC uptake at night; DOC uptake was  $7.58 \mu\text{mol C L}^{-1} \text{ h}^{-1}$  at 1200 and  $7.24 \mu\text{mol C L}^{-1} \text{ h}^{-1}$  at 0000 at PL (Fig. 18C and Table 16).

Glucose uptake was observed during all three phases of the bloom (Fig. 4C). Nighttime glucose uptake rates were significantly greater than daytime uptake rates during 4 of 9 experiments ( $p < 0.05$ , t-test) and were significantly lower than daytime rates during 3 of the 9 experiments ( $p < 0.05$ , t-test, Fig. 20B). During the 2003 bloom on 6/4 and 6/5, glucose uptake nearly doubled from 1200 to 1800 on 6/4 (Table 16) as PAR levels decreased (Fig. 17A and Table 1) and by midnight, glucose uptake was 8 times higher than that measured mid-day (Table 16). During the 2006 bloom, there was a substantial increase in glucose uptake at night during the 6/14 and 6/15 experiments. Glucose uptake increased from  $0.30 \mu\text{mol C L}^{-1} \text{h}^{-1}$  during the day to  $4.34 \mu\text{mol C L}^{-1} \text{h}^{-1}$  at night at GB (Table 16). At PL, glucose uptake increased from  $1.12 \mu\text{mol C L}^{-1} \text{h}^{-1}$  during the day to  $6.58 \mu\text{mol C L}^{-1} \text{h}^{-1}$  at night (Table 16). The nighttime glucose uptake observed on this date was also the highest glucose uptake rates measured during the study.

#### *Carbon uptake during late bloom conditions*

As for early and peak-bloom conditions, during the two late bloom experiments, bicarbonate uptake dominated C uptake during the day (Figs. 17B, 19D). Nighttime DOC uptake rates were about equal to daytime DOC uptake rates during 1 experiment (Fig. 17B) and were substantially higher than daytime DOC uptake rates during the other experiment (Fig. 19D). During 2006, total C uptake at 1200 ( $7.88 \mu\text{mol C L}^{-1} \text{h}^{-1}$  with 77% from bicarbonate) was about equal to total C uptake at 0000 ( $7.93 \mu\text{mol C L}^{-1} \text{h}^{-1}$  with 83% from glucose). During the 2003 bloom at GB, nighttime DOC uptakes rates ( $6.04 \mu\text{mol C L}^{-1} \text{h}^{-1}$ ) were similar to daytime rates ( $5.94 \mu\text{mol C L}^{-1} \text{h}^{-1}$ ) and DFAA was the dominant form of DOC taken up. Glucose and DFAA carbon was taken up at higher

rates than urea C at the end of *A. anophagefferens* blooms.

*Nitrogen uptake during early bloom conditions*

Absolute N uptake rates varied throughout this study interannually (Fig. 21, 22, and 23), with bloom stage, and over diel light cycles. During the first early bloom diel experiment at GB on 6/3 and 6/4 during 2004, total N uptake did not vary much during daylight hours (1200, 1800, and 0600) but was substantially lower at night (Fig. 22A).  $\text{NO}_3^-$  was the dominant form being utilized when light was available but was a smaller percentage of the total N uptake at night. The decrease in  $\text{NO}_3^-$  uptake was not compensated for with commensurate increases in  $\text{NH}_4^+$ , urea, or DFAA uptake. During the second early bloom experiment at GB on 5/18/06, total measured N uptake was very low with total N uptake rates of  $0.12 \mu\text{mol N L}^{-1} \text{h}^{-1}$  at 1200 and  $0.03 \mu\text{mol N L}^{-1} \text{h}^{-1}$  at 0000 (Fig 23 and Table 17) and DFAA was the dominant form of N taken up during the day and night (Fig. 23A).

*Nitrogen uptake during peak bloom conditions*

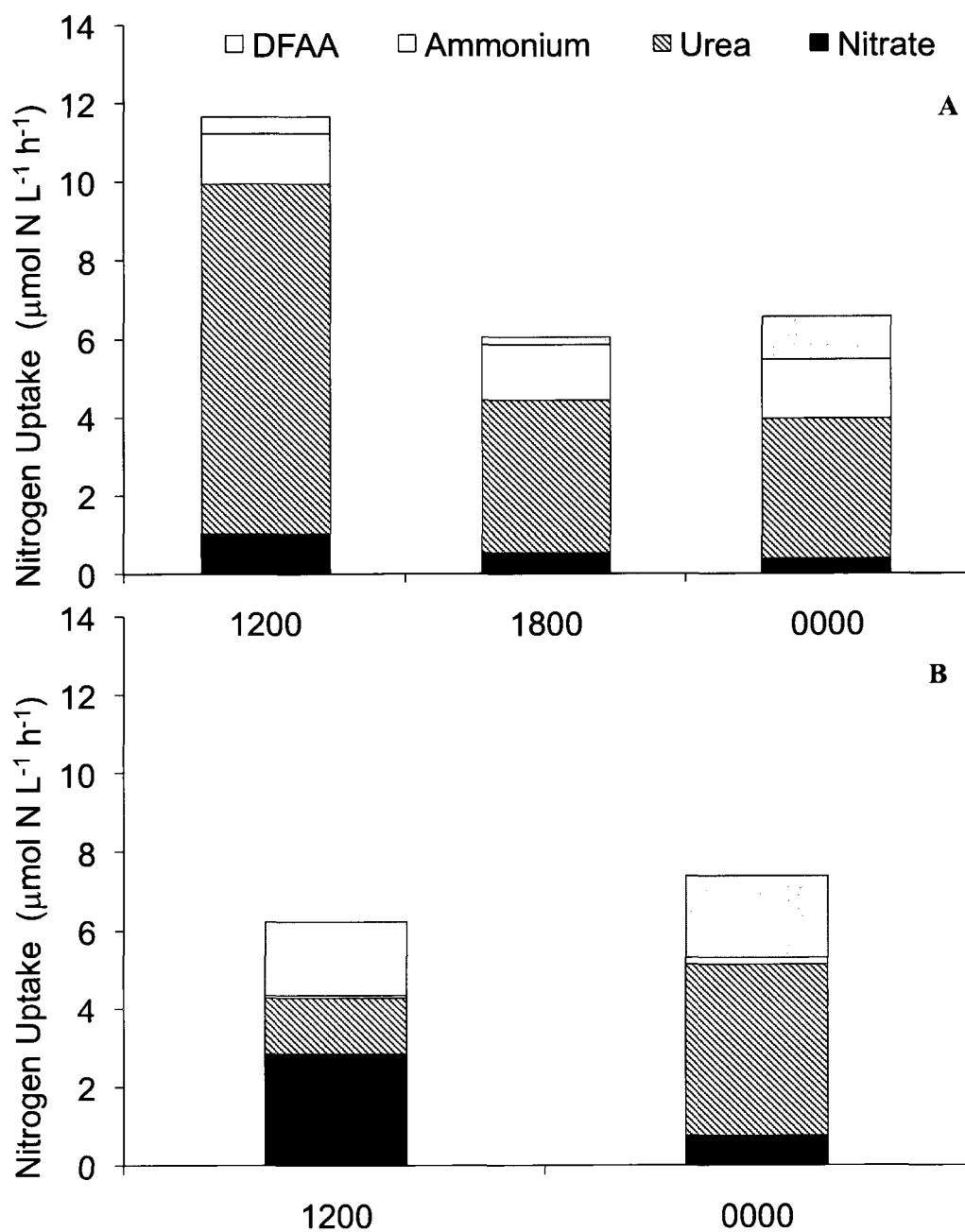
Absolute N uptake rates varied as did the relative contributions of the N compounds measured to total N uptake during peak bloom conditions in Chincoteague Bay. During 2003 at PL, and 2004 (at PL only), urea was the dominant form of N taken up during the peak of the bloom (Figs. 20A, 21C). In contrast, during 2004 at GB (Fig. 21B) and during 2006 at PL (Fig. 22B) and GB (Fig. 22C) ammonium and DFAA were the dominant sources of N uptake. During 2003, 2004, and 2006 the relative contribution of N compounds taken up did not vary much over diel light cycles; however, the relative magnitudes of N uptake did. Dark N uptake was nearly equal, less than or greater than N uptake during the light period.  $\text{NO}_3^-$  uptake was generally higher during the daylight

