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Availability of humic nitrogen to phytoplankton

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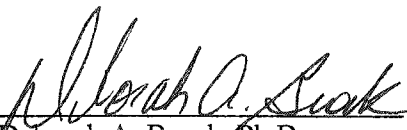This dissertation is submitted in partial fulfillment of the requirements for
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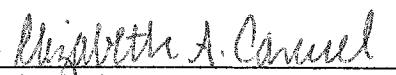
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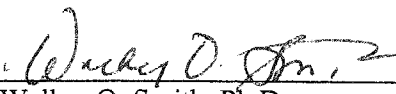
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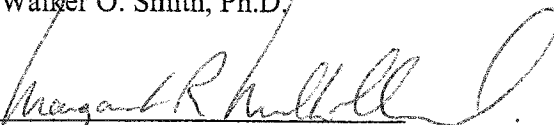
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DEDICATION

To my wife Kelly, whose love, support, and sacrifice made this project possible, and

to my parents, Ron and Donita, who instilled and nurtured my

love for the marine sciences.

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ABSTRACT

The chemical, physical, and biological factors affecting the bioavailability of humic nitrogen (N) to coastal phytoplankton were examined. Historically, humic substances have largely been considered biologically refractory, and humic-N is though to be unavailable biologically without prior oxidation via photochemical cleavage or remineralization by bacteria. This is due in part to the high aromaticity and low N content of humic substances. This dissertation investigates whether these assumptions are valid, and whether humic substances may be a more important source of N to the coastal phytoplankton community than previously believed. The research consisted of four main parts.

First, changes in the structure and N content of humic substances were monitored by forming humics in the laboratory and following the changes in structure and chemical composition as they aged. It was found that as humics age, they become more aliphatic and fulvic-like. It was also determined that the commonly used XAD-8 extraction technique may underestimate the N content of aquatic humics by stripping ammonium (NH_4^+) from the humic structure.

Second, whether or not this underestimation of humic-N has an effect on previously reported rates of photochemical N liberation from humic compounds was investigated. It was found that while the potential for the underestimation of photochemical release exists, previously reported rates are close to correct, largely due to physical and chemical interactions of humic substances with the surrounding environment.

Third, the bioavailability of humic-N was examined using a suite of coastal phytoplankton strains. In short, all coastal strains exposed to humic substances could take up humic-N in short-term incubations; an open ocean strain tested did not take up humic-N. Furthermore, younger humics appeared to be more labile than those aged for time periods greater than three months.

Finally, the significance of the salinity-mediated release was investigated and found to be a potentially important transport mechanism of NH_4^+ to the mid-saline regions of the estuary.

In conclusion, the combination of chemical, physical, and biological processes occurring in the estuarine and coastal ecosystems suggests that humic substances are highly dynamic, biologically active compounds and not the biologically recalcitrant molecules portrayed in current literature.

SECTION I:

BACKGROUND AND JUSTIFICATION

1

Humic substances can comprise a majority of the dissolved organic matter (DOM) found in aquatic environments, representing 10-75% of the total dissolved organic carbon (DOC) and 40-80% of the dissolved organic nitrogen (DON) pools in seawater (Beck et al. 1974, Thurman and Malcolm 1983, Thurman 1985, Alberts and Takács 1999). Despite the abundance of humic substances found within the coastal ecosystem, these compounds continue to remain one of the least understood classes in aquatic DOM, largely because their chemical structure is unknown. Rather, humic substances are a collection of colored, polyelectrolytic, organic acids characterized by large size and high molecular weight (HMW), ranging from 500 to 10^6 Daltons (Thurman 1985, Wershaw and Aiken 1985). Compounds found within this group are up to 3000 times the size of more readily assimilable compounds, such as amino acids and simple sugars (Moran and Hodson 1994b). Currently, humic substances are operationally defined by the means by which they are isolated. The current recommended operational definition of humic substances is the organic material that adheres to a macroporous resin (i.e. XAD-8) at a pH of 2 (Thurman 1985, Aiken 1988). Humic substances can be further categorized into fulvic acids, humic acids, and humin. Fulvic acids tend to be smaller in molecular weight (MW), ranging from 500-2000 Daltons, and are soluble in water at all pHs. Humic acids are much larger and often colloidal, typically ranging from 2000-5000 Daltons or larger and precipitate from solution at pHs lower than 2 (Thurman et al. 1982). Humins are insoluble at any pH.

Early works suggest that these large molecules are highly refractory, and therefore do not play a significant role as either an energy or nitrogen (N) source for

organisms (Fenchel and Blackburn 1979). This is due in part to the low N content of humic substances. In general, 0.5-6% of natural humic substances is N (Rashid 1985, Thurman 1985, Hedges and Hare 1987). This N can further be categorized into humic acids (2-6% N) and fulvic acids (<1-3% N; Schnitzer 1976 cited in Schnitzer 1985).

Humic substances may also alter the chemistry of the local environment either by the addition of nutrients or the removal of potential toxins. Carlsson et al. (1998) have shown the net release of ammonium (NH_4^+) and amino acids from isolated natural humic substances into the surrounding waters. Humic substances have also been shown to chelate biologically important metals, keeping them in solution and therefore making them potentially more available to phytoplankton (Prakash 1971, Prakash et al. 1973). Humics have also been shown to remove potential toxins from the environment thereby decreasing toxicity levels for phytoplankton and bacteria (Sunda and Lewis 1978, Toledo et al. 1980, Toledo et al. 1982).

The objective of this dissertation was to investigate whether the current assumptions that humic substances are highly recalcitrant, biologically refractory compounds were valid. A second focus of this dissertation was to investigate whether humics substances may be an important source of N to coastal phytoplankton. Four major hypotheses were examined.

1) The molecular weight distribution, C:N ratio, and chemical composition of humic substances vary with respect to their age since formation.

