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Multi-system analysis of nitrogen use by phytoplankton and heterotrophic bacteria

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Multi-System Analysis of Nitrogen Use by Phytoplankton and Heterotrophic Bacteria

A Dissertation
Presented to
The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

by
Paul B Bradley
2008
APPROVAL SHEET

This dissertation is submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Paul B Bradley

Approved, August 2008

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DEDICATION

A mi querida esposa, Silvia, por tu amor, apoyo, y paciencia durante esta aventura que has compartido conmigo, y

A mi hijo, Dominic, por tu sonrisa y tus carcajadas, que pueden alegrar hasta el más melancólico de los días
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ABSTRACT

Traditional measurements of phytoplankton N uptake have been confounded by bacterial retention on filters used in $^{15}$N uptake studies, and such methodological obstacles have limited our understanding of phytoplankton-bacterial interactions regarding N cycling. In this research, uptake of various inorganic and organic N substrates by phytoplankton and bacteria was measured in several marine ecosystems using two distinct approaches: size fractionation into phytoplankton and bacterial size classes, and flow cytometric (FCM) sorting of autotrophic cells. Comprehensive assessments of N uptake dynamics were conducted in Chesapeake Bay, the Mid-Atlantic Bight, and Raunefjord, Norway, with supplementary data collected from the York River, Virginia and the Gulf of Mexico.

In Chesapeake Bay, the composition of the dissolved N pool shifted from being dominated by dissolved inorganic N (DIN) in the upper bay to mostly dissolved organic N (DON) in the lower bay. Accordingly, phytoplankton nitrate uptake was highest near the head, whereas uptake of urea and dissolved free amino acids generally increased southward. Nonetheless, ammonium was the dominant form of N used by phytoplankton and bacteria throughout the bay.

In the Mid-Atlantic Bight, the surface layer was devoid of DIN but ambient urea concentrations were relatively high and this organic substrate supported a large majority of total measured N uptake. The dissolved N pool in the bottom water consisted of about two-thirds DIN, with ammonium contributing most to total uptake. Bacteria were especially active in the bottom water and contributed over half of the total DIN uptake, and there was evidence of bacterial urea uptake in the surface water.

In Raunefjord, a mesocosm approach was used to examine N uptake by a bloom of colonial Phaeocystis as well as the competition between phytoplankton and bacteria for limited N resources. Despite amending with nitrate, ammonium was the primary N form supporting the bloom. In the unfertilized mesocosm, bacteria were responsible for about half the urea uptake, most of the DFAA uptake, and at least a third of DIN uptake.

Overall, total dissolved N concentrations and total N uptake decreased from estuarine to oceanic waters, although uptake rates were highly variable within each ecosystem. The reduced N forms, ammonium and urea, were most important to phytoplankton N nutrition, and contrary to traditional belief, urea at times played an important role in bacterial N uptake. With respect to methodological approaches, traditional filtration resulted in significant overestimation of phytoplankton N uptake due to the inclusion of, and $^{15}$N enrichment in, bacterial biomass retained on filters.

This research represents the first comprehensive assessment of phytoplankton-specific N uptake across various ecosystems. It highlights not only the need for careful qualification of uptake rates measured using traditional approaches, but also the potential application of FCM sorting to more detailed examination of N uptake by phytoplankton in general, but also by specific taxa in various marine ecosystems.
MULTI-SYSTEM ANALYSIS OF NITROGEN USE BY PHYTOPLANKTON AND HETEROTROPHIC BACTERIA
CHAPTER 1

INTRODUCTION
Overview

The microbial food web has been the subject of increased attention over the past 30 years and research is continuously challenging traditional views of trophic processes in marine ecosystems. One example is the ecological roles that phytoplankton and heterotrophic bacteria (the latter hereafter referred to simply as bacteria) play in the microbial nitrogen (N) cycle, as well as the direct and indirect interactions between these two groups for shared N resources. Traditionally, phytoplankton were believed to use mostly dissolved inorganic nitrogen (DIN), whereas bacteria were thought of as strict remineralizers of dissolved organic nitrogen (DON). However, accumulating evidence indicates that this is not always the case. In many ecosystems, phytoplankton use DON extensively (e.g. Glibert et al., 1991; Sanderson et al., 2008) and bacteria contribute significantly to total DIN uptake (Middelburg and Nieuwenhuize, 2000; Allen et al., 2002; Rodrigues and Williams, 2002).

Most measurements of phytoplankton versus bacterial N uptake have been based on the use of various filters to separate these two groups, despite the fact that filters typically retain a mixed assemblage of autotrophs and heterotrophs. For example, most $^{15}$N tracer studies use GF/F filters to measure phytoplankton N uptake, although they retain about 50–75% of the bacterial community (Lee and Fuhrman, 1987; Lee et al., 1995; Gasol and Morán, 1999). Alternative approaches, which are discussed in more detail below, similarly suffer from methodological drawbacks. Therefore, quantitatively accurate data are currently lacking on the relative importance of DIN and DON to phytoplankton versus bacterial N nutrition across marine systems.
Nitrogen Uptake by Phytoplankton

The classical view of phytoplankton N uptake has been one dominated, until recently, by DIN utilization (Figure 1). Previously, ammonium (NH$_4^+$) and nitrate (NO$_3^-$) were viewed as the principal N forms supporting primary production in the marine environment, with NO$_3^-$ fueling new production and NH$_4^+$ being the dominant form of regenerated N (Dugdale and Goering, 1967). Although known as a source of new N, dinitrogen (N$_2$) fixation was not traditionally thought to contribute substantially to global primary production, but this view is being revised as estimates of global N$_2$ fixation increase (Capone et al., 2005). Also, evidence for urea and thus DON uptake by phytoplankton appeared as early as 1957 (Hattori, 1957), was shown to be important to phytoplankton in the 1970s (e.g. McCarthy, 1972a), but was not widely considered an important process in the marine N cycle until more recently (Berman and Bronk, 2003). This section focuses primarily on the use of DON by phytoplankton, which is a relatively new addition to our evolving understanding of the roles of phytoplankton and bacteria in N cycling (Figure 1).

Ammonium is often the dominant form of N used by phytoplankton in marine ecosystems because it requires comparatively little energy for assimilation. Nitrate and nitrite (NO$_2^-$), on the other hand, must be reduced to NH$_4^+$ before being incorporated into biomass. As such, NH$_4^+$ can inactivate or prevent the synthesis of assimilatory NO$_3^-$ reductase in phytoplankton (Syrett, 1988). The extent to which this occurs in the marine environment, however, is variable, and some studies have mistaken phytoplankton preference for NH$_4^+$ as inhibition of NO$_3^-$ uptake (Dortch, 1990). Cochlan and Bronk
(2003) found that these processes were happening simultaneously in the Ross Sea, but that the effect of ambient NH$_4^+$ concentrations on NH$_4^+$ uptake (i.e. preference) generally outweighed inhibition of NO$_3^-$ uptake. The relatively high affinity that phytoplankton express for NH$_4^+$ is often exemplified by an inverse relationship between uptake and availability. Low standing stocks of NH$_4^+$ are typically due to close coupling between N uptake and regeneration processes (Glibert, 1993).

Phytoplankton have a suite of physiological strategies that allow them to thrive on different nutrient sources. For example, large or rapid NO$_3^-$ inputs are known to stimulate blooms of diatoms, which are physiologically suited to take advantage of and grow quickly with the onset of NO$_3^-$-rich conditions (Goldman, 1993; Lomas and Glibert, 1999a; Berg et al., 2003). Dinoflagellates and cyanobacteria, on the other hand, are often associated with conditions of low ambient NO$_3^-$ and high concentrations of reduced N forms, such as NH$_4^+$ and urea (Berman and Chava, 1999; Lomas and Glibert, 1999b; Berg et al., 2003; Casey et al., 2007). Harmful algal bloom (HAB) species, which are discussed in more detail below, may be stimulated by DON availability (Anderson et al., 2002). Therefore, although phytoplankton are capable of exploiting various N sources, the increased availability of certain N forms could have dramatic effects on phytoplankton community structure, new production, and trophic transfer of energy.

As indicated above, the traditional view of the marine N cycle has largely ignored the importance of DON to phytoplankton N nutrition. In late winter/early spring at temperate to polar latitudes, for example, NO$_3^-$ tends to dominate the surface-water DIN pool, but is rapidly depleted with the onset of stratification and the spring phytoplankton bloom. Standing stocks of DIN are generally low during the N-limited summer, and are
often below detection despite high regeneration. In this case, phytoplankton could benefit from the ability to use organic N sources when DIN availability is decreased. Indeed, various researchers have shown not only that phytoplankton are able to use various organic N forms (reviewed in Antia et al., 1991; Bronk, 2002), but also that these substrates (primarily urea and amino acids) contribute substantially to phytoplankton N nutrition (Glibert et al., 1991; Veuger et al., 2004; Andersson et al., 2006; Sanderson et al., 2008).

Studies of DON uptake by phytoplankton have been limited by the availability of $^{15}$N-labeled DON substrates and also by the fact that much of the DON pool has not been characterized. Urea and amino acids are the most frequently studied DON forms, not only because they are important to phytoplankton and bacterial N nutrition, but also because they are readily available from commercial suppliers. In fact, $^{15}$N uptake studies have demonstrated that these two DON forms together represent an important N source to phytoplankton in various ecosystems (see below). Researchers have also examined the uptake of DON recently released from algae (Bronk and Glibert, 1993; Bronk et al., 2004; Veuger et al., 2004) and of N bound to humic substances (See et al., 2006); these studies demonstrate the potential importance of other organic N sources to autotrophs.

With respect to specific forms of organic N, many studies have examined the contribution of urea to phytoplankton N nutrition. Whether urea should be considered as an organic or inorganic N form has been the subject of some debate (e.g. Williams, 2000; Bronk, 2002); nonetheless, urea is treated here as an organic N form. McCarthy, who was among the first to quantify urea uptake by phytoplankton, found high urea uptake affinity coefficients among several diatom species in culture (1972b), but also demonstrated the
importance of urea to natural phytoplankton assemblages (1972a). Others have investigated urea uptake by phytoplankton in cultures, but these studies were mainly confined to only a few phytoplankton species (reviewed in Antia et al., 1991). More recent work has explored how entire phytoplankton communities use urea in natural systems, and the contribution of urea to total N uptake has been found to exceed 40% in many regions (Twomey et al., 2005; Andersson et al., 2006; Sanderson et al., 2008). In surface waters off the New Jersey coast, urea dominated N uptake by phytoplankton, comprising as much as 79% of total measured N uptake (see Chapter 3, this volume). Clearly, urea plays an important role in supplying N to marine phytoplankton. Although phytoplankton are capable of actively transporting amino acids across the cell membrane, they tend to rely less on these organic N molecules than the other N forms discussed above, and amino acids are often not included in studies of phytoplankton N uptake. However, several researchers have demonstrated the potentially significant role that dissolved amino acids, both free (DFAA) and combined (DCAA), may play. For example, Palenik and Morel (1990a; 1990b; 1991) found that cell-surface enzymes present in various algal species enables them to obtain N from organic substrates without direct assimilation. These enzymes catalyze the oxidation of amino acids and primary amines to produce NH$_4^+$, which can then be taken up by the algal cell. This mechanism has been shown to exist in a limited number of taxa, but few studies have examined the relative importance of the process to phytoplankton N nutrition. Subsequently, Mulholland et al. have shown that natural phytoplankton communities, including the brown tide alga *Aureococcus anophagefferens*, rely on amino acid oxidation and peptide hydrolysis to satisfy nutritional N demands (Mulholland et al.,
1998; 2002). In addition, photosynthetic dinoflagellates in a Chesapeake Bay tributary have been found to use cell-surface proteases to generate potential N sources in the form of amino acids, which can then either be transported into the cell or degraded extracellularly by amino acid oxidation to liberate NH$_4^+$ (Stoecker and Gustafson, 2003). It is uncertain, however, whether the NH$_4^+$ produced from these reactions is available for uptake by other cells, such as bacteria. Although DFAA have been known to contribute significantly to total N uptake by phytoplankton (up to 50%, Veuger et al., 2004), and DFAA uptake rates can exceed those of urea and NO$_3^-$ (Mulholland et al., 2004), this organic substrate is generally considered a fairly minor source of N for phytoplankton N nutrition (Bronk, 2002).

Harmful algal blooms represent an area of research receiving increased attention recently with respect to N dynamics. Of primary interest is the potential link between the increased frequency and intensity of HABs and coastal eutrophication (Anderson et al., 2002). Tremendous resources have been and are continuously being invested to try to determine the nutrient sources and other environmental variables that stimulate these often-toxic blooms. Organic N may play an important role in meeting the N needs of HAB species. Paerl (1988) showed a connection between increased inputs of dissolved organic matter (DOM) to nearshore waters and outbreaks of nuisance phytoplankton blooms, and Seitzinger and Sanders (1997) related estuarine eutrophication to large proportions of DON within the estuary’s N pool. When grown on urea, the toxic diatom species *Pseudo-nitzschia* produced domoic acid twice as rapidly as cells grown on NO$_3^-$ and three times as fast as those grown on NH$_4^+$ (Howard et al., 2007). Others have examined how DON affects growth of the brown tide alga *Aureococcus anophageferens*
in Long Island coastal waters (Berg et al., 1997; Gobler and Sañudo-Wilhelmy, 2001). Berg et al. (1997) concluded that up to 70% of the N utilized by *A. anophagefferens* was organic, particularly urea. Conversely, Gobler and Sañudo-Wilhelmy (2001) found that addition of urea did not significantly affect *A. anophagefferens* abundance in cultures and concluded that higher carbon (C)-containing DON compounds (e.g. amino sugars and acids) could contribute to the development of these brown tides. Increased organic N inputs may also contribute to the occurrence of HABs in Chesapeake Bay (Glibert et al., 2001). Finally, one other study showed a direct relationship between high urea concentrations (>1.5 μM N) and dinoflagellate blooms in estuarine aquaculture ponds, possibly even the onset of toxic *Karenia brevis* and *Pfiesteria piscicida* blooms (Glibert and Terlizzi, 1999).

The studies discussed here represent just a fraction of the research on N uptake by phytoplankton, but clearly demonstrate that the classical view of phytoplankton N use was missing an important piece of the marine N cycle. The current view depicted in Figure 1 includes most of the recent work showing DON use by phytoplankton, but may need to be revised in the future, when technological and methodological advances allow more organic N substrates to be examined and enzymatic processes to be quantified.

**Nitrogen Uptake by Bacteria**

Historically, the primary role of bacteria in N cycling has been viewed as the release of inorganic N (e.g. NH$_4^+$) during DOM decomposition, and thus bacteria were thought to be the nutrient regenerators for phytoplankton (Figure 1). The significant role
that bacteria play in DIN removal in the marine environment, although indirectly apparent, was largely ignored until the mid-1980s when it was clearly shown that bacteria also utilize NH$_4^+$ (Wheeler and Kirchman, 1986). Additional research has demonstrated that bacteria are capable of utilizing NO$_3^-$ as well (e.g. Allen et al., 2002). However, DFAA (as well as peptides and proteins) have traditionally been considered to be the preferred C and N substrates for bacteria in most marine settings, with alternative N substrates playing variable roles depending on ecosystem dynamics.

Whereas phytoplankton maintain their elemental composition by adjusting the rate of C fixation to the available nutrient supply, bacteria must assimilate or regenerate N to balance their consumption of relatively C- or N-rich organic matter, respectively. One can infer, therefore, that bacterial uptake rates of DIN in marine waters depend somewhat on organic C availability. Such evidence has been provided in numerous field and laboratory studies. Kirchman et al. (1990) found that bacterial growth in the subarctic Pacific was C-limited and that glucose additions stimulated NH$_4^+$ depletion. Goldman and Dennett (1991) studied bacterial C and N nutrition and found that when the substrate C:N was low, NH$_4^+$ uptake occurred only to balance glucose uptake, while additional NH$_4^+$ remained unused. Keil and Kirchman (1991) observed that a natural bacterial assemblage discontinued NH$_4^+$ uptake upon organic substrate depletion, but then resumed uptake with the addition of glucose. Such studies have led to the hypothesis that the efficiency of NH$_4^+$ utilization in the presence of amino acids depends upon the relative availability of the different substrates (e.g. DFAA, NH$_4^+$, glucose) and not their absolute availability (Kirchman et al., 1989; Goldman and Dennett, 1991; Hoch and Kirchman, 1995). Conversely, Williams (1995) argued that DIN limitation of bacteria could explain
the accumulation of dissolved organic carbon (DOC) during the summer in the North Atlantic and perhaps elsewhere. One important point that should be made, however, is that different groups within the bacterial assemblage might be using different chemical constituents of the DOM and DIN pools. For example, one group of bacteria may assimilate carbohydrates and NH$_4^+$ while another group uses amino acids and regenerates NH$_4^+$ (Kirchman, 2000). Such complexity in the community structure of bacterial assemblages and in the range of organic and inorganic substrates available to bacteria complicates the use of stoichiometry in studying bacterial N utilization.

Whereas DFAA are less important to phytoplankton than other N forms (see above), amino acids are well known to be an important N source to bacteria (Kirchman, 2000). Keil and Kirchman (1991), for example, showed that in the subarctic Pacific and the Delaware estuary the contribution of DFAA and NH$_4^+$ to bacterial N demand averaged 64% and 51%, respectively, which suggests that other DON sources were insignificant. Concentrations of DCAA tend to exceed those of DFAA in seawater (Bronk, 2002), and are a potentially important N source for bacteria (Coffin, 1989). Research suggests that the relative uptake of these two DON pools varies with their availability, but that DFAA are generally preferred over DCAA, with differing uptake mechanisms (Coffin, 1989).

Other studies have examined which N sources support bacterial growth and provide a variety of results. For example, Kroer et al. (1994) found that patterns of bacterial N utilization by oceanic, estuarine, and eutrophic riverine assemblages grown in batch cultures were fairly similar. Dissolved combined amino acids were most important in supporting bacterial growth, followed closely by NH$_4^+$, and then DFAA and dissolved
DNA. Bacterial NO$_3^-$ utilization occurred only in the oceanic cultures, but comprised up to 46% of the bacterial N demand. This is in contrast to the results of a study by Wheeler and Kirchman (1986) that examined N uptake by picoplankton. They found the $<$1 $\mu$m size fraction to use negligible amounts of NO$_3^-$ and urea, and concluded that bacteria may use a large portion of the NH$_4^+$ in the euphotic zone of marine waters. Additional studies, however, showed NO$_3^-$ to contribute significantly to marine bacterial growth (Kirchman et al., 1991; Kirchman and Wheeler, 1998; Allen et al., 2002). The importance of urea as a N source to bacterial communities is also variable. Although urea uptake by bacteria has traditionally been considered to be insignificant (Tamminen and Irmisch, 1996; Kirchman, 2000), several studies have shown otherwise (Jørgensen et al., 1999; Jørgensen, 2006; Sanderson et al., 2008). In particular, Sanderson et al. (2008) reported that urea comprised approximately 50% of total measured N uptake by the bacterial size class during an induced bloom of Phaeocystis pouchetii. Andersson et al. (2006) measured uptake of dual-labeled ($^{13}$C and $^{15}$N) urea by a mixed phytoplankton-bacterial assemblage and found that urea comprised roughly 10–40% of total N uptake, and that C uptake rates were, on average, about a third of N uptake rates. However, few studies have examined whether urea serves as C versus N source to bacteria in marine ecosystems.

Molecular approaches have also been used to describe bacterial N dynamics; with the increased use of gene probes and PCR primers have come new discoveries relating to processes ranging from N$_2$ fixation to NH$_4^+$ assimilation and DON metabolism (Zehr and Ward, 2002). For example, Allen et al. (2001) examined bacterial DIN use with molecular techniques and detected the presence of functional assimilatory NO$_3^-$ reductase genes in marine bacteria from various habitats. Their work suggests that bacteria capable
of using NO$_3^-$ are common throughout the world's oceans, but that the degree of NO$_3^-$ use needs to be clarified further.

In summary, bacteria rely predominantly on such labile organic N sources as amino acids, but bacterial affinity for specific N substrates varies significantly. The relative availability of different DIN or DON sources can affect uptake preferences (Kroer et al., 1994; Middelburg and Nieuwenhuize, 2000), and factors such as salinity, temperature, or oligotrophic versus eutrophic state can influence N uptake (Hoch and Kirchman, 1995; Reay et al., 1999). Furthermore, the C:N ratio of the substrate pool in relation to that of the bacterial cell likely affects the relative use of inorganic versus organic N by bacteria. It has recently been shown that generalist bacteria are favored in coastal waters with high variability in the composition and delivery of organic matter, and that physical processes and trophic interactions may exert more control over bacterial community structure than resource availability alone (Mou et al., 2008).

**Interactions between Phytoplankton and Bacteria**

Phytoplankton and bacterial N uptake dynamics are integral components of food web structure in marine ecosystems. Availability of different N forms affects not only which compounds phytoplankton and bacteria use preferentially, but also their community composition. For example, diatoms, which are typically favored under turbulent conditions with high and/or rapid NO$_3^-$ input (e.g. coastal upwelling; Kokkinakis and Wheeler, 1987), are relatively large and heavy, thus enhancing trophic level transfer efficiency and/or vertical export out of the euphotic zone (Goldman, 1993).
Conversely, flagellates and cyanobacteria, which are associated with uptake of reduced N forms (e.g. NH$_4^+$ and urea), require more trophic steps to reach higher consumers and therefore result in more energy loss to the system than diatoms do. Thus, it is important to thoroughly understand how the marine N cycle functions in order to evaluate such factors as phytoplankton community structure, new production, and energy transfer.

Phytoplankton and bacteria are often competing for limited DIN resources. Since bacteria are known to use NH$_4^+$, and likely do so more efficiently than phytoplankton (Kirchman 2000), competition for NH$_4^+$ may be quite high. Larger phytoplankton tend to have higher half saturation constants (K_s), and the high surface area to volume ratio of bacterial cells would enhance their ability to use DIN at low ambient concentrations (Valiela, 1995). Therefore, phytoplankton capable of using alternative N sources, such as urea and other organic N substrates, would have a competitive advantage over other algal species unable to compete effectively with bacteria for DIN. Indeed, research has shown significant uptake of NH$_4^+$ by marine bacteria when algae are competing for this substrate (Wheeler and Kirchman, 1986; Kirchman et al., 1994; Rodrigues and Williams, 2002). Although most bacteria are probably not strong competitors for NO$_3^-$ in oxic systems, Joint et al. (2002) showed that addition of glucose and NO$_3^-$ caused a large increase in bacterial activity concurrent with a decrease in chlorophyll and a change in the phytoplankton community structure. Thus, bacterial DIN use likely exerts some control over the size structure or community composition of phytoplankton assemblages, which would have tremendous implications for primary production and trophic interactions.

Despite over two decades of research, predicting the spatial and temporal conditions where autotrophs and heterotrophs compete for limiting nutrients remains a
major challenge and a comprehensive view of microbial N utilization has thus far not been achieved. To do so, one must elucidate the environmental controls on bacterial N uptake and remineralization in different marine systems. As mentioned above, C:N stoichiometry in the substrates used by bacteria and in the bacterial biomass itself have been argued to determine bacterial uptake versus regeneration of inorganic nutrients. Kirchman (1994) suggests that the phytoplankton community in oligotrophic environments is N-limited, and as a result, the DOM released by phytoplankton has a high C:N ratio (e.g. storage carbohydrates). Therefore, bacteria will require inorganic N in order to effectively break down the accumulating DOM. The converse of this can be found in estuarine environments where a greater supply of N leads to low C:N ratios in phytoplankton-released DOM (e.g. C:N of 3.4 - 4.5, Bronk et al., 1998), which would meet more of the bacterial N demand and decrease the dependence on inorganic N. Both of these hypotheses assume that bacteria are supported primarily by DOM derived directly and indirectly from phytoplankton. Although this may be true (e.g. Carlson, 2002), DOM often undergoes multiple transformations and degradation pathways before being utilized by bacteria (e.g. Benner, 2002; Mopper and Kieber, 2002), any of which may alter the C:N ratio of this material from that of its autotrophic source. Nonetheless, phytoplankton-derived material may in fact govern bacterial organic matter degradation in the open ocean, but this importance is diminished in coastal environments, where terrestrial organic matter and the benthos play significant roles.

While stoichiometry likely affects bacterial uptake and excretion of NH₄⁺ to some degree, it cannot be used exclusively to determine when and where these processes will occur. As nutrient availability changes temporally and spatially, so will phytoplankton
and bacterial community structure, growth efficiency, and C:N stoichiometry. Only by accounting for these important variants will one be able to predict the degree of competition between these groups for limiting nutrients.

Methodological Approaches for Separating Phytoplankton and Bacteria

In the past, numerous experimental approaches have been used to quantify phytoplankton versus bacterial N uptake, including size fractionation (Kirchman and Wheeler, 1998), inhibition of prokaryotic/eukaryotic protein synthesis (Middelburg and Nieuwenhuize, 2000), nutrient bioassays in mesocosm and laboratory experiments (Joint et al., 2002), and molecular analyses of N assimilation genes (Zehr and Ward, 2002; Allen et al., 2005). Drawbacks with each of these methods, however, prohibit accurate quantification of autotrophic versus heterotrophic N uptake (Bronk et al., 2007).

Most $^{15}$N uptake studies have used size-selective filtration to separate phytoplankton from bacteria, and this approach suffers from the indistinct size difference between these two groups. Glass fiber filters (e.g. Whatman GF/F; nominal pore size of 0.7 μm) are preferred for use in isotopic tracer experiments because they can be combusted to remove contaminant N and C, are compatible with isotopic analysis on a mass spectrometer, and are less expensive than other options (e.g. silver filters). In addition to capturing phytoplankton cells, GF/F (and filters of similar pore size) retain a considerable portion of the bacterial community due to the size overlap between these groups as well as a decrease in the effective pore size of the filter with increased particle load. Bacterial retention (by abundance) has been examined in numerous marine
ecosystems and is quite variable; in general, however, around half of the bacterial community is typically retained by GF/F filters (Table 1). Nonetheless, most N uptake rates measured using GF/F filters have been ascribed to phytoplankton alone rather than the actual mixed assemblage that includes bacteria.

Flow cytometric (FCM) sorting represents an alternative, but altogether underutilized approach to distinguish between the activity of various microbial groups, including phytoplankton and bacteria. Since the 1980s, FCM analyses have provided valuable insight into the structure and function of marine plankton communities (Yentsch et al., 1983; Olson et al., 1991; Veldhuis and Kraay, 2000). As opposed to the inadequate methods described above, FCM sorting can accurately separate phytoplankton from bacteria in natural samples, based on unique cellular properties, such as pigment autofluorescence (i.e. chlorophyll). This approach has been used previously to quantify primary production using radiocarbon (Rivkin et al., 1986; Li, 1994), and also to measure bacterial activity (Servais et al., 1999) as well as phytoplankton growth rates (Pel et al., 2004) and N uptake (Lipschultz, 1995).

Study Locations

The research presented here is based on data collected from several coastal marine systems. These study sites are intended to provide a means of comparing autotrophic and heterotrophic N utilization across ecosystems. The sites, which span broad spatial and temporal scales, are as follows:
Chesapeake Bay. With a watershed area of 165,760 km$^2$, a water volume of 74.4 km$^3$, and a length of almost 300 km, Chesapeake Bay is the largest estuary in the United States (Chesapeake Bay Program, 2004; Kemp et al., 2005). The Chesapeake Bay mainstem is relatively narrow (1 – 4 km) and deep (20 – 30 m), but the remainder is fairly shallow, with a mean depth of 6.5 m (Kemp et al., 2005). Chesapeake Bay also has numerous subestuarine tributary systems, including the James, York, Rappahannock, Potomac, Patuxent, Chester, and Choptank Rivers. The mainstem and tributaries are heavily influenced by anthropogenic nutrient loads, and a recent assessment ranked their eutrophic condition as either moderately high or high, with eutrophication symptoms that have not changed or have worsened over the past decade (Bricker et al., 2007). During two cruises in late summer 2004, N uptake by phytoplankton and bacteria was investigated along the mainstem of Chesapeake Bay in order to examine spatial variations in N utilization within the Bay. Uptake of various inorganic and organic N forms was measured using $^{15}$N tracers and either size-fractionation of phytoplankton and bacterial size classes or FCM sorting of autotrophic cells.

Mid-Atlantic Bight. The Long-term Ecosystem Observatory LEO-15 was established by the Mid-Atlantic Bight National Undersea Research Center in 1996 (Glenn et al., 1996). It is located in 15 m of water on the inner continental shelf offshore from the Rutgers University Marine Field Station (RUMFS) in Tuckerton, New Jersey. This region of the Mid-Atlantic Bight experiences recurrent upwelling typically lasting from days to weeks as a response to strong alongshore winds from the southwest (Glenn et al., 1996). The resulting cyclonic eddy entrains nutrient-rich bottom water from offshore into
the surface layer, thus stimulating phytoplankton blooms and oxygen depletion in the bottom layer following restratification (Hicks and Miller, 1980; Clemente-Colón, 2001; Vlahos et al., 2002). Using RUMFS as a base, two diel experiments were conducted in July 2002 to measure the uptake of $^{15}$N-labeled NH$_4^+$, NO$_3^-$, urea, and DFAA by various size fractions as well as FCM-sorted autotrophs in water samples taken from the surface and bottom (~14m) water.

**Raunefjord, Norway.** The University of Bergen’s Marine Biological Station is located on the Raunefjord at Espeland, approximately 20 km south of Bergen (60° 16’ N, 05° 14’ E). The Raunefjord is connected to an extensive fjordic network and runs north to south between the mainland and the island of Sotra in western Norway. The Marine Biological Station serves as the National Mesocosm Centre of Norway, with a floating mesocosm laboratory moored approximately 200 m offshore. A mesocosm study was conducted there in the spring of 2005 using four 11 m$^3$ (4.5 m deep, 2 m diameter) enclosures suspended from the pontoon structure. This study was designed to examine the uptake of $^{15}$N-labeled inorganic and organic N forms by phytoplankton and bacteria during an induced bloom of *Phaeocystis pouchetii*.

**Hypotheses**

The goals of this research were to: (1) quantify the uptake of DIN and DON by phytoplankton in diverse marine ecosystems; (2) quantitatively and qualitatively describe bacterial N use and its contribution to total N uptake; (3) apply FCM-sorted N uptake
rates in assessing the extent to which traditional filtration overestimates phytoplankton N uptake; and (4) evaluate the environmental factors influencing the relative uptake of various N forms by phytoplankton and bacteria. The following hypotheses are addressed:

1. In Chesapeake Bay (Chapter 2), reduced N (urea and NH₄⁺) forms comprise the majority of total N uptake by phytoplankton and DFAA represents a less utilized but still important N source to phytoplankton. Ammonium and DFAA are most important to bacterial N demand. As discussed above, heterotrophic processes dominate throughout Chesapeake Bay during summer, and regeneration of reduced N forms typically exceeds autotrophic demand despite low ambient concentrations (Smith and Kemp, 1995; Bronk et al., 1998). Amino acid uptake by phytoplankton in Chesapeake Bay and its tributaries has been previously demonstrated (Mulholland et al., 1998, 2003; Stoecker and Gustafson, 2003) and is likely significant in areas with limited DIN. However, DFAA uptake in estuarine and coastal waters is mostly bacterial (Glibert et al., 1991; Kirchman, 2000), and the availability of DFAA and DCAA increases during the summer due to biological production and riverine inputs (Jørgensen et al., 1999). Overall, the C:N ratio of DOM during summer is expected to be higher than during spring primarily as a result of degradation processes and decreased algal biomass present. Therefore, bacterial N nutrition is satisfied by DFAA, a combination of C-rich organic substrates, and NH₄⁺.
2. In the Mid-Atlantic Bight (Chapter 3), organic N substrates (e.g. urea) dominate total N uptake by phytoplankton in the surface layer under stratified conditions. Standing stocks of DIN are significantly higher below the pycnocline and support a large fraction of autotrophic N demand. Under stratified conditions, the surface-water N pool is expected to be dominated by DON, whereas the bottom waters are supplied with autochthonous DIN via remineralization and allochthonous DIN via advective processes. Therefore, phytoplankton rely primarily on organic N in the surface layer and bacteria contribute substantially to total uptake of DIN forms, whereas the bottom-water community relies predominantly on NH$_4^+$ and NO$_3^-$ for N nutrition.

3. In the Raunefjord mesocosm experiments (Chapter 4), I hypothesize that:

a. Diatoms dominate under high NO$_3^-$ and silicate (Si) conditions. Once Si has been depleted, Phaeocystis dominates over other algae and adapts well to a shift from high NO$_3^-$ availability (with amendment) to use of reduced N forms (NH$_4^+$, urea) in late spring/early summer. This research investigates the phytoplankton and bacterial response to inputs of NO$_3^-$ and urea in fjord mesocosms during initiation and decline of a Phaeocystis-dominated phytoplankton bloom. Published literature and results from a preliminary study suggest that available NO$_3^-$ is quickly removed from both control and amended mesocosms. The relative availability of NH$_4^+$ and urea increase over time due to regeneration
following an increase in bacterial and grazer production. These two reduced N forms consequently support an increasingly large proportion of phytoplankton N uptake.

**b. Bacteria in the Raunefjord, however, rely predominantly on DON forms under algal bloom conditions, but compete effectively with phytoplankton for limited DIN as the spring progresses.** Although bacteria are known to use NO$_3^-$, both diatoms and Phaeocystis are superior competitors under NO$_3^-$-replete conditions. Rather, bacteria prefer algal-released DON and rely predominantly on amino acids and ammonium as the bloom progresses from peak to senescence.

4. **Across all ecosystems studied (Chapter 5), I hypothesize that:**

**a. Nitrogen uptake by phytoplankton and bacteria is largely determined by availability rather than physiological affinity for specific N sources or the composition of DOM (e.g. C:N ratio).** Most temperate marine ecosystems undergo a seasonal transition in nutrient regime that reflects the change from dominance of new N supplied by either deep mixing (open ocean) or riverine and terrestrial sources (estuaries and coasts). Accordingly, new production and the dominance of larger phytoplankton such as diatoms in spring are replaced by regenerated production and the dominance of flagellates and cyanobacteria in summer. In other words, as N availability shifts from primarily NO$_3^-$ in spring to reduced N (NH$_4^+$ and
DON) during summer and fall, microbial nutrient preferences fluctuate and phytoplankton capable of using newly-available N forms are favored (e.g. Berg et al. 2003). Similarly, bacterial assemblages presumably shift away from an emphasis on labile algal-derived DON to a combination of relatively C-rich organic matter and DIN supplements.

b. Phytoplankton N uptake rates measured using traditional filtration (i.e. size fractionation) are significantly overestimated due to bacterial retention on filters (e.g. GF/F, 0.8 µm silver). Over half the bacterial community in coastal and estuarine systems is retained on GF/F filters, which artificially increases the phytoplankton PN concentration used to calculate absolute uptake rates. The degree to which filter-based uptake rates overestimate true phytoplankton uptake varies depending on the bacterial contribution to total community uptake. Substrates that are not favored by bacteria, such as NO$_3^-$ and urea, are overestimated to a lesser extent than NH$_4^+$ and DFAA, substrates for which bacterial affinity may exceed that of phytoplankton.