**Table 17** Nitrogen uptake rates during the 2003, 2004, and 2006 blooms

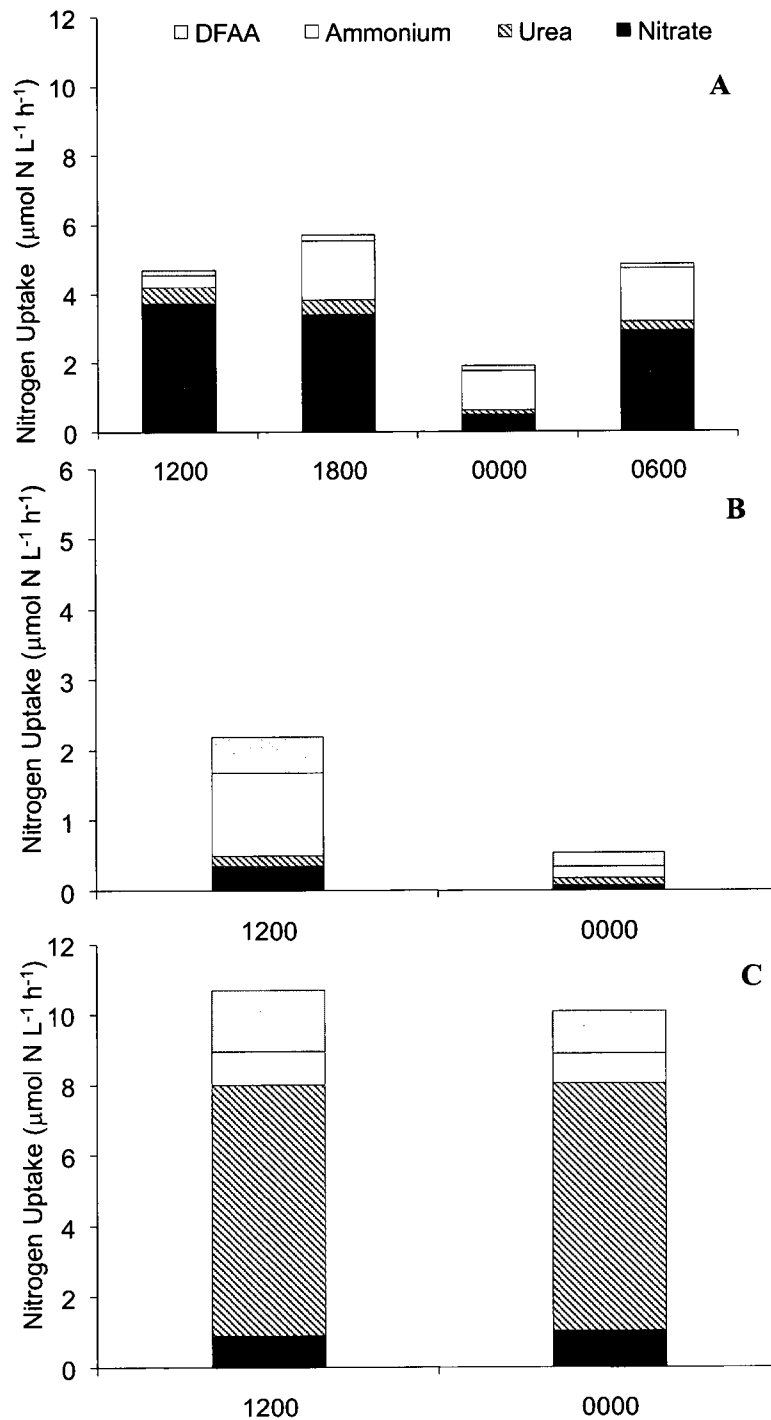
Date	Site	Time	NO <sub>3</sub> μmol N L <sup>-1</sup> h <sup>-1</sup>	Urea μmol N L <sup>-1</sup> h <sup>-1</sup>	NH <sub>4</sub> <sup>+</sup> μmol N L <sup>-1</sup> h <sup>-1</sup>	DFAA μmol N L <sup>-1</sup> h <sup>-1</sup>	Total μmol N L <sup>-1</sup> h <sup>-1</sup>	DON μmol N L <sup>-1</sup> h <sup>-1</sup>	% DON uptake
<b>2003:</b>									
04 June	PL	1200	1.01 (0.78)	8.95 (0.14)	1.62 (0.04)	0.42 (0.01)	11.67	9.37	80%
04 June	PL	1800	0.54 (0.02)	3.88 (0.67)	1.45 (0.08)	0.19 (0.01)	6.06	4.07	67%
05 June	PL	0000	0.37 (0.08)	3.59 (0.48)	1.52 (0.02)	1.10 (0.45)	6.59	4.69	71%
18 June	GB	1200	2.84 (0.25)	1.46 (0.06)	0.06 (0.00)	1.87 (0.10)	6.23	3.34	54%
19 June	GB	0000	0.75 (0.04)	4.39 (0.85)	0.16 (0.00)	2.08 (0.02)	7.38	6.47	88%
<b>2004:</b>									
03 June	GB	1200	3.73 (0.13)	0.48 (0.09)	0.35 (0.11)	0.13 (0.00)	4.69	0.61	13%
03 June	GB	1800	3.42 (0.27)	0.42 (0.04)	1.72 (0.15)	0.17 (0.07)	5.72	0.58	10%
04 June	GB	0000	0.50 (0.29)	0.13 (0.01)	1.13 (0.07)	0.16 (0.01)	1.92	0.29	15%
04 June	GB	0600	2.92 (0.56)	0.26 (0.01)	1.56 (0.14)	0.12 (0.03)	4.85	0.38	8%
10 June	GB	1200	0.35 (0.24)	1.18 (0.04)	0.16 (0.04)	0.51 (0.11)	2.20	1.69	77%
11 June	GB	0000	0.07 (0.01)	0.17 (0.02)	0.10 (0.02)	0.20 (0.04)	0.55	0.37	67%
10 June	PL	1200	0.91 (0.04)	7.12 (1.85)	0.96 (0.02)	1.72 (0.09)	10.71	8.84	83%
11 June	PL	0000	1.02 (0.06)	7.03 (0.28)	0.84 (0.08)	1.22 (0.08)	10.11	8.25	82%
<b>2006:</b>									
18 May	GB	1200	0.00 (0.00)	0.02 (0.00)	0.02 (0.00)	0.08 (0.01)	0.12	0.10	83%
19 May	GB	0000	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.02 (0.01)	0.03	0.02	67%
18 May	PL	1200	0.20 (0.01)	0.12 (0.00)	0.75 (0.01)	0.03 (0.00)	1.11	0.15	14%
18 May	PL	1800	0.11 (0.00)	0.12 (0.01)	0.57 (0.02)	0.50 (0.05)	1.31	0.63	48%
19 May	PL	0000	0.09 (0.00)	0.34 (0.01)	2.53 (0.35)	0.68 (0.20)	3.64	1.03	28%
14 June	GB	1200	1.42 (0.10)	0.17 (0.00)	1.54 (0.03)	2.59 (0.18)	5.72	2.76	48%
15 June	GB	0000	0.46 (0.01)	0.04 (0.00)	0.48 (0.04)	0.30 (0.05)	1.29	0.34	26%
14 June	PL	1200	0.43 (0.07)	0.20 (0.01)	0.36 (0.01)	0.28 (0.01)	1.27	0.48	38%
15 June	PL	0000	0.44 (0.02)	0.08 (0.00)	0.49 (0.13)	0.57 (0.12)	1.58	0.65	41%