Few studies have attempted to follow the chemical changes that occur during the formation and transport of humic substances. Moreover, the method of humic formation remains under debate. Stevenson (1994) noted that "The biochemistry of the formation of humic substances is one of the least understood aspects of humus chemistry and one of the most intriguing." While most agree that humic substances are derived from the degradation of plant material, there is little consensus on the mechanisms involved. Several theories exist concerning the formation of humic substances including lignin degradation, polyphenol polymerization, and sugar-amine condensation (Thurman 1985, Stevenson 1994). Aquatic humic substances are formed by a number of mechanisms including the leaching of organic matter from plants, algae, bacteria, and the terrestrial environment, ultraviolet oxidation, and polymerization of released biological products (Thurman 1985, Stevenson 1994). Harvey et al. (1983) propose a pathway by which marine lipids released by diatoms undergo oxidative cross-linking reactions resulting in the formation of fulvic acids. Harvey et al. (1984) and Harvey and Boran (1985) observed the formation of a synthetic fulvic acid from marine lipids and diatoms in seawater and found that the newly formed fulvic acids resemble fulvic acids isolated from the oceanic environment in both structure and function. Adhikari et al. (1986) measured the effects that different chemical oxidants have on the formation and structure of humic substances. Using hydroquinone and glycine in saline and non-saline media, they found that the chances for condensation and polymerization increase in saline media, resulting in synthetic humic substances of higher molecular weight. As a part of this study, humic substances were formed in the laboratory, and changes in the chemical

structure and molecular weight were monitored. Observation of changes in these parameters allows for a better understanding of how humic substances may be formed in the environment, how humics structure and molecular composition might change as they age in the environment, and how humic substances interact with differing molecules in the surrounding waters (i.e. NH_4^+).

2) A fraction of the N bound to humic substances is loosely associated as NH_4^+ and exchangeable; hence, an increase in salinity or exposure to ultraviolet light will result in the dissociation of this N from the humic structure.

Sholkovitz (1976) explored the effects of mixing riverine waters with seawater on the chemical characteristics of humic substances. He found that riverine humic substances flocculate and move from the dissolved phase into the particulate fraction at high rates between 15 and 22‰ salinity. Sieburth and Jensen (1970) and Prakash (1971) also noted that humic materials precipitate from solution when they come in contact with seawater. At intermediate salinities, humic substances sequester metal ions and these humic-metal complexes then precipitate out of solution (Sholkovitz 1976). In these previous studies only concentrations of metal ions, phosphorus, and humic substances were followed. It is likely that N also plays an important environmental role at these salinities.

Light energy, primarily in the ultraviolet (UV) spectrum, can break the chemical bonds of refractory organic molecules and result in the release of labile compounds into the surrounding water. Numerous studies have demonstrated a

release of bioavailable nitrogenous compounds including NH_4^+, amino acids, and nitrite (NO_2^-) from DOM after exposure to natural or simulated sunlight (Bushaw et al. 1996, Gardner et al. 1998, Bushaw-Newton and Moran 1999, Kieber 2000, reviewed in Bronk 2002). However, the release of N from DOM is not a universal occurrence, as a number of studies have shown either a loss or no change in the ambient NH_4^+ concentration after exposure of DOM to sunlight and/or UV light (Kieber et al. 1997, Jørgensen et al. 1998, Koopmans and Bronk 2002, McCallister 2002).

Current estimates of N delivery to the coastal ocean via photochemical release are dependant on rates of photoproduction measured using freshwater or riverine samples (e.g. Bushaw et al. 1996) assuming that photochemically active N reaches the coastal ocean where it is then released. However, if this loosely bound NH_4^+ can be removed by exposure to both light and increased salinity, it is possible that a majority of the photochemically reactive NH_4^+ will be dissociated within rivers and estuaries prior to reaching the coastal ocean. Thus, humic substances may be capable of acting as both a storehouse and a supplier of N for plants and microorganisms in the estuary (Schnitzer 1985).

3) Coastal phytoplankton can utilize N bound to humic substances;

and

4) Uptake rates of humic N vary and are dependent on humic age/composition; furthermore the mechanisms employed for humic N utilization will vary among phytoplankton strains.

Though considered N-poor, humics do have the potential to serve as a N source. Amino acids, amino sugars, NH_4^+, and nucleic acid bases comprise 46-53% of the humic acid associated N and 45-59% of fulvic acid N (Schnitzer 1985). The remaining N associated with humic substances, approximately 50% of the total humic-bound N, remains unidentified (Carlsson and Granéli 1993).

Early works largely suggest that HMW organics, such as humic substances, are highly refractory, and therefore do not play a significant role as either an energy or N source for organisms (Fenchel and Blackburn 1979). However, other studies suggest that HMW compounds are not as refractory as once believed (Amon and Benner 1994). Bacteria, for example, are capable of utilizing C from humic and humic-like substances for growth (Tranvik 1988, Moran and Hodson 1990, 1994a, b, Hunt et al. 2000). Furthermore, humic material isolated from a river and commercially available humic acid salts were shown to stimulate growth and primary production when added to natural plankton assemblages (Prakash and Rashid 1968, Prakash et al. 1973, Carlsson and Granéli 1993, Carlsson et al. 1993, Carlsson et al. 1995, Carlsson et al. 1998, Carlsson et al. 1999). Other works have also shown a similar response by phytoplankton cultures to the addition of humic substances (Prakash and Rashid 1968, Prakash et al. 1973). These studies did not investigate the mechanisms responsible for the enhanced growth, which could include chelation of metals, removal of toxins, or addition of N to the system. Direct uptake of ^{15}N-labeled humic substances by plankton in both riverine and coastal waters has also been observed (Bronk et al. 1999, Bronk et al. in prep.). However, it is unclear how the additions of these substances stimulate the observed utilization. Several

suggested mechanisms include the photochemical oxidation of humics into small more readily assimilable compounds (e.g. Bushaw et al. 1996, Bushaw-Newton and Moran 1999, Kieber 2000), direct uptake of the humic structure (Legrand and Carlsson 1998), or uptake of N moieties via cell surface enzymes as shown for dissolved amines (e.g. Palenik et al. 1988/1989, Palenik and Morel 1990).

To help answer these questions, natural aquatic humic substances were collected from the Satilla River in coastal Georgia on four cruises, from the Altamaha River, GA on a single research cruise, and from the York River, VA on three dates (Fig. 1). Upriver stations were sampled along each river. The Satilla River is a black water river. Large inputs of humic material originate in flood-plain swamps prior to entering the river. The Altamaha River has the second largest watershed in the United States with flow dominated by silt-laden waters from the upper coastal plain. The York River basin encompasses both piedmont and coastal plain topographies as its water travels to Chesapeake Bay. The three rivers examined have similar watersheds with if > 55% forested, > 25% agricultural, and < 5% urban land usage (T. Dai, pers. comm.). However humic concentrations vary significantly between the rivers. Concentrations are approximately 15, 6, and 1 mg humic-C L^{-1} and represent 75, 75, and 20% of the DOC pool in the Satilla, Altamaha, and York Rivers, respectively (this dissertation, Alberts and Takács 1999, Raymond and Bauer 2001). In addition to naturally extracted humics, commercially available humic acids (Aldrich Chemical Company) were also examined. Although available for purchase, these humic acids have been extracted from the soil in Germany.