Significance

This research is significant for a number of reasons. First, few studies have been able to accurately quantify phytoplankton versus bacterial N uptake in various marine ecosystems. Traditional views of their N preferences have typically relied on
measurements based on a mixed assemblage, rather than pure phytoplankton or bacterial populations. Nitrogen uptake measured using GF/F filters is often attributed solely to phytoplankton despite the considerable retention of bacteria as well. This research is the first to quantify how much GF/F filters overestimate phytoplankton uptake rates for various inorganic and organic substrates in a marine environment. Attributing N uptake by a mixed phytoplankton-bacterial assemblage on GF/F filters to phytoplankton alone can skew our understanding of coastal and oceanic systems. For example, natural resource managers seeking to reduce anthropogenic nutrient loading to coastal waters require accurate modeling of nutrient budgets, including the effects of different N forms on plankton communities. Furthermore, on a larger scale this research is central to the study of microbial food webs, whose structure determines the degree of new and regenerated production in the oceans, as well as energy transfer to higher trophic levels and organic matter export to the deep ocean, all of which ultimately affect the global C cycle. The common assumption that measured N uptake rates should be attributed to phytoplankton but not bacteria results in an inaccurate representation of oceanic CO₂ uptake in current models of the global C cycle.
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Table 1. Percent of bacterial abundance retained by GF/F filters, from studies representing numerous diverse ecosystems.

<table>
<thead>
<tr>
<th>Location</th>
<th>Retention efficiency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long Island Sound</td>
<td>43 – 65%¹</td>
<td>(Lee and Fuhrman, 1987)</td>
</tr>
<tr>
<td>subarctic Pacific</td>
<td>50 – 60%²</td>
<td>(Kirchman et al., 1989)</td>
</tr>
<tr>
<td>Chesapeake Bay</td>
<td>50%</td>
<td>(Glibert et al., 1995)</td>
</tr>
<tr>
<td>Long Island Sound</td>
<td>57 – 65%</td>
<td>(Lee et al., 1995)</td>
</tr>
<tr>
<td>Antarctic coastal waters</td>
<td>59 – 65%</td>
<td>(Lee et al., 1995)</td>
</tr>
<tr>
<td>NW Mediterranean Sea (coastal)</td>
<td>78 – 93%</td>
<td>(Gasol and Morán, 1999)</td>
</tr>
<tr>
<td>SW Mediterranean Sea (oceanic)</td>
<td>68 – 79%</td>
<td>(Gasol and Morán, 1999)</td>
</tr>
<tr>
<td>Atlantic (estuarine)</td>
<td>70%</td>
<td>(Gasol and Morán, 1999)</td>
</tr>
<tr>
<td>Atlantic (coastal)</td>
<td>67%</td>
<td>(Gasol and Morán, 1999)</td>
</tr>
<tr>
<td>Atlantic (oceanic)</td>
<td>29%</td>
<td>(Gasol and Morán, 1999)</td>
</tr>
<tr>
<td>Gulf of Riga (Baltic Sea)</td>
<td>32 – 69%</td>
<td>(Berg et al., 2001)</td>
</tr>
<tr>
<td>York River (Chesapeake Bay)</td>
<td>53 – 71%</td>
<td>(Bradley, P.B. unpubl. data)</td>
</tr>
</tbody>
</table>

¹ when filtering natural bacterial culture only
² when filtering the <1.0 μm fraction only
Figure 1. Classical and current views of the marine nitrogen (N) cycle in oligotrophic surface waters. Box sizes indicate relative proportions of dissolved inorganic and organic N forms. Dashed lines indicate transformations and processes included in the current view of N cycling: (A) Some phytoplankton use simple organic compounds as a N source; (B) Multiple species of N₂-fixing phytoplankton (cyanobacteria) exist in the open ocean; (C) Bacteria compete for NO₃⁻ and NH₄⁺; (D) Bacteria excrete urea and can also release high molecular weight DON; (E) Some bacterioplankton appear to fix N₂. Modified from Zehr and Ward (2002).
CHAPTER 2

PHYTOPLANKTON AND BACTERIAL NITROGEN USE IN CHESAPEAKE BAY MEASURED USING A FLOW CYTOMETRIC SORTING APPROACH

This chapter follows the format of Estuaries and Coasts
Two different approaches to measuring phytoplankton nitrogen (N) use were compared during a study conducted in late summer 2004 along the main axis of Chesapeake Bay. Uptake of $^{15}$N-labeled ammonium and nitrate and dual-labeled ($^{15}$N and $^{13}$C) urea and dissolved free amino acids (DFAA) was measured in surface water samples from upper, mid, and lower bay stations. Two distinct methods were used to separate phytoplankton from bacteria prior to isotopic analysis: (1) traditional filtration using Whatman glass fiber (GF/F) filters, and (2) flow cytometric (FCM) sorting of chlorophyll-containing cells. The concentration of dissolved inorganic nitrogen (DIN) decreased with distance south along the bay, primarily due to biotic removal and decreased N loads, whereas dissolved organic nitrogen (DON) concentrations were relatively constant. Phytoplankton relied more heavily on urea and DFAA as the ratio of DON:DIN increased toward the bay mouth, but ammonium was the dominant N form used throughout the transect. Overall, ammonium comprised $74 \pm 17\%$, urea $10 \pm 9\%$, DFAA $9 \pm 7\%$, and nitrate $7 \pm 12\%$ of total measured N uptake by phytoplankton. Results suggest that bacteria relied primarily on DFAA and NH$_4^+$ for N nutrition but also used N from urea at a rate similar to that of phytoplankton, whereas bacterial nitrate uptake was insignificant. Absolute N uptake rates measured using the traditional approach were greater than those of FCM-sorted phytoplankton. On average, phytoplankton uptake of ammonium, urea, and DFAA was overestimated by 61%, 53%, and 135%, respectively, as a result of bacterial retention on GF/F filters.
INTRODUCTION

Despite extensive research on the roles of phytoplankton and heterotrophic bacteria in nitrogen (N) cycling, relatively little is known about how these two groups interact when forced to share limited N resources. Traditionally, phytoplankton were believed to use primarily dissolved inorganic N (DIN), such as ammonium ($\text{NH}_4^+$) and nitrate ($\text{NO}_3^-$), to meet their N demand, followed by release of dissolved organic N (DON), which fueled bacterial production and remineralization (e.g., Pomeroy, 1974). However, DON substrates such as urea, dissolved free and combined amino acids (DFAA and DCAA, respectively), and humics often contribute substantially to phytoplankton N nutrition (Glibert et al., 1991; See et al., 2006; Bronk et al., 2007). Furthermore, heterotrophic bacteria supplement their DON consumption with uptake of $\text{NH}_4^+$ (Wheeler and Kirchman, 1986; Kirchman, 2000) or $\text{NO}_3^-$ (Kirchman and Wheeler, 1998; Kirchman, 2000; Allen et al., 2002).

The factors controlling DON versus DIN consumption by heterotrophic bacteria and its effect on phytoplankton dynamics are important but unresolved pieces of the marine N cycle. Bacterial DIN use, for example, may be relatively high in estuarine waters (e.g., Middelburg and Nieuwenhuize, 2000), depending on the supply of carbon (C)-rich, terrestrially-derived organic matter (Goldman and Dennett, 1991; Gardner et al., 1996). Others have suggested that bacterial $\text{NH}_4^+$ use increases from estuarine to coastal waters as amino acid availability decreases (Hoch and Kirchman, 1995; Kirchman, 2000). Regardless, competition between phytoplankton and bacteria for DIN in Chesapeake Bay likely peaks in late summer when surface water DIN concentrations are minimal and
phytoplankton productivity and biomass are N-limited (Fisher et al., 1999; Kemp et al., 2005). As such, increased bacterial DIN use may exacerbate phytoplankton N limitation, diminish primary productivity or biomass accumulation (e.g., Joint et al., 2002), or exert a selective pressure favoring phytoplankton taxa that can either compete effectively with bacteria for DIN or use available DON (Kirchman, 2000).

Phytoplankton and bacteria play distinct, yet equally significant roles in N cycling and energy transfer; however, the methodological difficulty in separating these two groups continues to limit our understanding of their N nutrition. The preferred technique to date has been filtration targeting the size difference between phytoplankton and bacteria (e.g., Wheeler and Kirchman, 1986; Kirchman and Wheeler, 1998; Allen et al., 2002), often using glass fiber filters (e.g., Whatman GF/F) to retain phytoplankton. However, GF/F filters, which have a nominal pore size of 0.7 μm, also typically retain over 50% of the bacterial community in coastal and estuarine waters (Lee and Fuhrman, 1987; Gasol and Morán, 1999). An alternative approach is to distinguish between prokaryotic and eukaryotic N assimilation using chemicals that selectively inhibit protein synthesis (Wheeler and Kirchman, 1986; Middelburg and Nieuwenhuize, 2000). The value of this method is weakened, however, by inadequate effectiveness and specificity of the inhibitors (Oremland and Capone, 1988), which can lead to inconsistent results (Veuger et al., 2004). Molecular techniques that identify the presence and expression of N assimilation genes in various microbial groups are promising (reviewed in Zehr and Ward, 2002), but provide qualitative rather than quantitative estimates of N uptake. Indeed, none of these approaches can accurately quantify phytoplankton-specific or bacteria-specific N use in marine ecosystems.
A newer approach to physically separating phytoplankton and bacteria is flow-cytometric (FCM) sorting of pigmented (i.e., autotrophic) cells from concentrated field samples. Flow cytometry was first applied to marine planktology over two decades ago to rapidly and precisely estimate pico- and nanophytoplankton abundance (Yentsch et al., 1983). Although FCM has been used extensively to describe microbial community structure by enumeration of phytoplankton (e.g., Campbell et al., 1994), heterotrophic bacteria (e.g., Monger and Landry, 1993), and marine viruses (Marie et al., 1999), it has also been used to analyze marine particulate organic matter (Minor et al., 1998) and estimate grazing rates by zooplankton and bivalves (reviewed in Olson et al., 1991). Using FCM sorting, one can isolate microorganisms of interest based on specific cellular properties, such as size or pigment autofluorescence. Paau et al. (1979) were the first to separate algal cells from bacteria using this approach, and others have similarly quantified primary production (Li, 1994), bacterial activity (Servais et al., 1999), phytoplankton growth rates (Pel et al., 2004), and N assimilation (Lipschultz, 1995) on a per-cell basis.

In the present study, we used $^{15}$N tracer techniques with both traditional filtration and FCM sorting of phytoplankton cells to measure uptake of different DIN and DON substrates in Chesapeake Bay. The goals were to: (1) examine the use of DIN and DON by phytoplankton in Chesapeake Bay surface waters during the N-limited late summer, and (2) use FCM sorting to evaluate the effect of bacterial retention on uptake rates measured using the traditional approach. We hypothesize that FCM sorting more accurately measures true phytoplankton uptake, whereas traditional filtration overestimates N uptake by phytoplankton. We also hypothesize that phytoplankton N use
shifts with availability, from DIN-based in the upper bay to more DON-based in the lower bay.

METHODS

Study site and sampling. During a cruise aboard the R/V Cape Henlopen, a total of six stations were sampled along the main axis of Chesapeake Bay from 29 August to 02 September 2004 (Fig. 1). At each station, water was collected near the surface (2 m) using a Niskin rosette, and depth profiles of salinity, temperature, oxygen, and fluorescence were characterized with a Sea-Bird Electronics 911 Plus CTD. Samples were taken in the morning at each station, starting with the two upper bay stations, 858 and 908, on 30 August, followed by the lower bay stations, 707 and 724, on 31 August, and the mid bay stations, 818 and 804, on 01 September.

Dissolved and particulate N and C concentrations. Samples for nutrient analyses were filtered through combusted (450°C for 2 h) Whatman GF/F filters and kept frozen at -20°C. Ammonium concentrations were measured manually with the phenol-hypochlorite method (Koroleff, 1983), and a Lachat QuikChem 8500 autoanalyzer was used with the Parsons et al. (1984) colorimetric technique to measure NO₃⁻ and nitrite (NO₂⁻) concentrations. Urea was determined using the manual monoxime method (Price and Harrison, 1987), whereas DFAA concentrations were measured as total DFAA using the fluorometric o-phthalaldehyde method (Parsons et al., 1984). Concentrations of DON were determined as the difference between total dissolved N (TDN) and DIN, and TDN
was measured using the persulfate oxidation technique (Bronk et al., 2000). A Shimadzu TOC-V was used with high-temperature combustion to measure dissolved organic C (DOC) concentrations (Hansell et al., 1997). Particulate N (PN) and organic C (POC) concentrations were determined from filters used to terminate isotopic tracer experiments on a Europa Geo 20/20 isotope ratio mass spectrometer equipped with an Automated Nitrogen and Carbon Analyzer for Solids and Liquids (ANCA-SL) sample processing unit.

**Uptake experiments.** Stable isotope tracer techniques were used to quantify uptake rates of inorganic and organic N by distinct components of the microbial community. To this end, the following four substrates were added separately to duplicate water samples: \(^{15}\)N-labeled NH\(_4^+\) and NO\(_3^-\) and dual-labeled \((^{15}\)N, \(^{13}\)C\) urea and DFAA (an algal extract containing sixteen amino acids; Cambridge Isotope Laboratories, Andover, MA). Tracer-level additions (<10% of ambient concentrations) of \(^{15}\)NH\(_4^+\) and \(^{15}\)NO\(_3^-\) were estimated from historic data provided by the Chesapeake Bay Program (www.chesapeakebay.net). Additions of labeled urea and DFAA were based on published data for Chesapeake Bay and adjacent coastal waters. Substrate isotopic enrichments for NH\(_4^+\) were corrected for isotope dilution by NH\(_4^+\) regenerated during the incubations (Glibert et al., 1982), following isolation of the NH\(_4^+\) pool using solid phase extraction (Dudek et al., 1986). Isotope dilution of the NO\(_3^-\), urea, and DFAA pools was not measured. Although uptake of these substrates may therefore be underestimated, the comparison between the two different methodological approaches that this paper focuses on is unaffected by isotope dilution. Furthermore, given the low \(^{15}\)N enrichment values
for cells in the NO₃⁻, urea, and DFAA incubations, even extensive dilution of these substrates would not change the primary conclusions presented here.

Immediately after sampling and CTD retrieval, eight 500 mL polyethylene (PETG) bottles (four substrates, in duplicate) were filled with surface water and spiked with ¹⁵N tracer. The PETG bottles were then incubated on deck for 1 – 3 h in flow-through incubators under simulated in situ light and temperature conditions. Incubations were terminated using varying filtration approaches to examine different components of the microbial community. A portion (150 mL) of each bottle was filtered through combusted 25 mm GF/F filters to obtain uptake rates for what has traditionally been referred to as phytoplankton, although as noted above, this fraction may also contain over half the bacterial community. Another 150 mL of sample was first passed through 35 μm mesh to remove larger plankton that could clog the flow cytometer, and these cells were then washed onto a GF/F filter in order to measure their biomass and isotopic enrichment. All GF/F filters were immediately frozen and stored at -20°C until isotopic analysis on the mass spectrometer. The <35 μm filtrate was concentrated over a 47 mm, 0.2 μm Supor filter to a final volume of 5 – 12 mL, which was then transferred to a centrifuge tube. The Supor filter was occasionally rinsed during concentration using a 10 mL pipette and also by placing the rolled filter into the centrifuge tube with the concentrated sample and inverting gently several times. The concentrated sample was preserved with paraformaldehyde at a final concentration of 0.2% (Campbell, 2001) and then frozen in liquid N.

An analysis of this concentration technique was conducted in the York River, a sub-estuary of Chesapeake Bay, and showed that little phytoplankton material, if any, is
lost to the Supor filter. In samples concentrated from 100 – 200 mL to 10 mL, the amount of chlorophyll a (Chl a) was, on average, 95% of whole-water (unconcentrated) Chl a, whereas the Supor filter retained 3% of Chl a. Increasing the concentration factor using an initial volume of 300 mL (twice that of this study) resulted in a greater loss of Chl a to the Supor filter, with 89% in the concentrated sample and 12% of the Chl a remaining on the Supor filter (Bradley, unpubl. data).

**FCM sorting of phytoplankton.** Duplicate samples for FCM sorting were kept at -80°C until analysis, whereupon they were thawed at room temperature. Phytoplankton cells were identified and sorted based on their chlorophyll autofluorescence using a Cytoperia inFlux V-GS flow cytometer located at the Bermuda Institute of Ocean Sciences. The inFlux V-GS is designed for stable, high speed sorting, which enabled high phytoplankton yield at sort rates of 2,000 to 10,000 cells s⁻¹. Phytoplankton cells were sorted into polypropylene tubes and filtered onto GF/F filters, which were stored at -20°C until isotopic analysis on the Europa mass spectrometer described above. To obtain N masses sufficiently above the Europa’s detection limit (≈ 1 μg N) for reliable ¹⁵N atom percent enrichment values, 1 – 2 μg N of carrier [(NH₄)₂SO₄] was added to the filters prior to analysis. A carrier correction was later performed to determine the isotopic enrichment in the original sample.

The accuracy of autotrophic sorting was periodically verified by collecting and analyzing the sorted and waste streams, and the presence of bacteria in sorted samples was quantified using acridine orange direct counts (Sherr et al., 2001). To evaluate the extent of any negative effects of the FCM sorting method on cellular integrity or retention
of $^{15}$N label, a simple experiment was conducted at three stations using boiling distilled water to deliberately rupture phytoplankton cells during filtration for both GF/F and FCM-sorted fractions. Before the GF/F or Supor filters went dry, 25 mL of boiling distilled water was added and the filtration or FCM concentration procedures were completed. These samples were later processed identically to those described above. To assess whether exposure to boiling water caused additional loss of internal N from preserved and sorted cells, absolute N uptake rates were calculated for GF/F and FCM samples as described below, but with the PN measured from filters analyzed on the mass spectrometer. If preservation and FCM sorting caused cells to rupture, the uptake rates for the control and boiled treatments would be statistically equal in the FCM-sorted samples and significantly different in the GF/F fraction.

**Calculation of N uptake rates.** Specific ($V$, h$^{-1}$) and absolute ($\rho$, $\mu$mol N L$^{-1}$ h$^{-1}$) N uptake rates were calculated using the equations of Dugdale and Goering (1967). Specific rates are useful for comparing the physiological affinity of phytoplankton and bacteria for specific substrates, whereas absolute rates illustrate how including bacterial biomass on GF/F filters can further skew measurements of phytoplankton N uptake.

To examine the accuracy of GF/F-based measurements of phytoplankton N use, total phytoplankton-only (Phyto) uptake rates were calculated by combining absolute uptake rates in the FCM and >35 $\mu$m fractions. Concentrations of PN were measured directly in the >35 $\mu$m and GF/F fractions; however, the FCM method precludes direct, accurate measurement of total autotrophic PN. Therefore, Phyto PN was estimated by correcting GF/F PN for bacterial biomass retained on these filters. Bacterial abundance
was measured in surface samples from each station using epifluorescence microscopy (K. Wang unpubl. data) and was conservatively converted to total bacterial biomass using a N content of 12 fg N cell$^{-1}$ (Vrede et al., 2002). Phyto PN was then obtained by subtracting 50% of total bacterial biomass from PN measured for the GF/F fraction.

The assumption that GF/F filters retained 50% of the bacterial biomass is supported in the literature for a variety of ecosystems (e.g., Lee et al., 1995; Gasol and Morán, 1999). In addition, Glibert et al. (1995) estimated that in Chesapeake Bay, 50% of bacteria are retained by GF/F filters, and recent measurements in the York River, a sub-estuary of Chesapeake Bay, ranged from 53 to 71% (Bradley, unpubl. data). Furthermore, 50% represents a conservative estimate because bacterial biomass in Chesapeake Bay tends to peak in late summer, and the above studies examined retention of bacteria by abundance rather than biomass. Presumably, larger bacteria are captured by the GF/F matrix, and have proportionally more biomass than smaller cells that pass through the filter.

RESULTS

Environmental conditions

Depth profiles captured by CTD varied considerably along Chesapeake Bay, with surface salinity increasing from 4 at Station 908 to 24 at the Bay mouth (Station 707). Surface temperatures varied relatively little throughout the Bay, from 25.7 to 27.6°C, with the coldest values measured at the mouth. Tropical Storm Gaston, which passed through the Bay on the night of 30 August, was a likely cause of stratification differences
between the three Bay regions. For example, Station 858, which was sampled the morning of 30 August, had a strong pycnocline at 9 to 12 m depth. In contrast, the lower bay stations, which were sampled the following morning, had virtually no change in salinity, temperature, or oxygen with depth. The mid bay stations, 818 and 804, were moderately stratified at depths of 15 – 20 m, and it seems likely that the storm had weakened and deepened these pycnoclines. The effects of this disruption on N transformations and bioavailability are discussed in further detail below.

**Dissolved and particulate N and C concentrations**

Total DIN concentrations ($\text{NH}_4^++\text{NO}_3^-+\text{NO}_2^-\text{)}$ decreased from $40.0 \pm 0.4 \mu\text{mol N L}^{-1}$ at the northernmost station (908) to $1.6 \pm 0.2 \mu\text{mol N L}^{-1}$ at the mouth (Station 707; Fig. 2). Accordingly, DIN comprised 76% and 11% of TDN at these two endpoints, respectively. Nitrate alone comprised 54% of TDN at Station 908, but decreased exponentially southward to a minimum of $0.2 \mu\text{mol N L}^{-1}$ at Station 707. Concentrations of $\text{NH}_4^+$ were also highest in the upper bay and were lowest ($0.5 \mu\text{mol N L}^{-1}$) at Station 804. Surface-water $\text{NO}_2^-$ concentrations were relatively high throughout the Bay and even dominated the mid bay DIN pool. Bottom waters in the mid bay region are typically hypoxic or anoxic during summer, creating low redox conditions in the sediment and water-column that enhance $\text{NH}_4^+$ flux from the former and inhibit nitrification in the latter (Kemp et al., 2005). The mixing, and subsequent nitrification, of $\text{NH}_4^+$-rich bottom water with oxygenated surface water during Gaston likely accounts for the high $\text{NO}_2^-$ concentrations (McCarthy et al., 1984; Bronk et al., 1998).
In contrast to DIN, DON concentrations varied relatively little along the bay, with an overall mean of $14.8 \pm 1.7 \, \mu\text{mol N L}^{-1}$ (Fig. 2). There were no spatial trends in urea concentrations, whereas DF AA concentrations were slightly lower in the upper bay and were less than $0.3 \, \mu\text{mol N L}^{-1}$ throughout. The contribution of DON to TDN increased southward, from 24% at Station 908 to 89% at Station 707, as available $\text{NH}_4^+$ and $\text{NO}_3^-$ were biologically removed in the upper and mid bay. Accordingly, the ratio of DIN to DON decreased exponentially from 3.2 at Station 908 to 0.1 at Station 707. The profile of DOC concentrations was similar to that of DON (data not shown), in that concentrations were highest in the mid bay ($282.4 \pm 5.5 \, \mu\text{mol L}^{-1}$), followed by the upper bay ($251.9 \pm 4.7 \, \mu\text{mol L}^{-1}$), and lower bay regions ($232.9 \pm 7.0 \, \mu\text{mol L}^{-1}$). Ratios of DOC to DON were roughly similar along the Bay, with values of 17.9, 17.7, and 16.7 for the upper, mid, and lower bay, respectively.

Particulate N concentrations did not exhibit any distinct trends along Chesapeake Bay (Fig. 3). Mean PN in the GF/F fraction was $9.9 \pm 2.0 \, \mu\text{mol N L}^{-1}$. Phytoplankton (Phyto) PN was 73 to 84% of GF/F PN and averaged $8.0 \pm 1.7 \, \mu\text{mol N L}^{-1}$. Particulate N concentrations in the $>35 \, \mu\text{m}$ fraction were considerably lower, with an overall mean of $0.9 \pm 0.3 \, \mu\text{mol N L}^{-1}$. Concentrations of POC were measured on GF/F filters from urea and DF AA uptake samples in order to calculate POC:PN ratios. Concentrations of POC in phytoplankton, however, were estimated from bacterial abundance and a cellular C content of 65 fg C cell$^{-1}$ (Fukuda et al., 1998 and references therein; Vrede et al., 2002), which resulted in a mean phytoplankton POC:PN ratio of $8.7 \pm 1.1$. The ratio of C:N in particulate matter on GF/F filters was $7.3 \pm 0.8$. 
FCM methodological considerations

In order to obtain sufficient N biomass for analysis on a mass spectrometer, a large number of phytoplankton cells (roughly $5 \times 10^6$) must be sorted. As such, FCM sorting requires a compromise between purity and yield in the sorted sample. In this study, we favored a higher yield of sorted phytoplankton, resulting in the inclusion of a small percentage ($7 \pm 3\%$) of the bacterial community. Using a conversion factor of 12 fg N cell$^{-1}$ (Vrede et al., 2002), this represents a bacterial biomass of $0.28 \mu\text{mol N L}^{-1}$, or 4% of phytoplankton PN, which was then diminished (by about 50% or more) with filtration of sorted samples onto GF/F filters. Therefore, bacterial influence on uptake rates calculated for FCM-sorted phytoplankton was insignificant.

An additional concern with the FCM method is that preserving, freezing, and sorting may damage cell membranes, thus decreasing apparent uptake rates due to loss of N that was taken up during the incubation but not yet utilized. If this were the case, FCM-sorted rates would be a function of assimilation (i.e., N incorporated into biomass) rather than N uptake, and would not differ between treatments. However, samples exposed to boiling distilled water had significantly lower ($p < 0.05$) estimated absolute uptake rates than the control treatments for both the GF/F and FCM-sorted fractions (data not shown). This suggests that the cellular integrity of FCM-sorted cells was similar to that of cells on GF/F filters and was only minimally compromised, if at all, by the method. Similarly, in methodological trials conducted using surface water collected from the York River, there was no significant difference ($p = 0.147$) in N uptake rates measured from FCM samples sorted fresh versus after preservation, freezing, and thawing (data not shown). These
results agree with the conclusions of Rivkin et al. (1986) that the cellular integrity of phytoplankton remains intact (i.e., no radioisotope is lost) during FCM sorting.

Specific and absolute uptake rates

Efforts to keep $^{15}$N tracer additions under 10% of ambient concentrations (Dugdale and Goering, 1967) had varying results. Addition of $^{15}$NO$_3^-$ ranged from 2 to 34% (mean of 17%), added urea label was 10 – 20%, and DFAA additions exceeded 40% of ambient concentrations at all stations. Although some of these enrichments could raise concerns about artificial stimulation of uptake rates due to increased availability, the amount of tracer added ($0.05 - 0.10 \mu$mol N L$^{-1}$) and the low uptake rates for these three substrates relative to those for NH$_4^+$ suggest that this was not a significant source of error. For example, $^{15}$NO$_3^-$ tracer additions were highest relative to ambient concentrations in the mid and lower bay, where NO$_3^-$ contributed less than 2% of total N uptake by the GF/F fraction. Similarly, $^{15}$N enrichment from DFAA tracer was highest in the upper bay, where DFAA uptake rates were lowest. Labeled NH$_4^+$ additions were 10% or less of ambient concentrations for all stations except 804 (43%). Due to relatively high regeneration rates, however, isotopic enrichment of the NH$_4^+$ substrate pool did not exceed 8% at any station.

Specific uptake rates in the GF/F and FCM fractions were not significantly different across all stations for NH$_4^+$ ($p = 0.969$) nor urea ($p = 0.915$), and although NH$_4^+$ and urea uptake rates by the $>35$ μm fraction were consistently lower than GF/F rates, the differences were not significant overall (NH$_4^+$: $p = 0.299$, urea: $p = 0.111$; Fig. 4A, C).
For NO₃⁻, specific uptake by FCM-sorted phytoplankton always equaled or exceeded that of the GF/F fraction, yet NO₃⁻ uptake rates were highest in the >35 μm fraction at Stations 908, 858, 804, and 707 (Fig. 4B). The opposite trend held true for DFAA; uptake rates in the GF/F fraction were significantly higher than both FCM uptake ($p < 0.05$) and >35 μm uptake ($p < 0.001$; Fig. 4D). Although spatial trends in specific uptake were largely absent, uptake of NO₃⁻ by the >35 μm fraction generally decreased toward the bay mouth, whereas uptake of urea by the FCM and >35 μm fractions generally increased southward, except for relatively low FCM uptake at Station 707.

In order to compare GF/F rates with those of phytoplankton only, absolute uptake rates were calculated for the Phyto fraction from the sum of FCM and >35 μm rates (Fig. 5). Except for NO₃⁻, absolute uptake rates were relatively insignificant in the >35 μm fraction due to low total biomass of these larger cells. Although absolute uptake rates for all substrates were consistently higher in the GF/F versus Phyto fraction, the differences across all stations were only significant for DFAA ($p < 0.01$), which had rates in the GF/F fraction that were two to four times those of Phyto. On average, absolute NH₄⁺ uptake rates were more than ten times higher than those of the other three substrates and comprised 74% of total measured N uptake in both the GF/F and Phyto fractions. With the exception of the northernmost station, 908, absolute NO₃⁻ uptake rates were the lowest of the four substrates used and generally decreased toward the bay mouth (Fig. 5B). Absolute uptake of urea and DFAA, on the other hand, was highest in the lower half of Chesapeake Bay (Fig. 5C-D). Overall, phytoplankton in the FCM and >35 μm fractions relied slightly more on NO₃⁻ and urea and slightly less on DFAA than did the mixed assemblage retained by GF/F filters (Fig. 6). Furthermore, the importance of NO₃⁻
to these two fractions tended to decrease southward; in contrast, urea and DFAA together comprised 9 – 17% of total Phyto uptake in the upper bay and 22 – 47% in the lower bay.

Dual-labeled ($^{15}$N and $^{13}$C) urea and DFAA tracers provided a means of calculating C uptake for these two organic substrates. Although phytoplankton and bacteria used the N from urea, there was no $^{13}$C enrichment in any of the samples, which suggests that the C was respired (as CO$_2$) following cleavage of N groups by urease. In contrast, $^{13}$C-DFAA uptake rates in the FCM fraction ranged from zero in the upper bay to 0.0283 μmol C L$^{-1}$ h$^{-1}$ in the lower bay, with a mean of 0.0120 ± 0.0120 μmol C L$^{-1}$ h$^{-1}$ (data not shown). Rates in the GF/F fraction were 0.0624 – 0.2704 μmol C L$^{-1}$ h$^{-1}$, with an overall mean of 0.1946 ± 0.0684 μmol C L$^{-1}$ h$^{-1}$.

**DISCUSSION**

Flow cytometric sorting is a powerful tool for distinguishing between phytoplankton and bacterial cellular activity. Nitrogen uptake rates measured using the traditional GF/F method, versus FCM sorting, are discussed below both in the context of how the former often overestimates phytoplankton N use, and also with respect to N cycling by phytoplankton and heterotrophic bacteria in Chesapeake Bay.

**Interpretation of FCM and GF/F uptake rates**

The traditional use of GF/F filters to separate phytoplankton from the microbial community can produce ambiguous results when determining autotrophic N uptake and
assimilation because it captures a mixed assemblage of phytoplankton and heterotrophic bacteria. This is significant because uptake rates measured using GF/F filters have traditionally been attributed to phytoplankton despite the fact that a substantial fraction of the bacterial community is also retained. The inclusion of bacterial biomass increases PN, and consequently absolute uptake rates, measured using GF/F filters, thereby potentially overestimating phytoplankton N uptake.

Specific N uptake rates provide a means of comparing the physiological ability of different size fractions or different types of cells to use $^{15}$N tracers and are not confounded by varying biomass, as are absolute uptake rates. Therefore, a unique perspective of phytoplankton and bacterial N use can be obtained by comparing specific uptake rates from the fractions studied here. If bacteria retained on GF/F filters have a strong affinity for a given N substrate compared to that of phytoplankton, specific N uptake rates calculated from these filters will be higher than those of phytoplankton-only (e.g., FCM fraction). Conversely, the inclusion of relatively $^{15}$N-deficient bacteria (due to low uptake of a substrate) on GF/F filters will dilute the isotopic signal in the PN pool and consequently underestimate specific N uptake by phytoplankton.

Application of this analysis to the data presented here for Chesapeake Bay provides insight that varies by substrate. The highest specific NO$_3^-$ uptake rates were measured in the FCM-sorted phytoplankton and $>$35 $\mu$m fractions (Fig. 4). Relatively low specific uptake rates in the GF/F fraction were due to a lack of bacterial NO$_3^-$ use and isotopic dilution of the PN pool by unlabeled ($^{14}$N) bacterial biomass on these filters. In contrast to NO$_3^-$, specific DFAA uptake rates were highest in the GF/F fraction as a result of strong bacterial affinity for this organic substrate relative to that of phytoplankton. The
trends for NH$_4^+$ and urea, however, were not as consistent across all samples. Overall, specific uptake rates of these two reduced N substrates were roughly equal between the GF/F and FCM fractions, suggesting that bacteria and phytoplankton utilized each substrate similarly at most stations.