DON uptake was calculated by summing the urea and DFAA uptake rates. Standard deviations are in parentheses. Shaded area indicates a dark sampling period

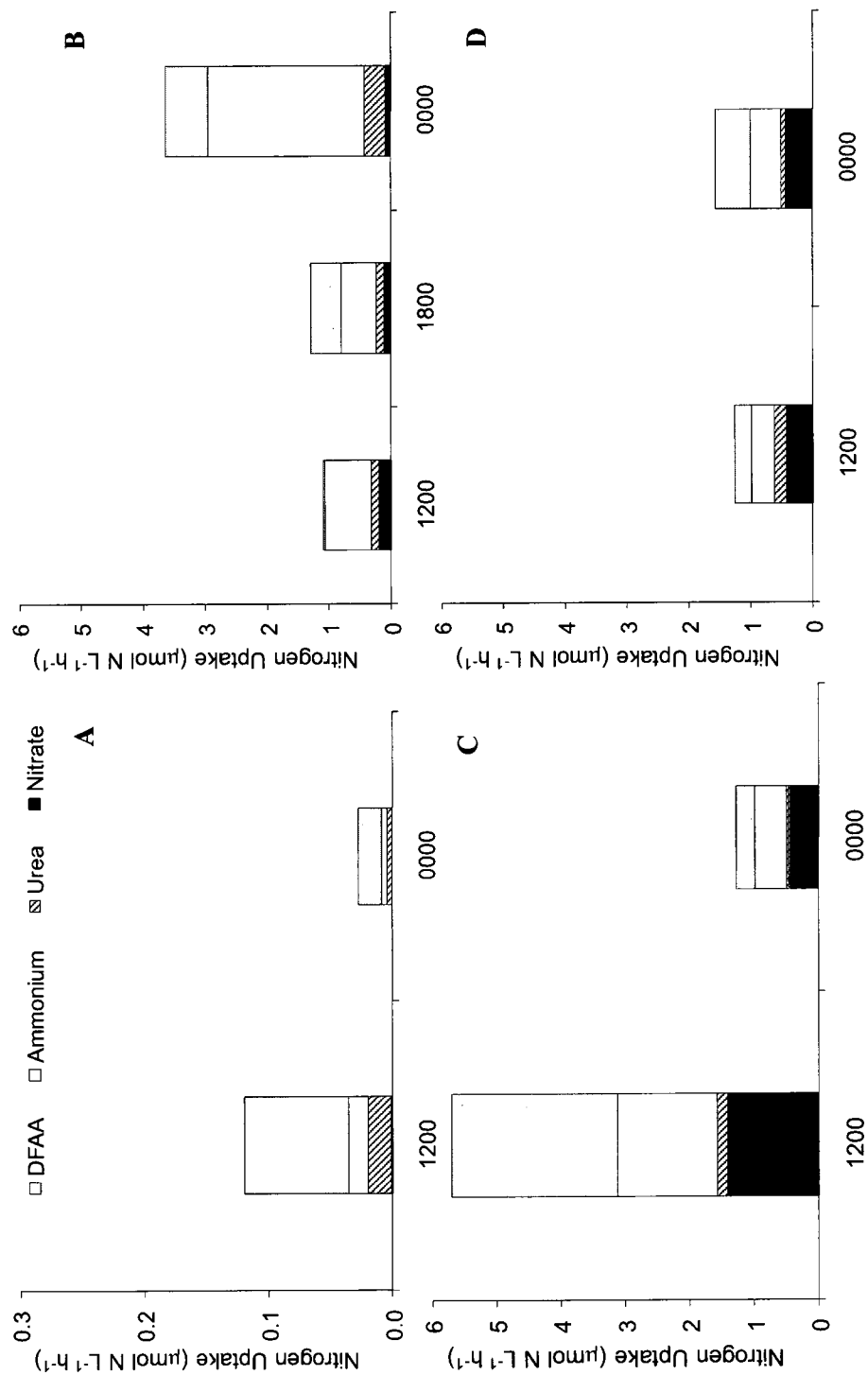




**Fig. 21** 2003 nitrogen uptake for (A) June 4 and 5 (peak bloom conditions) at PL and (B) June 18 and 19 (late bloom conditions) at GB



**Fig. 22** 2004 nitrogen uptake for (A) June 3 and 4 (early bloom conditions) at GB, (B) June 10 and 11 (peak bloom conditions) at GB and (C) June 10 and 11 (peak bloom conditions) at PL



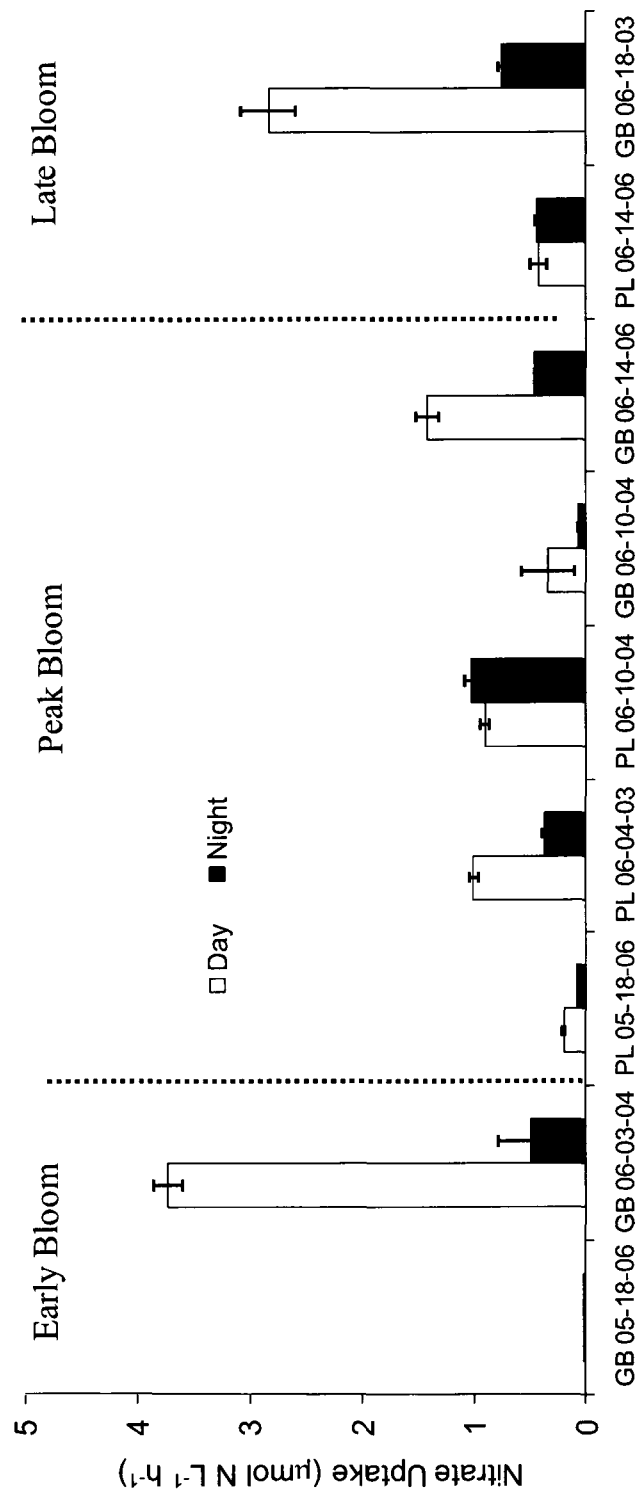
**Fig. 23** 2006 nitrogen uptake for (A) May 18 and 19 (early bloom conditions) at GB, (B) May 18 and 19 (peak bloom conditions) at PL, (C) June 18 and 19 (peak bloom conditions) at GB, and (D) June 18 and 19 (late bloom conditions) at PL