Figure 1. Sample sites examined throughout the dissertation. Humic substances were

extracted from A) the Satilla and Altamaha Rivers, GA, and B) the York

River, VA.

GEORGIA

Altamaha
River

Satilla
River

A

B

CHESAPEAKE BAY

York
River
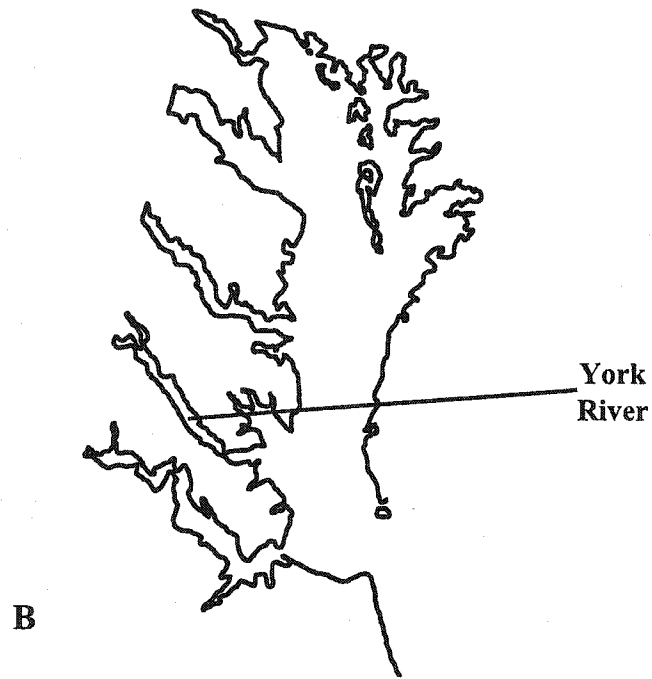
As part of this dissertation, several physical, chemical, and biological processes responsible for enhancing the bioavailability of humic-N were examined to gain a more complete understanding of the cycling of humic-N in an estuarine ecosystem. Although humic substances have historically been perceived to be biologically refractory due in part to their low N content, results presented in Section II suggest that humic substances in the natural environment are not as N-poor as the literature would suggest. This is followed in Section III by an examination of the photochemical reactivity of humic substances representative of those found in aquatic environments. Sections IV and V explore the availability of humic-N to coastal phytoplankton strains and potential mechanisms for utilization of this N. Section VI discussed in further detail the how increased salinity can force a release of NH_4^+ from humic substances and introduces the concept of the "humic shuttle" in which NH_4^+ bound to humic substances is released in higher salinities, providing a source of labile nitrogen to mid-saline phytoplankton communities. The final section is a brief conclusion outlining the significant findings of this dissertation.

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SECTION II

CHANGES IN MOLECULAR WEIGHT DISTRIBUTIONS, C:N RATIOS, AND

CHEMICAL STRUCTURES OF ESTUARINE HUMIC SUBSTANCES WITH

RESPECT TO SEASON AND AGE[†]

18

ABSTRACT

Humic substances were isolated from the Satilla and Altamaha Rivers, GA and the York River, VA via the commonly used XAD-8 extraction technique. They were also formed in the laboratory by allowing *Spartina alterniflora* plants to humify under controlled laboratory conditions over the course of one year. The chemical characteristics of the natural and laboratory produced humic substances were evaluated in a number of ways. First, chemical shifts in the atomic C:N ratio were monitored after exposing natural humic substances to environmentally relevant ammonium (NH_4^+) concentrations. Second, structural characteristics of natural humics and changes as humics aged were determined using both Fourier Transform Infrared (FTIR) spectroscopy and molecular weight size fractionation. Results indicate that the exposure of humic substances to environmentally relevant levels of NH_4^+ caused a significant decrease in the C:N ratios of the bulk, low molecular weight (LMW; < 3 kD), and high molecular weight (HMW; > 10 kD) size fractions, but not the intermediate size fraction (3-10 kD), for a majority of the samples. These data indicate that extraction of humic substances from the aquatic environment via XAD can cause an underestimation of humic nitrogen (N), suggesting that humic substances in the environment are more N-rich than previously thought. Structurally, as the humics aged both the LMW and HMW fractions increased, and FTIR spectrums suggest that they become more aliphatic and fulvic-like in character.

INTRODUCTION

Humic substances can comprise 10-75% of the dissolved organic carbon (DOC) and 40-80% of the dissolved organic nitrogen (DON) pool in seawater (Beck et al. 1974, Thurman and Malcolm 1983, Thurman 1985, Alberts and Takács 1999), and as much as 95% of the natural organic matter transported to coastal marshes by rivers are defined as humic substances (Alberts and Filip 1994). Humics are described as a heterogeneous mixture of organic molecules ranging in size from 500 to 10^6 Daltons that do not conform to any unique chemical structure (Thurman 1985, Wershaw and Aiken 1985). Humic substances consist of fulvic acids, humic acids, and humin. Fulvic acids tend to be smaller in molecular weight (MW), ranging from 500-2000 Daltons, and are soluble in water at all pHs. Humic acids are larger and often colloidal, typically ranging from 2000-5000 Daltons or larger and precipitate from solution at pHs lower than 2 (Thurman et al. 1982). Humins are insoluble at any pH.

Natural humic substances, isolated with XAD extraction, have been shown to contain 0.5 to 6% N (Rashid 1985, Thurman 1985, Hedges and Hare 1987). This N can further be categorized as N incorporated into humic acids, which are approximately 2 to 6% N, and fulvic acids, which are N poor at < 1 to 3% N (Schnitzer 1976). Amino acids, amino sugars, ammonium (NH_4^+), and nucleic acid bases makeup 46 - 53% of the N associated with humic acids and 45 - 59% of fulvic acid-N (Schnitzer 1985). The rest of the N associated with humic substances, approximately 50% of the total humic-bound N, remains unidentified (Carlsson and

Granéli 1993). Previous work indicates that the C:N ratio of aquatic humic substances, isolated with XAD resin, ranges from 18-30:1 for humic acids and 45-55:1 for fulvic acids, but can vary considerably (Thurman 1985).

Previous research suggests that in the aquatic environment humic substances associated with sediment particles can adsorb NH_4^+ (Rashid 1969). However, as more saline water passes over the sediments, due to transport of sediments downriver or tidal influx of saline waters, this adsorbed NH_4^+ can be desorbed from the sediment and released into the overlying waters as cation exchange sites on clay particles and organic matter in the sediment become occupied with seawater cations (Rosenfeld 1979, Gardner et al. 1991). Humic acids extracted from marine clay sediments can account for a large percentage of the cation exchange capabilities of these marine sediments and likely play an important role in NH_4^+ adsorption and release (Rashid 1969).