In contrast to specific uptake rates, two separate factors can affect measurements of absolute N uptake by phytoplankton using GF/F filters: (1) the enrichment or dilution of $^{15}$N in the PN pool due to bacterial uptake (or lack thereof), as previously discussed, and (2) the overestimation of phytoplankton PN as a result of bacterial retention on GF/F filters. The former can bias uptake rates in either direction, but the latter is unidirectional; in other words, retention of bacteria on a GF/F will always produce erroneously higher values of phytoplankton PN and therefore increase the reported absolute uptake rate. Furthermore, this overestimation of phytoplankton PN as a result of bacterial retention on GF/F filters offsets underestimations of phytoplankton uptake due to low bacterial N use. For example, specific uptake rates indicate that bacteria in Chesapeake Bay were not using $^{15}$NO$_3^-$ as much as phytoplankton were, yet absolute uptake rates were roughly equal between the GF/F and Phyto fractions at most stations (Fig. 5B) as a result of the compensatory effect of bacterial biomass on PN values from GF/F filters. Theoretically, however, absolute uptake by the Phyto fraction cannot exceed that of GF/F as it did at Stations 818 and 804; this may have been due to analytical error associated with isotopic measurements just above detection limits. Nonetheless, using specific uptake rates, one can examine the effect of both bacterial N use and bacterial biomass on absolute uptake rates determined from GF/F filters.
Overestimation of phytoplankton N uptake by GF/F filters

One goal for this research was to assess the extent to which GF/F filters overestimate autotrophic uptake of NH$_4^+$, NO$_3^-$, urea, and DFAA. This was calculated as:

\[
\% \text{ Overestimation} = \frac{\text{Absolute GF/F uptake} - \text{Absolute Phyto uptake}}{\text{Absolute Phyto uptake}} \times 100
\]

(Eq. 1)

On average, use of GF/F filters overestimated phytoplankton uptake of NH$_4^+$, urea, and DFAA by 61%, 53%, and 135%, respectively (Table 1). As discussed above, Phyto absolute uptake rates should not exceed those of GF/F filters (exceptions shown as negative values in Table 1). Although this was the case for NO$_3^-$ at every station except 707, Phyto absolute rates were only significantly higher than GF/F rates at Stations 804 and 818 ($p < 0.05$). Removal of these two stations results in an overestimation of phytoplankton NO$_3^-$ uptake of 5 ± 15% by GF/F filters.

Attributing N uptake by a mixed phytoplankton-bacterial assemblage on GF/F filters to phytoplankton alone can skew our understanding of coastal and oceanic ecosystems. For example, natural resource managers seeking to reduce anthropogenic nutrient loading to coastal waters require accurate modeling of nutrient budgets, including the effects of different N forms on plankton communities. In both coastal and oceanic systems, the efficiency with which energy is transferred to higher trophic levels depends partly on phytoplankton and bacterial dynamics and the N sources fueling their production. Furthermore, the $f$-ratio and vertical export of particulate matter in the ocean may be underestimated as a result of bacterial contributions to GF/F-measured uptake of reduced N, or potentially overestimated under conditions of increased bacterial NO$_3^-$ use.
Several conclusions can be drawn from the results of this study with respect to traditional views of phytoplankton and bacterial N preferences. Concentrations of DIN tend to vary considerably between Bay segments and across seasons, but our range of 1.6 – 40.0 μmol N L⁻¹ for DIN agrees well with historical measurements for summer (e.g., Harding, 1994). The TDN pool was dominated by NO₃⁻ at the northernmost station and by DON in the mid and lower bay, yet NH₄⁺ contributed most to total N uptake along the entire transect. Indeed, Chesapeake Bay surface waters are typically dominated by heterotrophic processes during summer, with regeneration of reduced N forms exceeding autotrophic uptake (Smith and Kemp, 1995; Bronk et al., 1998). Ammonium uptake was particularly high at Station 804 (3.6 μmol N L⁻¹ h⁻¹), but was balanced by a regeneration rate of 3.2 μmol N L⁻¹ h⁻¹ (data not shown). This uptake rate is higher than previous measurements in Chesapeake Bay for August (e.g., Bronk et al. 1998), but not as high as some rates measured elsewhere (Twomey et al., 2005). The nearly exclusive use of NH₄⁺ at Station 804 was also measured in samples taken from a dense algal bloom located just north of Station 818. Ammonium uptake rates measured using GF/F filters on samples from this algal bloom were nearly double those of Station 804 (6.7 ± 0.3 μmol N L⁻¹ h⁻¹) and comprised 94% of total measured N uptake. These results, which are not presented in further detail because samples for FCM sorting were not taken, suggest that the N dynamics at Station 804 may be symptomatic of a similar bloom. Flagellated algal blooms, especially those of the dinoflagellate *Prorocentrum minimum*, are common in Chesapeake Bay and its tributaries during summer (Glibert et al., 2001). The mean NH₄⁺
uptake rate from stations other than 804 was 0.3 \( \mu \text{mol N L}^{-1} \text{ h}^{-1} \), which is similar to rates from other studies for late summer (Glibert et al., 1991; Bronk et al., 1998).

Phytoplankton are believed to use \( \text{NH}_4^+ \) preferentially over \( \text{NO}_3^- \) when both are available, primarily due to the energetic cost of \( \text{NO}_3^- \) assimilation. Furthermore, \( \text{NH}_4^+ \) can inhibit the uptake and assimilation of \( \text{NO}_3^- \), particularly at \( \text{NH}_4^+ \) concentrations exceeding 1 \( \mu \text{mol N L}^{-1} \) (Dortch, 1990; Cochlan and Bronk, 2003). With the exception of the >35 \( \mu \text{m} \) fraction at Station 908, \( \text{NO}_3^- \) uptake was always lower than that of \( \text{NH}_4^+ \), even in the upper bay where \( \text{NO}_3^- \) was abundantly available. This may suggest that \( \text{NH}_4^+ \) was inhibiting \( \text{NO}_3^- \) uptake in Chesapeake Bay surface waters during this study; however, \( \text{NO}_3^- \) uptake was more closely related to \( \text{NO}_3^- \) availability than that of \( \text{NH}_4^+ \). Specific \( \text{NO}_3^- \) uptake rates in the >35 \( \mu \text{m} \) fraction were strongly correlated with ambient \( \text{NO}_3^- \) concentrations along the Bay (Pearson’s Correlation, \( r^2 = 0.99, p < 0.0001 \)). This relationship was also significant for the GF/F fraction (\( r^2 = 0.85, p < 0.01 \)), but not for FCM-sorted phytoplankton (\( r^2 = 0.29, p = 0.268 \)). In contrast, the relationship between specific \( \text{NO}_3^- \) uptake and ambient \( \text{NH}_4^+ \) concentrations was not significant for any fraction. These results suggest that although \( \text{NH}_4^+ \) may have inhibited \( \text{NO}_3^- \) uptake, it is also possible that only large phytoplankton (>35 \( \mu \text{m} \) and on GF/F filters) had a strong affinity for \( \text{NO}_3^- \), whereas smaller phytoplankton (i.e., FCM-sorted cells) preferred \( \text{NH}_4^+ \).

Concentrations of DON varied little along the transect relative to DIN, and our mean value of 14.8 \( \mu \text{mol N L}^{-1} \) was somewhat low compared to values in excess of 40 \( \mu \text{mol N L}^{-1} \) measured by Bronk et al. (1998), but was more consistent with those of other studies (McCarthy et al., 1977; Bronk, 2002). Although a large fraction of the DON pool is likely unavailable for phytoplankton use (Bronk et al., 2007), uptake of urea, for
example, has been shown to support phytoplankton N nutrition in Chesapeake Bay and its plume, especially under DIN-limited conditions (McCarthy et al., 1977; Glibert et al., 1991). Urea concentrations presented here were 0.50 – 1.03 μmol N L\(^{-1}\), and most values have historically fallen within this range for Chesapeake Bay (Lomas et al., 2002). Although urea availability generally decreased toward the bay mouth, the contribution of this organic substrate to total measured N uptake by the Phyto fraction increased from 4% at Station 908 to 22% at Station 707. Furthermore, there was a significant positive correlation between specific urea uptake by the >35 μm fraction and the ratio of DON:DIN \((r^2 = 0.83, p < 0.05)\). Together with previously discussed results, this suggests that urea, and perhaps other organic sources (see below), replace NO\(_3^-\) in the N nutrition of large phytoplankton as DON becomes relatively more abundant.

Amino acids are generally not considered to be important to autotrophic N nutrition, despite the fact that phytoplankton can actively transport DFAA into the cell (Antia et al., 1991; Bronk, 2002). There are other amino acid uptake mechanisms in addition to direct uptake, such as amino acid oxidation and peptide hydrolysis by means of proteolytic enzymes, and these pathways can play important roles in phytoplankton N nutrition (Palenik and Morel, 1990; Mulholland et al., 2003). Specific DFAA uptake rates presented here for the FCM and >35 μm fraction were not insignificant and actually exceeded those of NO\(_3^-\) in the FCM fraction at all but one station (908). Clearly, phytoplankton were using DFAA, albeit to a lesser extent than bacteria were, as indicated by comparison with the GF/F rates. There was no \(^{13}\)C enrichment from DFAA in the >35 μm fraction at all but one station (707), and C uptake from DFAA by the FCM fraction was relatively low compared to the \(^{15}\)N-DFAA uptake by FCM phytoplankton.
and the $^{13}$C-DFAA uptake by the GF/F fraction. In other words, phytoplankton were using the N, but not always the C, from the individual amino acids. This is likely a result of amino acid oxidation, whereby NH$_4^+$ is enzymatically cleaved from DFAA molecules and thus made available for cellular assimilation (Palenik and Morel, 1990).

Uptake of amino acid N by phytoplankton increased with distance south along the Bay and contributed most to total N uptake at the mouth. Furthermore, there was a strong correlation between the ratio of DON:DIN and absolute DFAA uptake by FCM-sorted phytoplankton ($r^2 = 0.72$, $p < 0.05$) and the $>35$ μm fraction ($r^2 = 0.85$, $p < 0.01$), but this relationship was not significant for the GF/F fraction ($r^2 = 0.44$, $p = 0.150$; Fig. 7). This pattern suggests that a physiological control may exist whereby phytoplankton DON use is triggered by the relative abundance of DON and DIN, a hypothesis supported by studies from various marine ecosystems, for both amino acids (Mulholland et al., 1998; Middelburg and Nieuwenhuize, 2000) and urea (Glibert et al., 1991; Mulholland et al., 2002, Bradley, P. B., unpubl.). Although it is possible that DFAA uptake by the $>35$ μm fraction was due to the activity of particle-attached bacteria rather than large phytoplankton, this is unlikely for several reasons. First, the rinsing procedures and GF/F filters used when collecting the $>35$ μm fraction may have removed the majority of any attached bacteria. Also, if bacteria were contributing significantly toward DFAA uptake by the $>35$ μm fraction, there would have been measurable $^{13}$C enrichment, as in the GF/F fraction. Finally, large phytoplankton are known to use amino acids in Chesapeake Bay (Mulholland et al., 2003; Stoecker and Gustafson, 2003).

Bacterial N use was not directly measured in this study using FCM sorting because of the lengthy processing required to obtain sufficient biomass for isotopic
analysis, as well as the difficulty in isolating bacteria from detritus and other background material. However, general conclusions can be drawn by comparing specific and absolute uptake rates between fractions. Bacteria typically use amino acids and NH$_4^+$ preferentially over other N forms, such as NO$_3^-$, urea, and dissolved DNA (Kirchman, 2000), and this was likely the case in the present study as well. On average, specific DFAA uptake by the GF/F fraction was roughly twice that of phytoplankton in the FCM and >35 μm fractions. Also, the percent contribution of DFAA to total absolute uptake increased from 9 ± 7% for phytoplankton-only to 13 ± 7% in the GF/F fraction as a result of bacterial use. The fact that specific uptake rates for NH$_4^+$ were generally equal between the GF/F and FCM fractions suggests that bacterial use of this substrate was on par with that of phytoplankton. Furthermore, at Station 908 NH$_4^+$ comprised 52% of phytoplankton N uptake but 64% of uptake by cells in the GF/F fraction, and similar results were found at Station 707, which indicates that in some cases bacteria outcompeted phytoplankton for available NH$_4^+$. Given that DOC:DON was relatively high (15.5 - 20.5), as was the ratio of POC:PN in the mixed GF/F assemblage (7.3 ± 0.8), bacteria likely required NH$_4^+$ to complement the respiration of relatively C-rich organic matter in Chesapeake Bay.

Therefore, NH$_4^+$ and DFAA were more important to bacteria than urea, whereas NO$_3^-$ uptake was insignificant. Contrary to traditional belief, urea can contribute significantly to bacterial N demand in marine ecosystems (Jørgensen, 2006; Sanderson et al., 2008, Bradley unpubl. data). Although it could not be quantified here, urea uptake by bacteria was significant relative to phytoplankton and the other substrates studied. Bacterial NO$_3^-$ uptake, on the other hand, was minimal, but is known to contribute
substantially to NO$_3^-$ uptake in other marine ecosystems (Kirchman and Wheeler, 1998; Kirchman, 2000; Allen et al., 2002).

**CONCLUSIONS**

Chesapeake Bay is a highly dynamic system with biogeochemical and ecological characteristics that vary over time and space (e.g., Kemp et al., 2005). This study sought not only to examine phytoplankton and bacterial N use along mainstem Chesapeake Bay during late summer, but also to compare true (FCM-sorted) phytoplankton N uptake rates with traditional GF/F-based measurements. Dissolved inorganic N comprised most of the TDN pool in the upper bay, but decreased rapidly toward the mouth due to biotic uptake. Ammonium was the dominant form of N used by phytoplankton and bacteria throughout the bay. Uptake of NO$_3^-$, on the other hand, was highest in the upper bay but relatively low overall. The uptake of urea and DFAA by phytoplankton increased as DON became relatively more abundant toward the bay mouth, and significant correlation between the ratio of DON:DIN and DFAA uptake suggests that this relative availability may trigger the use of DON by phytoplankton. Results suggest that phytoplankton and bacteria expressed similar affinity for NH$_4^+$ and urea, whereas bacterial DFAA use was higher, and NO$_3^-$ use generally lower, than that of phytoplankton. Using FCM sorting, it was determined that GF/F filters overestimated phytoplankton uptake of NH$_4^+$, urea, and DFAA by 61%, 53%, and 135%, respectively, as a result of bacterial retention. Future application of this FCM technique will allow for a more in-depth analysis of the ecological interactions between phytoplankton and bacteria with respect to N utilization under conditions of limited DIN availability.
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REFERENCES


Table 1. Percent overestimation of absolute ammonium (NH$_4^+$), nitrate (NO$_3^-$), urea and dissolved free amino acid (DFAA) uptake by phytoplankton using GF/F filters. Data were calculated by subtracting the Phyto (FCM + >35 μm) absolute uptake rate from that of the GF/F fraction, and expressing this difference as a percentage of the Phyto absolute uptake rate (see Eq. 1 in text). Positive values represent overestimations of phytoplankton N uptake by GF/F filters, while negative values are underestimations.

<table>
<thead>
<tr>
<th>Bay Segment</th>
<th>Station</th>
<th>NH$_4^+$</th>
<th>NO$_3^-$</th>
<th>Urea</th>
<th>DFAA</th>
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<td>114</td>
<td>-1</td>
<td>73</td>
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<td>858</td>
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<tr>
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<td>707</td>
<td>219</td>
<td>27</td>
<td>63</td>
<td>86</td>
</tr>
<tr>
<td>Mean ± s.d.</td>
<td>61 ± 90%</td>
<td>-19 ± 39%</td>
<td>53 ± 45%</td>
<td>135 ± 34%</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Stations sampled during a north-south transect of Chesapeake Bay. Dotted lines delineate the upper, mid, and lower bay regions.
Figure 2. Concentrations (\(\mu\text{mol N L}^{-1}\)) of dissolved inorganic (filled symbols, solid lines) and organic (open symbols, dashed lines) nitrogen forms measured along the main axis of Chesapeake Bay. Station numbers are shown at the top of the graph, and vertical dotted lines delineate the upper, mid, and lower bay regions. Error bars represent \(\pm 1 \text{ SD}\) of the mean.
Figure 3. Particulate N concentrations (μmol N L⁻¹) measured along the main axis of Chesapeake Bay, with station numbers shown at the top of the graph. Phytoplankton (Phyto) PN was derived from GF/F PN, corrected for retention of bacterial biomass on GF/F filters. Error bars represent ± 1 SD of the mean.
Figure 4. Specific uptake rates (h⁻¹) of (A) NH₄⁺, (B) NO₃⁻, (C) urea, and (D) DFAA for the GF/F, FCM, and >35 μm fractions. Specific uptake rates for the FCM fraction were calculated from FCM-sorted phytoplankton. Note the axis break and much higher scale for NH₄⁺. Error bars represent ± 1 SD of the mean.
Figure 5. Absolute uptake rates ($\mu$mol N L$^{-1}$ h$^{-1}$) of (A) NH$_4^+$, (B) NO$_3^-$, (C) urea, and (D) DFAA for the GF/F and phytoplankton-only (Phyto) fractions. Absolute uptake rates for the latter represent the combined uptake of FCM-sorted and >35 $\mu$m phytoplankton and were calculated using the estimated Phyto PN shown in Figure 3. Asterisks denote significant differences between fractions (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$). Note the difference in y-axis scales. Error bars represent ± 1 SD of the mean.
Figure 6. Percent contribution of $\text{NH}_4^+$, $\text{NO}_3^-$, urea, and DFAA to total measured N uptake by the (A) GF/F, (B) FCM-sorted phytoplankton, and (C) $>35 \, \mu\text{m}$ fractions in Chesapeake Bay surface waters. Vertical dotted lines delineate the upper, mid, and lower bay regions.
Figure 7. Correlation between the ratio of DON to DIN and absolute DAA uptake rates (µmol N L⁻¹ h⁻¹) in the GF/F, FCM-sorted phytoplankton, and >35 µm fractions in mainstem Chesapeake Bay surface waters.
CHAPTER 3

INFLUENCE OF SUMMER STRATIFICATION ON PHYTOPLANKTON NITROGEN UPTAKE IN A MID-ATLANTIC BIGHT UPWELLING REGION

This chapter follows the format of Estuarine, Coastal, and Shelf Science
Abstract

Little is known about the relative importance of inorganic and organic nitrogen (N) sources in fueling phytoplankton versus bacterial production on the continental shelf. This issue was addressed during two diel experiments conducted in the Mid-Atlantic Bight at the Long-term Ecosystem Observatory LEO-15 off southern New Jersey. Uptake of $^{15}$N-labeled ammonium ($\text{NH}_4^+$), nitrate ($\text{NO}_3^-$), and nitrite ($\text{NO}_2^-$), and dual-labeled ($^{15}$N and $^{13}$C) urea and dissolved free amino acids was measured in water taken from the surface and bottom mixed layers roughly every four hours over two 24-hour periods in July 2002. Two distinct methods were used to quantify $^{15}$N uptake rates: (1) traditional filtration into various phytoplankton and bacterial size classes, and (2) flow cytometric (FCM) sorting of autotrophic cells. Dissolved organic N (DON) comprised >99% of the total dissolved N (TDN) pool in surface waters; the bottom-water TDN pool, however, was divided between $\text{NH}_4^+$, $\text{NO}_3^-$, and DON. Urea was the dominant N form used by all fractions at the surface. Although phytoplankton >3 $\mu$m were responsible for most of the urea uptake, bacterial use was also significant. This finding is supported by sequence analysis of the ureC genes present; members of the Cyanobacteria and $\alpha$-Proteobacteria were the primary urea-utilizers <3 $\mu$m. In contrast, N uptake in the bottom layer was dominated by $\text{NH}_4^+$. The bacterial fraction was responsible for 20–49% of the size-fractionated $\text{NH}_4^+$ and $\text{NO}_3^-$ uptake in surface samples and 36–93% at the bottom. These results suggest that bacterial competition for available DIN may force phytoplankton to rely more on DON sources, such as urea, to meet their cellular N demands.
1. Introduction

Continental shelf ecosystems are characterized by dynamic, often transient conditions that can cause dramatic shifts in the supply of nitrogen (N) and other nutrients to the plankton community. Nitrogen sources to coastal waters include terrestrial runoff, riverine delivery, groundwater discharge, atmospheric deposition, biotic water column processes, upwelling, and sediment remineralization (Capone, 2000). Of these, coastal upwelling represents a significant, albeit ephemeral, source of new N to the surface water during summer months. Since coastal waters are often N-limited, the introduction of new N ultimately controls primary productivity and consequently ecosystem trophic state (Ryther and Dunstan, 1971; Eppley and Peterson, 1979; Howarth, 1988). Thus, these intermittent upwelling events can largely determine overall ecosystem productivity.

The diversity of N sources to coastal waters is reflected in the complexity of the total dissolved N (TDN) pool, which includes both inorganic and organic forms. Dissolved inorganic N (DIN) consists of ammonium (NH$_4^+$), nitrate (NO$_3^-$), and nitrite (NO$_2^-$). Dissolved organic N (DON), which typically comprises the majority of the TDN pool (roughly 60–70% in coastal and oceanic surface waters), is a complex mixture of compounds, including urea, dissolved free amino acids (DFAA), dissolved combined amino acids (DCAA: oligopeptides, proteins), amino sugars, nucleic acids, and complex macromolecules such as humics (Antia et al., 1991; Bronk, 2002).

In the traditional view of the marine N cycle, phytoplankton use DIN while bacteria remineralize DON into the inorganic forms supporting primary production. Research over the past three decades, however, has shown that bacteria balance their
DON consumption with uptake of DIN (Wheeler and Kirchman, 1986; Kirchman, 2000; Allen et al., 2002), but also that phytoplankton use DON to meet cellular N demands (Bronk et al., 2007). In fact, DON uptake has been shown to satisfy a large proportion of the N requirement of autotrophs (Berman and Bronk, 2003; Bronk et al., 2007), including harmful algal species (e.g. Mulholland et al., 2004).

Studies of phytoplankton versus bacterial N uptake have been hampered by inadequate methodology for precisely separating these two groups (Bronk et al., 2007). Phytoplankton N uptake rates have typically been measured using glass fiber filters with a pore size small enough to retain the phytoplankton community (e.g. Whatman GF/F, 0.7 µm nominal pore size) because they can be precombusted to remove contaminant N and are amenable to analysis on a mass spectrometer. However, GF/F filters retain 40–75% of the bacterial community, on average (Lee and Fuhrman, 1987; Lee et al., 1995; Gasol and Morán, 1999), thus making it difficult to attribute N uptake rates measured on GF/F filters to phytoplankton alone. Alternatively, several studies have examined N uptake by the bacterial size fraction (e.g. <0.8 µm) and estimated the contribution of larger phytoplankton by subtraction from uptake rates derived from unfiltered water samples (Bury et al., 2001; Sanderson et al., 2008). However, size-fractionation approaches cannot exclusively separate autotrophic and heterotrophic cells. Analyses of N assimilation genes (e.g. NO₃⁻ reductase, urease) have improved our understanding of which microbial groups are playing a role in uptake of various N forms. Unfortunately, molecular assays cannot quantitatively determine N uptake rates for these microbes.

A promising approach for avoiding these methodological problems is flow cytometric (FCM) sorting, whereby phytoplankton cells are physically separated from
heterotrophic bacteria and protists, detritus, and other particulate matter based on the presence of chlorophyll or accessory pigments. Researchers have used FCM sorting to isolate cells for measurements of primary production (Li, 1994), bacterial activity (Servais et al., 1999), phytoplankton growth rates (Pel et al., 2004) and N assimilation (Lipschultz, 1995; Casey et al., 2007) on a cellular scale.

This study represents a component of the Geochemical Rate-RNA Integration Study (GRIST), which was a pilot experiment designed to examine the relationship between gene expression in complex bacterial and phytoplankton communities and relevant biogeochemical rate processes (Kerkhof et al., 2003; Corredor et al., 2004; Gibson et al., 2006). The goal of the study presented here was to examine N use by phytoplankton and heterotrophic bacteria at a site in an upwelling region of the Mid-Atlantic Bight continental shelf using multiple approaches, including $^{15}$N tracer techniques along with size fractionation and FCM sorting, as well as sequence analysis of the urease ($ureC$) genes present in surface waters.

2. Materials and methods

2.1 Study site and field sampling

The LEO-15 site is located in 15 m of water on the inner continental shelf, just offshore from the Rutgers University Marine Field Station (RUMFS) in Tuckerton, New Jersey (Glenn et al., 1996). Using RUMFS as a base, two diel experiments were conducted, hereafter referred to as Diel 1 (20–21 July 2002) and Diel 2 (22–23 July
2002). At roughly four-hour intervals, water was collected from the surface (1 m) and bottom (~14 m) of the water column using a pump and hose apparatus into 20 L acid-washed HDPE carboys, which were shaded with neutral-density screen and transported to RUMFS for nutrient analyses and $^{15}\text{N}$ uptake experiments within 45 minutes of collection. Samples for molecular analyses were filtered and flash frozen on station aboard the R/V Arabella. Due to rough seas during both diel periods, full 24 h sampling was not possible.

### 2.2 Nutrient analyses

At each time point, water from both depths was filtered through Whatman GF/F filters (precombusted at 450°C for 2 h), frozen, and later analyzed to determine dissolved nutrient concentrations. Filtered samples for the determination of NH$_4^+$ concentrations were refrigerated (4°C) after addition of the phenol-alcohol reagent, which binds available NH$_4^+$, and analyzed at RUMFS within 24 h of collection using the manual phenol-hypochlorite method (Koroleff, 1983). Nitrate and NO$_2^-$ concentrations were measured colorimetrically on an O.I. Analytical AlpKem Flow Solution IV AutoAnalyzer (Parsons et al., 1984), urea was measured using the manual monoxime method (Price and Harrison, 1987), and total DFAA concentrations were determined as the individual amino acids using high-performance liquid chromatography with o-phthaldialdehyde (Lindroth and Mopper, 1979). Concentrations of DON were calculated as the difference between TDN and DIN, with TDN measured using the persulfate oxidation technique of Valderrama, as described in Bronk et al. (2000).
2.3 Uptake experiments

At each sampling time point, water for uptake experiments was transferred from the 20 L carboys to separate 500 ml PETG bottles. The following five substrates were added to replicate water samples from each depth: $^{15}$N-labeled NH$_4^+$, NO$_3^-$, and NO$_2^-$, and dual-labeled ($^{15}$N, $^{13}$C) urea and DFAA (an algal extract consisting of 16 amino acids; Cambridge Isotope Laboratories, Andover, MA). Despite the availability of a suite of $^{15}$N-labeled organic substrates, most studies of DON uptake in marine ecosystems have used urea and amino acids as proxies for DON utilization because of their known importance to microbial N nutrition (McCarthy, 1972a; McCarthy, 1972b; Pomeroy, 1974) and their commercial availability.

When possible, tracer additions of less than 10% of ambient concentrations were estimated from published data, and the initial isotopic enrichment of the substrate pool was later calculated as in Bronk et al. (1998). After addition of labeled substrates, the samples were incubated for approximately one hour in flow-through coolers kept at representative in situ light and temperature conditions. Incubations were terminated either by filtration onto 25 mm GF/F or silver membrane filters, or by cell concentration for FCM sorting (see below).

To examine N preferences within the phytoplankton community, incubations were divided into four size fractions: >5μm (Diel 1 only) or >3 μm (Diel 2 only), GF/F, >0.8 μm (NH$_4^+$ and NO$_3^-$ only), and 0.2-0.8 μm (NH$_4^+$ and NO$_3^-$ only). The latter two fractions were targeted to examine the role of heterotrophic bacteria in DIN utilization within the plankton community. At both a day and night time point during each diel,
samples for FCM sorting were prepared by gently filtering 150–250 ml down over a 47 mm, 0.2 μm Supor membrane filter to a final concentrated volume of 5–10 ml, which was then preserved with paraformaldehyde (0.2% final concentration) and frozen in liquid N (Campbell, 2001). In tests of this concentration technique conducted in the relatively turbid York River (Virginia), samples concentrated down from 100–200 ml to 10 ml contained 95 ± 3% of whole-water (unconcentrated) chlorophyll a (Chl a), whereas the Supor filter retained 3 ± 1%. Increasing the initial volume to 300 ml resulted in a greater loss of phytoplankton to the Supor filter, with 89 ± 3% of Chl a in the concentrated sample and 12 ± 3% remaining on the filter (Bradley, unpublished results).

The GF/F and silver membrane filters (5 μm, 3 μm, 0.8 μm, and 0.2 μm) used to terminate the incubations were kept frozen at -20°C until analysis on a Europa GEO 20/20 isotope ratio mass spectrometer with an Automated Nitrogen and Carbon Analyzer for Solids and Liquids (ANCA-SL) to determine both particulate N (PN) concentrations and isotopic atom percent enrichments in the PN pool for each substrate and size fraction. Specific and absolute N uptake rates were calculated as described by Dugdale and Goering (1967). The NH₄⁺ pool was isolated using solid phase extraction (Dudek et al., 1986; Brzezinski, 1987), and NH₄⁺ uptake rates were corrected for isotope dilution due to NH₄⁺ regenerated during the course of the incubation, as described in Glibert et al. (1982). Rates of NO₃⁻, urea, and DFAA uptake were not corrected for isotope dilution.
2.4 FCM sorting of autotrophic cells

Samples for FCM sorting were stored frozen (-80°C) and thawed at room temperature prior to analysis. Phytoplankton cells were sorted based on their chlorophyll autofluorescence using a Beckman-Coulter Epics Altra flow cytometer at an average sort speed of approximately 1,000 cells s⁻¹. The sorted cells were then filtered onto 25mm 0.2 μm silver membrane filters, which were stored at -20°C prior to mass spectrometric analysis (see section 2.3). A small amount (1 to 2 μg N) of potassium nitrate carrier was added to each pelletized sample filter to produce total N masses sufficiently above the Europa's detection limit (~1 μg N) for reliable ¹⁵N atom percent enrichment values. A mathematical carrier correction was performed when calculating the isotopic enrichment of the sample particulate matter.

Purity of the sorted phytoplankton was assessed using bacterial enumeration via both FCM and acridine orange direct microscopic counts (Sherr et al., 2001). On average, sorted samples contained 5 ± 2% of whole-water (unsorted) bacterial abundance. The extent to which this FCM method negatively affects cellular integrity or retention of ¹⁵N label has been examined previously and is described in depth elsewhere (see Chapter 2). Cellular integrity of sorted phytoplankton cells was minimally compromised, if at all, by the method. Rivkin et al. (1986) also found that phytoplankton cellular integrity remains intact (i.e., no radioisotope was lost) during FCM sorting following primary production incubations.
2.5 Statistical analyses

Differences in mean uptake rates between Diels 1 and 2 or between surface and bottom within a diel period were evaluated for significance using Student’s t-tests. Pearson’s Correlation was used to determine whether there was a significant correlation between uptake rates and time of day, or between uptake of a substrate by two fractions (e.g. 0.2-0.8 μm and GF/F). Results of these statistical tests are reported as p values and $r^2$ correlation coefficients, where appropriate.

2.6 ureC gene analysis

The goal of the GRIST pilot study was to correlate concurrently measured biogeochemical flux rates with the expression of genes involved in such pathways. To date, little is known about the phylogenetic diversity of genes responsible for urea assimilation in coastal zones, especially through the use of cultivation-independent approaches. The bacterial and picoeukaryotic taxa capable of utilizing urea were determined using sequence analysis of the ureC genes present in surface waters. The ureC gene analysis work described herein was conducted by M.E. Frischer, J.E. Brofft, and M.G. Booth (Skidaway Institute of Oceanography; Bradley et al., in prep.).

Genomic DNA was purified from cells collected in the 0.2-0.8 μm and 0.8-3.0 μm size classes by filtration. Thirty liters of seawater was filtered through a 3.0 μm Versapor pleated capsule and then sequentially passed through 142 mm, 0.8 μm and 0.2 μm Supor filters, which were immediately frozen and later pulverized before extracting genomic
DNA using the UltraClean mega soil DNA kit (MoBio). PCR amplification of ureC genes took place in 25 μl reactions consisting of 12.5 μl Qiagen HotStar master mix, 0.5 μM of each primer, and 10 ng of genomic DNA. The forward primer (ureCnineF) was paired with either the ureCfiveRev or ureCsixRev reverse primer to form products of approximately 926 or 917 bp, respectively. The ureC gene of Silicibacter pomeroyi (Moran et al., 2004) was successfully amplified with either primer set under these conditions. Priming sites were chosen to maximize inclusiveness and amplify a large portion of the gene. Based on inspection of ureC gene and amino acid alignments, the primers designed here target nearly all available sequences of most Gram-negative bacteria and eukaryotic algae. Since the ureC sequences of eukaryotic algae available in GenBank at this time (Pseudoisochrysis paradoxa [AF432601], Tetraselmis sp. CCMP1613 [AF432600], Rhodomonas salina [AF432599], Phaeodactylum tricornutum [AF432598], and Chlamydomonas sp. CCMP 222 [AF432597]) are partial in length, it is unknown whether they are compatible with the forward primer. However, at least one of the reverse primers matches the ureC genes of each species. The PCR products generated using a 52°C annealing temperature and 35 cycles were agarose gel-extracted using a Freeze N’ Squeeze™ spin column (Bio-Rad Laboratories), then cloned using the TOPO TA cloning vector for sequencing kit (Invitrogen). For sequencing, plasmids were purified using the High Pure plasmid isolation kit (Roche) and sequenced using the M13F (5’ tgt aaa acg aec gcc agt) and M13R (5’ age gca taa caa ttt cac aca gga) primers by capillary electrophoresis using the CEQ™ DTCS Quick start sequencing kit and analyzed using a CEQ™ 8000 8-channel capillary sequencer (Beckmann Coulter, Inc.). Four total libraries were constructed; one library was generated from each DNA sample (0.2-0.8 μm
and 0.8-3.0 μm fractions of a sample taken at 1 m depth on 18 July 2002 at 20:00) using both primer sets (ureCnineF/ureCfiveRev and ureCnineF/ureCsixRev). Fifteen clones from each library were extracted, sequenced and phylogenetically analyzed as described elsewhere (Allen et al., 2002). These ureC sequences were deposited in GenBank and are represented by the accession numbers DQ286064 through DQ286116.

3. Results

3.1 Environmental conditions

The Mid-Atlantic Bight region around LEO-15 is often subjected to strong southerly winds that drive episodic upwelling typically lasting from days to weeks (Glenn et al., 1996). This upwelling entrains nutrient-rich bottom water from offshore into the surface layer, thus stimulating phytoplankton blooms and organic matter accumulation (Hicks and Miller, 1980; Clemente-Colón, 2001; Vlahos et al., 2002). Temperature and fluorescence profiles indicate an upwelling event around 10–12 July, with a possible smaller mixing event from 18–20 July. However, stratification strengthened at the start of Diel 1 on 20 July and was maintained through Diel 2, with a thermocline at 6–8 m depth. Surface water temperatures increased from 19°C to 22°C during Diel 1, then ranged between 22°C and 24°C during Diel 2 as stratification increased. Bottom water temperatures were from 15–17°C and 16–18°C for Diels 1 and 2, respectively. Salinity remained relatively constant during Diel 1, increasing slightly from 31.6 at the surface to 32.0 in the bottom water, and fluctuated very little through Diel 2. Chlorophyll
measurements, corroborated by fluorometry data from LEO-15 node A, indicated a small but distinct bloom that appeared to intensify during Diel 1, and peaked in Diel 2 (see Fig. 2 in Corredor et al., 2004).