than at night (Fig. 24).

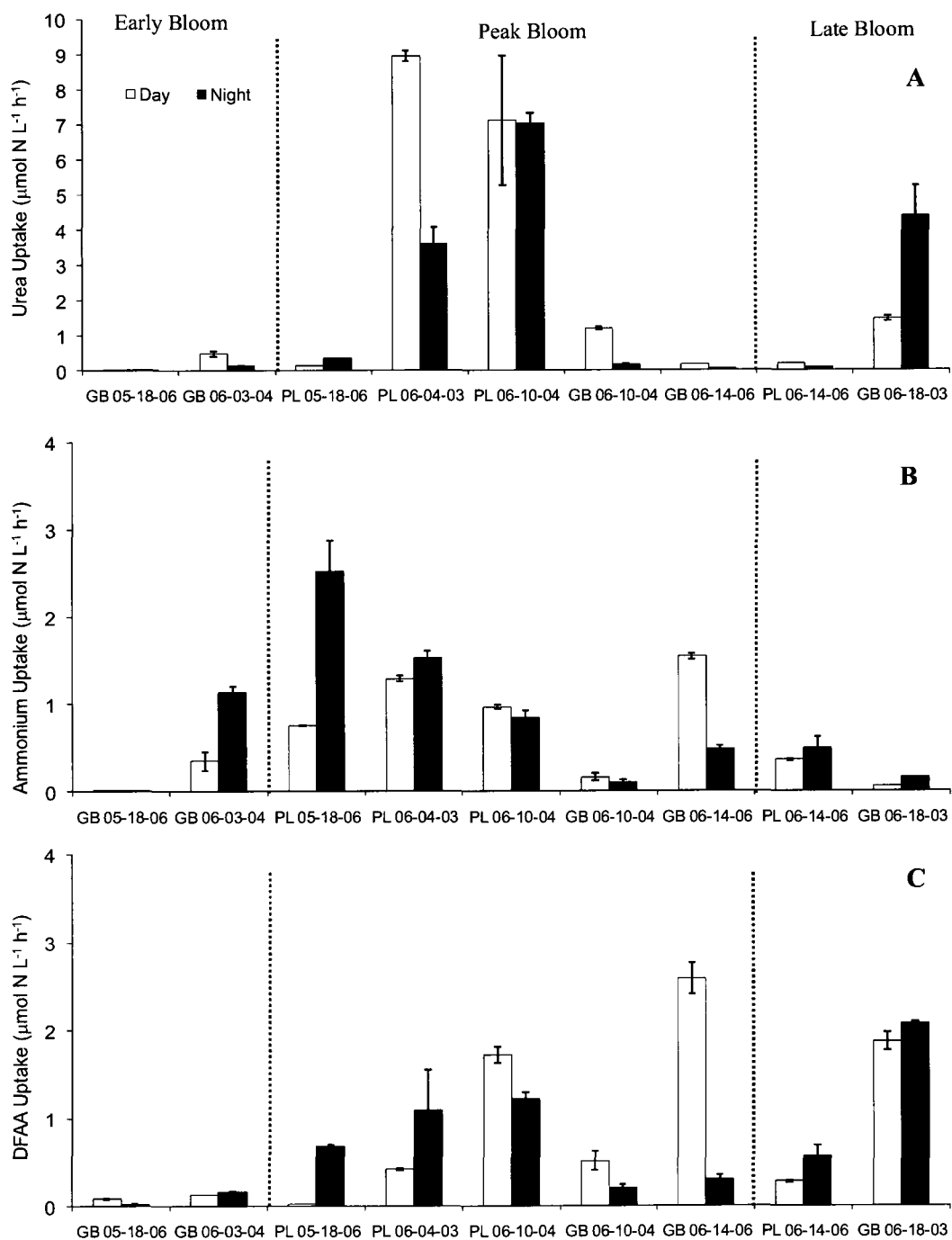
Urea N uptake during the peak bloom experiments was greater than nighttime uptake on 4 of the 5 sampling days (Fig. 25A). On the one date that this was not the case, there was no significant difference between night and day uptakes. The highest  $\text{NH}_4^+$  uptake rates measured during this study were observed during peak bloom conditions (Fig. 25B). On all the days when  $\text{NH}_4^+$  uptake was detected, there was always both daytime and nighttime uptake. During peak bloom conditions, nighttime  $\text{NH}_4^+$  uptake rates were either greater or significantly greater than daytime rates. The only time this was not the case was on 6/14 and 6/15 during the 2006 bloom at GB (Fig. 25B). On this date, daytime  $\text{NH}_4^+$  uptake rates were  $1.54 \mu\text{mol N L}^{-1} \text{h}^{-1}$  and nighttime rates were  $0.48 \mu\text{mol N L}^{-1} \text{h}^{-1}$  (Table 17). Although urea N uptake was always detectable throughout the study, two experiments during the peak of the bloom stand out. At PL, on 6/4-6/5 in 2003 and 6/10-6/11 in 2004, daytime urea rates were  $8.95 \mu\text{mol N L}^{-1} \text{h}^{-1}$  and  $7.12 \mu\text{mol N L}^{-1} \text{h}^{-1}$  respectively (Table 17). These were the highest urea N uptake rates observed during the study (Fig. 25A). While there was no significant difference between day and night urea N uptake rates on 6/10 and 6/11, nighttime uptake rates on 6/5 were more than 50% less than daytime rates. During the day on 6/4, the total nitrogen uptake rate was actually higher than the carbon uptake rate.