Humic substances extracted from rivers and estuaries are also able to adsorb NH_4^+. This NH_4^+ can be re-released into the surrounding water as salinity increases (see Section VI, this volume). In the laboratory it was observed that the traditional humic isolation technique, extraction onto XAD-8 resin (Aiken 1985), strips adsorbed NH_4^+ from the humic structure (Fig. 1). Based on this observation, it was hypothesized that the atomic C:N ratio of humics in the natural environment, prior to XAD isolation, may be more N-rich than currently thought. To test this hypothesis, this study: 1) quantified changes in C:N ratios of humic substances before and after exposure to NH_4^+ at concentrations commonly observed in natural waters; 2) determined to which MW fraction of humic substances (LMW, < 3 kD; intermediate

Figure 1. The C:N ratio of humic substances enriched at 0.048 μmol N:μmol humic C

for Aldrich humic acids and Satilla River humic substances before (□) and

after (■) extraction onto DAX-8 resin measured in this study. * represents

significant differences in C:N ratio at $p < 0.05$, and ** represents significance

at $p < 0.01$. Error bars represent ± 1 standard deviation.
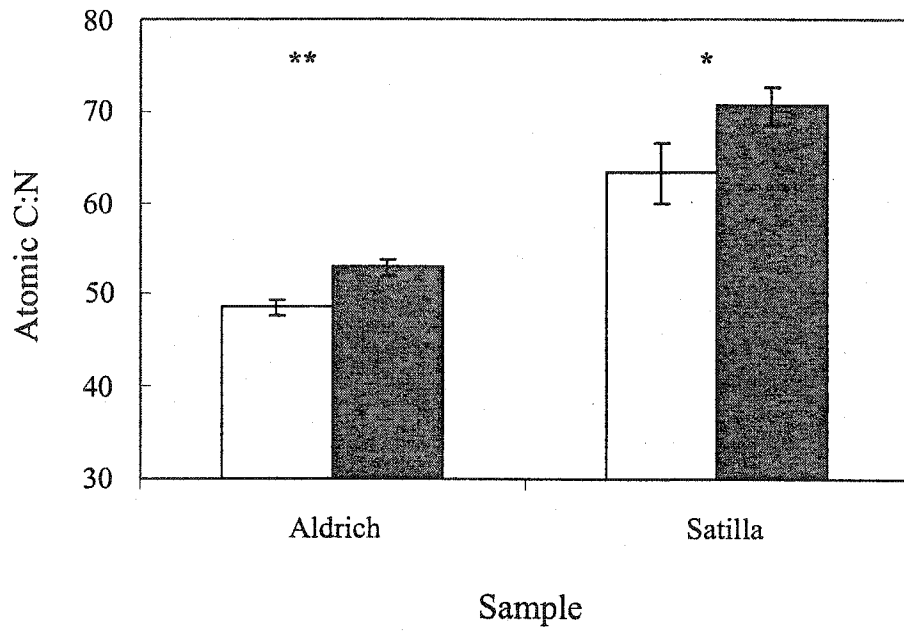
MW, 3-10 kD; HMW> 10 kD) the NH_4^+ adsorbs; and 3) quantified structural changes that occur within a humic molecule as it ages utilizing wet chemical and Fourier Transform Infrared (FTIR) spectroscopy techniques. In brief, it was found that: 1) NH_4^+ readily adsorbs to a wide range of MW fractions resulting in a decrease in the C:N ratio; 2) that as humics age, a higher portion of the C can be found in the LMW and HMW fractions, but not the intermediate range; and 3) humic substances become more fulvic-like in character.

MATERIALS AND METHODS

Sample sites

Natural aquatic humic substances were collected from upriver stations on the Satilla River in coastal Georgia on four cruises, from the Altamaha River on a single research cruise, and from the York River on three dates (Table 1). The Satilla River is a black water river receiving large inputs of humic material originating in flood-plain swamps. The Altamaha River has the second largest watershed in the United States, with its flow is dominated by silt-laden water from the upper coastal plain, and the York River basin encompasses both piedmont and coastal plain topographies en route to Chesapeake Bay. While all three rivers are similar with average annual land use averages of > 55% forested, > 25% agricultural, and < 5% urban (T. Dai, pers. comm.), humic concentrations vary significantly. Concentrations are approximately 1250, 500, and 83 µM humic-C and represent 75, 75, and 20% of the DOC pool in the

Table 1. Dates and locations of estuarine sampling.

River	Date	Longitude	Latitude
Satilla	November 2000	81°40'76" W	30°57'21" N
Satilla	April 2001	81°40'85" W	30°57'26" N
Satilla	June 2001	81°40'85" W	30°57'24" N
Satilla	December 2001	81°41'46" W	30°57'15" N
Altamaha	November 2000	81°26'50" W	31°19'93" N
York	September 2001	76°53'27" W	37°33'17" N
York	January 2002	76°53'27" W	37°33'17" N
York	April 2002	76°53'27" W	37°33'17" N

Satilla, Altamaha, and York Rivers respectively (Alberts and Takács 1999, Raymond and Bauer 2001).

Humic isolation

Humic substances were extracted on Supelco DAX-8 resin, as previously described by Aiken (1985) for Amberlite XAD-8 resin. Note that DAX-8 and XAD-8 resins function similarly in isolating bulk humic solutes from aquatic sources, producing mixtures with similar chemical compositions (Peuravuori et al. 2002). With this protocol, organic acids are adsorbed to the resin in the protonated form. Accordingly, samples were first acidified to a pH of 1.8 with concentrated hydrochloric acid (HCl) and then passed through a glass column packed with acidified DAX-8 resin. The column of DAX-8 resin was then rinsed with deionized water (DIW), until the eluate reached a pH > 5, thereby removing any remaining salts from the resin. Following the rinse, the column was back flushed with two bed volumes of 0.2 N sodium hydroxide (NaOH) to elute bound humic substances from the resin.

XAD-8 resins bleed small amounts of organic molecules with the eluate (Aiken 1988). Therefore, prior to extraction of the humic substances, the DAX-8 resin was cleaned using a Soxhlet extraction procedure (solvents include ether, acetonitrile, and methanol) followed by extensive rinses of HCl, NaOH, and DIW (Thurman and Malcolm 1981, Aiken 1985). Before samples were extracted, DIW water was extracted to establish background levels of DON, NH_4^+, and NO_3^- as well

as DOC that may leach from the DAX-8 resin (Parsons et al. 1984, Peltzer et al. 1996, Hansen and Koroleff 1999, Bronk et al. 2000).