3.2 Dissolved and particulate N concentrations

Concentrations of $\text{NH}_4^+$ and $\text{NO}_x^-$ ($\text{NO}_3^- + \text{NO}_2^-$) in the surface layer were at or below detection (0.05 and 0.03 $\mu$M N, respectively; Fig. 1) and DON comprised 99–100% of the TDN pool with mean concentrations of 7.4 and 8.7 $\mu$M N for Diel 1 and 2, respectively (data not shown). Specifically, the surface TDN pool consisted of 26% urea, 3% DFAA, and 70–71% unidentified DON (Fig. 2).

The TDN composition differed substantially in the bottom water. The mean TDN concentration was 12.6 $\mu$M N for both Diel 1 and 2, and was roughly divided into thirds between $\text{NH}_4^+$, $\text{NO}_x^-$, and DON (Fig. 2). Dissolved inorganic N comprised up to 67% of the ambient TDN, whereas urea and DFAA concentrations represented just 13–18% and 1% of the TDN pool, respectively. The entire DON pool averaged 38% of bottom-water TDN during Diel 1 and 44% during Diel 2.

Measured PN concentrations were highest in the GF/F and $>0.8$ $\mu$m size fractions (Fig. 3 and Table 1). Differences in PN between these two fractions are best explained not only by the small difference in filter pore size, but also by their structure. The matrix composition of GF/F filters tends to enhance bacterial retention, as opposed to the silver filter’s membrane structure. Consequently, bacterial retention on GF/F filters typically exceeds 50% in coastal and estuarine waters (see Table 1 in Chapter 2). Literature values
for bacterial retention on 0.8 μm silver filters, however, do not exist. The percent of bacterial abundance retained by 0.8 μm filters was measured using surface water from the York River, Virginia and found to average 35 ± 15% across a range of filtered volumes (Bradley, unpubl. data). Therefore, phytoplankton-only (Phyto) PN was estimated by subtracting half the 0.2-0.8 μm PN from the >0.8 μm PN concentrations, which is equivalent to assuming that 33% of bacterial biomass is retained on the 0.8 μm silver filters. This calculation removes the contribution of bacterial biomass in approximating phytoplankton PN. The Phyto PN concentrations generally equaled or slightly exceeded those of the >5 μm and >3 μm size classes, which indicates that smaller autotrophs were present at LEO-15 during this study. Rather than present both the GF/F and >0.8 μm data, the following sections are confined to discussing the more commonly used GF/F rates to facilitate literature comparisons. However, PN and uptake rate data from these two fractions are compared in Table 1.

3.3 Absolute N uptake – traditional GF/F

Reflecting nutrient availability, N uptake by cells retained on GF/F filters was dominated by urea at the surface, with absolute uptake rates that were 3–4 times those of NH₄⁺ and 10–50 fold greater than those of NO₃⁻, NO₂⁻, and DFAA (Fig. 4). Urea uptake comprised as much as 79% of total measured N uptake at the surface, followed by NH₄⁺ (15–40%), and NO₃⁻, NO₂⁻, and DFAA, each of which contributed an average of 5% or less to total uptake (Fig. 2, Table 2). Although absolute uptake rates for all substrates increased between diel experiments, this trend was only significant for NO₃⁻ and DFAA
There were no clear diel patterns observed in surface-water N uptake for any of the five substrates.

As with dissolved N concentrations, NH$_4^+$ dominated absolute N uptake by the GF/F fraction in the bottom water; together with NO$_3^-$, DIN accounted for up to 84% of the total GF/F uptake (Fig. 2, Table 2). However, uptake rates of all substrates were over ten times higher, on average, in the surface water. Due to incomplete data, NO$_2^-$ is not presented in Table 2. When included in total N uptake, NO$_2^-$ made up 3 ± 1% and 6 ± 2% of total GF/F uptake in the surface and bottom water, respectively. Regarding DON use in the bottom water, urea and DFAA combined to represent 33% of the total measured N uptake during Diel 1 and 22% during Diel 2. Mean uptake rates of these two DON forms were roughly similar across both diel experiments. In contrast to the surface, there were diel trends in the bottom water (Fig. 4). Uptake of NH$_4^+$ by the GF/F fraction decreased significantly from morning to night during each diel (Diel 1: $r^2 = 0.91, p < 0.05$; Diel 2: $r^2 = 0.92, p < 0.01$). Although NO$_x^-$ uptake rates did not correlate similarly with time of day, the contribution of NO$_3^-$ to total bottom-water N uptake increased significantly over each diel (Diel 1: $r^2 = 0.99, p < 0.01$; Diel 2: $r^2 = 0.81, p < 0.05$).

3.4 Absolute N uptake – >5 μm and >3 μm fractions

Absolute N uptake by the >5 μm (Diel 1) and >3 μm (Diel 2) size classes, as in the GF/F fraction, was dominated by urea at the surface, followed by NH$_4^+$. However, unlike the GF/F fraction, the larger phytoplankton favored NO$_3^-$ over DFAA in the surface water ($p < 0.0001$; Fig. 5, Table 2). Compared to the GF/F fraction, the >5 μm or
>3 μm fraction relied more on urea and less on DFAA to meet their N nutrition, in the surface as well as the bottom water. Mean absolute surface-water uptake rates in these larger fractions were up to seven times lower than GF/F rates.

In the bottom water, NH$_4^+$ was the dominant N source to the >5 and >3 μm fractions, comprising 31–68% of total measured N uptake. Whereas NO$_3^-$, urea, and DFAA contributed equally to bottom-water GF/F uptake, urea was preferred over NO$_3^-$ and DFAA by the larger phytoplankton (Table 2). Uptake of NO$_3^-$, which was measured during Diel 1 only and therefore excluded from Table 2, represented 3 ± 1% and 10 ± 1% of surface- and bottom-water uptake by the larger phytoplankton. On average, bottom-water uptake rates were five times lower in the >5 and >3 μm versus the GF/F fractions, partly due to their difference in PN concentrations.

3.5 Specific N uptake – FCM-sorted vs. size-fractionated

To investigate the individual roles of phytoplankton and bacteria in N dynamics at LEO-15, we compared uptake rate profiles for various plankton assemblages and the respective change in the contribution of each $^{15}$N substrate to total measured N uptake. In this section, we report N-specific uptake rates ($V$, h$^{-1}$) rather than absolute rates ($\rho$, μM N h$^{-1}$), to better compare the physiological N metabolism of the plankton community.

**Surface.** Overall, the hierarchy of urea $> NH_4^+ > NO_3^- \approx$ DFAA for contribution to total surface-water N uptake was fairly consistent across all fractions for both diel experiments. With few exceptions, specific uptake rates for all four substrates in the surface water were highest in the GF/F fraction, and FCM rates matched or exceeded
those of the >5 and >3 μm fractions in most samples (Fig. 6). These trends indicate N uptake by bacteria and smaller autotrophs in the GF/F fraction. As with the >5 and >3 μm size classes, FCM-sorted phytoplankton relied more on urea uptake than the GF/F fraction did, but only during Diel 1 (Table 3). Specific urea uptake rates from both diel experiments, in both the surface and bottom water, were strongly correlated between the GF/F and >5 or >3 μm fractions ($r^2 = 0.84, p < 0.0001$).

Specific NH$_4^+$ uptake rates in the GF/F and 0.2-0.8 μm (bacterial) fractions were strongly correlated in the surface water during both diel experiments ($r^2 = 0.75, p < 0.01$), whereas they were not for the GF/F and >5 or >3 μm fractions ($r^2 = 0.31, p = 0.092$). Also, specific NH$_4^+$ uptake by the 0.2-0.8 μm fraction exceeded FCM rates during Diel 1 (0.2-0.8 μm data are not shown in Fig. 6 because only NH$_4^+$ and NO$_3^-$ uptake were measured in this fraction). The contribution of NH$_4^+$ to total N uptake changed little between day and night of Diel 2 in the FCM fraction, but roughly doubled in the GF/F and >3 μm size fractions (Table 3). Similarly, NH$_4^+$ uptake by the bacterial fraction more than doubled between day and night of Diel 2.

Specific NO$_3^-$ uptake in the surface layer was considerably lower than that of urea and NH$_4^+$ and varied relatively little over both diel experiments (Fig. 6). Nitrate uptake rates tended to be highest in the GF/F fraction, but did not significantly exceed those of the FCM and >5 or >3 μm fractions and contributed about equally to total N uptake (Tables 2 and 3). Specific NO$_3^-$ uptake rates were lowest in the 0.2-0.8 μm fraction.

Amino acids were generally the least important substrate in the surface water (Fig. 6, Tables 2 and 3). Specific uptake of DFAA was quite variable in the GF/F fraction, but relatively constant in the FCM, >5 and >3 μm fractions. Mean DFAA uptake
rates were also significantly higher in the GF/F fraction than in the >5 μm (p < 0.05) or >3 μm fractions (p < 0.01).

**Bottom.** Although NH$_4^+$ dominated specific N uptake by all fractions in the bottom water (Fig. 6), the importance of $^{15}$N substrates to total uptake was more variable between fractions than at the surface. Also in contrast with the surface, GF/F uptake rates often did not exceed those of other fractions, with some noteworthy exceptions.

In contrast to the surface, specific bottom-water NH$_4^+$ uptake rates correlated well in the GF/F and >5 or >3 μm fractions ($r^2 = 0.51$, $p < 0.05$), but not in the GF/F and 0.2-0.8 μm fractions. On average, NH$_4^+$ uptake by the 0.2-0.8 μm fraction comprised 61% and 52% of total NH$_4^+$ uptake during Diel 1 and 2, respectively. Uptake of NH$_4^+$ decreased from day to night during Diel 2 in the GF/F and FCM fractions, but not the >3 μm size class (Fig. 6, Table 3).

The decrease in uptake between surface and bottom water was smallest for NO$_3^-$, most likely because of the increased role of bacteria at the bottom. Specific NO$_3^-$ uptake by the 0.2-0.8 μm fraction dominated in the bottom water during Diel 1, comprising 55–93% (mean of 73%) of total NO$_3^-$ uptake, but decreased significantly between diel studies ($p < 0.05$). Although bottom-water NO$_3^-$ uptake rates were low in the GF/F, FCM, and >5 or >3 μm fractions relative to other substrates (Fig. 6), these fractions relied more on NO$_3^-$ for N nutrition in the bottom water than at the surface (Tables 2 and 3).

Specific uptake of urea by all fractions was 1–2 orders of magnitude lower in the bottom water than at the surface (Fig. 6). Due to sampling problems, data are not available for bottom-water urea uptake by the FCM fraction during Diel 1. Specific urea uptake rates were highest in the >3 μm fraction during Diel 2, and despite relatively low
urea uptake rates in the bottom water, this DON substrate contributed substantially to N uptake in all fractions (Tables 2 and 3).

Specific DFAA uptake rates in the bottom water were strongly correlated between the GF/F and >5 or >3 μm fractions ($r^2 = 0.79, p < 0.01$) over both diel experiments (data not shown), yet uptake rates were 2–3 times higher in the GF/F fraction versus the FCM and >5 or >3 μm fractions. Accordingly, the contribution of DFAA to total bottom-water uptake during Diel 1 was highest for the GF/F fraction and lowest for FCM-sorted phytoplankton, although DFAA uptake by the latter comprised a greater percentage of total N uptake during Diel 2.

3.6 *ureC* diversity

To determine the diversity of microbes capable of utilizing urea in the <3 μm size class, we designed and applied PCR primers targeting the gene (*ureC*) that encodes for the large catalytic $\alpha$ subunit of the urease enzyme (Mobley et al., 1995). A total of 53 sequences derived from four clone libraries were recovered from a surface sample; each recovered sequence was distinct from those present in GenBank (Fig. 7). The GenBank-derived *ureC* sequences that were potentially amplifiable with our primer sets fell into 10 clades (arbitrarily referred to as 1–10 in Fig. 7), six of which contained LEO-15 sequences and four of which contained sequences recovered from the Sargasso Sea metagenomic library (Venter et al., 2004). Similar to the Sargasso Sea *ureC* genes, the majority of the LEO-15 sequences were affiliated with those of the *Cyanobacteria* (47%) and the alpha *Proteobacteria* (30%). The *Cyanobacteria* clade consisted of *ureC*
sequences from eleven cultivated species. Based on a criterion of 98% amino acid identity, two types of Cyanobacteria-like sequences were recovered among the LEO-15 clones; one group consisted of 23 highly similar sequences that were approximately 95% identical at the amino acid level to two highly similar LEO-15 sequences. Both groups were most similar (95–96% amino acid identity) to the ureC genes of Synechococcus sp. WH7805 and WH8102 and to two Sargasso Sea clones (EAI52258 and EAJ32162). A comparatively higher diversity of alpha Proteobacteria-like ureC sequences was recovered. Sixteen LEO-15 clones associated with this group share an amino acid identity ranging between 80–100% and are composed of seven distinct sequence types based on a 98% amino acid identity cut-off. These sequences were most closely related to those of the bacteria Silicibacter pomeroyi and Silicibacter sp. TM1040 (up to 93.5% amino acid identity) and several Sargasso Sea clones (up to 96.4% amino acid identity). The organisms corresponding to the ureC sequences in this clade are all members of the alpha Proteobacteria subphylum. The remaining LEO-15 sequences (23%) were affiliated with four distinct clades and did not share high sequence identity with any ureC sequence present in GenBank (77–84% amino acid identity). As a result, the phylogenetic group of the corresponding organisms cannot be inferred.

The largest proportion of LEO-15 sequences recovered from the 0.2-0.8 μm size class were members of the alpha Proteobacteria (46%) cluster, while the sequences in the 0.8-3.0 μm fraction consisted primarily of Cyanobacteria (70%), regardless of the primer pair used. There was extensive redundancy in the types of ureC sequences recovered from each size fraction. The vast majority (92.5%) of 0.8-3.0 μm sequences shared at least 98% amino acid identity with at least one sequence isolated from the 0.2-0.8 μm
libraries. Unfortunately, no molecular data from cells retained by the >3 μm filter exists to complement the uptake data for the >3 μm fraction.

4. Discussion

4.1 Surface-water uptake of organic and inorganic N

Urea has been recognized as a source of N nutrition to marine phytoplankton for decades (Hattori, 1957; McCarthy, 1972b), but was largely neglected as such until more recently. In situ measurements of urea concentrations and uptake by the plankton community have increased, but data remain relatively sparse, especially as a percentage of total N uptake. Urea concentrations and uptake rates measured in the surface water at LEO-15 are quite high relative to published values from other marine ecosystems, including some anthropogenically-impacted estuaries (Bronk, 2002; Glibert et al., 2005). For example, urea concentrations measured along Chesapeake Bay between 1972 and 1998 rarely exceeded 1.5 μM N (Lomas et al., 2002), compared to concentrations ranging from 1.4 to 2.7 μM N at LEO-15. The source of such elevated urea concentrations is unclear, but potential sources include fish and zooplankton excretion, phytoplankton exudation, bacterial regeneration, terrestrial runoff, and atmospheric deposition (Berman and Bronk, 2003). These sources must have been sufficiently high to maintain a supply of urea capable of supporting elevated uptake by the plankton community.

Urea uptake rates averaged 1.34 ± 0.49 μM N h⁻¹ in the surface water, which exceeds those of most other marine environments (Bronk, 2002), including those cited by
Lomas et al. (2002) for the 26-year period in Chesapeake Bay (<1 μM N h⁻¹). Some studies have reported absolute urea uptake rates of up to 10 μM N h⁻¹ (Kristiansen, 1983; Mulholland et al., 2004; Twomey et al., 2005); however, such elevated rates often result from either high PN (e.g. bloom conditions), rather than high specific uptake rates, or from ¹⁵N tracer additions far in excess of 10% of ambient concentrations. Furthermore, urea uptake at LEO-15, while relatively high, was not corrected for isotope dilution and therefore was likely underestimated (Hansell and Goering, 1989; Bronk et al., 1998). Regardless, these results suggest that the phytoplankton community at LEO-15 is well-adapted to use urea and emphasize the importance of including phytoplankton urea utilization in N budgets and models of nutrient dynamics in marine ecosystems. Other organic N substrates that were not studied here but were likely present in the DIN-depleted surface layer may have also played an important role in autotrophic N nutrition. Uptake of urea and other DON forms by phytoplankton may be the result of an inability to compete effectively with bacteria for limited DIN.

Concentrations and uptake rates of NH₄⁺ and NO₃⁻ at LEO-15 are consistent with published data from various marine ecosystems, if not specifically the inner continental shelf. For example, the absence of a standing stock of DIN in the surface water resembles an oligotrophic oceanic gyre, yet the surface uptake rates are comparable to results from some coastal and estuarine systems (Bronk et al., 1998; Bronk and Ward, 1999; Berg et al., 2001; Veuger et al., 2004). Concentrations and absolute uptake of DFAA at LEO-15 were generally at the upper end of values reported elsewhere (Bronk 2002).

Due to the virtual absence of ambient NH₄⁺, NO₃⁻, and NO₂⁻ in the surface water, tracer additions of 0.1–0.2 μM, albeit small, still represented 65–100% of ambient
concentrations. This may have enhanced surface-water uptake of these DIN substrates, and thus underestimated the relative importance of urea. However, surface-water uptake of NO$_3^-$ and NO$_2^-$ was low relative to urea, and any stimulatory effect of tracer additions would not have changed the major findings reported here. The excess $^{15}$NH$_4^+$ addition was minimized by relatively high NH$_4^+$ regeneration rates, which were as high as 1.71 μM h$^{-1}$ and averaged 0.85 ± 0.51 μM h$^{-1}$ in the surface layer (data not shown).

DFAA tracer additions ranged from 15–213% of ambient concentrations (mean of 99%), and although such high enrichment of the available pool may have artificially enhanced DFAA uptake rates, this may have been offset to some degree by DFAA regeneration.

4.2 Phytoplankton versus bacterial N uptake

Phytoplankton and bacterial N uptake have been studied in various marine ecosystems (Bronk et al., 2007), but distinguishing their affinity for and use of DIN and DON remains a significant challenge. Using traditional methods along with more modern approaches, namely FCM cell sorting and molecular assays, we contrasted the N uptake and affinity patterns of various plankton constituents in the surface and bottom mixed layers at LEO-15.

Larger phytoplankton were responsible for the majority of urea uptake at LEO-15, as evidenced by the strong correlation between GF/F and >5 or >3 μm specific rates for both depths and diel studies, as well as the increased importance of urea to total uptake by the larger phytoplankton fractions (Table 2). However, specific urea uptake rates were often greatest in the GF/F fraction, particularly during Diel 1, which suggests that either
picophytoplankton or bacteria were also using urea. This conclusion is supported by the ureC assay results (see below). In general, urea is believed to play a greater role as a N source for phytoplankton than for bacteria (Berman and Bronk, 2003), and there is evidence that picophytoplankton, especially cyanobacteria, are important in urea uptake (Berg et al., 2003; Glibert et al., 2004). However, FCM uptake rates, which include all phytoplankton, were also less than GF/F rates, indicating that bacteria were responsible for the difference between GF/F and larger phytoplankton uptake. Additional support for this conclusion is provided by the $^{13}$C in the dual-labeled urea tracer. Although $^{13}$C-urea uptake is not presented here due to a lack of noteworthy results, it is worth mentioning that GF/F filters from both depths of Diel 1 were enriched in $^{13}$C from urea, whereas the FCM and >5 or >3 µm fractions were not (data not shown). Not only does this suggest that bacterial urea use was significant during Diel 1, it also highlights a functional difference between how bacteria and phytoplankton metabolize urea.

The ureC sequences recovered from the surface water at LEO-15 were diverse and represent microbes whose ureC genes have not been deposited in GenBank to date. The high proportion of 0.8-3.0 µm-derived sequences having >98% amino acid identity to those of the bacterial fraction implies that the majority of ureC genes retrieved in this study were bacterial. Based on our phylogenetic analysis, the Cyanobacteria and members of the alpha Proteobacteria appear to represent two major groups capable of urea assimilation in the surface waters of the LEO-15 site. Furthermore, many marine cyanobacteria, including Synechococcus spp., are known to possess ureC genes and can utilize urea as a sole N source (Collier et al., 1999; Moore et al., 2002). The fact that both groups represent the majority of ureC sequences in the LEO-15 libraries and the Sargasso
Sea metagenomic database suggests that they may be significant constituents of the urea-assimilating community in marine systems. These sample sets represent very distinct systems: relatively N-rich, turbid, shallow coastal waters versus N-poor, clear open ocean, and the ureC genes were collected by very different means (primer-based amplification of genomic DNA versus direct cloning). While these data only indicate organisms capable of urea uptake at LEO-15, and not necessarily those actually using it, they support our finding of bacterial influence on urea uptake by the GF/F fraction.

Although the use of urea by heterotrophic bacteria is generally considered insignificant relative to that of DFAA and NH$_4^+$ (Hoch and Kirchman, 1995; Kirchman, 2000), urea has been shown to contribute substantially to bacterial N demand in some systems (Jørgensen et al., 1999; Middelburg and Nieuwenhuize, 2000; Jørgensen, 2006). Furthermore, uptake of urea by bacteria can be stimulated by addition of labile organic carbon (Tamminen and Irmisch, 1996; Gobler and Sañudo-Wilhelmy, 2001). Although DOC concentrations were not measured prior to the start of Diel 1, movement of relatively DOC-rich water into the surface layer could possibly explain the increased role of bacteria in urea removal. The similarity in urea uptake by all fractions during Diel 2 and the lack of $^{13}$C uptake by the GF/F fraction suggest that bacterial urea use had diminished by that time, perhaps due to a decrease in excess labile organic C.

In general, phytoplankton tend to prefer NH$_4^+$ over other N sources because this reduced substrate requires the least amount of energy to assimilate. However, under DIN-limited conditions, N availability, rather than N preference, can regulate uptake (Tamminen and Irmisch, 1996). Furthermore, the high surface area to volume ratio of bacteria gives them an advantage over phytoplankton when competing for limited NH$_4^+$,
such as in the surface water at LEO-15, where \( \text{NH}_4^+ \) regeneration and uptake were tightly coupled. Therefore, it is not surprising that bacterial uptake comprised as much as 49% of total \( \text{NH}_4^+ \) uptake in the surface water and up to 72% at the bottom, which are probably conservative estimates, since some bacterial \( \text{NH}_4^+ \) use was likely captured on the 0.8 \( \mu \text{m} \) filter. This enhanced bacterial affinity for \( \text{NH}_4^+ \), and to a lesser degree \( \text{NO}_3^- \) (see below), could conceivably exert a selective pressure on phytoplankton that can either compete effectively for limited DIN or use alternative N sources, such as urea and other labile DON. Regardless, the phytoplankton community at LEO-15 was clearly capable of exploiting the elevated urea concentrations.

Differences between DFAA uptake rates in the GF/F versus the FCM, >5 \( \mu \text{m} \), and >3 \( \mu \text{m} \) fractions suggest that bacterial DFAA use was high in both the surface and bottom water (Fig. 6). However, the contribution of DFAA to total N uptake was essentially the same for these fractions in the surface water, with even more ambiguous results in the bottom water (Tables 2 and 3). Therefore, it is unclear to what extent bacteria relied on DFAA for N nutrition at LEO-15. Nonetheless, the finding that DFAA represented as much as 17% of phytoplankton N uptake is significant because amino acids have traditionally been neglected as a source of N to autotrophs, despite evidence that they directly use DFAA to varying degrees, and even indirectly via extracellular enzymatic processes (Palenik and Morel, 1990; Mulholland et al., 2002; Mulholland et al., 2003; Stoecker and Gustafson, 2003).

In addition to the use of urea, \( \text{NH}_4^+ \), and presumably DFAA (to some extent), \( \text{NO}_3^- \) was also a significant N source to the bottom-water bacterial community. This is evident in the dominance of bacterial \( \text{NO}_3^- \) uptake rates, which comprised an average of
73% of total NO\textsubscript{3}\textsuperscript{-} uptake in the bottom water during Diel 1, and an increase in the contribution of NO\textsubscript{3}\textsuperscript{-} to total uptake in the GF/F versus >3 μm fraction during Diel 2. Despite the dogma that marine bacteria are not significant consumers of NO\textsubscript{3}\textsuperscript{-}, various researchers have indeed shown, as is the case here, that NO\textsubscript{3} can support growth of heterotrophic bacteria in a number of marine ecosystems (Kirchman et al., 1991; Kroer et al., 1994; Kirchman and Wheeler, 1998; Allen et al., 2002; Allen et al., 2005).

4.3 Ecosystem dynamics and N sources at LEO-15

The Mid-Atlantic Bight upwelling region surrounding LEO-15 is a dynamic environment featuring transient upwelling events and mesoscale physical processes that enhance chemical and biological variability. Identifying the predominant nutrient sources to phytoplankton and bacteria as well as the factors governing interaction between these groups remains a significant challenge. Phytoplankton production at LEO-15 during these two summer diel experiments was dominated by regenerated N forms (urea, NH\textsubscript{4}\textsuperscript{+}) in the surface layer, and both new (NO\textsubscript{X} \textsuperscript{-}) and regenerated (NH\textsubscript{4} \textsuperscript{+}) N sources in the bottom water. Results suggest that a brief upwelling event prior to the start of Diel 1, followed by increased stratification, may have triggered a small phytoplankton bloom and resulted in substantial surface to bottom differences in N dynamics. Removal of DIN and subsequent release of DON by phytoplankton into the surface layer during such a bloom could account for the observed N uptake. Accordingly, DON concentrations were significantly higher at the surface than at the bottom (8.0 ± 1.8 μM vs. 5.1 ± 0.6 μM; \(p < 0.001\)), whereas the reverse was true for TDN (surface: 8.0 ± 1.8 μM, bottom: 12.6 ± 1.1 μM;
Relatively high NH$_4^+$ concentrations and significant uptake of NH$_4^+$ and NO$_3^-$ by the 0.2-0.8 μm fraction in the bottom water suggest that bacterial activity was high. This could have been due to enhanced delivery of particulate matter from the surface layer, but bottom-water samples were collected from 1 m above the sediment surface, and interactions between benthic and pelagic environments were likely a major influence on bottom-water dynamics.

Although upwelling-stimulated phytoplankton production may have taken place during this study period, it is also possible that physical circulation moved water masses of varying biological and chemical properties around LEO-15 during the two diel experiments. Other researchers have attributed changes in phytoplankton cell concentrations (Sosik et al., 2003) and pigments (Corredor et al., 2004) at LEO-15 to physical processes. However, based on groups evident in FCM analyses, there is no evidence to suggest that large shifts in the plankton community composition (i.e. species present) took place during this study, although a detailed assessment was not conducted.

5. Conclusions

These results show the complexity that distinguishes coastal ecosystems with respect to N availability and uptake by phytoplankton and bacteria, and also demonstrate the utility of combining traditional (size fractionation) and more modern (FCM and ureC assays) methods in such investigations. Urea clearly supported the bulk of phytoplankton N nutrition in the surface water, followed (distantly) by NH$_4^+$, with NO$_3^-$ and DFAA playing minor roles in phytoplankton N uptake. In contrast, NH$_4^+$ supported most of the
phytoplankton N demand in the bottom water, followed by urea. These results were likely driven primarily by N availability, and secondly by N affinity and ability to compete with heterotrophic bacteria.

Contrary to most studies of bacterial N use, the bacterial community at LEO-15 showed evidence of significant urea utilization. Although we were unable to quantify their relative importance, all four N substrates examined contributed to bacterial N demand during this study. The factors regulating uptake of various N sources, both inorganic and organic, by phytoplankton and bacteria in coastal ecosystems, as well as the interaction between these groups under N-limited conditions, are undoubtedly complex.

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Table 1. Particulate N (PN) concentrations (μM N) and specific NH₄⁺ and NO₃⁻ uptake rates (V: h⁻¹) for the GF/F (nominal pore size of 0.7 μm) and >0.8 μm size fractions. Also shown is the ratio between the GF/F and >0.8 μm data. Diel 1 and 2 are abbreviated D1 and D2, respectively, while “S” and “B” refer to surface and bottom water, respectively.

The five sampling times are abbreviated T1 through T5.

<table>
<thead>
<tr>
<th>PN (μM N)</th>
<th>V: NH₄⁺</th>
<th>V: NO₃⁻</th>
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<tbody>
<tr>
<td></td>
<td>GF/F 0.8 μm Ratio</td>
<td>GF/F 0.8 μm Ratio</td>
</tr>
<tr>
<td>D1 S T1</td>
<td>9.65</td>
<td>7.61</td>
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<tr>
<td>D1 S T2</td>
<td>10.87</td>
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<tr>
<td>D1 S T4</td>
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<td>D1 S T5</td>
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<tr>
<td>D2 S T1</td>
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<td>D2 S T4</td>
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<td>D2 B T5</td>
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Table 2. Percent contribution (mean ± standard deviation) of each substrate to total measured N uptake in the GF/F and >5 or >3 μm size fractions, averaged across all time points of both diel studies. Asterisks indicate significant differences between the two size fractions for a given substrate. Due to incomplete data, NO$_2^-$ was excluded from these calculations. The sum of the means may differ from 100% as a result of rounding.

<table>
<thead>
<tr>
<th></th>
<th>NH$_4^+$</th>
<th>NO$_3^-$</th>
<th>Urea</th>
<th>DFAA</th>
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<tr>
<td></td>
<td>GF/F 5/3 μm</td>
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<td>GF/F 5/3 μm</td>
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<tr>
<td>Surface</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Diel 1</td>
<td>20 ± 5% 13 ± 4% *</td>
<td>4 ± 1% 5 ± 1%</td>
<td>74 ± 7% 80 ± 3%</td>
<td>4 ± 2% 3 ± 1%</td>
</tr>
<tr>
<td>Diel 2</td>
<td>23 ± 9% 19 ± 7%</td>
<td>5 ± 1% 6 ± 1%</td>
<td>66 ± 9% 72 ± 6%</td>
<td>5 ± 1% 3 ± 1% ***</td>
</tr>
<tr>
<td>All Surface</td>
<td>22 ± 7% 17 ± 6%</td>
<td>4 ± 1% 5 ± 1%</td>
<td>69 ± 8% 75 ± 6% *</td>
<td>5 ± 2% 3 ± 1% ****</td>
</tr>
<tr>
<td>Bottom</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diel 1</td>
<td>51 ± 5% 54 ± 16%</td>
<td>15 ± 5% 14 ± 11%</td>
<td>17 ± 5% 21 ± 3%</td>
<td>16 ± 6% 11 ± 4%</td>
</tr>
<tr>
<td>Diel 2</td>
<td>61 ± 6% 57 ± 6%</td>
<td>16 ± 6% 10 ± 7%</td>
<td>11 ± 6% 26 ± 5% ***</td>
<td>12 ± 4% 7 ± 2% **</td>
</tr>
<tr>
<td>All Bottom</td>
<td>57 ± 7% 56 ± 11%</td>
<td>16 ± 5% 12 ± 9%</td>
<td>14 ± 6% 24 ± 5% ***</td>
<td>14 ± 5% 9 ± 4% **</td>
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* p < 0.1; ** p < 0.05; *** p < 0.01
Table 3. Percent contribution of each substrate to total measured N uptake for various phytoplankton assemblages at 12:50 (Day) and 02:10 (Night) of Diel 1 and 11:55 (Day) and 22:55 (Night) of Diel 2. Due to incomplete data, NO$_2^-$ has been excluded from these calculations. Night data from Diel 1 bottom not available (n.a.) due to a sampling error.

Due to rounding, the sum of the means may differ from 100%.