#### *Nitrogen uptake during late bloom conditions*

During late bloom periods, multiple N sources were taken up (Fig. 21 B and 23D). Once again nighttime  $\text{NO}_3^-$  uptake was less than or equal to daytime  $\text{NO}_3^-$  uptake rates (Fig. 24). Despite the lower  $\text{NO}_3^-$  uptake at night, nighttime N uptake was higher during both late bloom experiments. Urea N uptake was responsible for the highest



**Fig. 24** Day (white bars) and night (dark bars) nitrogen uptake for nitrate



**Fig. 25** Day (white bars) and night (dark bars) nitrogen uptake for urea (A) ammonium (B) and DFAA (C)

nighttime N uptake rates during late bloom conditions. On 6/18 and 6/19 during the 2003 bloom at GB, urea N rates went from  $1.46 \mu\text{mol N L}^{-1} \text{h}^{-1}$  during the day to  $4.39 \mu\text{mol N L}^{-1} \text{h}^{-1}$  during the night (Table 17). DFAA N uptake also appeared to increase as the bloom progressed with some of the highest uptake rates ( $2.08 \mu\text{mol N L}^{-1} \text{h}^{-1}$ ) being measured during peak and late bloom conditions (Fig. 25C).

## Discussion

### *Light and dark carbon uptake*

During this study, bicarbonate was usually the dominant form of C taken up during the day during all phases of the bloom. This was expected given that this organism is photosynthetic and has been observed in previous brown tide blooms in Chincoteague Bay (Mulholland et al. 2009; Chapter II). The uptake of bicarbonate also followed a diel pattern with the highest rates measured at mid-day, lower rates early or late in the day and no uptake at night. High bicarbonate uptake was also measured during all phases of the bloom, even when cell densities were high and light availability may have been limited due to self shading. This is consistent with previous observations that *A. anophagefferens* is well adapted to low light environments (Yentsch et al. 1989; MacIntyre et al. 2004). The ability to photosynthesize at similar rates even as light becomes limited due to high cell abundance and self shading during blooms could give *A. anophagefferens* an advantage over other species that are not adapted to low light environments.

While photosynthetic C uptake accounted for a large fraction of the total C uptake, DOC uptake was also detected during all phases of the bloom, during the day and during the night, suggesting that *A. anophagefferens* supplements DIC uptake with DOC.

This could contribute to its ability to out-compete other strictly photosynthetic species and bloom in the environment. On average, 21% of the total carbon uptake was glucose and glucose uptake was detected during all phases of the bloom. Glucose uptake accounted for 0-83% of the total carbon uptake and 0-90% of the total DOC uptake (Table 16). Other studies have observed glucose uptake in cultures of *A. anophagefferens* (Dzurica et al. 1989) and in the field during blooms (Mulholland et al. 2009a; Chapter II). Gobler and Sanudo-Wihelmy (2001a) found that the addition of glucose during an *A. anophagefferens* bloom in West Neck Bay, stimulated *A. anophagefferens* growth relative to other phytoplankton, causing *A. anophagefferens* abundances to increase from 31 to 97% of the total algal biomass. Glucose uptake by other phytoplankton groups, including diatoms (Rivkin and Putt 1987; Paerl et al. 1991; Kamjunke et al. 2008) and *Prochlorococcus* (Gómez-Baena et al. 2008), has been observed previously. Uptake of organic C may augment photosynthetic C acquisition and thereby allow for additional growth beyond that supported by photoautotrophy.

During this study, glucose uptake was higher at night than during the day for 4 of the 9 experiments and on average ( $1.9 \mu\text{mol C L}^{-1} \text{h}^{-1}$  and  $0.7 \mu\text{mol C L}^{-1} \text{h}^{-1}$ , during the night and day, respectively), suggesting that DOC uptake might be enhanced at night when light is unavailable for photosynthesis. Kamjunke et al. (2008) found that glucose uptake by phytoplankton was enhanced in the dark. In contrast, Andersson et al. (2006) and Middleburg and Nieuwenhuize (2000) observed that DOC uptake rates in turbid estuaries were not higher during the dark. These incubations, however, were conducted on samples collected during the day and incubated in the dark and so may have been physiologically distinct from populations collected during the night. Similar results were



also observed in a eutrophic reservoir where irradiance levels had no effect on DOC uptake by phytoplankton (Znachor and Nedoma 2009). However, these authors found that the addition of glucose affected phytoplankton growth by causing a decrease in chlorophyll fluorescence in the diatoms present, suggesting a switch from autotrophic to heterotrophic metabolism.

#### *Light and dark DIN uptake*

Throughout this study, *A. anophagefferens* took up multiple nitrogen compounds during all phases of the bloom. This is consistent with a recent study of the *A. anophagefferens* genome that identified genes to facilitate transport or uptake of as many as 8 different forms of N (Berg et al. 2008). The ability to take up multiple nitrogen sources over a full 24-hour diurnal cycle might be an advantage for *A. anophagefferens* if co-occurring organisms are limited to taking up particular N compounds during the day. For example, during this study,  $\text{NO}_3^-$  uptake was generally higher during the day but was always detected, even at nighttime (Fig. 24). On two occasions daytime and nighttime  $\text{NO}_3^-$  uptake rates were not significantly different ( $p > 0.05$ , t-test). The enzyme needed to reduce  $\text{NO}_3^-$  intracellularly, nitrate reductase, requires ATP and is light dependent (Berges and Mulholland 2008). Berges et al. (1995) found that nitrate reductase activity peaked during the middle and end of the light cycle. The  $\text{NO}_3^-$  uptake results presented here are also consistent in part with those reported for an *A. anophagefferens* bloom in South Africa, when dark uptake was only a fraction of light uptake of  $\text{NO}_3^-$  (Probyn et al. 2010). During a Chesapeake Bay plume study,  $\text{NO}_3^-$  uptake was higher during the day than at night (Glibert and Garside 1992). In a culture study, when *Heterosigma carterae* was kept in the dark for over 24 hours,  $\text{NO}_3^-$  uptake ceased entirely (Clark and Flynn

2002).

Studies have also found that N limitation is an important factor controlling dark uptake of  $\text{NO}_3^-$ . Cochlan et al. (1991) found that dark  $\text{NO}_3^-$  uptake increased under N-limited conditions. Similarly, when cultures of *Karenia brevis* were exposed to  $\text{NO}_3^-$  depleted conditions, enhanced dark  $\text{NO}_3^-$  uptake rates were observed (Sinclair et al. 2006a). During this study, although mean DIN concentrations were low ( $0.98 \mu\text{mol L}^{-1}$ ), neither DIN nor DON were depleted.

The uptake and assimilation of  $\text{NH}_4^+$  requires less cellular energy and therefore is less light dependent than  $\text{NO}_3^-$  uptake by photoautotrophs (Lipschultz et al. 1985) and results presented here seem to confirm this. Cochlan et al. (1991) found that  $\text{NH}_4^+$  uptake decreased at night but not as much as  $\text{NO}_3^-$  and attributed this to the greater energy required for  $\text{NO}_3^-$  uptake. In cultures of *H. carterae* dark uptake of  $\text{NH}_4^+$  could be greater than 50% of light uptake even when cells were N depleted (Clark and Flynn 2002). Similar to the culture results and those presented here, during an *A. anophagefferens* bloom in South Africa, Probyn et al. (2010) found that dark uptake of  $\text{NH}_4^+$  was 50% of the maximum light uptake rates but also noted that  $\text{NH}_4^+$  uptake was negatively impacted at high irradiances.