Producing aged humics in the laboratory

Coastal humics were produced in the laboratory by allowing *Spartina alterniflora* to humify in the presence of coastal bacteria. *Spartina alterniflora* was chosen as a source for humic formation because it is the dominant primary producer within most salt marshes of the southeastern United States and is responsible for up to 80% of marsh primary production (Pomeroy et al. 1981). *Spartina alterniflora* plants were collected in April 2000 at the Skidaway Institute of Oceanography (SkIO) and then grown in a bucket over a period of 3 months. The plants were harvested by cutting the leaves off at soil level, dried in an oven for one week at 40 $^{\circ}$C, and shredded in a Wiley mill (60 mesh). The shredded *S. alterniflora* (42 g) was divided equally into six 1L flasks of coastal seawater collected from SkIO; the humic substances had been removed from the seawater using DAX-8 extraction. The flasks were then inoculated with coastal bacteria and placed in the dark, where they were stirred with a magnetic stir bar. One-liter samples (n=1) were removed from the dark over the period of one year at time points of 1 week, 2 weeks, 1 month, 3 months, 6 months, and 1 year. At each time point, the humic substances were isolated via DAX-8, as previously described, neutralized with HCl, and frozen for future experiments. A true T_0 was not possible as the starting material was a solid *S. alterniflora* shoot, so 1 week was chosen as a baseline.

Enrichment of humics with NH4+

Humic substances were enriched with NH_4^+ by swirling humic samples

(100 mL, 833 μM humic-C; n=3) overnight with both low and high concentrations of

NH_4^+, as NH_4Cl, to give final ratios of 0:1, 0.048:1, and 0.48:1 μmol NH_4^+:μmol

humic-C. Unfortunately, humic concentrations are rarely measured in a typical suite

of environmental parameters, and when quantified, they usually are reported in units

of C. Concentrations of humics and NH_4^+ are also rarely measured together making it

difficult to get a robust estimate of environmentally realistic NH_4^+: humic-C ratios.

However, due to a lack of humic-C data, DOC was used as a conservative tracer for

humic-C (10-75% of total DOC; Alberts and Takács 1999, Thurman 1985).

Accordingly, concentrations of NH_4^+ were chosen based on data from the study sites

and previously published ratios of NH_4^+: DOC in aquatic systems (Table 2). The

sample pH was increased to 10 with 1.0 N NaOH; subsequently the sample was

degassed to remove residual unbound NH_4^+ by spinning in a SpeedVac concentrator

and applying a strong vacuum (1 torr) to the headspace over the sample for 7-8 hours;

the sample was kept below room temperature throughout this procedure. Each

sample was then reneutralized with 5% HCl, allowed to warm to room temperature,

and reconstituted to 100 mL with DIW. The concentrations of NH_4^+ in DIW blanks

run through the procedure were determined manually via the phenol-hypochlorite

technique modified for colored/turbid water to determine if NH_4^+ was introduced

during the isolation procedure (Koroleff 1983).

Table 2. Ratio of μM NH_4^+ to μM DOC in coastal estuaries.
Where available, ranges are reported. Data for Dog Creek and
Oyster Creek are reported as mean ± standard error.

Location	NH_4^+ (μM)	DOC (μM)	NH_4^+:DOC
Streams			
Highland, Scotland[1]	1.1 - 1.2	500 - 517	0.0024
Cairngorn, Scotland[1]	0.6 - 2.1	450 - 675	.0012 - .0048
Southwest Scotland[1]	2.4	533 - 933	.0024 - .0048
Tweed, Scotland[1]	0.8 - 1.3	400 - 483	.0012 - .0036
Dog Creek[2]			
High Flow	6.2 ± 0.7	1325	0.0060
Low Flow	5.5 ± 0.4	933	0.0048
Oyster Creek[2]			
High Flow	7.0 ± 0.5	2142	0.0036
Low Flow	15.6 ± 0.8	2208	0.0072
			Range = 0.0012 to 0.0072
Rivers			
Altamaha River, GA[3]	0.4 - 4.5	300 - 475	0.0010 - 0.0120
Changjiang, China[4,5]	14.6	142 - 400	0.0360 - 0.1032
Huanghe, China[4,6]	5.3	167 - 333	0.0216
Lena River, Siberia[7]	0.01 - 0.3	308 - 1042	0.0000 - 0.0005
Mura River, Slovenia[8]	6.4 - 85.7	150 - 708	0.0240 - 0.2424
Ogeechee River, GA[3]	0.3 - 1.02	267 - 535	0.0048
Satilla River, GA[3]	0.2 - 8.3	525 - 3133	0.0012 - 0.0024
Savannah River, GA[3]	0.7 - 7.0	225 - 417	0.0024 - 0.0228
Zhujiang, China	11.5	142	0.0816
Sapelo Island, GA[9,10]			
Summer	0.4 - 40.6	8 - 908	0.0012 - 0.0996
Winter	1.8 - 73.4	50 - 292	0.0204 - 0.4680
			Range = 0.0000 to 0.4680
Mangroves			
Coral Creek[11]	0.1 - 0.8	92 - 125	0.0007 - 0.0084
Taylor River[12]	0.6 - 4.5	708 - 1533	0.0006 - 0.0060
Caeté Estuary, Brazil[13]			
Dry Season	2.5 - 14.0	292 - 400	0.0072 - 0.0444
Rainy Season	3.0 - 19.5	283 - 558	0.0096 - 0.0504
			Range = 0.0006 to 0.0504

[1]Chapman et al. (2001); [2]Wahl et al. (1997); [3]This study;
[4]Cauwet & Mackenzie (1993); [5]Zhang cited in Zhang et al. (1999);
[6]Zhang (1996); [7]Lara et al. (1998); [8]Brodnjak-Vončina et al. (2002);
[9]Haines (1979); [10]Alberts & Takács (1999); [11]Boto and Wellington (1988);
[12]Davis et al. (2001); [13]Dittmar and Lara (2001)

C:N determination

The concentrations of DOC and total dissolved N (TDN) of the extracted humic substances were determined via high temperature oxidation using a Shimadzu TOC-V$_{cn}$ equipped with a TNM-1 total N detector. Samples were combusted at 720°C after injection onto a platinum catalyst, converting all C to CO_2, which was measured via IR fluorescence. N was converted to nitric oxide (NO) during combustion and measured via chemofluorescence after mixing with ozone gas.