<table>
<thead>
<tr>
<th></th>
<th>NH$_4^+$</th>
<th></th>
<th>NO$_3^-$</th>
<th></th>
<th>Urea</th>
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<th>DFAA</th>
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<td>Diel 1 Night</td>
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<td>Diel 2 Day</td>
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<td>5% 6% 7%</td>
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<td>Diel 2 Night</td>
<td>40% 32% 31%</td>
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<td>Diel 1 Day</td>
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<td>Diel 2 Day</td>
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Figure 1. Dissolved nutrient concentrations measured in surface and bottom waters at LEO-15 during two diel experiments in July 2002. Note the two-fold increase in scale between surface and bottom. Error bars denote ± 1 SD of the mean. Shaded bars indicate dark periods. Surface concentrations of \( \text{NH}_4^+ \), \( \text{NO}_3^- \), and \( \text{NO}_2^- \) were typically below detection (0.05, 0.03, 0.03 \( \mu \text{M} \), respectively) and thus are not distinguishable from zero. Concentrations of “other DON” (DON other than urea and DFAA) are not shown, but ranged from 4.0–8.6 \( \mu \text{M} \) N at the surface and from 2.3–4.0 \( \mu \text{M} \) N in the bottom water.
Figure 2. Percent contribution of substrates to total dissolved N (TDN) concentrations and total measured GF/F uptake in surface and bottom waters at LEO-15. Data represent the means across both diel experiments.
Figure 3. Particulate nitrogen (PN) concentrations measured in surface and bottom waters at LEO-15 during two diel experiments in July 2002. Note the four-fold decrease in scale between surface and bottom. Error bars denote ± 1 SD of the mean. Shaded bars indicate dark periods. Data from Diel 1 Bottom, time point five, does not appear here or in any other figures due to a sampling error. See the text for an explanation of Phyto PN.
Figure 4. Absolute nitrogen uptake rates (\( \rho; \mu M \text{ N h}^{-1} \)) measured using GF/F filters from two diel experiments at LEO-15 in July 2002. Note the difference in scale between surface and bottom uptake rates. Error bars denote \( \pm 1 \) SD of the mean, as determined using propagation of error. Shaded bars indicate dark periods. The \( \text{NH}_4^+ \) uptake rate for Diel 1 Surface, first time point could not be corrected for isotope dilution and therefore is not shown here.
Figure 5. As in Figure 4, but for the >5 μm (Diell) and >3 μm (Diel 2) fractions. Note that the scale of the y-axis is half that of Figure 4.
Figure 6. Specific uptake rates (V: h⁻¹) in the GF/F, FCM-sorted phytoplankton, and >5 or >3 μm fractions at 12:50 (Day) and 02:10 (Night) during Diel 1 and 11:55 (Day) and 22:55 (Night) of Diel 2. Night uptake rates are denoted by hatched bars. Note the ten-fold difference in scale between surface and bottom rates. Nitrite uptake rates are excluded due to incomplete data, and n.a. denotes data not available due to sampling error.
Figure 7. Dendrogram (~338 amino acids) displaying inferred phylogenetic relationships between LEO-15 clones and related ureC sequences recovered from GenBank. Sequences from LEO-15 are designated LEO and are surrounded by a box. Sequences recovered from libraries generated with primer pairs ureCnineF/ureCfiveRev or ureCnineF/ureCsixRev are designated as 95 (and an open circle) or 96 (and a filled circle), respectively. Sequences recovered from the 0.2-0.8 μm or 0.8-3.0 μm fraction end in .2 (open box) or .8 (shaded box), respectively. Identical sequences are listed adjacent to one another. Significant bootstrap values (>50%) are listed at the nodes of the tree. The ureC sequence from the fungal species Schizosaccharomyces pombe was used as the outgroup. GenBank accession numbers for the LEO clones are DQ286064–DQ286116X; accession numbers for the remaining ureC sequences are shown in parentheses.
CHAPTER 4

NITROGEN USE BY PHYTOPLANKTON AND BACTERIA DURING AN INDUCED *PHAEOCYSTIS POUCHETII* BLOOM, MEASURED USING SIZE FRACTIONATION AND FLOW CYTOMETRIC SORTING APPROACHES

*This chapter follows the format of Aquatic Microbial Ecology*
The uptake of inorganic and organic nitrogen (N) by phytoplankton and bacteria was measured during a mesocosm study conducted in Raunefjord, Norway in April 2005. Two mesocosms were batch fertilized with nitrate and phosphate at a ratio of 16:1 and maintained separately in the light and in the dark, while two unamended light and dark mesocosms served as controls. Dissolved nutrients, phytoplankton and bacterial biomass, and phytoplankton community composition were monitored throughout the four-week experiment. Uptake of $^{15}$N-labeled ammonium and nitrate, and dual-labeled ($^{15}$N and $^{13}$C) urea and dissolved free amino acids (DFAA) was measured for phytoplankton and bacteria using two methods: size fractionation into $>0.8 \ \mu m$ and $0.2-0.8 \ \mu m$ size classes and flow cytometric sorting based on chlorophyll autofluorescence. Prior to fertilization, dissolved inorganic N concentrations were low and comprised about 5% of total dissolved N. Added nitrate was removed from the amended mesocosm in the light within ten days, stimulating a large bloom of colonial Phaeocystis pouchetii. Ammonium contributed over half of total measured N uptake by phytoplankton and bacteria in both lighted mesocosms, while nitrate and urea each supplied roughly 10 – 25%. Overall, DFAA were a negligible N source to phytoplankton and contributed 11% to total bacterial uptake. Bacterial uptake represented a significant portion of total uptake for all N forms, but contributed most to urea and DFAA uptake. Comparison of the two methods for measuring phytoplankton versus bacterial uptake demonstrates how using 0.8 \ \mu m filters can lead to significant overestimation of phytoplankton N uptake.
INTRODUCTION

Phytoplankton biomass accumulation in marine ecosystems at high northern latitudes is initially limited in spring by insufficient light. Spring blooms of phytoplankton typically develop once the mixed layer depth is shallow enough for photosynthetic gains to exceed respiratory losses (Sverdrup, 1953). Ultimately, however, the magnitude and duration of the spring bloom are limited by the availability of nutrients, particularly nitrate ($\text{NO}_3^-$), phosphate ($\text{PO}_4^{3-}$), and silicate (Si). In NE North Atlantic waters, chain-forming diatoms (e.g. *Skeletonema costatum* and *Chaetoceros* spp.) dominate early during the spring bloom, and are generally followed by *Phaeocystis* spp. (Erga and Heimdal, 1984; Lancelot and Mathot, 1987; Erga, 1989). This typical diatom-*Phaeocystis* succession of dominance is likely due to the competitive ability of diatoms to exploit high $\text{NO}_3^-$ availability, but only as long as Si concentrations are $>2$ μmol L$^{-1}$ (Reid et al., 1990; Egge and Aksnes, 1992). However, others have observed concurrent diatom and *Phaeocystis* blooms in the North Sea and argued that the latter only develops under nutrient-replete conditions once a daily irradiance threshold has been met (Bakker et al., 1990; Peperzak et al., 1998).

Coastal eutrophication has become a global concern, and extensive research has focused on how increased, predominantly anthropogenic nutrient loads are affecting marine biota (Nixon, 1995; Cloern, 2001). Of particular significance, perhaps more so than the absolute quantity of nutrient loads, is the relative supply of macronutrients. Human activities not only have increased the delivery of nitrogen (N) and phosphorus (P) to coastal waters, but also have caused a decrease in Si loads in many regions (Humborg
et al., 2000), resulting in elevated N:Si ratios. This shift toward Si limitation puts diatoms at a competitive disadvantage in favor of non-siliceous phytoplankton (Officer and Ryther, 1980; Conley et al., 1993). For example, over a 23-yr period in the German Bight, increasing N and decreasing Si concentrations resulted in a four-fold increase in N:Si and a shift from diatom to flagellate (*Phaeocystis*) dominance (Radach et al., 1990). Similarly, a correlation between abundance and duration of *Phaeocystis* blooms and increased nutrient loading has been suggested (Cadée and Hegeman, 2002), but others argue that eutrophication has not been a major cause of long-term variation in *Phaeocystis* dynamics (e.g. Gieskes et al., 2007).

Although the physiology and ecology of *Phaeocystis* have been studied extensively, relatively little is known about how well this alga can adapt to varying nutrient regimes during the bloom period. In late winter, NO$_3^-$ dominates the total dissolved N (TDN) pool in the North Sea region and fuels the spring bloom. Diatoms tend to outcompete other algae for available NO$_3^-$, and can rapidly deplete the NO$_3^-$ stock in the surface mixed layer. *Phaeocystis*, on the other hand, appears to benefit from a flexible N uptake strategy, whereby colonies form under NO$_3^-$-replete conditions, but maintain high biomass into the N-limited early summer period (e.g. Lancelot, 1995). Ammonium (NH$_4^+$) uptake by *Phaeocystis*-dominated blooms has been shown to increase either as NO$_3^-$ concentrations decrease to low or undetectable levels (Smith, 1993; Rodrigues and Williams, 2002), or as NH$_4^+$ concentrations increase with peak bloom biomass (Gentilhomme and Lizon, 1998; Tungaraza et al., 2003). These results suggest that *Phaeocystis* is capable of exploiting reduced N forms as they become increasingly available in the late bloom stages; however, few studies have investigated the extent to
which dissolved organic N (DON) can support *Phaeocystis* blooms. A mesocosm study similar to that presented here was conducted in 2003 and included uptake rate measurements for two DON substrates (urea and dissolved free amino acids, DFAA). After depleting amended NO$_3^-$ stocks, the *Phaeocystis*-dominated assemblage relied on urea for the majority (up to 80%) of its N demand as ambient concentrations of this reduced form increased (Sanderson et al., 2008).

Relative to other algae, *Phaeocystis* competes well for N (Riegman et al., 1992), and the persistence of colonial blooms into the typically N-limited early summer suggests that it can either compete equally as well against heterotrophic bacteria for limited dissolved inorganic N (DIN), or rely on alternative N forms (e.g. urea). In theory, however, the small size and large surface area to volume ratio of heterotrophic bacteria should give them the competitive advantage over *Phaeocystis*. Indeed, Rodrigues & Williams (2002) attributed up to 68% of the total DIN uptake to heterotrophic bacteria during the peak *Phaeocystis* bloom. Mesocosm experiments in Danish coastal waters have also shown that bacteria can outcompete phytoplankton for available DIN, but required addition of labile carbon (C; glucose) to do so (Jacquet et al., 2002; Joint et al., 2002; Havskum et al., 2004).

Studies examining the interaction between phytoplankton and heterotrophic bacteria for shared N resources have suffered from inadequate methodology for quantifying their individual activity (Bronk et al., 2007). Nutrient uptake by phytoplankton has typically been measured using glass fiber filters (e.g. Whatman GF/F) that retain nearly all autotrophs, but also a significant fraction of the bacterial community (Gasol and Morán, 1999). This same limitation applies to size fractionation as a means of
measuring the contribution of bacteria to total uptake. Metabolic inhibitors have been used to discriminate between prokaryotic and eukaryotic activity (e.g. Veuger et al., 2004), but their lack of effectiveness and specificity limits the value of this approach (Oremland and Capone, 1988). One underutilized technology capable of surmounting these methodological obstacles is flow cytometric (FCM) sorting, which enables the isolation of planktonic groups based on unique cellular properties, such as chlorophyll autofluorescence in autotrophs (e.g. Lipschultz, 1995; Zubkov and Tarran, 2005).

This study represents part of a larger project designed to examine the correlation between uptake of DIN and DON by phytoplankton and heterotrophic bacteria and expression of the genes that regulate assimilation of these N sources. Here we describe the results of $^{15}$N uptake experiments conducted over the course of a 4-week mesocosm study in a coastal fjord of western Norway during spring 2005. The goals of this study were: (1) to induce a bloom of Phaeocystis pouchetii by addition of NO$_3^-$ and PO$_4^{3-}$; (2) to compare the uptake of $^{15}$N-labeled DIN and DON substrates by phytoplankton and bacteria; (3) to investigate the potential role of various plankton taxa in uptake of the different N forms; and (4) to compare the use of traditional filtration versus FCM sorting in accurately quantifying phytoplankton N use; and (5) to compare these results with those from a previous study conducted in early spring 2003 under different initial nutrient conditions and plankton community structure.
MATERIALS AND METHODS

Mesocosm design and sampling. Experiments were conducted from 1 to 27 April 2005 in the Raunefjord at the University of Bergen’s Marine Biological Field Station in western Norway (60° 16' N, 05° 14' E). Four 11 m³ (4.5 m deep, 2 m diameter) enclosures were suspended from a pontoon dock 200 m offshore. Two light mesocosms were composed of transparent polyethylene and kept open at the surface to allow for penetration of approximately 90% of photosynthetically active radiation (PAR). The two dark mesocosms were composed of opaque polyethylene and kept covered at the surface to limit light penetration.

The mesocosms were filled in situ on 31 March by pumping unfiltered fjord water from 5 m depth, and then kept well mixed throughout the experiment using a 40 L min⁻¹ airlift system. Furthermore, 10% of mesocosm volume was renewed daily with fjord water (3 m depth) to allow for new species to be introduced, avoid large shifts in pH, and compensate for removal of sampled water. Additional details about the mesocosm design are provided in Nejstgaard et al. (2006). One light (M2) and one dark mesocosm (M4) were amended with NaNO₃ and KH₂PO₄ at concentrations of 16 μmol L⁻¹ and 1 μmol L⁻¹, respectively, after initial sampling on 1 April. The remaining two mesocosms, one light (M1) and one dark (M3), were not amended. Samples for chlorophyll a (Chl a), NOₓ⁻ (NO₃⁻ + NO₂⁻, nitrite), and PO₄³⁻ were removed daily from each mesocosm, whereas water for remaining nutrient analyses (see below), particulate N (PN) and particulate organic C (POC) concentrations, plankton counts, and uptake rate measurements was
sampled every other day in a staggered pattern (M1 and M2: even-numbered days; M3 and M4: odd-numbered days).

**Biomass and community composition.** Chlorophyll \( a \) concentrations were determined by filtering 20 to 100 ml of sampled water, in triplicate, onto 25mm, 0.45 \( \mu \)m cellulose-acetate filters (Sartorius), which were then extracted in 90% acetone overnight at 4°C and analyzed on a Turner Design 10-AU fluorometer according to Parsons et al. (1984). Particulate N (PN) and particulate organic C (POC) concentrations were measured on a Europa Geo 20/20 isotope ratio mass spectrometer equipped with an Automated Nitrogen and Carbon Analyzer for Solids and Liquids (ANCA-SL) sample processing unit, from filters used to terminate isotopic tracer experiments (see below). Phytoplankton were identified and enumerated by A.F. Sazhin, as described in Sazhin et al. (2007). Briefly, *Phaeocystis* colonies and non-motile cells within colonies were counted using light microscopy, and motile *Phaeocystis* cells and other microplankton were enumerated using epifluorescence microscopy.

**Nutrient analyses.** After collection, samples for nutrient analyses were filtered through Whatman GF/F filters (precombusted at 450°C for 2 h) and frozen immediately in either acid-washed polypropylene tubes (\( \text{NH}_4^+ \) and urea) or acid-washed HDPE bottles (all others). All samples were analyzed in triplicate except for urea (duplicate). Concentrations of \( \text{NH}_4^+ \) and \( \text{PO}_4^{3-} \) were measured on-site within 5 d of collection and analyzed colorimetrically on a Shimadzu UV-160 spectrophotometer using the manual phenol hypochlorite technique (Koroleff, 1983) and the manual \( \text{PO}_4^{3-} \) technique.
Concentrations of $\text{NO}_3^-$, $\text{NO}_2^-$, and Si were measured at the University of Bergen using a Skalar autoanalyzer. Urea concentrations were determined using the manual monoxime method (Price and Harrison, 1987), and DFAA concentrations were analyzed as dissolved primary amines (DPA) according to the $\text{o-phthaldialdehyde}$ method (Parsons et al., 1984). Kirchman et al. (1989) showed that DFAA and DPA are about equal when $\text{NH}_4^+$ concentrations are low ($<1 \mu\text{mol L}^{-1}$ in this case); therefore, they are referred to as DFAA here. Concentrations of DON were determined as the difference between TDN and DIN, with TDN measured using the persulfate oxidation technique (Bronk et al., 2000). Standard deviations for mean DON values were calculated using propagation of error. A Shimadzu TOC-5000 Analyzer was used with the high-temperature combustion method (Hansell et al., 1997) to measure dissolved organic C (DOC) concentrations.

**Uptake rate experiments.** Net uptake rates of $^{15}\text{N}$-labeled $\text{NH}_4^+$ and $\text{NO}_3^-$ and dual-labeled ($^{15}\text{N}, ^{13}\text{C}$) urea and DFAA (an algal extract consisting of sixteen amino acids; Cambridge Isotope Laboratories, Andover, MA) were measured in samples taken from all four mesocosms as described above. Eight 1 L polyethylene bottles were filled with water from each mesocosm (four substrates in duplicate), spiked with labeled substrates (see above), and then incubated for roughly 3 h at *in situ* light and temperature conditions in the fjord.

Incubations were terminated using filtration; however, the filter type varied in order to examine different components of the microbial community. A portion of each bottle (35–200 ml) was filtered initially through a 25 mm, 0.8 $\mu\text{m}$ silver membrane filter,
this $>0.8 \mu m$ fraction represents the traditional approach to measuring phytoplankton N uptake. The $0.8 \mu m$ filtrate was then passed through a 25 mm, $0.2 \mu m$ silver filter; this $0.2-0.8 \mu m$ fraction represents the bacterial size class. A second volume of sample (80-200 ml) was first screened through 35 $\mu m$ mesh to remove Phaeocystis colonies and other plankton large enough to clog the flow cytometer orifice. The retained cells were then washed onto a 25 mm GF/F filter using $0.2 \mu m$-filtered fjord water. The $<35 \mu m$ filtrate was concentrated over a 47 mm, $0.2 \mu m$ Supor filter to a volume of 5-13 ml, which was preserved with paraformaldehyde at a final concentration of 2% (modified from Campbell, 2001) and frozen in liquid N for FCM sorting. This concentration technique was analyzed in a relatively turbid Chesapeake Bay tributary to determine how much phytoplankton biomass is lost (i.e. stuck) to the Supor filter. In samples concentrated down from 100-200 ml to 10 ml, Chl $a$ averaged $95 \pm 3\%$ of whole-water (unconcentrated) Chl $a$ values, whereas the Supor filter retained $3 \pm 1\%$ of Chl $a$, on average (Bradley, unpubl. data).

The GF/F and silver filters were kept frozen at -20°C until 1 d prior to analysis and then thawed and dried at 40°C overnight. A Europa GEO 20/20 isotope ratio mass spectrometer with an in-line Automated Nitrogen Carbon Analyzer for Solids and Liquids (ANCA-SL) was used to determine PN and POC concentrations as well as $^{15}N$ and $^{13}C$ isotopic enrichments from each sample. Specific N uptake rates ($V: \text{h}^{-1}$) were calculated by dividing the excess $^{15}N$ in the particulate matter by the initial $^{15}N$ enrichment of the dissolved substrate pool per incubation time. Absolute uptake rates ($\rho: \mu \text{mol N L}^{-1} \text{ h}^{-1}$) were calculated as the product of $V$ and PN (Dugdale and Goering, 1967). Ammonium was isolated by solid phase extraction (Dudek et al., 1986) to correct
NH$_4^+$ uptake rates for isotope dilution and to measure NH$_4^+$ regeneration rates (Glibert et al., 1982). Rates of NO$_3^-$, urea, and DFFA uptake were not corrected for isotope dilution.

The percent of bacterial biomass retained on 0.8 μm silver filters was estimated for M1 and M2 to determine the phytoplankton-only (Phyto) PN. Bacterial abundance measured during this study was converted to total bacterial biomass using a cellular N content of 12 fg N cell$^{-1}$, which was determined by Vrede et al. (2002) for bacterial isolates from Raunefjord. The bacterial biomass retained by 0.8 μm filters was calculated as the difference between total bacterial biomass and 0.2–0.8 μm PN. On average, 24 ± 14% and 58 ± 21% of total bacterial biomass was retained on 0.8 μm filters in M1 and M2, respectively. Therefore, Phyto PN was estimated from the >0.8 μm and 0.2–0.8 μm PN using bacterial retention values of 25% and 50% for M1 and M2, respectively. Furthermore, these percentages represent conservative estimates of bacterial retention based on the lower conversion factor of 12 fg N cell$^{-1}$ (for C- or P-limited cells) rather than 35 fg N cell$^{-1}$ for bacteria in exponential growth (Vrede et al., 2002).

**FCM sorting of autotrophs.** Concentrated samples were kept frozen at -80°C and thawed at room temperature prior to sorting on a Beckman-Coulter Epics Altra flow cytometer. Phytoplankton cells were discriminated based on their chlorophyll autofluorescence and sorted at rates ranging from 300 to 1,200 cells s$^{-1}$. The waste stream was periodically collected and analyzed using epifluorescence microscopy to verify the accuracy of the autotrophic sort, and the purity of the sorted phytoplankton samples was assessed using bacterial enumeration. On average, 94 ± 2% of the bacteria were removed during the sorting process. Based on bacterial abundance from M1 and M2 and a N
content of 12 fg N cell\(^{-1}\) (Vrede et al., 2002), bacterial biomass contributed 4% of Phyto PN in the final sorted sample. Sorted phytoplankton cells were then filtered onto 25 mm GF/F filters. As GF/F filters retain roughly 50% of bacteria (see Chapter 1), the bacterial contribution to Phyto uptake measured in FCM-sorted samples is considered negligible. The filters were stored at -20°C until analysis on the mass spectrometer as described above. A small carrier addition of 1 μg N [as \((\text{NH}_4\text{)}_2\text{SO}_4\)] and 8 μg C (as sucrose) to each pelletized sample was done to produce total N masses sufficiently above the Europa’s detection limit (~1 μg N) for reliable \(^{15}\text{N}\) atom percent enrichment values. A mathematical carrier correction was performed when calculating the final \(^{15}\text{N}\) isotopic enrichment.

Potential negative effects of the sorting method on retention of \(^{15}\text{N}\) tracer have been examined previously and are described in depth elsewhere (see Chapter 2). Briefly, uptake rates measured from FCM-sorted phytoplankton were compared with those from filters exposed to boiling water to examine whether preserving, freezing, and sorting cells causes phytoplankton to lose N taken up but not yet assimilated. Significantly lower uptake rates in the boiled versus FCM-sorted samples confirmed that the integrity of sorted phytoplankton cells was minimally compromised, if at all, by our methodology. This conclusion agrees with previous research demonstrating that phytoplankton cellular integrity remains intact (i.e. no radioisotope was lost) in samples sorted following primary production incubations (Rivkin et al., 1986).

**Statistical analyses.** The significance of apparent differences in mean uptake rates between either plankton fractions or mesocosms was assessed using Student’s \(t\)-
tests. Potential relationships between measured variables (e.g. uptake rates and ambient concentrations) were evaluated for significance using Pearson’s Correlation. Results of these statistical tests are reported as $p$ values and $r^2$ correlation coefficients, where appropriate.

RESULTS

Biomass and community composition

A large phytoplankton bloom developed in M2, the amended mesocosm with light, whereas phytoplankton biomass did not increase substantially in any of the other three mesocosms. Concentrations of Chl $a$ in M2 increased to a maximum of 32.3 μg L$^{-1}$ on 13 April, then decreased rapidly over the final two weeks (Fig. 1A). In M1, the unamended mesocosm with light, Chl $a$ increased slightly to a small peak of 2.4 μg L$^{-1}$ on 4 April, but then declined gradually. Chlorophyll $a$ in the dark mesocosms generally decreased throughout the experiment.

Trends in total PN (>0.8 μm + 0.2–0.8 μm) concentrations resembled those of Chl $a$ in that PN increased in M2 over the first two weeks and decreased slowly throughout the study in the other mesocosms (Fig. 1B). Unlike Chl $a$, however, total PN decreased gradually in M2 over the last two weeks and stayed relatively high, likely due to the presence of detrital PN. Phyto PN concentrations were 86% of >0.8 μm PN in both M1 and M2. The >35 μm PN, which consists of Phaeocystis colonies, peaked at 9.5 μmol L$^{-1}$ in M2, the only mesocosm with a colonial bloom.
Ratios of PN:Chl a were initially about 3–4 and decreased over the first few days (Fig. 1C). As the bloom intensified in M2, PN:Chl a decreased further to a minimum of 0.8, then steadily returned to its initial value over the final two weeks. Although PN:Chl a was significantly higher in M1 than in M2 ($p < 0.01$), their profiles were similar. In the dark mesocosms, PN:Chl a fluctuated between 2 and 5 over the first three weeks, then increased sharply in the last week of the experiment to a final value of 19 (Fig. 1C).

The phytoplankton community composition in M1 was initially dominated by *Phaeocystis* solitary cells, which then gave way to small phototrophic flagellates (other than *Phaeocystis*) and cyanobacteria (Fig. 2A). Diatom abundance in M1, on the other hand, remained at relatively low levels. In M2, *Phaeocystis* dominated throughout the study, shifting from solitary motile cells over the first ten days to colonial cells thereafter (Fig. 2B). Diatom abundance in M2 was greatest during the first week, whereas cell numbers of other autotrophs (mainly flagellates) generally increased during the study. Based on microscope counts, the abundance of heterotrophic ciliates and flagellates was noticeably higher in M2 than in M1.

**Nutrients**

Concentrations of TDN were relatively constant in M1, but nearly doubled over the course of the experiment in the dark control (M3; Fig. 3A, C). Addition of NO$_3^-$ to M2 and M4 raised the TDN concentrations in these mesocosms to 22 µmol N L$^{-1}$, which rapidly declined to a background of ~ 6 µmol N L$^{-1}$ in M2 due to biotic uptake, but held steady in M4 (Fig. 3B, D). Undefined DON (un-DON: DON other than urea and DFAA)
was the largest pool of fixed N throughout most of the study in M1, M2, and M3, whereas NO$_X^-$ dominated TDN in M4 (Fig. 4).

The plankton community in the light mesocosms quickly removed any available NO$_3^-$. Concentrations of NO$_3^-$ were ≤0.2 µmol N L$^{-1}$ in M1, and decreased to 0.01 µmol N L$^{-1}$ after initial amendment in M2 (Fig. 3A–B). Except for a short-lived peak, NO$_3^-$ concentrations in M3 were typically ≤0.6 µmol N L$^{-1}$ (Fig. 3C) and declined only slightly in M4 (Fig. 3D). With little variation, NO$_2^-$ concentrations were ≤0.06 µmol N L$^{-1}$ in all mesocosms. In the light mesocosms, NH$_4^+$ concentrations were ≤0.9 µmol N L$^{-1}$ and highest during the final two weeks of the study. In the dark mesocosms, NH$_4^+$ rose steadily to 3–4 µmol N L$^{-1}$ (Fig. 3E–H).

Concentrations of DON varied little in the four mesocosms (Fig. 3A–D); however, the mean concentration in M2 (5.9 ± 0.5 µmol N L$^{-1}$) was significantly higher than in M1 (5.0 ± 0.5 µmol N L$^{-1}$; $p < 0.001$), whereas mean DON values in the dark mesocosms were equal. Urea concentrations held steady at 0.3–0.7 µmol N L$^{-1}$ in all mesocosms, except for an increase to 1.2 µmol N L$^{-1}$ in M3 (Fig. 3E–H). Concentrations of DFAA were also <0.7 µmol N L$^{-1}$ in all mesocosms, but varied considerably. On average, urea and DFAA together comprised 13 ± 3% of DON and ~ 10% of TDN in all mesocosms but M4, where they were only 4% (Fig. 4). Concentrations of DOC were fairly constant at ~ 100 µmol C L$^{-1}$ in M1, M3, and M4, but increased in M2 to a maximum of 251.3 µmol C L$^{-1}$ on 26 April (Fig. 5A). Ratios of DOC:DON consequently increased from about 16 to 37 in M2, and were roughly 15–20 in the other three mesocosms.
In the two control mesocosms, $\text{PO}_4^{3-}$ concentrations were relatively low, with a small spike in M3 on 12 April (Fig. 5B). Amended $\text{PO}_4^{3-}$ was quickly consumed in M2 but remained at $\sim 1$ μmol L$^{-1}$ in M4. The initial ratio of N:P (DIN:$\text{PO}_4^{3-}$) was about 7 in all mesocosms. In M1, N:P only exceeded the Redfield ratio of 16 on 12 April, when maximum $\text{NO}_3^-$ and minimum $\text{PO}_4^{3-}$ concentrations coincided, with a N:P of 96 (data not shown). Otherwise, the mean N:P was 8.4 ± 6.0. In M2, N:P decreased from 14.0 on 2 April to 0.6 on 10 April, then increased to about 3 before dropping again to 0.7 on the last day. The mean N:P was 5.7 ± 5.2 in M2, 20.8 ± 9.6 in M3, and 16.0 ± 1.0 after initial amendment in M4.

Initial concentrations of Si were $\sim 1$ μmol L$^{-1}$ in all mesocosms except M4 (Fig. 5C). Furthermore, Si was significantly higher in the dark versus light control ($p < 0.0001$) but did not differ significantly between the amended mesocosms ($p = 0.132$). In M2, Si initially decreased to a minimum of 0.4 μmol L$^{-1}$, then increased with the *Phaeocystis* bloom to $\sim 1.5$ μmol L$^{-1}$ over the final two weeks. The ratio of DIN:Si was always $\leq$1.0 in M1 and rose from 0.6 to 2.3 in M3 during the experiment. Nitrate additions to M2 and M4 increased DIN:Si to approximately 16, but this decreased to 0.4 by 10 April and remained at <0.7 thereafter.

Ammonium regeneration rates in M1 and M2 generally increased to a peak on 18–20 April, and were significantly higher in M2 ($p < 0.05$; Fig. 6). The mean $\text{NH}_4^+$ regeneration rates in M1 and M2 were 0.604 ± 0.413 μmol N L$^{-1}$ h$^{-1}$ and 0.324 ± 0.169 μmol N L$^{-1}$ h$^{-1}$, respectively. Ammonium regeneration rates were roughly equal in the dark mesocosms and increased steadily from about 0.240 μmol N L$^{-1}$ h$^{-1}$ on 1 April to
almost 1 μmol N L⁻¹ h⁻¹ on 27 April, with a mean of approximately 0.650 μmol N L⁻¹ h⁻¹ in both (data not shown).

**Specific N uptake**

Specific uptake rates are presented here for three fractions: >35 μm, FCM (phytoplankton <35 μm), and 0.2–0.8 μm, which is composed primarily of bacteria. Because N uptake in the two dark mesocosms (M3 and M4) was generally insignificant, this section focuses on results from the two light mesocosms (M1 and M2). However, specific DFAA uptake rates in M3 and M4, although low, were similar in magnitude to those measured in the light mesocosms (data not shown). Addition of NO₃⁻ to M4 resulted in significantly higher specific NO₃⁻ uptake by the 0.2–0.8 μm fraction (nearly four-fold relative to the control; p < 0.0001).

Specific uptake rates describe the physiological ability of cells to assimilate N and are not influenced by biomass. Therefore, they are valuable in comparing how different plankton fractions use a particular N substrate, and can also be used to examine whether N fertilization affects the ability of phytoplankton and bacteria to use N. For example, specific uptake rates were similar in magnitude between the two light mesocosms (Fig. 7), although addition of NO₃⁻ to M2 resulted in significantly higher specific NO₃⁻ uptake by the >0.8 μm fraction (p < 0.05) and lower urea uptake (p < 0.05) by the 0.2–0.8 μm fraction, relative to M1.

In M1, specific uptake rates for NH₄⁺ and urea were not statistically different between the FCM and 0.2–0.8 μm fractions (NH₄⁺: p = 0.496; urea: p = 0.890). In fact,
urea uptake by the 0.2–0.8 μm fraction was relatively high over the final two weeks of the study (Fig. 7). Overall, NO₃⁻ uptake by the FCM fraction was twice that of the 0.2–0.8 μm size class ($p = 0.051$), although NO₃⁻ uptake rates in these two fractions converged over time. Specific DFAA uptake by the 0.2–0.8 μm fraction was significantly higher than that of the FCM fraction throughout the study in M1 ($p < 0.0001$).

It is important to note that specific uptake rates in the >35 μm fraction were negligible in M1 due to the absence of large phytoplankton (e.g. colonial *Phaeocystis*). Similarly, low uptake of all substrates was measured in the >35 μm fraction in M2 during the week preceding the colonial bloom (Fig. 7). Consequently, specific uptake of NO₃⁻ and urea by the FCM fraction in M2 declined after 1 wk as *Phaeocystis* was increasingly retained by the 35 μm mesh. However, uptake of NH₄⁺ and DFAA by the FCM fraction was not likewise affected by the removal of *Phaeocystis* colonies to the >35 μm fraction. In M2, specific uptake rates of NH₄⁺, NO₃⁻, and urea were about equal between the FCM and 0.2–0.8 μm fractions (Fig. 7). After bloom initiation (~ April 8), N uptake by the >35 μm fraction was significantly higher than that of the bacterial size class for NH₄⁺ ($p < 0.05$), NO₃⁻ ($p < 0.001$), and urea ($p < 0.0001$). In contrast, DFAA uptake rates were higher in the 0.2–0.8 μm fraction than the FCM ($p < 0.01$) or >35 μm ($p < 0.05$) fractions, despite a six-fold increase in the latter over the final week of the study.

**Absolute N uptake**

Absolute uptake rates in the Phyto fraction were calculated from the sum of absolute rates in the FCM (<35 μm) and >35 μm fractions, and are compared here with
those of the >0.8 μm fraction (Table 1). Nitrogen uptake by the >0.8 μm fraction, as with uptake measured using GF/F filters, is typically attributed to phytoplankton despite a presumed, but largely unknown, influence of bacteria on these rates. Although absolute uptake rate profiles were generally similar for these two fractions (data not shown), uptake by the >0.8 μm fraction was 2–5 fold higher than that of Phyto in M1 and up to 2-fold higher than Phyto uptake in M2 (Table 1).

Absolute uptake rates were calculated for the total bacterial community (Bact) to account for the N uptake by bacteria retained on 0.8 μm filters. These rates were derived using specific uptake rates in the 0.2–0.8 μm fraction, assuming that the $^{15}$N enrichment of bacteria on 0.8 μm filters equaled that of cells in the smaller size class, and the total bacterial PN calculated from the 0.2–0.8 μm PN and percent retention of bacterial biomass on 0.8 μm filters (25% and 50% for M1 and M2, respectively; see Methods). Absolute uptake rates in the Phyto and Bact fractions provide a more accurate means of assessing how N use differed between phytoplankton and bacteria than do rates from filters with mixed (0.8 μm) or partial (0.2–0.8 μm) assemblages.

In M1, phytoplankton uptake of NH$_4^+$, NO$_3^-$, and urea generally exceeded that of bacteria over the first ten days; subsequently, however, bacterial uptake either equaled (NH$_4^+$ and NO$_3^-$) or exceeded (urea and DFAA) that of phytoplankton (Fig. 8). Due to the higher phytoplankton biomass in M2, absolute uptake rates in the Phyto fraction dominated over those of Bact for all substrates but DFAA. Uptake of DFAA measured in the Phyto fraction increased exponentially over the last two weeks in M2, while rates in the Bact fraction generally decreased.
Whereas specific uptake rates describe the physiological capacity of different plankton fractions to utilize a given N substrate, absolute uptake rates describe the bulk consumption of a substrate by a particular fraction. For example, absolute uptake of NH$_4^+$, NO$_3^-$, urea, and DFAA by the Phyto fraction was, on average, 15-, 20-, 10-, and 12-fold higher, respectively, in M2 versus M1, primarily due to increased biomass in M2. In contrast, absolute uptake by the Bact fraction was roughly equal between mesocosms for NH$_4^+$ ($p = 0.211$) and urea ($p = 0.936$), but significantly higher in M2 for NO$_3^-$ and DFAA ($p < 0.05$ for both). Absolute uptake rates in both dark mesocosms were relatively low ($\leq 0.025 \mu$mol N L$^{-1}$ h$^{-1}$) for all fractions as a result of minimal specific uptake combined with small biomass, and therefore are not described here.

Differences between the Phyto and $>0.8 \mu$m fractions were small with respect to the relative importance of N substrates to total uptake in each mesocosm. The former relied slightly more on NO$_3^-$ than the latter in both M1 and M2, whereas the $>0.8 \mu$m fraction relied slightly more on NH$_4^+$ in M1 and urea in M2 (Fig. 9, Table 2). Overall, however, NH$_4^+$ was the most important substrate to the plankton community in the light mesocosms, contributing 69 ± 14% and 59 ± 19% to total N uptake by the $>0.8 \mu$m fraction in M1 and M2, respectively. Urea was more important to the $>0.8 \mu$m fraction than NO$_3^-$ in M1 ($p < 0.05$), but these two contributed equally (~ 20%) to total N uptake in M2. Amino acids were not a significant N source for phytoplankton, with DFAA comprising just 2% of total N uptake in the $>0.8 \mu$m fraction. The contribution of NO$_3^-$ to total uptake by the $>0.8 \mu$m fraction was negatively correlated with time in both light mesocosms (M1: $p < 0.01$, $r^2 = 0.62$; M2: $p < 0.05$, $r^2 = 0.45$), whereas NH$_4^+$ comprised more of total uptake over time (M1: $p < 0.05$, $r^2 = 0.51$; M2: $p < 0.05$, $r^2 = 0.46$).
The bacterial fraction also relied more on NH$_4^+$ than any other substrate in both M1 and M2 (Fig. 9, Table 2). Compared to the >0.8 μm fraction, DFAA played a significantly greater role in total N uptake by bacteria ($p < 0.01$), comprising 11% of total N uptake, on average, in both light mesocosms. The 0.2–0.8 μm fraction in M2 relied slightly more on NO$_3^-$ and slightly less on urea than in M1; nonetheless, urea was the second most important N substrate (of those studied) to bacteria in both mesocosms.