In contrast to the observations above, during the present study, nighttime  $\text{NH}_4^+$  uptake was 3 times higher than daytime rates during 2 of the 9 experiments. On 5/18/06 at PL,  $\text{NH}_4^+$  concentrations doubled from noon to midnight (Table 14). On 5/18, strong SW winds throughout the day, with wind gusts as high as 25 knots (Wallops Island Airport), caused white caps to be visible throughout the day and likely causing concomitant increases in  $\text{NH}_4^+$  concentration due to sediment resuspension in this shallow

water column. Horrigan et al. (1990) found that storm could lead to an increase in  $\text{NH}_4^+$  concentrations and microbial activity due to the mixing of water and sediments.

#### *Light and dark DON uptake*

While some studies have observed a diel pattern of urea N uptake with higher uptake during the day (Bronk et al. 1998; Fan and Glibert 2005), urea uptake is less light dependent than  $\text{NO}_3^-$  uptake (Lipschultz et al. 1985). Fan et al. (2003) reported higher urease activity for *A. anophagefferens* during the day. In this study, daytime N urea uptake rates were significantly higher than nighttime rates on 5 out of the 9 experiments performed. Although daytime uptake rates for urea N were usually higher, nighttime uptake was also observed during this study and significantly higher urea N uptake was observed at night on 2 occasions ( $p < 0.05$ , t-test). Other studies have also found that *A. anophagefferens* takes up urea N in the dark. During a brown tide bloom in South Africa, Probyn et al. (2010) found that, like  $\text{NH}_4^+$ , dark urea N uptake was 50% of the maximum light uptake rates. In that study, populations were not sampled at night but rather light levels were manipulated during daytime incubations. The fact that the cells were not preconditioned to darkness, however, may have influenced the results since many cells have diurnal rhythms of enzyme synthesis and activity.

Urea was an important source of N for *A. anophagefferens* during this study and on average, N uptake from urea was 30% of the total N uptake and could be as high as 77%. Since the production of urease requires energy, it is thought that most phytoplankton prefer less energetically costly forms of N such as  $\text{NH}_4^+$  (Bronk et al. 2007). However, *A. anophagefferens* has been shown to have a high affinity for urea (Lomas et al. 1996; Berg et al. 1997) and has been shown to take up urea N at high rates

(Dzurica 1989; Lomas et al. 1996; Berg et al. 1997; Mulholland et al. 2002, Mulholland et al. 2009; Chapter II). Cultures of *A. anophagefferens* grow equally well on urea or  $\text{NO}_3^-$  as sole sources of N (Dzurica 1989, Pustizzi et al. 2004). Consequently, urea appears to be a preferred source of N for this organism.

The highest urea N uptake rates were observed on a day when total nitrogen uptake rates exceeded total carbon uptake rates and the turnover time for PN (Table 14) due to measured N uptake on this date (June 10-11, 2004 at PL) was 4 h (total N uptake during the day and night were about equal) (Table 17). These high N turnover times exceed that expected based on maximum growth rates reported for *A. anophagefferens* (Gobler and Sañudo-Wilhelmy 2001; Gobler et al. 2002; Kana et al. 2004). One reason for this might be that a carbon source, not measured as part of this study, was being utilized. Additionally, after being nitrogen starved, phytoplankton can take up urea at rates greater than is needed for growth (Antia et al. 1991). It is possible that the high urea uptake rate were a response to a sudden input of urea after a period of N starvation. Studies have also shown that some diatoms, such as *Ditylum brightwellii*, are capable of excess N uptake (in excess of what is required for growth) when N is supplied in pulses (Stolte and Riegman 1995). Clark et al. (2002), demonstrated that a large capacity for dark N assimilation in diatoms may be required to maintain daily growth rates. The authors suggest that excessive dark N uptake is needed to balance daytime C fixation.

*A. anophagefferens* took up DFAA during the day and night with no apparent diel pattern. During the 9 experiments, daytime DFAA N uptake was significantly greater 4 times ( $p < 0.05$ , t-test), nighttime DFAA N uptake was significantly greater 4 times

( $p < 0.05$ , t-test), and day and nighttime DFAA N uptake was not significantly different on one occasion ( $p > 0.05$ , t-test, Fig. 25C). Other studies have also reported conflicting results for DFAA uptake over day-night cycles. Kamjunke and Tittel (2008) found that dark uptake rates of leucine could at times exceed light uptake rates. Leucine was used as a proxy for the amino acid pool in this study as well and these results are consistent with their observation that phytoplankton take up leucine. This has important implications for the interpretation of bacterial productivity estimates made using this compound (Mulholland et al., accepted). Other studies however have reported light-dependent uptake of amino acids (Rivkin and Putt 1987; Mary et al. 2008).

*C:N uptake ratio for dissolved organic compounds*

While urea was used as an N source during brown tide blooms, urea also contributed C for *A. anophagefferens* growth on many of the sampling dates. Low urea C uptake was observed during several brown tide blooms in New York, Maryland, and Virginia (Chapter II; Lomas et al. 1996; Mulholland et al. 2002; Mulholland et al. 2009a). When urea is taken up by phytoplankton, urease breaks urea down into  $\text{NH}_4^+$  and  $\text{CO}_2$  intracellularly, and in a photoautotroph growing in DIC replete environments, the  $\text{CO}_2$  may be released and the  $\text{NH}_4^+$  assimilated by cells (Anita et al. 1977). This process was used to explain the production of  $^{13}\text{C}$ -DIC when dually labeled  $^{15}\text{N}$  and  $^{13}\text{C}$  urea was added to a benthic microbial community (Veuger and Middelburg 2007).

Although urea C was only a small fraction of the total C uptake measured during *A. anophagefferens* blooms sampled, stoichiometrically, most or all of the C from urea was taken up by *A. anophagefferens* on some occasions. Balanced urea uptake would have a C:N uptake ratio of 0.5, since there is 1 C atom to every 2 N atoms in urea. With

some notable exceptions, the highest C:N uptake ratios from urea were measured at night (Table 18), and the C:N uptake ratio for urea averaged 1.4, suggesting that more urea C than N was taken up at night. During 4 of the 9 diel experiments, C:N uptake ratios increased from  $<0.5$  during the day, to  $> 0.5$  at night and during another of the experiments, urea N uptake was not detected and so urea C:N uptake ratios could not be calculated even though urea C uptake rates were high during this experiment (Table 18).

During a brown tide bloom in Quantuck Bay NY, Lomas (2004) found that although carbon uptake from urea was generally insignificant, when light levels were low, carbon uptake from urea could be as high as 40% of the bicarbonate uptake. During this study, on average, urea C accounted for 13% of the total DOC uptake at noon and 18% at night. We speculate that at low light levels or when DIC is drawn down and becomes limiting during blooms, production of  $\text{CO}_2$  by urease could supply  $\text{CO}_2$  that could be readily assimilated via the enzyme Rubisco.