Molecular weight determination

To obtain MW information, as well as to determine if NH_4^+ binds preferentially to humics of differing MW, triplicate samples were separated by size using Amicon Centriprep centrifugal filter devices (Millipore). These columns are composed of two chambers separated by a regenerated cellulose ultrafiltration membrane. Centrifugation allows for molecules with a MW smaller than the nominal pore size to pass through the membrane. The use of a reverse filtration mechanism, in which ultrafiltration occurs in the opposite direction of centrifugal force, allows particles to sediment out thereby reducing the number of clogged membrane pores. For this experiment, YM-3 and YM-10 Centriprep membranes were used to separate out the 3 kD and 10 kD MW compounds, respectively. As these membranes contain trace amounts of glycerin, extensive rinses (5-10) with DIW and 1% HCl were necessary prior to sample addition to prevent DOC contamination of the samples. Each rinse required 95 min centrifugation for YM-3 membranes and 45 minutes for the YM-10 columns. These columns are also designed for single use and the

extensive rinses can damage the membrane surface or seal and affect performance.

Therefore, care must be taken when handling and preparing the columns for use.

Blanks for DOC MW samples contained 63 ± 21 μM C; TDN blanks were below

detection limits.

Structural determination

      Samples isolated from the Satilla River and humic substances formed in the

lab via *S. alterniflora* degradation were analyzed by FTIR spectroscopy to determine

the differences in the major structural components of the humic substances both

seasonally and as they aged. Analyzing aquatic humics in their aqueous state is

advantageous, as drying of the samples can cause changes in bonds and cross-linking

of humic compounds (MacCarthy et al. 1975). Humic samples (1 mL) were

analyzed on a Shimadzu FTIR-8300, utilizing a Pike Horizontal Attenuated Total

Reflectance (HATR) sample attachment. The HATR allows for analysis of samples

in the liquid state and multiple beam passes through the sample, which increases

resolution for dilute samples. Prior to analysis, humic samples were concentrated

10X in a SpeedVac concentrator (Thermo Savant) to enhance resolution. Humic

spectra were obtained by subtracting the water spectrum from the sample spectrum as

described by MacCarthy et al. (1975) using Hyper-IR software (Shimadzu Scientific

Inc.).

Data analysis

Differences in humic C:N ratio, %C, and %N values were compared via one-way analysis of variance (ANOVA) using SAS statistical analysis software. Samples were considered to be significantly different at a p-value of 0.05 or less.

RESULTS

C:N ratio of enriched humic substances

In all Satilla River samples examined, exposing humic substances to NH_4^+ resulted in a decrease of the bulk atomic C:N ratio of the humic material. The decrease in the C:N ratio between the field sample extracted by DAX-8 and the sample enriched with 0.048 μmol NH_4^+:μmol humic-C was significant at the $p < 0.05$ level in both the spring and winter samples (Fig. 2A). There was also a decrease in the C:N ratio between the 0.048 μmol and 0.48 μmol NH_4^+:μmol humic-C in autumn and summer, but it was not statistically significant ($p > 0.3$).

The C:N ratio also decreased with increasing enrichment in the Altamaha and York River samples. The C:N ratio of the Altamaha River decreased approximately 27% after enriching with 0.048 μmol NH_4^+:μmol humic-C (51.8 to 37.6, $p < 0.01$; Fig. 2B). Although all three York River samples showed a decrease in the C:N ratio, the decrease was significant only in the winter for the 0.048 μmol NH_4^+:μmol humic-C enrichment ($p < 0.05$; Fig 2C).

Figure 2. The atomic C:N ratio of humic substances after enrichment with increasing

concentrations of NH_4Cl. Humics were extracted from A) the Satilla River,

GA, during spring, summer, fall, and winter, B) the Altamaha River, GA

during autumn, and C) the York River, VA during spring, autumn, and winter.

Data for 0.48 μmol N:μmol humic C for the autumn York River sample was

lost due to instrument error, and the York River 0 μmol N enrichment spring

sample was lost due to contamination. Error bars represent ± 1 standard

deviation.

Saturation (μmol N:μmol humic C⁻¹)
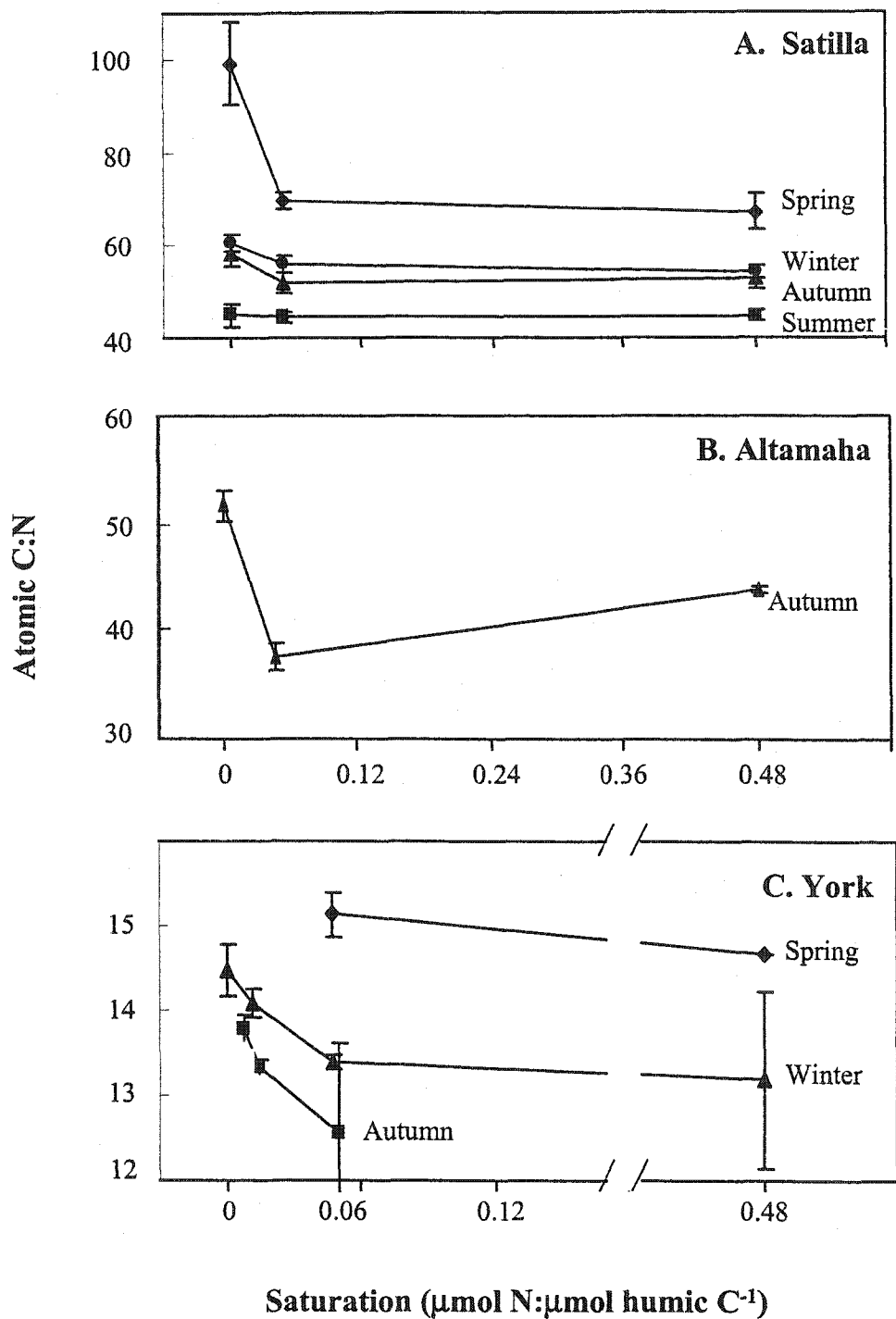
Aging of humic substances