**DISCUSSION**

The ability of *Phaeocystis* to maintain bloom density under varying nutrient regimes suggests that this alga competes well against other phytoplankton, and potentially bacteria, for available N under both N-replete and N-limited conditions. We examined this phenomenon in mid-spring 2005 by inducing a bloom dominated by *Phaeocystis* using nutrient-manipulated mesocosms. Although the bloom only occurred when NO$_3^-$ and PO$_4^{3-}$ were added, NO$_3^-$ dominated total N uptake on only one of eleven sampling days (6 April). Overall, NO$_3^-$ contributed as much to phytoplankton N use as did urea (~20%). Of the four substrates used here, NH$_4^+$ supplied the majority of N to phytoplankton and bacteria (up to 88% and 85%, respectively) in both light mesocosms.

In M2, specific NH$_4^+$ uptake was strongly correlated with ambient NH$_4^+$ concentrations for the >35 μm ($p < 0.0001$, $r^2 = 0.87$) and FCM ($p < 0.001$, $r^2 = 0.86$) fractions. Ambient NH$_4^+$ concentrations in M2 were also closely related to NH$_4^+$ regeneration rates ($p < 0.01$, $r^2 = 0.67$), but none of these relationships were significant in M1. Elevated NH$_4^+$ regeneration rates in the amended mesocosm could have been due to
bacterial remineralization of algal-exudated DON or via sloppy feeding and excretion by grazers. Grazer-mediated processes tend to be the dominant source of regenerated NH$_4^+$ in marine ecosystems (Bronk and Steinberg, 2008). However, given the lack of DON accumulation in M2 with bloom decay, as well as the predator defense mechanisms of colonial Phaeocystis, such as its large size and general unpalatability (Nejstgaard et al., 2007), bacterial NH$_4^+$ remineralization may have been high. The profile of specific NH$_4^+$ uptake by the 0.2–0.8 µm fraction resembled that of the FCM and >35 µm fractions, which indicates that bacteria were also using regenerated NH$_4^+$. Therefore, it is possible that one component of the bacterial community was remineralizing labile DON while another assemblage was complementing degradation of C-rich Phaeocystis exudates (e.g. mucous) with NH$_4^+$ uptake. Regardless, NH$_4^+$ production and consumption processes appear to have been closely coupled in the amended mesocosm.

Results from the control mesocosm resembled ecosystem dynamics that might be expected for North Sea coastal waters in late spring, with a decreasing importance of NO$_3^-$ to phytoplankton N nutrition concurrent with decreasing biomass and an increased dependence on NH$_4^+$. These dynamics are analogous to the transition that characterizes most temperate marine ecosystems: a shift from new production (and diatom dominance) in spring to regenerated production (flagellates and cyanobacteria) in summer.

**Phaeocystis and phytoplankton versus bacterial N use**

Based on initial DIN to PO$_4^{3-}$ ratios of ~ 7, phytoplankton in the Raunefjord appear to have been N-limited rather than P-limited prior to the start of this experiment.
Furthermore, relative to the control, NO$_3^-$ and PO$_4^{3-}$ amendment significantly increased specific uptake of NO$_3^-$ only, whereas an increase in the specific uptake of all N forms would have been expected if P-limitation was relieved by fertilization. Although phytoplankton were apparently N-limited before amendment, the rapid removal of added PO$_4^{3-}$ highlights the importance of this nutrient to the *Phaeocystis* bloom in M2.

The dominance of NH$_4^+$ uptake, even after addition of NO$_3^-$, was unexpected based on results from a similar study, in which urea replaced NO$_3^-$ as the dominant N form used during an induced *Phaeocystis* bloom (Sanderson et al., 2008, see below). The importance of NH$_4^+$ over other N forms has been reported elsewhere, however. In a mesocosm study conducted in Danish coastal waters in late spring, Joint et al. (2002) reported greater NH$_4^+$ uptake relative to NO$_3^-$, despite NO$_3^-$ fertilization. Similarly, Smith (1993) found an inverse relationship between uptake of NH$_4^+$ and NO$_3^-$ during a spring bloom dominated by *Phaeocystis* in the Greenland Sea, with NH$_4^+$ uptake exceeding that of NO$_3^-$ in May. One possible explanation is that the phytoplankton community in Raunefjord, including *Phaeocystis* solitary cells, was adapted to low N availability following depletion of ambient NO$_3^-$ earlier in the spring, and as such could use regenerated NH$_4^+$ more efficiently than added NO$_3^-$. Inhibition of autotrophic NO$_3^-$ uptake by ambient NH$_4^+$ is another potential explanation for the higher NH$_4^+$ uptake rates measured here (Dortch, 1990; Cochlan and Bronk, 2003). However, this was clearly not the case in M2, where added NO$_3^-$ was quickly removed, and there was no significant relationship between specific NO$_3^-$ uptake rates and ambient NH$_4^+$ concentrations in M1 ($p = 0.065, r^2 = 0.33$). Therefore, autotrophic preference for NH$_4^+$, rather than inhibition of NO$_3^-$ use, seems likely.
Although the environmental factors that stimulate *Phaeocystis* colony formation remain uncertain, it has been suggested that this process may be a strategic response to low nutrient availability and thus gives colonies a competitive advantage over other algae (Lancelot, 1995). The dominance of *Phaeocystis* in the bloom described here once again demonstrates its competitive ability to exploit both high N (e.g. added NO$_3^-$) and low N (e.g. after 10 April) conditions. The respective roles that *Phaeocystis* colonies versus solitary flagellate cells played in this outcome, however, are not clear. The relatively high specific NH$_4^+$ uptake rates in the FCM fraction in M2, versus the sharp decline in NO$_3^-$ uptake rates during colony formation suggest that colonies relied more on NO$_3^-$, whereas NH$_4^+$ was more important to solitary cells (and other phytoplankton). Furthermore, the fact that FCM and >35 μm specific NH$_4^+$ uptake rates were equal to or greater than those of the 0.2–0.8 μm fraction in both mesocosms suggests that *Phaeocystis* can compete well against heterotrophic bacteria for available NH$_4^+$. Differences in N use by phytoplankton and bacteria are best demonstrated using the absolute uptake rates calculated for the Phyto and Bact fractions (Fig. 8), which account for the bacterial biomass and activity captured on 0.8 μm filters. Absolute uptake rates estimated for all bacteria (Bact) were roughly 40% higher than in the 0.2–0.8 μm fraction in M1 and 90% higher in M2. In M1, nutrient availability was limited and phytoplankton were initially better able to use available N than bacteria (except for DFAA). Starting in mid-April, however, phytoplankton could no longer outcompete bacteria for available DIN, and bacterial uptake of urea and DFAA exceeded that of phytoplankton. Differences between phytoplankton and bacterial uptake of NH$_4^+$, NO$_3^-$, and urea in M2 indicate that phytoplankton, especially *Phaeocystis*, can exploit new N
sources to form large blooms, but can also persist on regenerated N once new N has been exhausted. Under these conditions, bacterial growth appears to be supported more by phytoplankton-derived organic N than in M1, thus relieving the need for phytoplankton and bacteria to compete for limited N resources.

Bacterial uptake comprised a substantial portion of total uptake of all N forms in the control mesocosm (Table 2). Urea is typically not recognized as an important N source to bacteria (Tamminen and Irmisch, 1996; Kirchman, 2000), although several studies have found otherwise (Jørgensen et al., 1999; Jørgensen, 2006; Sanderson et al., 2008). In this study, bacterial uptake represented about 50% of total urea uptake in M1. Lower bacterial contribution to total uptake in the amended mesocosm was primarily a result of high phytoplankton biomass, since specific uptake rates in the 0.2–0.8 μm fraction were relatively high (Fig. 7). Overall, however, bacteria had a greater affinity for DFAA and lesser affinity for NO₃⁻ than did phytoplankton. This finding is not surprising given that bacteria tend to prefer DFAA over NO₃⁻ in marine environments (Kirchman, 2000), and also given the ability of Phaeocystis to compete well for NO₃⁻ under N-limited conditions (Riegman et al., 1992). However, a rapid increase in DFAA uptake by the >35 μm fraction was measured in the amended mesocosm during the last week of the experiment (Fig. 7). Although prymnesiophytes, such as Phaeocystis, are known to use DFAA via extracellular enzymatic processes (Palenik and Morel, 1990b; Berman and Bronk, 2003), this also may have been due to the elevated activity of heterotrophic bacteria attached to senescent Phaeocystis colonies (Thingstad and Billen, 1994).
Comparison with the 2003 bloom

A similar study was conducted at the same location in 2003 (Nejstgaard et al., 2006; Sanderson et al., 2008), but with largely dissimilar results. The most substantial difference between these two studies is that the 2003 experiment began on 28 February (versus April 1 here), and therefore captured different ecosystem dynamics. In 2003, Si and NO$_3^-$ were still relatively abundant on day 1 of the experiment, and the typical diatom-*Phaeocystis* succession was observed. The depletion of Si and NO$_3^-$, which are crucial to diatom dominance, in the fjord prior to initiating the present study probably explains the absence of a diatom bloom in any of the mesocosms.

Sanderson et al. (2008) reported that urea dominated uptake by the >0.8 \( \mu \)m fraction during the induced *Phaeocystis* bloom, likely a result of increased urea availability following the decline of the early diatom and flagellate bloom. In contrast, NH$_4^+$ was the dominant N form used during the *Phaeocystis* bloom in 2005, also due to increased supply by regeneration. The increase in urea concentrations in 2003, but not in 2005, may have been due to excretion by grazers feeding on diatoms and flagellates, which were relatively more abundant in the former study (Sanderson et al. 2008). In 2005, regeneration of NH$_4^+$ rather than urea suggests that energy was transferred indirectly through the microbial loop rather than from diatoms to zooplankton. Together, the results from these two studies demonstrate that although NO$_3^-$ is key to initiating *Phaeocystis* blooms, reduced N forms play an important role in sustaining the bloom once NO$_3^-$ has been depleted, and they also illustrate the versatility of *Phaeocystis* with respect to its ability to exploit various N sources as they become available.
Size-fractionated vs. FCM-sorted N uptake

To date, studies of phytoplankton N use have typically relied on filtration as a means of distinguishing autotrophic from heterotrophic activity, despite the size overlap between these groups and clogging effects on filters. These drawbacks have been demonstrated (Lee and Fuhrman, 1987; Gasol and Morán, 1999; Berg et al., 2001), but are often overlooked in studies attributing uptake rates measured on GF/F filters (nominal pore size of 0.7 μm), for example, to phytoplankton. Here we used FCM sorting to measure phytoplankton uptake without the confounding effect of bacteria, but also to examine how traditional filtration can overestimate phytoplankton N uptake.

We hypothesized that differences between absolute uptake rates in the >0.8 μm and Phyto (FCM + >35 μm) fractions would be greatest in the amended mesocosm as a result of high biomass and increased clogging effects on 0.8 μm filters. However, absolute uptake rates in the Phyto and >0.8 μm fractions were not statistically different in the amended mesocosm, although these results are somewhat complicated by the fact that the >35 μm fraction represented over half of the Phyto biomass, which is uncommon for most systems. In the control mesocosm, however, uptake rates in the >0.8 μm fraction were significantly greater than those of the FCM fraction (>35 μm uptake was negligible) for NH₄⁺ (p < 0.01), urea (p < 0.05), and DFAA (p < 0.0001), but not NO₃⁻ (p = 0.119). There are two, non-mutually exclusive ways in which bacterial retention on 0.8 μm silver filters could have caused overestimation of absolute phytoplankton N uptake rates: (1) if bacteria were relatively more enriched in ¹⁵N than phytoplankton (i.e. used more substrate), and (2) overestimating phytoplankton PN due to retention of bacterial
biomass. Evidence for the former should appear in the specific uptake rates. The only substrate for which >0.8 μm specific rates significantly exceeded those of the FCM fraction was DF AA (p < 0.05), which is not surprising given bacterial affinity for this labile organic substrate. Nonetheless, this suggests that the contribution of bacterial biomass to >0.8 μm PN was the primary cause of higher uptake rates in this fraction versus the FCM fraction in the control mesocosm. These results demonstrate that the use of 0.8 μm filters (and presumably GF/F filters) may result in significant overestimation of phytoplankton N uptake.

CONCLUSION

Addition of NO₃⁻ and PO₄³⁻ to M2 resulted in a large bloom of Phaeocystis pouchetii, whereas no bloom occurred in the unamended mesocosm (M1). Although NO₃⁻ was clearly important to fueling the Phaeocystis bloom, ammonium was the predominant form of N used by phytoplankton and bacteria. Nitrate and urea each contributed roughly 20% to phytoplankton N uptake in both mesocosms. Amino acids, however, were a negligible N source to phytoplankton, but comprised about 11% of total bacterial N uptake. Overall, bacteria contributed significantly to total N uptake in M1, and although phytoplankton N uptake dominated over that of bacteria in M2, N metabolism of these two groups were closely coupled.

Results from the control mesocosm indicate that bacteria were able compete effectively with phytoplankton for limited N resources. In the amended mesocosm, on the other hand, Phaeocystis was able to exploit new N and rapidly form a colonial bloom,
then switch to regenerated N forms to sustain high biomass. Previous studies of phytoplankton and bacterial N uptake have been hampered by methodological obstacles. The use of flow cytometric sorting of autotrophs in this study demonstrated how bacterial retention can lead to significant overestimation of phytoplankton N uptake in the >0.8 μm fraction, and underestimation of bacterial uptake in the 0.2–0.8 μm fraction. The environmental conditions leading to the development of colonial *Phaeocystis* blooms versus diatom- or bacteria-dominated communities are undoubtedly complex; however, accurate quantification of the N utilization patterns of these plankton groups will help clarify their ecological interactions.

**ACKNOWLEDGEMENTS**

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LITERATURE CITED


Table 1. Ratio of >0.8 μm to phytoplankton (Phyto) absolute uptake rates for ammonium (NH$_4^+$), nitrate (NO$_3^-$), urea, and dissolved free amino acids (DFAA).

M1: unamended, in light; M2: nitrate and phosphate added, in light. The mean ± SD and (min – max) are given.

<table>
<thead>
<tr>
<th>Mesocosm</th>
<th>NH$_4^+$</th>
<th>NO$_3^-$</th>
<th>Urea</th>
<th>DFAA</th>
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<tr>
<td></td>
<td>3.8 ± 2.5</td>
<td>2.1 ± 0.6</td>
<td>2.8 ± 1.1</td>
<td>4.8 ± 4.8</td>
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<td>(1.3 - 9.0)</td>
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<td>M2</td>
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Table 2. Percent contribution of each $^{15}$N-labeled substrate to total measured uptake by phytoplankton and bacteria, as well as the bacterial contribution to total uptake (phytoplankton + bacteria) of each substrate. The phytoplankton (Phyto) and bacterial (Bact) fractions are described in the text. M1: unamended, in light; M2: nitrate and phosphate added, in light. The mean ± SD and (min – max) are given.

<table>
<thead>
<tr>
<th>Mesocosm</th>
<th>NH$_4^+$</th>
<th>NO$_3^-$</th>
<th>Urea</th>
<th>DFAA</th>
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<tr>
<td>Phyto</td>
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<td>16 ± 21%</td>
<td>19 ± 10%</td>
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<td>(3 – 70)</td>
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<td>Bacterial contribution</td>
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<tr>
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<td>(4 – 70)</td>
<td>(6 – 25)</td>
<td>(0 – 10)</td>
</tr>
<tr>
<td>Bact</td>
<td>56 ± 20%</td>
<td>15 ± 10%</td>
<td>18 ± 11%</td>
<td>11 ± 5%</td>
</tr>
<tr>
<td>Bacterial contribution</td>
<td>19 ± 17%</td>
<td>14 ± 12%</td>
<td>20 ± 15%</td>
<td>58 ± 27%</td>
</tr>
<tr>
<td></td>
<td>(5 – 59)</td>
<td>(3 – 37)</td>
<td>(6 – 52)</td>
<td>(9 – 88)</td>
</tr>
</tbody>
</table>
Figure 1. Concentrations of (A) chlorophyll $a$ and (B) total particulate nitrogen (PN), as well as (C) the ratio of PN:Chl $a$ in control (M1 and M3; circles) and amended (M2 and M4; squares) mesocosms. Open symbols represent mesocosms exposed to light (M1 and M2), and filled symbols represent those kept in the dark (M3 and M4). Error bars in (A) and (B) denote ± 1 SD of the mean, and in some cases are smaller than the symbols.
Figure 2. Plankton community composition in numbers of cells ($\times 10^7$ L$^{-1}$) in (A) M1 (control, in light) and (B) M2 (amended, in light) mesocosms. The “other autotrophs” fraction consisted mostly of small, autotrophic flagellates and cyanobacteria in M1 and the former in M2.
Figure 3. Concentrations of (A–D) total dissolved nitrogen (TDN), dissolved organic nitrogen (DON), and nitrate + nitrite (NO$\text{}_x^-$), and (E–H) ammonium (NH$_4^+$), urea, and dissolved primary amines (DPA). M1: control, in light; M2: amended, in light; M3: control, in dark; M4: amended, in dark. Note the difference in y-axis scale between plots. Error bars represent ± 1 SD of the mean ($n = 2–3$) and may be smaller than the symbols.
Figure 4. Percent contribution of $\text{NH}_4^+$, $\text{NO}_3^-$, urea, DFAA, and undefined DON (un-DON: DON other than urea and DFAA) to total dissolved nitrogen. M1: control, in light; M2: amended, in light; M3: control, in dark; M4: amended, in dark.
Figure 5. Concentrations of (A) dissolved organic carbon (DOC), (B) phosphate (PO$_4^{3-}$), and (C) silicate (Si) in the control (circles) and amended (squares) mesocosms. Open symbols represent mesocosms exposed to light, and filled symbols represent those kept in the dark. Error bars represent ± 1 SD of the mean (n = 2−3) and may be smaller than the symbols.
Figure 6. Rates of NH$_4^+$ regeneration in the control (M1) and amended (M2) mesocosms with light. Error bars represent ± 1 SD of the mean (of duplicate incubations), and are sometimes smaller than the symbols.
Figure 7. Specific uptake rates of \( \text{NH}_4^+ \), \( \text{NO}_3^- \), urea, and DFAA by the >35 \( \mu \text{m} \) (larger phytoplankton), flow cytometer-sorted phytoplankton <35 \( \mu \text{m} \) (FCM), and 0.2–0.8 \( \mu \text{m} \) (bacterial) fractions in the control and amended mesocosms with light. Error bars represent ± 1 SD of the mean (of duplicate incubations), and are sometimes smaller than the symbols.
Figure 8. Absolute uptake rates of $\text{NH}_4^+$, $\text{NO}_3^-$, urea, and DFAA by phytoplankton (Phyto) and bacteria (Bact) in the control (M1) and amended (M2) mesocosms with light. The Phyto and Bact fractions are described in the text. Note the difference in y-axis scale between plots. Error bars represent ± 1 SD of the mean (of duplicate incubations), and are sometimes smaller than the symbols.
Figure 9. Percent contribution of $^{15}$N-labeled substrates to total measured N uptake by the Phyto (phytoplankton-only), >0.8 μm (phytoplankton + bacteria), and 0.2–0.8 μm (bacteria) fractions. The Phyto fraction represents the combined uptake of the flow cytometer-sorted phytoplankton <35 μm (FCM) and >35 μm fractions. M1: control, in light; M2: amended, in light.
CHAPTER 5

CROSS-SYSTEM COMPARISON OF PHYTOPLANKTON AND BACTERIAL NITROGEN UPTAKE MEASUED USING FLOW CYTOMETRIC SORTING VERSUS TRADITIONAL FILTRATION

*This chapter follows the format of* Limnology and Oceanography
Abstract

Traditional measurements of phytoplankton nitrogen (N) uptake have been confounded by bacterial retention on filters used in $^{15}$N uptake studies (e.g. Whatman GF/F), and this methodological obstacle has limited our understanding of phytoplankton-bacteria interactions with respect to N cycling. The importance of various inorganic and organic N substrates to phytoplankton and bacteria was examined in several marine ecosystems using two distinct methods: (1) size fractionation into the phytoplankton and bacterial size classes, and (2) flow cytometric (FCM) sorting of autotrophic cells. The ecosystems studied include Chesapeake Bay, the York River (a subestuary of Chesapeake Bay), the Mid-Atlantic Bight, Raunefjord (Norway), and the oligotrophic Gulf of Mexico. Total dissolved N concentrations and total N uptake decreased from estuarine to oceanic waters, although uptake rates were highly variable within each ecosystem.

Overall, ammonium comprised the majority (54 ± 28%) of total measured N uptake by phytoplankton, followed by urea (29 ± 26%), nitrate (12 ± 16%), and dissolved free amino acids (5 ± 5%). Estimates of N uptake indicate that, at times, bacteria were responsible for over half of the total uptake of each substrate, and that urea represented a significant N source to bacteria, despite traditional views to the contrary. On average, filter-based N uptake rates overestimated actual phytoplankton uptake (measured using FCM sorting) by a factor of 1.41 ± 1.50 for ammonium, 0.94 ± 1.38 for nitrate, 1.25 ± 1.24 for urea, and 2.22 ± 1.60 for amino acids. These results highlight the need for an improved understanding of the distinct roles that phytoplankton and bacteria play in cycling N and how they interact under conditions of limited inorganic N availability.
Introduction

Since the seminal contributions of Pomeroy (1974) and Azam et al. (1983), much progress has been made in understanding the ecological roles of phytoplankton and heterotrophic bacteria (hereafter simply referred to as bacteria) in the marine environment. In particular, conceptual models of phytoplankton-bacteria interactions with respect to nitrogen (N) cycling have evolved from the traditional view that phytoplankton rely almost exclusively on ammonium (NH$_4^+$) and nitrate (NO$_3^-$), whereas bacteria remineralize algal-released organic matter. Nitrogen pathways that were previously either unknown or considered insignificant have since become primary components of the modern view of microbial N cycling (Zehr and Ward, 2002). For example, dissolved organic N (DON), once considered relatively unimportant to phytoplankton N nutrition, can provide significant N to primary producers (Glibert et al., 1991; Bronk, 2002; Sanderson et al., 2008). In addition, bacteria require dissolved inorganic N (DIN) to maintain elemental stoichiometry within the cell, such as when respiring relatively carbon (C)-rich organic matter (Kirchman, 2000), and thus compete with phytoplankton for DIN. The effects of anthropogenic N sources on phytoplankton-bacteria dynamics have also been examined and will likely be the subject of increasing attention (Cloern, 2001; Duce et al., 2008).

Despite such advances, a clear understanding of the environmental factors that determine the composition of and interaction between phytoplankton and bacterial communities, as well as their respective roles in nutrient cycling, is lacking. This is partly due to methodological challenges in distinguishing between autotrophic and
heterotrophic activity. Numerous approaches have been used to examine phytoplankton versus bacterial N uptake, including metabolic inhibitors (Wheeler and Kirchman, 1986; Middelburg and Nieuwenhuize, 2000), nutrient bioassays (Gobler and Sañudo-Wilhelmy, 2001), and molecular analyses of genes involved in N assimilation (Zehr and Ward, 2002; Allen et al., 2005). Weaknesses in each of these methods, however, preclude accurate quantification of phytoplankton versus bacterial N uptake (Bronk et al., 2007). The most common approach to date has been size-selective filtration that targets the vague size difference between phytoplankton and bacteria. Glass fiber filters (e.g. Whatman GF/F, 0.7 μm nominal pore size) are typically used in 15N uptake studies because they can be combusted to remove contaminants, are amenable to isotopic analysis on a mass spectrometer, and are relatively inexpensive. However, due to some size overlap between these two microbial groups and clogging effects inherent with filtration, a variable portion of the bacterial community is also retained. Several studies have quantified bacterial retention (by abundance) on GF/F filters, and although the results vary, over half of the bacterial community is generally retained (Table 1). Despite this fact, most N uptake measurements using GF/F filters have been attributed to phytoplankton alone rather than the mixed assemblage actually involved.

An alternative, yet underutilized approach is flow cytometric (FCM) sorting. For decades, flow cytometry has been a valuable tool for enumerating and assessing marine plankton communities (Yentsch et al., 1983; Olson et al., 1991; Veldhuis and Kraay, 2000). In contrast to traditional filtration, which uses an imperfect size-based classification, FCM sorting can identify the functional difference between phytoplankton and bacteria (i.e. pigment autofluorescence) and therefore more accurately isolates one
from the other in natural samples. This alternative approach has been used to measure bacterial activity (Servais et al., 1999), primary production (Rivkin et al., 1986; Li, 1994), phytoplankton growth rates (Pel et al., 2004), and N uptake (Lipschultz, 1995).

Although many studies have quantified N uptake by phytoplankton using traditional filtration, few, if any, have evaluated these measurements in consideration of the effect bacteria may have on reported rates. Similarly, measurements of bacterial N uptake using size fractionation do not account for uptake by bacteria retained on the fractionating filter (e.g. GF/F, 0.8 μm silver). In the present study, $^{15}$N uptake rates were measured using both FCM sorting and size-selective filtration approaches to examine the accuracy of traditional filtration-based measurements of phytoplankton N uptake. In addition, the relative importance of various inorganic and organic N substrates to phytoplankton and bacterial N nutrition, as well as the bacterial contribution to total uptake, were assessed in the following ecosystems: Chesapeake Bay, the York River, Virginia (a tributary of Chesapeake Bay), the Mid-Atlantic Bight, Raunefjord, Norway, and the oligotrophic Gulf of Mexico.

Methods

Study sites and sampling. Sampling locations, dates, depths, and experimental methods are described briefly in Table 2. Water was collected from near the surface (1 – 2 m), and occasionally deeper in the water column as well, using a Niskin rosette, pump, or acid-washed bucket. Samples from Raunefjord, Norway were part of a
mesocosm experiment in which one mesocosm was not amended, whereas the other had NO$_3^-$ and phosphate (PO$_4^{3-}$) initially added at 16 $\mu$mol N L$^{-1}$ and 1 $\mu$mol L$^{-1}$, respectively.

**Dissolved N analyses.** Samples for dissolved nutrient analyses were filtered through precombusted (450°C for 2 h) GF/F filters and stored (-20°C) in acid-washed polypropylene tubes (NH$_4^+$ and urea) or acid-washed HDPE bottles (all others). All nutrient analyses were conducted in triplicate, except for urea, which was measured in duplicate. Ammonium concentrations were measured colorimetrically using the manual phenol-hypochlorite technique (Koroleff, 1983). Concentrations of NO$_3^-$ and NO$_2^-$ were determined using either an Alpkem Flow Solution IV (O.I. Analytical) or Lachat QuikChem 8500 autoanalyzer with the colorimetric method of Parsons et al. (1984). Urea concentrations were measured according to the manual monoxime method (Price and Harrison, 1987). Dissolved free amino acid (DFAA) concentrations were determined for Mid-Atlantic Bight samples as the sum of individual AA concentrations measured with high-performance liquid chromatography and $o$-phthalaldehyde (Lindroth and Mopper, 1979). All other DFAA samples were analyzed as dissolved primary amines using the $o$-phthalaldehyde method of Parsons et al. (1984). Dissolved primary amines are approximately equal to DFAA concentrations in waters with relatively low ambient NH$_4^+$ concentrations (Kirchman et al., 1989). Total dissolved N (TDN) concentrations were measured using persulfate oxidation (Bronk et al., 2000), and DON values were calculated as the difference between TDN and DIN (sum of NH$_4^+$, NO$_3^-$, and NO$_2^-$). Particulate N concentrations were measured from filters used to terminate $^{15}$N uptake experiments (see below), on a Europa Geo 20/20 isotope ratio mass spectrometer.

$^{15}$N uptake experiments. Nitrogen uptake rates were measured using the following stable isotopic tracers: $^{15}$N-labeled NH$_4^+$ and NO$_3^-$ and dual-labeled ($^{15}$N, $^{13}$C) urea and DFAA (an algal extract comprised of 16 amino acids; Cambridge Isotope Laboratories). For each substrate used, duplicate clear polyethylene (PETG) bottles were filled with sampled water, spiked with $^{15}$N-labeled tracer, and incubated for 1 – 4 h at in situ light and temperature conditions (Table 2). In Chesapeake Bay and Raunefjord, incubation bottles were filled with unfractionated (i.e. whole) water. However, in the York River, Mid-Atlantic Bight, and Gulf of Mexico, water used to fill incubation bottles was first screened through a 53 $\mu$m mesh to remove larger plankton. When possible, tracer additions of 10% (or less) of ambient concentrations were estimated from published data.

Incubations were terminated using filtration; however, the protocols and filters varied according to the method or size fraction of interest, depending on the system (Table 2). In all samples, an aliquot of incubated water was filtered through either a GF/F (nominal pore size of 0.7 $\mu$m) or 0.8 $\mu$m silver filter to collect what has traditionally been referred to as the phytoplankton fraction (see caveats above). Except for the Chesapeake Bay samples, the GF/F or <0.8 $\mu$m filtrate was subsequently passed through a 0.2 $\mu$m silver filter to collect the bacterial size class. The filters were frozen immediately and stored at -20°C until isotopic analysis. In Chesapeake Bay and Raunefjord, a separate volume of incubated water was initially passed through a 35 $\mu$m mesh to remove larger plankton capable of clogging the flow cytometer orifice. This >35 $\mu$m fraction was then
washed onto a GF/F filter using 0.2 μm-filtered seawater, and the filter was frozen and stored for isotopic analysis as above.

Samples for FCM sorting were prepared by concentrating the <35 μm filtrate (Chesapeake Bay and Raunefjord) or the <53 μm incubated water (York River, Mid-Atlantic Bight, Gulf of Mexico) down over a 47 mm, 0.2 μm Supor filter, to a final volume of 5 – 12 ml. Using a 10 ml pipette, the Supor filter was periodically rinsed with retentate during the concentration process in order to keep the cells in suspension. The concentrated sample and Supor filter were transferred to a polypropylene centrifuge tube, which was then inverted gently several times to further rinse the filter. After removing the Supor, the sample was preserved with paraformaldehyde at a final concentration of 0.2 – 2% (Campbell, 2001), frozen in liquid N, then stored at -80°C.

To determine whether phytoplankton biomass was adequately removed from the Supor filters following concentration of FCM samples, chlorophyll a (Chl a) was measured both before and after concentrating, using surface water taken from the relatively turbid York River. Sample volumes of 100 ml, 200 ml, and 300 ml were filtered, each in triplicate, through GF/F filters. Equivalent volumes, also in triplicate, were concentrated to 10 ml samples using 0.2 μm Supor filters as above, which were then filtered onto GF/F filters. The GF/F filters, as well as the Supor filters used for concentrating, were extracted overnight in 90% acetone and analyzed on a Turner Design 10-AU fluorometer according to Parsons et al. (1984). On average, 96 ± 3%, 94 ± 3%, and 89 ± 3% of ambient Chl a concentrations were retained in 10 ml samples concentrated from 100 ml, 200 ml, and 300 ml, respectively (Table 3). Chlorophyll a concentrations on the Supor filters accounted for 3 ± 1%, 4 ± 1%, and 12 ± 3% of the
100, 200, and 300 ml ambient Chl $a$ values, respectively. These results indicate that even with moderate sample concentration (e.g. 200 ml to 10 ml) in a turbid estuary, an insignificant amount of phytoplankton biomass is lost to the Supor filter.

**FCM sorting of autotrophs.** Concentrated FCM samples from the York River, Mid-Atlantic Bight, and Raunefjord were sorted on an Epics Altra flow cytometer (Beckman-Coulter), whereas those from Chesapeake Bay and the Gulf of Mexico were sorted using an inFlux V-GS flow cytometer (Cytopeia). Following daily instrument alignment and calibration, the samples were thawed at room temperature, and autotrophs were sorted using Chl $a$ fluorescence as the gating criterion. Sort speeds varied depending on the density of concentrated samples, but were generally 300 – 1,200 cells s$^{-1}$ on the Epics Altra and from 1,000 to 10,000 cells s$^{-1}$ on the inFlux, which is designed for stable, high-speed sorting. Due to the fairly high number of phytoplankton cells needed for isotope analysis on a mass spectrometer (roughly $1 - 5 \times 10^6$), a compromise between yield and purity was necessary. Cells sorted from Mid-Atlantic Bight and Gulf of Mexico samples were collected on 0.2 μm silver filters to ensure retention of picophytoplankton, whereas those from all other sites were collected on GF/F filters. The filters were then stored at -20°C until isotopic analysis.

The sorting accuracy was periodically confirmed by collecting and reanalyzing the sorted and waste streams using both flow cytometry and epifluorescence microscopy. The purity of the sorted samples was assessed using bacterial enumeration by flow cytometry and by acridine orange direct counts (Sherr et al., 2001). Overall, $6.4 \pm 3.3\%$ of bacteria were included in the autotrophic sorted population when averaged across all
study sites. This bacterial abundance in sorted samples, when converted to biomass using a cellular N content of 5 fg N cell$^{-1}$ for Gulf of Mexico samples and 12 fg N cell$^{-1}$ for all other locations (e.g. Vrede et al. 2002), represents 3.4 ± 0.7% of sorted phytoplankton biomass. Roughly half of this bacterial biomass would have passed through the GF/F filters used to collect the FCM-sorted samples. Therefore, the influence of bacteria on phytoplankton N uptake rates measured using FCM was insignificant.