During a *P. minimum* bloom, Fan and Glibert (2005) found that  $<1\%$  of total C uptake came from urea, despite the observation that urea N was an important source of N during these blooms. Stoichiometrically however, the C:N urea ratio during the bloom averaged 2.3, indicating that more C from the urea was being used than N. The amount of C being used from urea doubled over the course of the bloom and the authors suggested that this may have been because as the bloom progressed, DIC was drawn down, resulting in concomitant increases in pH levels (9-9.5) and bicarbonate limitation. During the present study, the highest urea C uptake rates were observed during the peak of the bloom, when cell density and DIC drawdown were also high. Based on continuous monitoring results from the Maryland Department of Natural Resources

**Table 18** C:N uptake ratios for urea, DFAA, DOM, and total uptake during the 2003, 2004, and 2006 blooms

Date	Site	Time	Urea Uptake C:N	DFAA Uptake C:N	DOM Uptake C:N	Total Uptake C:N
<b>2003:</b>						
04 June	PL	1200	0.0	1.0	0.1	0.6
04 June	PL	1800	0.0	0.3	0.1	0.4
05 June	PL	0000	0.6	2.6	1.6	1.2
18 June	GB	1200	0.1	3.0	1.8	3.1
19 June	GB	0000	0.1	2.7	0.9	0.8
<b>2004:</b>						
03 June	GB	1200	0.1	4.3	1.3	1.5
03 June	GB	1800	0.6	5.0	3.1	0.9
04 June	GB	0000	2.8	7.1	8.6	1.3
04 June	GB	0600	0.0	4.6	1.4	0.7
10 June	GB	1200	2.7	2.8	3.9	8.4
11 June	GB	0000	6.9	3.8	7.4	4.9
10 June	PL	1200	0.3	2.2	0.9	1.7
11 June	PL	0000	0.4	2.6	0.9	0.7
<b>2006:</b>						
18 May	GB	1200		11.4	44.7	60.5
19 May	GB	0000		14.5	84.5	56.3
18 May	PL	1200	0.0	3.0	2.6	5.5
18 May	PL	1800	0.0	1.3	1.4	2.1
19 May	PL	0000	0.0	1.2	1.1	0.3
14 June	GB	1200	0.0	2.1	2.0	6.0
15 June	GB	0000	0.5	3.5	15.9	4.2
14 June	PL	1200	0.1	2.6	3.9	6.2
15 June	PL	0000	0.0	2.4	12.2	5.0

(<http://mddnr.chesapeakebay.net/newmontech/contmon/index.cfm>), pH during the 2006 bloom (<8.4) did not approach the levels observed during the Fan and Glibert (2005) study (no continuous monitoring data from 2003 and 2004).

Previous studies have also observed an uncoupling of urea C and N uptake in benthic microbial communities (Veuger and Middelburg 2007), in cultures studies of *Thalassiosira pseudonana* (Price and Harrison 1998) and during *A. anophagefferens* blooms (Mulholland et al. 2002). Other studies have found that urea C is taken up faster rate than urea N. In the turbid Scheldt estuary, urea was used primarily as a C source, especially in months when light was limited (Andersson et al. 2006). Similarly, in the eastern Canadian Arctic, uptake of urea C was faster rate than uptake of urea N. Results presented here show that *A. anophagefferens* is capable of taking up C from urea. Although *A. anophagefferens* often assimilates more urea C than N, urea C contributes only a minor fraction of the total C uptake measured (bicarbonate, glucose, and DFAA).

Like urea, DFAA were used as both a C and N source. As for other compounds, a consistent diel cycle was not observed even in samples organized by bloom phase. On average, DFAA uptake accounted for 47% of the total measured DOC uptake, which was higher than uptake rates of glucose and urea at this time, making it a very important source of C during brown tide blooms. DFAA N uptake averaged 26% of the total N uptake during the blooms and could be as high as 70%.

Other studies have also observed DFAA being used as both a C and N source (Mulholland et al. 2002; Mulholland et al. 2003; Andersson et al. 2006). In the turbid Scheldt estuary, amino acids were used as both a C and N source by cells collected onto GF/F filters (nominal pore size of 0.7  $\mu\text{m}$ ) and DFAA uptake was highest in months



when light and temperature were low (November, January, and April) (Andersson et al. 2006). During an *A. anophagefferens* bloom in Quantuck Bay, both C and N from DFAA were taken up, however on average, the C:N uptake ratio from DFAA was 2, suggesting that not all the C was being utilized (Mulholland et al. 2002). Higher C uptake rates were observed during the bloom and in the stationary phase of *A. anophagefferens* cultures. One suggestion to explain the decoupling between N and C uptake from amino acids is extracellular amino acid oxidation whereby  $\text{NH}_4^+$  is liberated from the amino acid and taken up leaving the C behind (Mulholland et al. 2002, 2003). This could in part explain the uncoupling of the DFAA C:N uptake ratio during this study. C:N uptake ratios ranged from 0.2 to 3.1 and averaged 1.2 during the day and 1.3 at night (Table 18). This suggests that although both C and N from DFAA's were taken up, DFAA were primarily used as an N source and most of the C was not used.

### Conclusions

I hypothesized that *A. anophagefferens* would be capable of taking up organic carbon at night and results from this study demonstrate that *A. anophagefferens* actively takes up both organic and inorganic C and N during the day and at night. Although photosynthetic C uptake usually dominated C uptake during the day, organic C was also taken up during the day as well as at night. On several occasions during this study, DOC uptake at night was comparable to daytime DIC uptake rates. DOC uptake may augment DIC uptake and allow *A. anophagefferens* to continue to grow at night, allowing it to outcompete other species that are strictly autotrophic and can acquire C only during daylight hours.

*A. anophagefferens* is also capable of taking up a wide variety of N sources

during all phases of a bloom, including  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea, and DFAA. While  $\text{NO}_3^-$  uptake was generally lower at night,  $\text{NH}_4^+$ , urea and DFAA uptake at night was often comparable or even higher than daytime uptake rates. The ability to take up both organic and inorganic N during the day and at night could again give *A. anophagefferens* a competitive advantage over species that take up N primarily during daylight hours and help explain why these blooms form and persist even when light levels are low due to self shading.

## CHAPTER V

### CONCLUSIONS AND FUTURE DIRECTIONS

#### Conclusions

Since brown tide blooms were first observed in 1985, numerous studies have been undertaken to describe, and understand the causes and impacts of these blooms (Lomas and Gobler 2004). While these studies have given us great insights, many questions still remain unanswered. Although there are studies that provide data describing bloom impacts, nutrient controls on *A. anophagefferens* growth, C and N uptake by *A. anophagefferens*, and grazing on *A. anophagefferens*, there have been no process-oriented, multi-year studies comparing C and N uptake and bloom dynamics at the same site over multiple years. This study was designed to address some of these unresolved questions and to examine them over a multiyear period to determine whether there were common factors contributing to the formation of brown tide blooms. *A. anophagefferens* blooms have been attributed to a number of factors including organic N enrichment and DIN depletion (LaRoche et al. 1997; Lomas et al. 2001; Gobler and Sañudo-Wilhelmy 2001b; Gobler et al. 2002; Kana et al. 2004). However, most of these assertions come from studies conducted during individual blooms at a single site rather than long-term assessments of bloom dynamics.

I undertook a multi-year study of brown tide blooms in Chincoteague Bay, VA and MD, to examine interannual differences in nutrient dynamics during brown tide blooms. Results indicate that from 2002 to 2007, there was an increase in bloom intensity and duration and an overall accumulation of DOC in Chincoteague Bay

(Chapter II). This has important implications for the overall health of the bay and may lead to changes in ecosystem structure and metabolism, trophic status, and food web interactions.