In the aging experiment, the C:N ratio of bulk humic substances formed in the laboratory decreased by a factor of two (from 38 to 19) between weeks 1 and 2 ($p < 0.05$, Fig. 3A). This was due to a significant increase in the concentration of DON from 17.9 to 34.3 μM N over this time period while C concentrations were held constant (data not shown). After week 2 age appeared to have little influence on the C:N ratio of the bulk humics (Fig. 3A). C:N ratios were also calculated for each size fraction from the laboratory-formed humic substances. Overall, the C:N ratio in the LMW size fraction decreased by more than half during the year of sampling (from 37 to 16; Fig. 3B), although this decrease was not constant. An initial decrease occurred between 1 week and 2 weeks, followed by an increase through the 3 month sampling, and concluding with a decrease to the 1 year sampling. A decrease was also observed in the C:N ratio of the HMW size fraction between the 1 week and 1 month samplings (from 36 to 16; Fig. 3C), followed by a net increase, rising to approximately 28 after 6 months ($r^2 = 0.91$; $p < 0.01$). The C:N ratio of the HMW size fraction dropped to 25 at the 1 year time point, but this decrease was not significant ($p > 0.05$).

The MW distribution of the humic substances also changed considerably as samples were aged in the laboratory (Fig. 4). There were several trends evident within the data set. In the LMW fraction the % C decreased between the first and second week of formation; for the remainder of the experiment there was no overall change in the % C values for the LMW size fraction (Fig. 4A). However, an increasing trend in LMW % C was detected from 3 months to 1 year ($r^2 = 0.83$, $p < 0.005$). The % N in the LMW fraction increased significantly through time ($r^2 = 0.77$,

Figure 3. Atomic C:N ratios of laboratory aged humic substances isolated from

degraded *S. alterniflora*: the bulk sample (A), the LMW (< 3 kD) size fraction

(B), and HMW (> 10 kD) size fraction (C). Error bars represent ± 1 standard
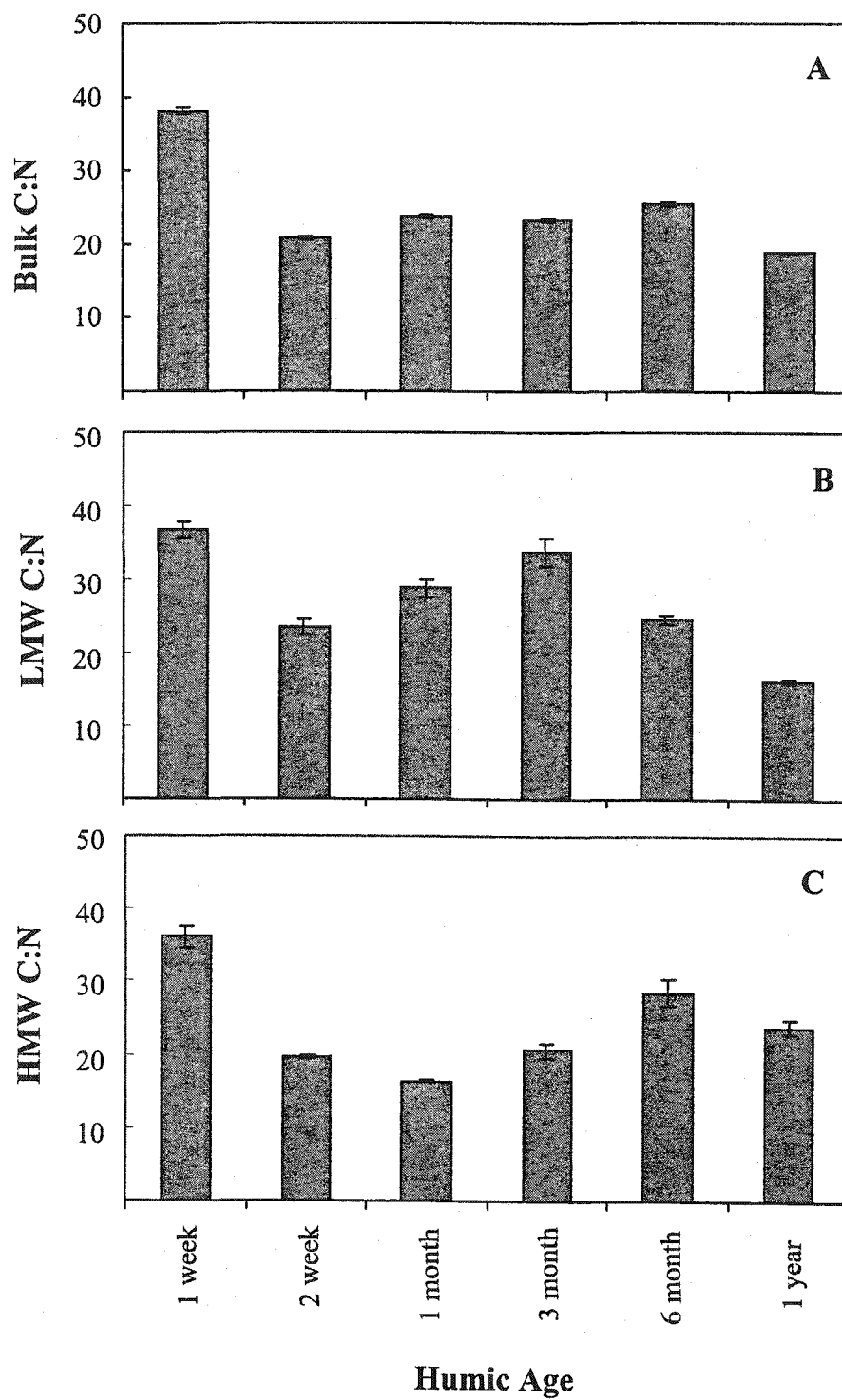
deviation.