**Calculation of $^{15}$N uptake rates.** Using the Europa mass spectrometer described above, PN concentrations and $^{15}$N enrichment values were measured on filters used to terminate the tracer experiments (see above). To ensure reliable measurement of $^{15}$N enrichment in FCM-sorted cells, 1 – 2 $\mu$g N of carrier ((NH$_4$)$_2$SO$_4$) was added to these filters immediately before analysis, and the $^{15}$N enrichment in the original sample was later determined using a carrier correction. Specific N uptake rates ($V$, h$^{-1}$) were calculated by dividing the excess $^{15}$N in the particulate matter by the $^{15}$N enrichment of the dissolved substrate pool per hour of incubation time. Absolute N uptake rates ($\rho$, $\mu$mol N L$^{-1}$ h$^{-1}$) were calculated as the product of $V$ and PN (Dugdale and Goering, 1967). The NH$_4^+$ pool was isolated using solid phase extraction (Dudek et al., 1986) and analyzed to correct NH$_4^+$ uptake rates for isotope dilution caused by NH$_4^+$ regenerated during the incubation (Glibert et al., 1982). Uptake rates for NO$_3^-$, urea, and DFAA were not corrected for isotope dilution.

To compare absolute N uptake rates measured using traditional filtration versus FCM sorting, the PN concentration for phytoplankton only (Phyto) was estimated and multiplied by specific uptake rates for FCM-sorted autotrophs. In contrast to the size
fractions summarized in Table 2, preparation and sorting of FCM samples precluded the direct analysis of Phyto PN on the mass spectrometer. Therefore, Phyto PN was calculated by correcting the GF/F and >0.8 μm PN values for bacterial biomass retained on these filters. Unless noted otherwise, a retention efficiency of 50% (of bacterial biomass) was used for GF/F filters (Table 1) and 33% was used for 0.8 μm filters (Bradley, P.B. unpubl. data). In York River and Mid-Atlantic Bight samples, bacterial biomass was calculated from PN concentrations in the 0.2μm-GF/F and 0.2-0.8 μm size classes, respectively. In Chesapeake Bay and the Gulf of Mexico, bacterial abundance was converted to bacterial biomass using conversion factors of 12 fg N cell$^{-1}$ (Vrede et al., 2002) and 5 fg N cell$^{-1}$, respectively. The former was chosen to represent bacteria from a relatively nutrient-rich estuarine environment, whereas the latter reflects the lower N content of nutrient-limited oceanic bacteria (e.g. Fukuda et al., 1998). In Raunefjord, the percent of bacterial biomass retained on 0.8 μm filters was calculated separately for the control and amended mesocosms by subtracting the 0.2-0.8 μm PN from the total bacterial biomass, which was calculated from bacterial abundance using 12 fg N cell$^{-1}$. The resulting retention values, 24 ± 14% for the control mesocosm and 58 ± 21% for the amended mesocosm, were conservatively rounded to 25% and 50%, respectively, for simplicity in calculating Phyto PN as follows:

\[
\text{Phyto PN (control)} = \text{> 0.8μm PN} - \left[ \frac{(0.2 - 0.8 \mu m \text{ PN})}{3} \right] \quad (\text{Eq. 1})
\]
Phyto PN (amended) = > 0.8μm PN – (0.2 - 0.8 μm PN)  \hspace{1cm} (Eq. 2)

In other words, the proportion of bacterial biomass in the 0.8 μm versus 0.2-0.8 μm fraction is 25/75 for M1 (Eq. 1) and 50/50 for M2 (Eq. 2), and the above equations remove the bacterial biomass on 0.8 μm filters accordingly to obtain Phyto PN. The Phyto PN concentrations, less the >35 μm PN when measured (Chesapeake Bay and Raunefjord), were multiplied by V for the FCM-sorted phytoplankton to obtain absolute uptake rates for the FCM fraction. In Chesapeake Bay and Raunefjord, absolute uptake rates for total phytoplankton were calculated as the sum of the FCM and >35 μm rates.

Despite substantial evidence that GF/F filters retain at least 50% of the bacterial biomass (Table 1), as well as this author’s assessment of 0.8 μm silver filters (Bradley, P.B. unpubl. data), the assumption that these filters retain 50% and 33% of the bacterial biomass, respectively, was tested with data from the York River and Mid-Atlantic Bight. Using bacterial abundance data and PN concentrations from the relevant fractions (Table 2), the same approach described above for Raunefjord yielded bacterial retention values of 61 ± 7% for GF/F filters (York River) and 34 ± 11% for 0.8 μm filters (Mid-Atlantic Bight).

Results

_Nitrogen availability._ Ambient nutrient concentrations varied considerably within and between ecosystems. In general, however, TDN concentrations decreased from Chesapeake Bay (estuarine ecosystem) to the Mid-Atlantic Bight (coastal ecosystem) to
the Gulf of Mexico (oceanic ecosystem; Fig. 1). Although the Raunefjord is a coastal environment, it is fairly oligotrophic with a nutrient regime in the control mesocosm that most resembled that of the Gulf of Mexico. As a percent of the TDN concentrations, DON and DIN were about equal in Chesapeake Bay, but DON became increasingly dominant along the estuarine to open ocean gradient, comprising over 75% of TDN in the Raunefjord and Gulf of Mexico (Table 4). Rates of NH$_4^+$ regeneration followed the same trend between ecosystems as TDN concentrations, with highest rates in Chesapeake Bay and the lowest regeneration rates measured in the Gulf of Mexico (Fig. 1).

In Chesapeake Bay, DIN dominated the TDN pool in the upper bay and particularly in the bottom water, but was biotically removed during transport toward the mouth. As a result, the TDN pool in the lower bay was mostly composed of DON (Table 4). In the Mid-Atlantic Bight, standing stocks of DIN had been depleted from the surface layer of a stratified water column prior to the two diel experiments conducted at the Long-term Ecosystem Observatory LEO-15. However, DIN was relatively abundant in the bottom water, where the TDN pool was roughly split in thirds between NH$_4^+$, NO$_x^-$, and DON. Urea concentrations in both layers were relatively high and comprised as much as 40% of TDN. Ambient N dynamics in the Raunefjord prior to initiating the mesocosm experiments were dominated by DON (>85% of TDN). Added NO$_3^-$ was quickly removed from the amended mesocosm, at which point the composition of the TDN pool reverted back to a dominance of DON over DIN. In the Gulf of Mexico, samples taken from near the surface were virtually devoid of DIN, but NO$_x^-$ concentrations were higher in samples taken from the deep chlorophyll maximum or the bottom of the euphotic zone.
Urea, however, represented the most abundant form of identified N and comprised 10% of TDN overall.

_Nitrogen Uptake_. Total N uptake by both the phytoplankton-only (i.e. FCM-sorted) and mixed auto- and heterotrophic assemblages (i.e. phytoplankton and bacteria on GF/F and 0.8 μm filters) followed the same trend as for TDN and NH₄⁺ regeneration rates. Total N uptake rates for phytoplankton averaged 0.58 ± 0.99, 0.44 ± 0.59, 0.22 ± 0.32, and 0.04 ± 0.04 μmol N L⁻¹ h⁻¹ in Chesapeake Bay, Mid-Atlantic Bight, Raunefjord, and Gulf of Mexico samples, respectively. The York River results are not included in this comparison because uptake of only one or two of the four total substrates was measured (Table 2). The variability in measured uptake rates within each ecosystem was much greater than the differences in total N uptake between the above environments. See Chapters 2, 3, and 4 for a detailed discussion of how N uptake rates varied within these ecosystems. This chapter, on the other hand, examines: (1) how the relative uptake of N substrates varied between ecosystems, (2) which substrates were generally important to phytoplankton versus bacteria, and (3) the extent to which filters (e.g. GF/F or 0.8 μm) overestimate phytoplankton N uptake rates.

_Relative importance of N substrates_. Phytoplankton relied mostly on reduced N forms (NH₄⁺ and urea) at the four sites described here. In particular, NH₄⁺ was the dominant N substrate used by phytoplankton everywhere except the Mid-Atlantic Bight surface water, where urea was the primary N form used (Table 5). Overall, NO₃⁻ was significantly less important to phytoplankton than urea (p < 0.0001), and DFAA supplied
just 5% of the phytoplankton N demand, although on occasion these two substrates contributed substantially to total N uptake.

In Chesapeake Bay, NH$_4^+$ uptake rates exceeded those of the other substrates in all samples regardless of location, depth, or dissolved N concentrations. Nitrate uptake was greatest in the upper bay, where ambient concentrations were as high as 80 μmol N L$^{-1}$. However, DIN availability decreased rapidly toward the mouth, where urea and DFAA uptake rates were highest and supported a greater proportion of total phytoplankton N uptake.

In the DIN-depleted surface waters of the Mid-Atlantic Bight, high ambient urea concentrations supported the majority of phytoplankton N demand, followed by NH$_4^+$. Uptake of NO$_3^-$ and DFAA were relatively minor in the surface water. Concentrations of NH$_4^+$ and NO$_x^-$ in the bottom water, however, comprised about two-thirds of the TDN pool and supported the majority of phytoplankton N uptake, although urea uptake was also significant.

The addition of NO$_3^-$ to the amended mesocosm in the Raunefjord experiments had a minor effect on the relative importance of N substrates (Table 5, see also Chapter 4). Ammonium comprised 60% and 64% of total phytoplankton uptake in the amended and control mesocosms, respectively. Urea and NO$_3^-$ each contributed 15 – 25% of total uptake by phytoplankton, which relied more on urea than NO$_3^-$ in the control mesocosm, whereas NO$_3^-$ uptake was slightly higher in the amended mesocosm. Phytoplankton use of DFAA was insignificant in both enclosures.

In the oligotrophic outer shelf waters of the Gulf of Mexico, NH$_4^+$ uptake rates tended to exceed those of the other N substrates in the surface water, although urea was
nearly as important to phytoplankton N nutrition. However, in samples taken from the deep Chl maximum (approximately 50 m) or the bottom of the euphotic zone (~90 m), NO$_3^-$ was the dominant N form used by phytoplankton while NH$_4^+$ and urea comprised much less of the total N uptake than at the surface. As in other ecosystems, amino acids were a relatively unimportant source of N to phytoplankton in the Gulf of Mexico.

Filter- versus FCM-based N uptake rates. Specific and absolute N uptake rates measured from FCM-sorted phytoplankton were compared with rates measured using traditional filtration in order to assess the extent to which the latter approach overestimates phytoplankton uptake, and also to examine the use of N substrates by bacteria. With few exceptions, filter-based (e.g. GF/F and 0.8 μm) absolute uptake rates equaled or exceeded those from FCM-sorted samples (Figs. 2 and 3). On average, filter-based rates exceeded FCM phytoplankton uptake by a factor of 1.41 ± 1.50 for NH$_4^+$, 0.94 ± 1.38 for NO$_3^-$, 1.25 ± 1.24 for urea, and 2.22 ± 1.60 for DFAA. However, due to the large variation in uptake rates within and between ecosystems, the overall mean rates calculated for the GF/F- versus FCM-based datasets were not statistically different for NH$_4^+$ ($p = 0.284$), NO$_3^-$ ($p = 0.175$), or urea ($p = 0.897$), but the difference was significant for DFAA uptake rates ($p < 0.0001$). Although the results varied for each substrate, the difference in rates between the two methods tended to be smallest in Chesapeake Bay and greatest in the Mid-Atlantic Bight.

The percent contribution of individual N substrates to total uptake measured using these two approaches were relatively similar, but with some key differences that are useful in interpreting phytoplankton-bacterial dynamics. In Chesapeake Bay, NO$_3^-$
contributed more to total uptake by FCM-sorted phytoplankton than it did to uptake by the GF/F fraction, but the difference was not significant (Table 5). However, DFAA comprised a significantly greater proportion of total N uptake by the GF/F fraction in the upper bay ($p < 0.0001$). In the Mid-Atlantic Bight, the GF/F fraction used significantly more NO$_3^-$ and less urea in the bottom water than FCM-sorted phytoplankton did. In the surface water, on the other hand, the GF/F fraction relied less on urea and more on DFAA than phytoplankton alone did. In the Raunefjord experiments, the only significant difference between the two fractions was for DFAA, which comprised a significantly larger percentage of total uptake in the GF/F fraction. Finally, differences in the relative importance of these N forms to FCM and GF/F uptake in the Gulf of Mexico were more substantial than in any other ecosystem, although the results were quite variable and based on a limited number of samples. Nonetheless, FCM-sorted phytoplankton relied primarily on NH$_4^+$ and NO$_3^-$, whereas urea contributed the greatest to N uptake by the GF/F fraction, suggesting substantial use by bacteria.

\textit{Bacterial N uptake.} Nitrogen uptake rates were occasionally measured for the bacterial size class (0.2-0.8 $\mu$m, 0.2 $\mu$m-GF/F), not only to quantify bacterial N use, but also to examine whether bacterial retention on GF/F or 0.8 $\mu$m silver filters leads to a significant underestimation of bacterial N uptake using this size fractionation approach. To this end, bacterial abundance was converted to biomass using a cellular N content of 12 fg N cell$^{-1}$ (Vrede et al., 2002), and this biomass was multiplied by the specific uptake rates measured for the bacterial size class to obtain absolute N uptake rates for the whole bacterial community. The bacterial contribution to total uptake of each substrate was then
calculated by dividing these bacterial rates by the summed uptake of bacteria and phytoplankton (i.e. FCM-based rates).

Values for the bacterial contribution to total uptake were calculated in the York River, Mid-Atlantic Bight, and Raunefjord. In the York River, bacterial uptake comprised $10 \pm 1\%$ of total $\text{NH}_4^+$ uptake and $16 \pm 2\%$ of total urea uptake. Due to a lack of bacterial abundance data, the bacterial contribution could not be calculated for other York River samples (e.g. $\text{NO}_3^-$). In the Mid-Atlantic Bight, results varied between the surface and bottom mixed layers. Bacterial uptake of $\text{NH}_4^+$ and $\text{NO}_3^-$ in the surface water represented $28 \pm 13\%$ and $21 \pm 12\%$ of total uptake of these substrates, respectively. In the bottom water, however, bacteria were responsible for $56 \pm 16\%$ of total $\text{NH}_4^+$ uptake and $52 \pm 20\%$ of total $\text{NO}_3^-$ uptake. In the Raunefjord experiments, results varied between the control and amended mesocosms due to significant differences in phytoplankton biomass, which is a component of absolute uptake rates. In the control mesocosm, bacterial uptake comprised $42 \pm 12\%$, $32 \pm 12\%$, $49 \pm 14\%$, and $80 \pm 9\%$ of total $\text{NH}_4^+$, $\text{NO}_3^-$, urea, and DFAA uptake, respectively. In the amended mesocosm, however, the bacterial contribution to total uptake was $19 \pm 17\%$ for $\text{NH}_4^+$, $14 \pm 12\%$ for $\text{NO}_3^-$, $20 \pm 15\%$ for urea, and $58 \pm 27\%$ for DFAA.

Discussion

The decreasing trend in TDN concentrations from Chesapeake Bay to the Gulf of Mexico exemplifies the transition that characterizes an estuarine to open ocean gradient in allochthonous nutrient supply. Estuaries receive large N inputs from diverse sources,
including agricultural and urban runoff, sewage overflow, wastewater discharge, riverine delivery of distant sources, and atmospheric deposition. Although the relatively shallow depth of estuaries enhances benthic-pelagic coupling and nutrient remineralization, allochthonous sources typically comprise the majority of supplied N (e.g. Cloern 2001). Coastal/continental shelf ecosystems are heterogeneous, dynamic environments with variable rates and magnitude of nutrient supply. Terrestrially-derived N sources still play a critical role in the productivity of shelf ecosystems, but new N is introduced from offshore as well and autochthonous sources become increasingly important. The open ocean, however, receives little allochthonous N supply, with atmospheric deposition being perhaps the greatest source of external N to these systems (Duce et al., 2008). The decrease in TDN concentrations from Chesapeake Bay to the Mid-Atlantic Bight and Gulf of Mexico follows the same trend reported by Bronk (2002), who compiled an extensive list of published TDN and DON data from estuarine, coastal, and oceanic waters. However, in the research presented here, the percent contribution of DON to the TDN pool increased from shore to sea, whereas the reverse trend was reported by Bronk (2002).

Although the Raunefjord is technically a coastal ecosystem, N concentrations and composition at this location most resembled an oceanic environment (Table 4). For example, DON concentrations measured prior to initiating the Raunefjord experiments averaged 5.5 ± 0.7 μmol N L⁻¹ h⁻¹, which compares well to that reported by Bronk (2002) for oceanic surface waters (5.7 ± 2.0 μmol N L⁻¹ h⁻¹). Whereas coastal waters in the southern bight of the North Sea (e.g. Wadden Sea) are shallow and heavily impacted by nutrient loads, coastal fjords of western Norway, such as Raunefjord, are deep (up to
200 m), have shorter retention times, and are not as affected by nutrient loading via riverine delivery and terrestrial runoff. Furthermore, the relatively deep mixing depth and short growing period limit the magnitude of phytoplankton biomass accumulation, relative to Chesapeake Bay or the Mid-Atlantic Bight, for example.

Changes in overall productivity between ecosystems, as indicated by total N uptake rates, followed the same decreasing trend from estuary to open ocean that is discussed above, but were overshadowed by the extensive variability within each system. For example, uptake rates in Chesapeake Bay were occasionally even less than those measured in the Gulf of Mexico, but were much higher on the whole. As an indication of how dynamic marine ecosystems can be, the range in NH$_4^+$ uptake rates measured in Chesapeake Bay spanned over two orders of magnitude (0.05 - 6.71 μmol N L$^{-1}$ h$^{-1}$). Therefore, generalizations about differences between marine environments must be interpreted with an appreciation for the variability that characterizes individual ecosystems.

**Relative importance of N substrates to phytoplankton.** On average, NH$_4^+$ and urea comprised 83% of total measured N uptake across the ecosystems described here. The importance of these reduced N forms to phytoplankton N nutrition is not surprising given that the studies were conducted predominantly during N-limited periods with low NO$_3^-$ availability. Nitrate tends to play the greatest role in phytoplankton N uptake during the spring (Berg et al., 2003; Tungaraza et al., 2003; Twomey et al., 2005), whereas NH$_4^+$ and urea comprise the bulk of N uptake during the summer (Glibert et al., 1991; Bronk et al., 1998; Andersson et al., 2006).
Although urea is increasingly being recognized as an important N source to phytoplankton, its role in supporting primary producers has traditionally been underappreciated and perhaps underestimated as well. In the research described here, urea contributed 29 ± 26% of total phytoplankton N uptake, compared to the 19 ± 15% reported by Bronk (2002) for a range of marine systems, and also exceeded the average contribution of NO$_3^-$ (12 ± 16%; $p < 0.0001$). When relatively abundant (e.g. Mid-Atlantic Bight surface water), urea uptake represented as much as 83% of total measured N uptake. Furthermore, urea uptake rates presented here, like the overwhelming majority of published rates, were not corrected for isotope dilution caused by urea regeneration and therefore may underestimate the importance of urea to phytoplankton N nutrition.

There are multiple pathways by which phytoplankton can use dissolved free and combined (e.g. oligopeptides) amino acids, including active transport, amino acid oxidation, and peptide hydrolysis (Bronk et al., 2007 and references therein). Despite limited research, the importance of this organic N substrate to autotrophs has been demonstrated in estuarine and coastal waters (Berg et al., 2003; Veuger et al., 2004; Andersson et al., 2006), and its role in sustaining certain harmful algal bloom species has been shown as well (Berg et al., 1997; Mulholland et al., 2002). Bronk (2002) provided a value of 23 ± 24% of total uptake as DFAA, but this value includes uptake rates measured for bacteria only, as well as other studies in which bacterial DFAA use was unavoidably captured on filters used to retain phytoplankton (e.g. GF/F). Amino acid uptake by phytoplankton is not routinely measured in $^{15}$N uptake studies, and reported values for the percent contribution of DFAA to total uptake may be further skewed by studies that examine this process under circumstances in which it is expected to occur
significantly. In fact, DFAA comprised as much as 23% of total measured N uptake by FCM-sorted phytoplankton in this study, but in general represented a minor N source (5%) to phytoplankton N nutrition.

To examine the relationship between phytoplankton N nutrition and changes in the TDN pool composition, data were aggregated into “High N input” (i.e. eutrophic) and “Low N input” (oligotrophic) ecosystems as described in Table 6. As the contribution of DON to TDN increased from the upper estuary to the coastal and open ocean, NH$_4^+$ and NO$_3^-$ contributed significantly less to total phytoplankton uptake ($p < 0.01$ and $p < 0.05$, respectively), while urea comprised more of total phytoplankton uptake in the Low N ecosystems (Table 6). Furthermore, within the High N ecosystems, the contribution of NO$_3^-$ to total uptake was significantly higher for FCM-sorted phytoplankton than for the mixed phytoplankton-bacteria assemblage ($p < 0.05$), whereas the reverse was true for DFAA in both High N and Low N ecosystems ($p < 0.0001$ for both). These results indicate that phytoplankton exploited NO$_3^-$ when it was available, were able to compete effectively with bacteria for limited DIN in various marine ecosystems, and that urea became an important N source to phytoplankton once DIN availability had declined.

*Controls on N uptake and relative importance of N substrates.* The presence of ambient NH$_4^+$ has been found to inhibit NO$_3^-$ uptake by phytoplankton, although the mechanism whereby this occurs is quite variable in the marine environment (Dortch, 1990; Cochlan and Bronk, 2003). The results presented here would suggest that NH$_4^+$, which typically dominated total N uptake, inhibited NO$_3^-$ uptake rates, which were generally rather low. However, there was no correlation between NO$_3^-$ uptake (either
specific uptake rates or as a percent of total uptake) and either ambient NH$_4^+$ concentrations or NH$_4^+$ regeneration rates. Dortch (1990) cautioned that care must be taken to distinguish between inhibition of NO$_3^-$ uptake by NH$_4^+$ and preference for NH$_4^+$ over NO$_3^-$. Clearly, the importance of NH$_4^+$, and relative unimportance of NO$_3^-$, to phytoplankton during the N-limited conditions predominantly sampled here are the result of physiological affinity (i.e. preference) for the reduced N substrate rather than NO$_3^-$ inhibition. Even in samples with relatively high NO$_3^-$ concentrations (e.g. upper Chesapeake Bay, amended Raunefjord mesocosm), NH$_4^+$ uptake rates exceeded those of NO$_3^-$. The phytoplankton community is probably well-adapted to assimilate NH$_4^+$ during N-limited conditions in order to fully exploit this substrate as it becomes available via regenerative processes, especially considering the energetic expense of NO$_3^-$ assimilation.

This apparent preference for NH$_4^+$ was also evident in the disproportionality between NH$_4^+$ uptake (as a percent of the total) and relative abundance (i.e. as a percent of TDN). Overall, NH$_4^+$ comprised 54% of total measured N uptake, but only 12% of the TDN pool. Despite this apparent disconnect between NH$_4^+$ uptake and availability, the relative importance of NH$_4^+$ uptake increased as NH$_4^+$ comprised more of the TDN pool, although the correlation between these two parameters was relatively weak (Pearson’s $r^2 = 0.28$, $p < 0.0001$). Interestingly, the percent of total uptake as NH$_4^+$ decreased when urea was more abundant, both in terms of absolute concentrations (Pearson’s $r^2 = 0.37$, $p < 0.0001$) and as a percent of TDN (Pearson’s $r^2 = 0.47$, $p < 0.0001$). This may suggest that autotrophic preference for NH$_4^+$ does not govern uptake dynamics alone, but rather that availability of various N sources plays an important role. Alternatively, these findings may be the result of specific phytoplankton taxa expressing a stronger affinity
for urea than NH$_4^+$. In support of the former hypothesis, specific urea uptake rates for phytoplankton were positively correlated with both absolute and relative urea concentrations (Pearson’s $r^2 = 0.46$ and 0.69, respectively, $p < 0.0001$ for both relationships; Fig. 4A). Also, urea uptake rates increased significantly with the ratio of DON:DIN (Pearson’s $r^2 = 0.78$, $p < 0.0001$; Fig. 5A). This finding suggests that the relative availability of DON versus DIN substrates is of some significance to phytoplankton, and that increased use of urea (and potentially other organic N forms) by phytoplankton is triggered as DIN is depleted. This was clearly the case in Chesapeake Bay, where urea and DFAA were much more important to total uptake in the lower bay, once the majority of ambient DIN had been removed in transit.

_Bacterial N use._ Bacteria have been thought to rely predominantly on amino acids (free and combined) and NH$_4^+$ to support N demand (Kirchman, 2000). Nitrate, while known to contribute significantly to bacterial N uptake on occasion (Kroer et al., 1994; Kirchman and Wheeler, 1998; Allen et al., 2002), is generally not considered as an important N source to bacteria. Bacterial assimilation of urea has traditionally been viewed as negligible (Tamminen and Irmisch, 1996; Kirchman, 2000), although more recent research has shown that this may not always be the case (Jørgensen et al., 1999; Jørgensen, 2006; Sanderson et al., 2008). Sanderson et al. (2008), for example, found that urea contributed roughly 40 – 60% of total measured N uptake by the bacterial size class (0.2–0.8 μm). Direct and indirect evidence from the ecosystems described here also suggest that bacteria rely on urea for N nutrition, and that bacterial uptake of urea may represent a significant portion of total urea uptake.
Measurement of N uptake by the bacterial size class provides the most direct assessment of the relative importance of N substrates to bacteria. However, this is not possible for every ecosystem presented here because bacterial uptake of all four substrates was only measured in the Raunefjord experiments and to a limited extent in the Gulf of Mexico. Nonetheless, in the Raunefjord, most (~56%) of the total N uptake by the 0.2–0.8 μm fraction was in the form of NH$_4^+$. Urea, however, was more important than both NO$_3^-$ and DFAA, which each contributed about 11–12% of total bacterial uptake, compared to approximately 20% for urea.

Calculated values for the percent contribution of bacteria to total N uptake indicate that bacteria were responsible for a significant fraction of measured N uptake. The finding that 80% of DFAA use in the Raunefjord control mesocosm is not surprising given bacterial affinity for amino acids. Similarly, large contributions that bacteria made to total NH$_4^+$ uptake (see above) agree with conventional views of the importance of this DIN source (Kirchman, 2000), particularly during N-limited periods such as those sampled here. However, bacteria also contributed over 50% of total NO$_3^-$ uptake in Mid-Atlantic Bight bottom water and 32% in the control mesocosm at Raunefjord. Perhaps most importantly though, 16%, 20%, and 49% of total urea uptake in the York River, amended mesocosm (Raunefjord), and control mesocosm, respectively, was by bacteria. This result is in stark contrast to traditional dogma regarding bacterial N uptake, and is examined in more detail below.

Additional, albeit indirect, evidence for significant urea uptake by bacteria exists in several data sources. First, the difference between the relative importance of N substrates to FCM-sorted versus filter-based uptake indicates the role of bacteria in
uptake measured using the traditional filtration approach. Although urea did not contribute more to total uptake when bacteria were present, the fact that this substrate contributed equally to the two fractions suggests that bacterial urea use was significant; otherwise, the contribution of urea to total uptake would have decreased in samples containing bacteria. Moreover, the correlation between urea uptake and availability was equally significant when bacteria were present (i.e. on GF/F and 0.8 μm filters) as for phytoplankton alone (see p. 21). Specific urea uptake rates increased with ambient urea concentrations and also with urea as a percent of TDN (Pearson’s $r^2 = 0.73, p < 0.0001$; Fig. 4B). A lack of urea uptake by bacteria retained on filters would have confounded this relationship and resulted in a weaker correlation in these data relative to those of FCM-sorted phytoplankton. The inverse correlation between NH$_4^+$ uptake (as a percent of the total) and urea concentrations was stronger when bacteria were present (Pearson’s $r^2 = 0.41, p < 0.0001$) than for phytoplankton alone (Pearson’s $r^2 = 0.37, p < 0.0001$), and the same was true for relative NH$_4^+$ uptake and relative urea availability ($r^2 = 0.55$ versus 0.47). When combined with a significant positive correlation between urea uptake and the ratio of DON:DIN (Pearson’s $r^2 = 0.70, p < 0.0001$; Fig. 5B), these results suggest that bacteria prefer NH$_4^+$ as a supplemental N source to DOM oxidation, but that urea is relied upon increasingly as DIN availability declines.

*Overestimation of phytoplankton N uptake by traditional filtration.* As hypothesized, retention of bacterial biomass on GF/F and silver filters resulted in an overestimation of phytoplankton PN, and consequently an overestimation of absolute N uptake by phytoplankton. However, overestimation of phytoplankton uptake rates were
ostensibly enhanced by the fact that retained bacteria were actively using various N sources, and the difference in the degree to which filters overestimate phytoplankton uptake reflect the relative importance of these substrates to bacteria. For example, the difference between FCM-sorted and filter-based uptake rates was largest for DFAA, followed by NH$_4^+$, urea, and lastly NO$_3^-$, and this hierarchy generally follows the conventional view, although most researchers would probably expect bacterial NO$_3^-$ use to exceed that of urea.

There are significant implications regarding the overestimation of phytoplankton uptake (and underestimation of uptake by the bacterial size class) by GF/F filters. Measurements of new production in the oceans are used in global carbon models to estimate the flux of atmospheric CO$_2$ into the oceans, and its subsequent removal into the deep ocean. If phytoplankton uptake of regenerated N forms (e.g. NH$_4^+$ and urea) is overestimated, these values for new production and the $f$-ratio will be underestimated. There is a substantial difference in energy transfer to higher trophic levels depending on the relative importance of phytoplankton versus bacterial N use; in other words, if the proportion of N used by bacteria is underestimated, then more energy is being inefficiently processed by the microbial loop, relative to a more direct pathway from larger phytoplankton to grazers and fish. In coastal ecosystems, the relative availability of N substrates may affect phytoplankton community structure and overall ecosystem function and health. Accurate knowledge of the microbial fate of N in coastal ecosystems may assist resource managers target nutrient load reductions more efficiently within the watershed.
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References


Table 1. Percent of bacterial abundance retained by GF/F filters, from studies representing numerous diverse ecosystems.

<table>
<thead>
<tr>
<th>Location</th>
<th>Retention efficiency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long Island Sound</td>
<td>43 – 65%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(Lee and Fuhrman, 1987)</td>
</tr>
<tr>
<td>subarctic Pacific</td>
<td>50 – 60%&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(Kirchman et al., 1989)</td>
</tr>
<tr>
<td>Chesapeake Bay</td>
<td>50%</td>
<td>(Glibert et al., 1995)</td>
</tr>
<tr>
<td>Long Island Sound</td>
<td>57 – 65%</td>
<td>(Lee et al., 1995)</td>
</tr>
<tr>
<td>Antarctic coastal waters</td>
<td>59 – 65%</td>
<td>(Lee et al., 1995)</td>
</tr>
<tr>
<td>NW Mediterranean Sea (coastal)</td>
<td>78 – 93%</td>
<td>(Gasol and Morán, 1999)</td>
</tr>
<tr>
<td>SW Mediterranean Sea (oceanic)</td>
<td>68 – 79%</td>
<td>(Gasol and Morán, 1999)</td>
</tr>
<tr>
<td>Atlantic (estuarine)</td>
<td>70%</td>
<td>(Gasol and Morán, 1999)</td>
</tr>
<tr>
<td>Atlantic (coastal)</td>
<td>67%</td>
<td>(Gasol and Morán, 1999)</td>
</tr>
<tr>
<td>Atlantic (oceanic)</td>
<td>29%</td>
<td>(Gasol and Morán, 1999)</td>
</tr>
<tr>
<td>Gulf of Riga (Baltic Sea)</td>
<td>32 – 69%</td>
<td>(Berg et al., 2001)</td>
</tr>
<tr>
<td>York River (Chesapeake Bay)</td>
<td>53 – 71%</td>
<td>(Bradley, P.B. unpubl. data)</td>
</tr>
</tbody>
</table>

<sup>a</sup> when filtering natural bacterial culture only

<sup>b</sup> when filtering the <1.0 μm fraction only
Table 2. Sampling locations, dates, depths sampled, and a brief summary of experimental setup. Substrates include ammonium (N4), nitrate (N3), urea (U), and a mixture of dissolved free amino acids (AA).

<table>
<thead>
<tr>
<th>Location</th>
<th>Dates sampled</th>
<th>Depth(s) sampled (m)</th>
<th>$^{15}$N substrates</th>
<th>0.2 μm–GF/F</th>
<th>GF/F</th>
<th>0.2–0.8 μm</th>
<th>&gt;0.8 μm</th>
<th>FCM</th>
<th>&gt;35 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chesapeake Bay</td>
<td>28–29 Jul 2004</td>
<td>2, ~9$^c$</td>
<td>N4, N3, U, AA</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 Aug – 1 Sep 2004</td>
<td>2, ~9$^c$</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>York River, Virginia</td>
<td>26 Mar 2004</td>
<td>1</td>
<td>N3</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>28 Jun 2004</td>
<td>1</td>
<td>N4</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>04 Mar 2005</td>
<td>1</td>
<td>N3</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>21 Mar 2005</td>
<td>1</td>
<td>N3</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>09 Oct 2006</td>
<td>1</td>
<td>N4, U</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mid-Atlantic Bight$^d$</td>
<td>20–21 Jul 2002</td>
<td>1 and 14$^d$</td>
<td>N4, N3, U, AA</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22–23 Jul 2002</td>
<td>1 and 14$^d$</td>
<td>N4, N3, U, AA</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raunefjord, Norway</td>
<td>1 – 27 Apr 2005</td>
<td>2</td>
<td>N4, N3, U, AA</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gulf of Mexico$^f$</td>
<td>14 Jul 2002</td>
<td>2, ~50$^g$, ~90$^h$</td>
<td>N4, N3, U, AA</td>
<td>X</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

$^a$ FCM samples were prescreened with a 35 μm mesh after incubating with $^{15}$N and before concentration

$^b$ FCM samples were filtered through a 53 μm mesh before incubating with $^{15}$N substrates

$^c$ Below the pycnocline

$^d$ At the Long-term Ecosystem Observatory LEO-15

$^e$ Just above the seafloor

$^f$ On the West Florida shelf

$^g$ Chlorophyll maximum

$^h$ Chlorophyll maximum
Table 3. Chlorophyll *a* (Chl *a*) concentrations (µg L⁻¹) in whole (unconcentrated) sample, samples concentrated from 100, 200, or 300 ml to a final volume of 10 ml using a 0.2 µm Supor filter, and the concentration of Chl *a* left on the Supor filter after concentration. Water was collected from the surface (<1 m) of the York River, Virginia. Mean ± 1 SD (*n* = 3 per volume filtered) is given for both Chl *a* concentration and percent of whole Chl *a* value.