Results from this study confirm molecular results (Berg et al. 2008) showing that *A. anophagefferens* is nutritionally versatile and able to use a wide range of nitrogen and carbon sources to meet its nutritional demands. During a 2002 bloom at PL in Chincoteague Bay, N uptake was dominated by urea (Mulholland et al. 2009a). In 2006,  $\text{NH}_4^+$  was the dominant source of N taken up while in 2007,  $\text{NO}_3^-$  uptake dominated the total measured N uptake. This was somewhat unexpected because blooms of *A. anophagefferens* have been attributed to their ability to use organic N and thrive at high DON:DIN ratios (LaRoche et al. 1997; Gobler and Sañudo-Wilhelmy 2001b; Gobler et al. 2002) and further, they have been shown to have a high affinity for urea and  $\text{NH}_4^+$  (Lomas et al. 1996; Berg et al. 1997) but not  $\text{NO}_3^-$  (Lomas et al. 1996; Mulholland et al. 2002). Overall, results show that  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea, and DFAA's were taking up simultaneously during blooms and the dominant source of N varied between years (Chapter II), over the course of blooms (Chapters II and IV), and over diel light cycles (Chapter IV), suggesting that *A. anophagefferens* has a flexible metabolism that allows it to exploit many nitrogen sources and this flexibility may be a key to its success. Results from this study demonstrate that *A. anophagefferens* actively takes up  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea, and DFAA's during blooms. However, a recent study of the *A. anophagefferens* genome has reported that *A. anophagefferens* has the ability to take up at least eight different forms of N (Berg et al. 2008). The authors showed that *A. anophagefferens* is capable of utilizing nitrite ( $\text{NO}_2^-$ ) as an N source. During this study,  $\text{NO}_2^-$  uptake was measured

during the 2007 bloom (results not shown) and this compound contributed only a small fraction of the total N uptake.

The ability to take up both organic and inorganic N over during the day and at night could give *A. anophagefferens* a competitive advantage over other species that cannot, and help explain why these blooms form and persist. Because no single N compound was responsible for fueling brown tide growth, the total N load and retention of that load within the system may be key factors contributing to brown tides rather than inputs of any particular form of N.

I also confirmed results from other studies that *A. anophagefferens* is mixotrophic, acquiring C both auto- and heterotrophically. Any strategy for managing nutrient loads to prevent blooms should also take into account the ability of *A. anophagefferens* to take up both inorganic and organic N and C. Organic C uptake subsidized C acquisition from photosynthesis during all of the blooms examined during this study. Although bicarbonate uptake was usually higher than organic carbon uptake during the day, sampling and rate measurements were generally made at mid-day when PAR availability was at its peak (Chapter IV). Nighttime organic C uptake was detected in every diel experiment performed during this study and on several occasions during this study, DOC uptake at night was comparable to daytime DIC uptake. This ability allows *A. anophagefferens* to acquire C over the entire 24-hour light cycle and may help it outcompete other species that are strictly autotrophic, acquiring C only during the light period. The finding that *A. anophagefferens* is actively taking up both organic C and organic and inorganic N over the 24-hour light cycle is critical for understanding the N and C nutrition of this organism because current dogma is that C uptake is limited to

daylight hours and N uptake at night is low and limited to particular N compounds and environmental conditions (Chapter IV).

This study also examined potential competitive interactions between *A. anophagefferens* and heterotrophic bacteria and I demonstrated that it is possible to distinguish taxon-specific uptake of C and N by *A. anophagefferens* and heterotrophic bacteria during incubations of natural assemblages using stable isotopes as tracers coupled with flow cytometry. Bacterial and *A. anophagefferens* cell-specific uptake rates confirm that *A. anophagefferens* uses a wide range of N sources during blooms including  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea, and DFAA-N and that it, and not bacteria, are capable of being the dominant consumers of these resources during a bloom (Chapter III). Results also confirm that *A. anophagefferens* supplements photosynthetic C uptake with the uptake of organic C compounds. *A. anophagefferens* C uptake from glucose, DFAA, and urea was demonstrated in this study

I showed that although bacteria are thought to be the primary consumers of amino acids such as leucine, the amino acid employed as a tracer during this study, *A. anophagefferens* took up both C and N from this amino acid at much higher rates than bacteria. This finding has important implications for bacterial productivity studies that assume that bacteria are the primary consumers of leucine in the environment.

### **Future Directions**

During this study, C and N uptake rates were not always balanced on a variety of time scales. One possible explanation is that other forms of N and C were taking up besides the compounds measured during this study. Tuchman et al. (2006) examined the uptake of 95 organic compounds by diatoms. The authors report that diatoms were

capable of taking up 68% of these compounds during the day and 94% in dark conditions. *A. anophagefferens* has now been shown to have a diverse metabolism and a surprising genomic capability for uptake of a diverse complement of organic compounds (Berg et al. 2008). Gaining a better understanding of controls on the uptake of N and C and identifying and measuring the contribution of the full suite of compounds taken up by this organisms is crucial for controlling nutrient inputs that contribute to blooms of this organism.

During blooms, *A. anophagefferens* may be utilizing additional sources of DOC and DON other than those measured here. Over 70% of total DON in surface waters may be bioavailable to phytoplankton (Seitzinger et al. 2002). The substrates tested in this study only represented a fraction (<10%) of the DON present in the environment. While much of this DON is still uncharacterized, there may be ways to examine its uptake. Bronk and Glibert (1993) created algal-derived labeled DON to measure DON uptake in Chesapeake Bay. Veuger et al. (2004) also used algal-derived labeled DON to measure microbial uptake in Randers Fjord, an estuary in Denmark. To examine the full contribution of co-occurring phytoplankton and the DOM they produce to brown tide nutrition. I recommend isotopically labeling a phytoplankton species found in Chincoteague Bay, such as *Skeletonema*, by growing it on  $^{13}\text{C}$  bicarbonate and  $^{15}\text{NO}_3^-$  and then extracting the algal DOM to measure its uptake by *A. anophagefferens*.

Results from this study clearly show the need to consider both daytime and nighttime uptake of N and C in nutrient budgets for this species. In addition, although bacteria are generally thought not to exhibit a diel uptake pattern, a recent study found increased amino acid uptake by bacteria in the light (Mary et al. 2008). More detailed

analyses regarding phytoplankton versus bacterial uptake of organic C and N compounds is needed. One new technique coming on line that might be useful to employ is stable isotope probing wherein natural populations are incubated with stable isotopes and then genetic material is examined to determine which species or groups actively incorporated the isotope (Warwick et al. 2009).

Like Long Island embayments where *A. anophagefferens* blooms were first observed during 2001, Chincoteague Bay may be susceptible to invasion by other potentially harmful or disruptive algal bloom species. Gobler et al. (2008) observed that embayments that currently or formerly experienced brown tides are now experiencing *Cochlodinium polykrikoides* blooms. *C. polykrikoides* also takes up a wide range of N and C compounds and like *A. anophagefferens*, is also capable of hydrolyzing peptides and taking up dipeptides (Mulholland et al. 2009b). Gobler et al. (2008) notes that these blooms are achieving biomasses that are 5 times greater than what was observed during brown tide blooms. Although *Cochlodinium* blooms tend to occur in tributaries more than open bays (Gobler et al. 2008), and currently occur in the lower Chesapeake Bay tributaries, Chincoteague Bay may become susceptible to *C. polykrikoides* blooms in the future. Shifts in the dominant phytoplankton groups can affect aquatic food webs and a better understanding of causes of these shifts is necessary to better predict the long-term and perhaps irreversible impacts of cultural eutrophication in aquatic systems. There are likely thresholds of nutrient loading or retention within aquatic systems that induce such system-wide shifts and identifying these thresholds is key to our understanding the long-term impacts of eutrophication on coastal systems.



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