<table>
<thead>
<tr>
<th></th>
<th>Original volume</th>
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<tbody>
<tr>
<td></td>
<td>100 ml</td>
</tr>
<tr>
<td>Whole sample</td>
<td>5.1 ± 0.0</td>
</tr>
<tr>
<td>Concentrated sample</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>(96 ± 3%)</td>
<td>(94 ± 3%)</td>
</tr>
<tr>
<td>0.2 µm Supor filter</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>(3 ± 1%)</td>
<td>(4 ± 1%)</td>
</tr>
</tbody>
</table>
Table 4. Total dissolved nitrogen (TDN) concentrations (μmol N L⁻¹) and the percent contribution of ammonium (NH₄⁺), nitrate + nitrite (NOₓ⁻), urea, dissolved free amino acids (DFAA), and undefined DON (un-DON; DON other than urea and DFAA) to TDN. Mean ± 1 SD and (min – max) are given.

<table>
<thead>
<tr>
<th></th>
<th>[TDN]</th>
<th>NH₄⁺</th>
<th>NO₃⁻</th>
<th>Urea</th>
<th>DFAA</th>
<th>un-DON</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chesapeake Bay (upper)</td>
<td>50.3 ± 20.1</td>
<td>33 ± 23%</td>
<td>38 ± 30%</td>
<td>2 ± 1%</td>
<td>0.2 ± 0.2%</td>
<td>27 ± 14%</td>
</tr>
<tr>
<td></td>
<td>(34.5 – 95.9)</td>
<td>(9 – 70)</td>
<td>(5 – 88)</td>
<td>(1 – 3)</td>
<td>(0 – 0.4)</td>
<td>(3 – 40)</td>
</tr>
<tr>
<td>Chesapeake Bay (middle)</td>
<td>25.3 ± 4.5</td>
<td>22 ± 28%</td>
<td>15 ± 11%</td>
<td>3 ± 1%</td>
<td>0.8 ± 0.5%</td>
<td>59 ± 17%</td>
</tr>
<tr>
<td></td>
<td>(19.6 – 35.4)</td>
<td>(2 – 68)</td>
<td>(1 – 32)</td>
<td>(1 – 5)</td>
<td>(0.1 – 1.4)</td>
<td>(30 – 76)</td>
</tr>
<tr>
<td>Chesapeake Bay (lower)</td>
<td>16.7 ± 2.6</td>
<td>9 ± 1%</td>
<td>7 ± 7%</td>
<td>3 ± 0%</td>
<td>1.4 ± 0.1%</td>
<td>80 ± 6%</td>
</tr>
<tr>
<td></td>
<td>(13.6 – 19.2)</td>
<td>(8 – 9)</td>
<td>(2 – 12)</td>
<td>(3.4 – 3.5)</td>
<td>(1.3 – 1.5)</td>
<td>(76 – 84)</td>
</tr>
<tr>
<td>Mid-Atlantic Bight (bottom)</td>
<td>12.6 ± 1.1</td>
<td>30 ± 5%</td>
<td>29 ± 4%</td>
<td>14 ± 1%</td>
<td>1 ± 1%</td>
<td>25 ± 6%</td>
</tr>
<tr>
<td></td>
<td>(6.1 – 11.7)</td>
<td>(22 – 37)</td>
<td>(23 – 33)</td>
<td>(13 – 18)</td>
<td>(1 – 3)</td>
<td>(18 – 35)</td>
</tr>
<tr>
<td>Mid-Atlantic Bight (surface)</td>
<td>8.0 ± 1.7</td>
<td>0.1 ± 0.3%</td>
<td>0.2 ± 0.3%</td>
<td>26 ± 6%</td>
<td>3 ± 2%</td>
<td>71 ± 5%</td>
</tr>
<tr>
<td></td>
<td>(10.2 – 13.9)</td>
<td>(0 – 0.9)</td>
<td>(0 – 0.9)</td>
<td>(20 – 40)</td>
<td>(1 – 6)</td>
<td>(59 – 75)</td>
</tr>
<tr>
<td>Raunefjord, Norway (amended)</td>
<td>9.4 ± 5.7</td>
<td>4 ± 3%</td>
<td>1 ± 2% a</td>
<td>7 ± 1%</td>
<td>3 ± 2%</td>
<td>83 ± 6%</td>
</tr>
<tr>
<td></td>
<td>(5.8 – 21.7)</td>
<td>(1 – 10)</td>
<td>(0 – 4)</td>
<td>(5 – 11)</td>
<td>(2 – 10)</td>
<td>(72 – 90)</td>
</tr>
<tr>
<td>Raunefjord, Norway (control)</td>
<td>5.4 ± 0.7</td>
<td>5 ± 5%</td>
<td>19 ± 31%</td>
<td>5 ± 2%</td>
<td>4 ± 2%</td>
<td>67 ± 24%</td>
</tr>
<tr>
<td></td>
<td>(4.8 – 7.2)</td>
<td>(0 – 16)</td>
<td>(0 – 73)</td>
<td>(2 – 10)</td>
<td>(1 – 8)</td>
<td>(24 – 88)</td>
</tr>
<tr>
<td>Gulf of Mexico</td>
<td>5.8 ± 0.4</td>
<td>7 ± 13%</td>
<td>10 ± 10%</td>
<td>9 ± 3%</td>
<td>2 ± 1%</td>
<td>71 ± 14%</td>
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<tr>
<td></td>
<td>(5.3 – 6.6)</td>
<td>(0 – 30)</td>
<td>(2 – 25)</td>
<td>(6 – 13)</td>
<td>(1 – 3)</td>
<td>(56 – 88)</td>
</tr>
<tr>
<td>Average</td>
<td>50.3 ± 20.1</td>
<td>12 ± 17%</td>
<td>14 ± 20%</td>
<td>10 ± 9%</td>
<td>2 ± 2%</td>
<td>61 ± 24%</td>
</tr>
</tbody>
</table>

*Includes NO₃⁻ addition of 16 μmol N L⁻¹ to the amended mesocosm
Table 5. Percent contribution of ammonium ($\text{NH}_4^+$), nitrate ($\text{NO}_3^-$), urea, and dissolved free amino acids (DFAA) to total measured N uptake by phytoplankton-only (e.g. FCM) and by phytoplankton and bacteria retained on GF/F and 0.8 μm filters. Mean ± 1 SD and (min – max) are given for each ecosystem. Asterisks indicate whether the contribution of each substrate was significantly different between fractions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

<table>
<thead>
<tr>
<th>Ecosystem</th>
<th>Phytoplankton</th>
<th>Phytoplankton + Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{NH}_4^+$</td>
<td>$\text{NO}_3^-$</td>
</tr>
<tr>
<td>Chesapeake Bay (upper)</td>
<td>72 ± 24%</td>
<td>19 ± 23%</td>
</tr>
<tr>
<td></td>
<td>(29 - 93)</td>
<td>(0 - 66)</td>
</tr>
<tr>
<td>Chesapeake Bay (middle)</td>
<td>80 ± 19%</td>
<td>6 ± 8%</td>
</tr>
<tr>
<td></td>
<td>(45 - 98)</td>
<td>(1 - 5)</td>
</tr>
<tr>
<td>Chesapeake Bay (lower)</td>
<td>68 ± 11%</td>
<td>3 ± 2%</td>
</tr>
<tr>
<td></td>
<td>(52 - 75)</td>
<td>(1 - 5)</td>
</tr>
<tr>
<td>Mid-Atlantic Bight (bottom)</td>
<td>53 ± 12%</td>
<td>11 ± 8%</td>
</tr>
<tr>
<td></td>
<td>(31 - 68)</td>
<td>(2 - 29)</td>
</tr>
<tr>
<td>Mid-Atlantic Bight (surface)</td>
<td>18 ± 7%</td>
<td>5 ± 1%</td>
</tr>
<tr>
<td></td>
<td>(9 - 32)</td>
<td>(3 - 7)</td>
</tr>
<tr>
<td>Raunefjord, Norway (amended)</td>
<td>60 ± 22%</td>
<td>23 ± 20%</td>
</tr>
<tr>
<td></td>
<td>(15 - 86)</td>
<td>(4 - 70)</td>
</tr>
<tr>
<td>Raunefjord, Norway (control)</td>
<td>64 ± 23%</td>
<td>16 ± 21%</td>
</tr>
<tr>
<td></td>
<td>(15 - 88)</td>
<td>(3 - 70)</td>
</tr>
<tr>
<td>Gulf of Mexico</td>
<td>44 ± 33%</td>
<td>19 ± 26%</td>
</tr>
<tr>
<td></td>
<td>(9 - 91)</td>
<td>(1 - 67)</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>54 ± 28%</td>
<td>12 ± 16%</td>
</tr>
</tbody>
</table>
Table 6. Percent contribution of ammonium (NH$_4^+$), nitrate (NO$_3^-$), urea, and dissolved free amino acids (DFAA) to total measured N uptake by phytoplankton (e.g. FCM) and by phytoplankton + bacteria (e.g. GF/F and 0.8 μm size fractions). Data were aggregated into High N Input (i.e. Eutrophic) and Low N Input (i.e. Oligotrophic) categories according to TDN concentration. Mean ± 1 SD and (min – max) are given for each ecosystem.

<table>
<thead>
<tr>
<th></th>
<th>Phytoplankton</th>
<th></th>
<th>Phytoplankton + Bacteria</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NH$_4^+$</td>
<td>NO$_3^-$</td>
<td>Urea</td>
<td>DFAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High N Input$^a$</td>
<td>68 ± 20%</td>
<td>18 ± 17%</td>
<td>9 ± 7%</td>
<td>5 ± 5%</td>
</tr>
<tr>
<td>(Eutrophic)</td>
<td>(29 - 93)</td>
<td>(0 - 66)</td>
<td>(1 - 25)</td>
<td>(0 - 19)</td>
</tr>
<tr>
<td>Low N Input$^b$</td>
<td>48 ± 29%</td>
<td>10 ± 15%</td>
<td>37 ± 27%</td>
<td>5 ± 5%</td>
</tr>
<tr>
<td>(Oligotrophic)</td>
<td>(9 - 98)</td>
<td>(0 - 70)</td>
<td>(1 - 83)</td>
<td>(0 - 24)</td>
</tr>
<tr>
<td></td>
<td>66 ± 18%</td>
<td>9 ± 9%</td>
<td>11 ± 10%</td>
<td>14 ± 8%</td>
</tr>
<tr>
<td></td>
<td>(29 - 93)</td>
<td>(0 - 30)</td>
<td>(1 - 39)</td>
<td>(1 - 35)</td>
</tr>
<tr>
<td></td>
<td>44 ± 24%</td>
<td>10 ± 10%</td>
<td>36 ± 27%</td>
<td>10 ± 7%</td>
</tr>
<tr>
<td></td>
<td>(7 - 96)</td>
<td>(0 - 37)</td>
<td>(0 - 86)</td>
<td>(1 - 38)</td>
</tr>
</tbody>
</table>

$^a$ Upper half of Chesapeake Bay and Raunefjord amended mesocosm

$^b$ Lower half of Chesapeake Bay, Mid-Atlantic Bight, Raunefjord control mesocosm, and Gulf of Mexico
Figure 1. Variations in (A) total dissolved nitrogen (TDN) concentrations and (B) ammonium (NH$_4^+$) regeneration rates across the five ecosystems studied. Concentrations of TDN were not determined (n.d.) in the York River. The mean ± 1 SD and (min - max) are shown for each ecosystem. Undefined DON (un-DON) represents DON other than urea and dissolved free amino acids (DFAA).
Figure 2. Absolute uptake rates of (A) ammonium and (B) nitrate, measured using flow cytometric sorting of autotrophic cells versus filtration with GF/F or 0.8 µm silver filters. Note the difference in log scale between the plots. The equations for the linear regressions are given, and the dotted lines provide a 1:1 slope reference.
Figure 3. Absolute uptake rates of (A) urea and (B) dissolved free amino acids, measured using flow cytometric sorting of autotrophic cells versus filtration with GF/F or 0.8 μm silver filters. Note the difference in log scale between the plots. The equations for the linear regressions and coefficient of correlation are given, and the dotted lines provide a 1:1 slope reference.
Figure 4. Correlation between relative urea availability (as a percent of total dissolved nitrogen) and specific urea uptake rates for (A) phytoplankton only and (B) for a mixed assemblage of phytoplankton and bacteria. The equations for the linear regression and coefficient of correlation are given. Error bars represent ± 1 standard deviation of the mean (of duplicate incubations).
Figure 5. Correlation between the ratio of DON to DIN and specific urea uptake rates for (A) phytoplankton only and (B) for a mixed assemblage of phytoplankton and bacteria on GF/F and 0.8 μm filters. The equations for the linear regression and coefficient of correlation are given. Error bars represent ± 1 standard deviation of the mean (of duplicate incubations).
CHAPTER 6

CONCLUSION
The goals of this research were to: (1) quantify the uptake of DIN and DON by phytoplankton in several marine ecosystems with varying nutrient regimes; (2) use size-fractionated and FCM-sorted uptake results to quantitatively and qualitatively describe bacterial N use and the contribution of bacteria to total N uptake; (3) apply FCM-sorted N uptake rates in assessing the extent to which traditional filtration overestimates phytoplankton N uptake; and (4) evaluate the environmental factors influencing the relative uptake of various N forms by phytoplankton and bacteria. This chapter summarizes the principal findings regarding these research objectives as they apply to the three ecosystems described in Chapters 2, 3, and 4. In addition, data from these three sites and two others (York River and Gulf of Mexico) are synthesized into an overall description of how phytoplankton and bacteria use N resources in various environments and the accuracy of the methods used to quantify these phenomena.

Chesapeake Bay is a highly dynamic system characterized by considerable temporal and spatial variability. For example, inputs of freshwater, nutrients, and sediment vary substantially during the year and also throughout the bay as a result of biotic and abiotic processes. This study sought to examine the changes in availability and uptake of N sources by phytoplankton and bacteria along the main axis of Chesapeake Bay during late summer. Concentrations of DIN were relatively high in the upper bay, but decreased exponentially with distance south as these N forms were used by phytoplankton and bacteria. In most temperate marine ecosystems, standing stocks of DIN are depleted by late summer due to biotic uptake (Valiela, 1995). However, N loads to Chesapeake Bay are substantial, and ambient concentrations of NH$_4^+$ and NO$_3^-$ remain
relatively high in the upper bay during summer. Furthermore, most N delivered to the Bay is inorganic in composition, but N exported at the mouth consists mostly of organic N forms due to biotic transformations en route (e.g. planktonic DIN uptake and subsequent release of organic N via exudation and grazing, Kemp et al., 2005). Such was the case during this study, as NH$_4^+$ and NO$_3^-$ concentrations decreased exponentially southward and the contribution of DIN to TDN decreased from 76% at the northernmost station to 11% near the mouth. Accordingly, the ratio of DON:DIN was strongly correlated with salinity ($r^2 = 0.94$, $p < 0.01$). Urea concentrations were highest in the upper bay, but did not exhibit any clear spatial trends. Concentrations of DFAA, on the other hand, increased toward the bay mouth.

Despite such distinct transitions in the absolute and relative availability of N resources, NH$_4^+$ was the dominant N form used throughout the Bay, and NH$_4^+$ uptake was not correlated with either salinity, ambient NH$_4^+$ concentrations, or NH$_4^+$ regeneration rates. Nonetheless, there were clear spatial trends in the uptake of other N substrates. For example, phytoplankton uptake$^1$ of NO$_3^-$ decreased toward the bay mouth and was significantly correlated with ambient NO$_3^-$ concentrations ($r^2 = 0.72; p < 0.05$). Nitrate uptake by larger phytoplankton (>35 µm) in particular was strongly related to availability ($r^2 = 0.995; p < 0.0001$). In contrast to NO$_3^-$, phytoplankton uptake of urea and DFAA generally increased southward, but the only significant relationship between urea uptake and N availability was for specific uptake by the >35 µm and the ratio of DON:DIN ($r^2 = 0.83; p < 0.05$). Urea regeneration, which was not measured during this

$^1$ When discussing uptake rate results in this chapter, the term ‘phytoplankton’ is used in reference to FCM-based measurements of autotrophic N uptake (plus >35 µm uptake when appropriate). The term ‘uptake’ refers to absolute uptake rates. Specific uptake rate results are noted as such.
study, may have been at least partly responsible for the observed uptake dynamics. Phytoplankton DFAA uptake, however, was significantly correlated to both ambient DFAA concentrations ($r^2 = 0.85; p < 0.01$) and the ratio of DON:DIN ($r^2 = 0.79; p < 0.05$). The relationship between DFAA uptake and availability was even more significant for the GF/F fraction ($r^2 = 0.96; p < 0.001$), perhaps due to bacterial affinity for amino acids. These results indicate that absolute abundance ($\text{NO}_3^-$) and relative availability (i.e. DON:DIN) can affect N uptake by phytoplankton and bacteria. However, these factors do not always explain variations in phytoplankton N use. Although $\text{NO}_3^-$ uptake was governed largely by absolute availability, dynamics of $\text{NH}_4^+$ uptake were too complex for such a simplistic explanation.

Estuarine ecosystems such as Chesapeake Bay, with relatively high abundances of bacteria and detrital matter, present a substantial challenge to researchers whose aim is to measure phytoplankton N uptake using GF/F filters. Particulate N concentrations used to calculate phytoplankton uptake rates also include PN from bacteria and detritus, which thus confounds any attempt to quantify the autotrophic component. Phytoplankton-only uptake rates were calculated from FCM-sorted autotrophic cells and used to determine how much GF/F filters overestimated phytoplankton uptake along the main axis of Chesapeake Bay. The enrichment of $^{15}\text{N}$ from $\text{NO}_3^-$ was low for bacteria retained on GF/F filters throughout the Bay and this offset the overestimation of phytoplankton PN due to retention of bacterial biomass. Therefore, despite the fact that phytoplankton PN was overestimated due to retention of bacterial biomass on GF/F filters and that specific $\text{NO}_3^-$ uptake rates were underestimated with GF/F filters due to low bacterial use, $\text{NO}_3^-$ uptake rates were roughly equivalent between the filtration- and FCM-based methods.
Bacterial affinity for $\text{NH}_4^+$ and urea, on the other hand, was on par with that of phytoplankton and even higher than phytoplankton for DFAA. Consequently, GF/F filters overestimated phytoplankton uptake of $\text{NH}_4^+$, urea, and DFAA by 61%, 53%, and 135%, respectively, as a result of bacterial retention. These results illustrate the need for improved methodological approaches to distinguishing between phytoplankton and bacterial N use. Furthermore, future application of this FCM technique will allow for a more in-depth analysis of the ecological interactions between phytoplankton and bacteria with respect to N utilization under conditions of limited DIN availability.

Results from the Mid-Atlantic Bight demonstrate the complexity that distinguishes coastal ecosystems with respect to N availability and uptake by phytoplankton and bacteria. Data from the LEO-15 monitoring node indicated the occurrence of upwelling approximately two weeks prior to this study, but the water column had since re-stratified and DIN had been removed from the surface layer before the experiment was initiated. Upwelling events common to the LEO-15 region of the Mid-Atlantic Bight introduce new N to the surface and stimulate phytoplankton production. Such a scenario could explain the increasing phytoplankton biomass observed at LEO-15 over the course of this study, as well as the relatively high standing stocks of urea, which may have been the product of enhanced grazing. Regardless, the surface water was virtually devoid of inorganic N, and undefined DON (DON other than urea and DFAA) comprised 71% of TDN, on average. With respect to the N forms discussed here, ambient urea concentrations were high and dominated the available N pool (26% of TDN). Following availability, urea supported over two-thirds of phytoplankton N nutrition in the surface water. The uptake of DIN, particularly $\text{NH}_4^+$,
was disproportionately high relative to availability, which suggests that uptake and remineralization processes were tightly coupled and also that the phytoplankton community was well-adapted to exploit new N sources (i.e. tracer additions). The importance of DFAA to phytoplankton N nutrition in the surface was fairly minor.

In contrast, the bottom-water TDN pool was more evenly split between inorganic and organic N forms and NH$_4^+$ supplied the majority of N used by phytoplankton, followed by NO$_x^-$ and urea. As in the surface layer, DFAA were the least utilized of the N forms studied, but contributed significantly (~10%) to phytoplankton nutrition in the bottom water. Relative to the surface layer, uptake rates were substantially lower in the bottom water, most likely as a result of lower phytoplankton biomass and light attenuation.

Nitrogen uptake by the bacterial community was assessed using direct measurement via size-fractionation, interpretive analysis of FCM-sorted uptake rates, and molecular assays for the ureC gene. Although a quantitative analysis of the relative importance of the N forms studied was not possible, the results suggest that all five N substrates (including NO$_2^-$) contributed to bacterial N demand during these diel experiments. Contrary to dogma, the bacterial community at LEO-15 showed evidence of significant urea utilization, particularly in the surface water during the first diel experiment. The contribution of bacteria to total specific uptake of NH$_4^+$ and NO$_3^-$ was roughly 20 – 30% in the surface water and over 50% of total uptake in the bottom water. These results indicate that bacteria were competing effectively with phytoplankton for DIN, perhaps forcing some algal species to rely on urea as an alternative N source. The relatively high concentrations of NO$_2^-$ in the bottom water suggest that nitrifying (or
denitrifying) bacteria were actively using this transient intermediate N compound. Finally, differences in DFAA uptake between the larger phytoplankton and GF/F size fractions indicate that bacteria were actively using amino acids, and more so in the bottom versus surface water.

The factors regulating uptake of various N sources, both inorganic and organic, by phytoplankton and bacteria in coastal ecosystems, as well as the interaction between these groups under N-limited conditions, are undoubtedly complex. These results were likely driven primarily by N availability; however, differences in the composition of microbial communities and their affinity for various N sources may have also contributed to observed surface to bottom differences in N uptake dynamics. This research also demonstrates the utility of combining traditional (size fractionation) methods with more modern (FCM and ureC assays) approaches in investigations of microbial N use.

As in most coastal areas around the world, the North Sea region has suffered from increased anthropogenic pressure in the form of nutrient enrichment over the past few decades. Increased N and phosphorus loads, concomitant with a decrease in silica loads due to human activities inland, are causing a shift in the phytoplankton community composition in favor of flagellates over diatoms. Phaeocystis, a phytoplankter that appears well-adapted to exploit these changes in nutrient dynamics, has been characterized as a HAB species, forms vast gelatinous colonial blooms that are not readily consumed by grazers, and produces dimethyl sulfide, which may play an important role in the global climate. Phaeocystis is unique in its ability to morph between solitary flagellate and colonial life cycle stages, and blooms typically consist of the latter.
In a mesocosm experiment in the Raunefjord, western Norway, addition of \( \text{NO}_3^- \) and \( \text{PO}_4^{3-} \) resulted in a large bloom of colonial \textit{Phaeocystis pouchetii}. \textit{Phaeocystis} is known to take advantage of high \( \text{NO}_3^- \) conditions, and added \( \text{NO}_3^- \) was removed from the water column within ten days of amendment. There were no distinct trends in either absolute concentrations of dissolved N forms or in their availability relative to one another in the amended mesocosm. Once added \( \text{NO}_3^- \) had been depleted in the amended mesocosm, undefined DON comprised the bulk (80%) of TDN, and \( \text{NH}_4^+ \), urea, and DFAA each contributed roughly 5 – 10%. In the amended mesocosm, \textit{Phaeocystis} was able to exploit new N and rapidly form a colonial bloom, then switch to regenerated N forms to sustain high biomass. Phytoplankton uptake of \( \text{NH}_4^+ \) was strongly correlated with ambient \( \text{NH}_4^+ \) concentrations \( (r^2 = 0.82; \ p < 0.001) \), and \( \text{NH}_4^+ \) concentrations were driven by \( \text{NH}_4^+ \) regeneration rates \( (r^2 = 0.67; \ p < 0.01) \). Although \( \text{NO}_3^- \) was clearly important to fueling the bloom, \textit{Phaeocystis} (both solitary cells and colonies) relied largely on \( \text{NH}_4^+ \) throughout the study and was able to compete effectively with other algae and bacteria for limited \( \text{NH}_4^+ \) as it became available via regeneration. It is possible, therefore, that the phytoplankton community had adapted to N-limited conditions prior to the experiment and as such expressed a greater affinity for regenerated \( \text{NH}_4^+ \) than other N forms, even when supplied in abundance as \( \text{NO}_3^- \). Uptake rates for the other N forms were relatively low and were not correlated with ambient concentrations. Nitrate and urea each contributed roughly 20% to phytoplankton N uptake, and DFAA were a negligible N source to phytoplankton. Although phytoplankton N uptake dominated over that of bacteria in the amended mesocosm, N metabolism of these two groups were closely coupled.
No bloom occurred in the unamended control mesocosm, and results indicate that bacteria were able to compete effectively with phytoplankton for limited N resources. In the control mesocosm, bacterial uptake of NH$_4^+$, NO$_3^-$, and urea equaled or exceeded that of phytoplankton over the second half of the experiment, whereas bacterial DFAA uptake rates were approximately four times those of phytoplankton throughout the study. Overall, bacteria contributed 80% and 49% of total (phytoplankton + bacterial) DFAA and urea uptake in the control mesocosm, respectively, 42% of NH$_4^+$ uptake, and 32% of NO$_3^-$ uptake.

The use of flow cytometric sorting of autotrophs in this study demonstrated how bacterial retention can lead to significant overestimation of phytoplankton N uptake in the >0.8 μm fraction, and underestimation of bacterial uptake in the 0.2–0.8 μm fraction. Approximately 58% and 24% of bacterial biomass was retained on 0.8 μm filters in the amended and control mesocosms, respectively. However, because bacterial uptake of all four N forms studied was relatively high, percent overestimation values for filter-based uptake rates were generally much higher than these amounts. Elucidating the environmental conditions that lead to the development of colonial *Phaeocystis* blooms versus diatom- or bacteria-dominated communities remains a significant challenge; however, accurate quantification of the N utilization patterns of these plankton groups will help clarify their ecological interactions.

Overall, the relative importance of different N substrates to phytoplankton N nutrition appeared to depend primarily on availability and secondly on physiological affinity. For example, NH$_4^+$ and NO$_3^-$ dominated total N uptake when dissolved
concentrations of these DIN forms were relatively high, such as in the upper Chesapeake Bay, Mid-Atlantic Bight bottom water, and Gulf of Mexico deep Chl maximum, or also upon addition of NO$_3^-$ in the Raunefjord mesocosms. Furthermore, phytoplankton relied more on urea and DFAA (and potentially other, unidentified organic N sources) when these substrates were relatively abundant, such as in the lower Chesapeake Bay, the Mid-Atlantic Bight surface water, and following depletion of added NO$_3^-$ in the Raunefjord experiments. These results demonstrate the capability of phytoplankton to switch between different metabolic pathways for N assimilation depending on substrate availability. Opportunistic phytoplankton taxa with flexible N uptake strategies may thus be favored over specialist species that are well-adapted to use a particular substrate.

However, NH$_4^+$ uptake rates dominated total measured N uptake throughout the ecosystems studied and were disproportionately higher than ambient NH$_4^+$ concentrations as a percent of total uptake and TDN, respectively. This fact, combined with relatively high NH$_4^+$ regeneration rates, indicates that NH$_4^+$ was recycled rapidly via heterotrophic processes such as bacterial remineralization, zooplankton grazing and bacterivory. Therefore, phytoplankton affinity for this reduced N form may have expressed greater control over autotrophic N nutrition in some cases than relative availability of N substrates.

For decades, NO$_3^-$ and NH$_4^+$ have been viewed as the principal N nutrients supporting primary production in the marine environment. This research builds on previous studies that have demonstrated the importance of organic N sources, particularly urea, to phytoplankton N nutrition. In a compilation of published DON uptake rates, Bronk (2002) reported that urea and DFAA (or dissolved primary amines) represented
19% and 23%, respectively, of total measured N uptake in numerous marine ecosystems. The respective values across the ecosystems presented here are 29% and 11% for GF/F and 0.8 μm filters and 29% and 5% for FCM-sorted phytoplankton (see Table 4 in Chapter 5). Clearly, urea represents a significant N source to phytoplankton and should be included in all examinations of N uptake by autotrophs. The percent of uptake as DFAA cited by Bronk (2002) is substantially higher than that provided here, in large part because the studies represented in that average were generally describing either a mixed assemblage of autotrophs and heterotrophs, or just bacterial uptake alone. Furthermore, DFAA uptake has not been measured routinely in studies of phytoplankton N use, despite evidence to suggest that amino acids can contribute significantly to phytoplankton N nutrition (see Chapter 1). In the research described here, DFAA contributed as much as 22% to phytoplankton uptake in Chesapeake Bay, for example, but overall represented a fairly minor N source to autotrophs.

Although bacterial N uptake was not directly measured in all samples, a qualitative analysis of bacterial N affinity was possible through the use of specific uptake rates for different fractions (e.g. GF/F versus FCM). The most in-depth examination of bacterial N uptake was conducted in the Raunefjord experiments. Ammonium was the most important N substrate to bacteria, comprising 56% of total uptake on average. In contrast to traditional views, urea contributed more to bacterial N uptake (22%) than DFAA or NO₃⁻ (11% each). The reasons why urea was favored over DFAA are not clear, although it is possible that bacteria were consuming C-rich *Phaeocystis* exudates (e.g. polysaccharides) and required N from NH₄⁺, NO₃⁻, and urea to maintain stoichiometric balance. Furthermore, ambient urea concentrations were nearly twice those of DFAA
(p < 0.0001), which suggests that overall availability may have played a role. There was
evidence to suggest that urea contributed significantly to bacterial N demand elsewhere
(e.g. Chesapeake Bay, Mid-Atlantic Bight), but the relative importance of N substrates is
not clear from these ecosystems. Nonetheless, in the studies described here, bacteria
appear to prefer DFAA, NH$_4^+$, and urea over NO$_3^-$.

An additional analysis of bacterial N preferences is possible using the averaged percent contribution of individual substrates to
total uptake in GF/F versus FCM-sorted samples. Since GF/F samples are affected by
bacteria and FCM-sorted samples are not, differences between these two results give an
indication of the nature of bacterial influence. For example, across all ecosystems, FCM-
sorted phytoplankton used slightly more NO$_3^-$ than the GF/F fraction did, although the
difference was not significant (p = 0.143; see Table 5 in Chapter 5). Within the upper
Chesapeake Bay and amended Raunefjord mesocosm (“High N input” systems),
phytoplankton used significantly more NO$_3^-$ than the mixed assemblage did (p < 0.05).

Uptake of DFAA, on the other hand, contributed significantly more to uptake by the
GF/F fraction than for phytoplankton alone (p <0.0001). The contribution of urea to total
N uptake was equal between fractions, which indicates that bacterial use was sufficiently
high to maintain this percentage; the value for the GF/F fraction would have been
significantly lower had bacteria not been using this substrate.

A primary goal for this research was to evaluate the extent to which traditional
filter-based measurements overestimate phytoplankton N uptake due to bacterial
retention. Averaged across all samples, DFAA uptake rates by phytoplankton were
overestimated most dramatically (by a factor of 2.2 ± 1.6), followed by NH$_4^+$ (1.4 ± 1.5),
urea (1.3 ± 1.2) and NO$_3^-$ (0.9 ± 1.4). As discussed previously, GF/F and 0.8 μm silver
filters overestimate phytoplankton N uptake primarily as a result of PN overestimation due to retention of bacterial biomass, but relatively high bacterial uptake will enhance this level of inaccuracy. As such, one could conclude that DFAA were the most important N substrate to bacteria, followed by NH$_4^+$ and urea, whereas NO$_3^-$ was only marginally significant to bacterial N demand.

Because of the relative similarity in the importance of N substrates to phytoplankton and bacteria, there was no significant difference between f-ratios calculated using uptake rates measured from FCM-sorted phytoplankton versus those computed for the mixed assemblage captured by GF/F and 0.8 μm filters. This result should be interpreted cautiously, however, since it represents coastal rather than oceanic environments and is focused on measurements made largely during N-limited summer periods. During spring blooms in temperate oceanic environments and upwelling in coastal areas such as the Peruvian shelf, f-ratios are likely underestimated as a result of an overestimation of regenerated primary production. More research using an approach such as FCM sorting is needed to obtain accurate estimates of new production in the oceans, especially given the implications regarding sequestration of anthropogenic CO$_2$ in the deep ocean.

Phytoplankton use numerous inorganic and organic N substrates to meet their nutritional demands, and affinity for different N forms varies between taxa. Diatoms, for example, are known to exploit available NO$_3^-$ to form blooms that generally promote healthy ecosystems. Harmful algal blooms, on the other hand, have been associated with the use of reduced N forms, particularly DON. Whereas certain phytoplankton are consumed directly by higher trophic levels (e.g. mesozooplankton), primary production
from other taxa tends to be diverted through the microbial loop before reaching higher
trophic levels. An improved understanding of the nutrient dynamics that determine
phytoplankton community composition would enable coastal managers to target nutrient
load reductions more effectively to those types of N that are more detrimental on an
ecosystem scale. For example, urea-based fertilizer may favor bacteria (and low energy
transfer) or nuisance/harmful algae, whereas NO$_3^-$-based fertilizer may favor
phytoplankton taxa that are more palatable to grazers and transfer energy more efficiently
to higher trophic levels.

This research was designed to investigate the relative importance of different
inorganic and organic N forms to phytoplankton and bacteria in marine ecosystems with
differing nutrient regimes, using an approach that provides accurate quantification of
phytoplankton-only uptake rates. To this end, results were interpreted in the context of
both the traditional view of microbial N cycling as well as evolving, current views on
phytoplankton and bacterial N use. This work also sought to quantify, for the first time,
how much GF/F-based uptake rate measurements overestimate phytoplankton N uptake.
Our understanding of the ecological roles that phytoplankton and bacteria play in the
microbial nitrogen (N) cycle as well as larger-scale ecosystem dynamics has been limited
by an inability to accurately distinguish between the activity of these two groups. Flow
cytometric sorting represents a powerful means of investigating phytoplankton N uptake
without the confounding effect of bacteria. Improved methodology and technological
advances will undoubtedly provide new tools with which researchers can probe the
structure and function of microbial communities.
References


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Born Paul Bradley Burt in Dover, New Hampshire on April 3, 1977. Graduated from Bourne High School (Bourne, Massachusetts) in 1995. Earned a B.S. in Marine Biology in May 1999 (Magna Cum Laude) from Roger Williams University in Bristol, Rhode Island. Worked as a lab technician at Springborn Laboratories (Wareham, Massachusetts) and Syngenta Biotechnology, Inc. (Research Triangle Park, North Carolina) from 1999 until entering the doctoral program in the School of Marine Science at The College of William and Mary in 2001. Served as a Dean John A. Knauss Marine Policy Fellow (Sea Grant) in the Office of Legislative Affairs at the National Oceanic and Atmospheric Administration (NOAA) in Washington, DC from 2007 to 2008.