Figure 4. Mean % total C (□) and N (■) by weight in the < 3 kD size fraction (A) and 3 – 10 kD size fraction (B) for humics of increasing age. Error bars represent ±1 standard deviation.
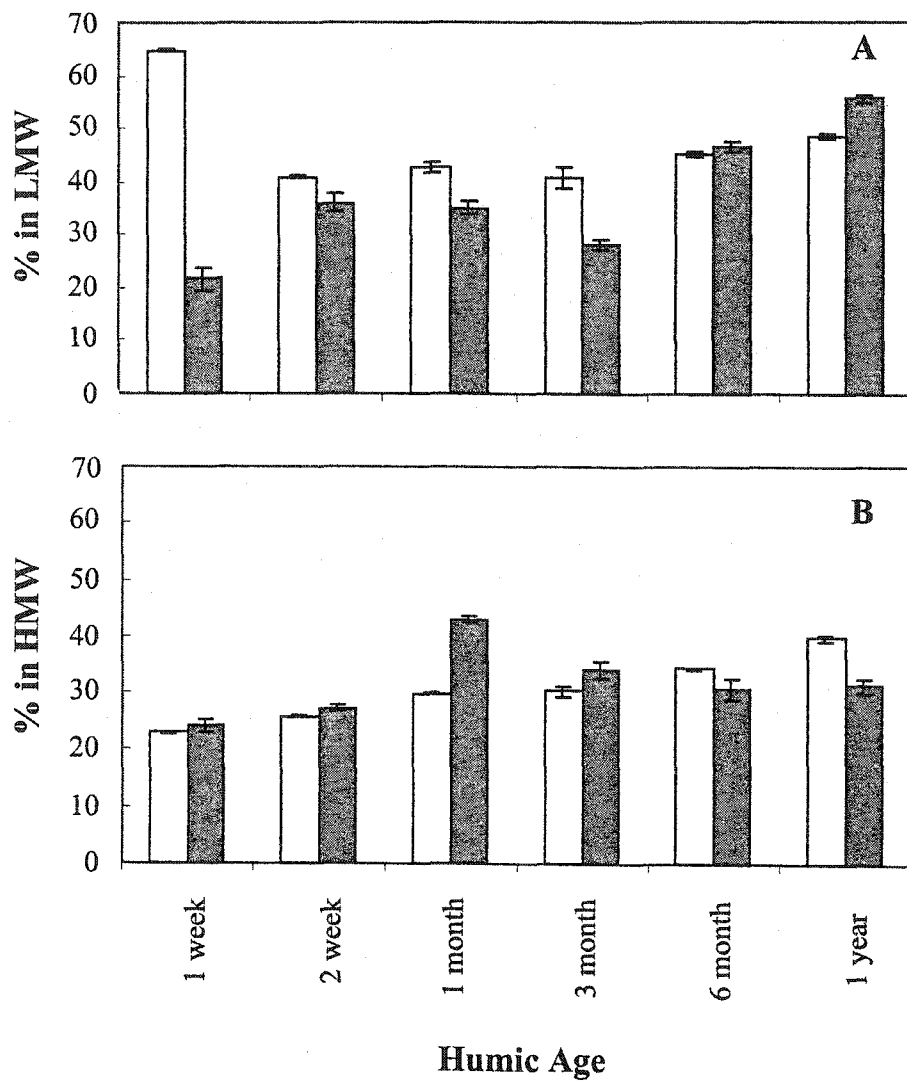
p < 0.0001). In the HMW fraction the % C increased steadily throughout the experiment (Fig. 4; $r^2 = 0.89$, p < 0.0001), and the % N increased by a smaller but significant amount (p < 0.05), with a large increase between 2 weeks and 1 month.

FTIR structural data

All samples showed similar patterns in FTIR spectra and resembled a Type III spectrum, typical of aquatic humics (Stevenson and Goh 1971). Strong absorption bands were evident near 3400, 2920, 1720, 1580, 1540, and 1200 cm^{-1} (spectra not shown). The wide 3400 cm^{-1} absorption band is due to O-H stretching (and trace N-H stretching). The absorption at 2900 cm^{-1} is created due to aliphatic C-H stretching, while the 1720 cm^{-1} band is C=O bonds in COOH. High 1600 cm^{-1} absorption is created by aromatic C=C bonds, 1540 cm^{-1} absorption by COO^- symmetric stretching, and the 1200 cm^{-1} band is due to C-O stretching and OH deformation of COOH (Stevenson 1994). Structural differences between all Satilla River samples were minor and could not be used to differentiate NH_4^+ binding patterns based solely on FTIR data.

Humic substances that were formed in the lab by degrading *S. alterniflora* were also examined via FTIR to determine changes in the structure as they aged (Fig. 5B). These data indicate an increase in either O-H or N-H bonds (3400 cm^{-1}) as the samples aged.

Figure 5. (A) Sample FTIR spectrum of humics isolated from the Satilla River, GA in April 2001. (B) Changes in the FTIR spectrum of *S. alterniflora* derived humics produced in the laboratory as they age from 1 week to six months.

Figure showing FTIR spectra (A and B) with Transmittance on the y-axis and Wavelength (cm⁻¹) on the x-axis.

Panel A labels: O-H Stretching, Aliphatic C-H Stretching, Carboxyl C=O bonds, Aromatic C=C bonds, C-H stretching of phenolic O-H, C-O stretching of polysaccharides. Peaks labeled: 3400, 2920, 1720, 1580, 1390, 1050. Marked **A**.

Panel B labels: 1 week, 6 month. Marked **B**.

x-axis values: 4000, 3500, 3000, 2500, 2000, 1750, 1500, 1250, 1000, 750

Wavelength (cm⁻¹)

DISCUSSION

Here three aspects of this study are discussed: the underestimation of C:N ratios of natural humic substances, changes in the chemical characteristics, and changes in the structural composition of humic substances as they age.

Underestimation of C:N ratios of natural humic substances

Two concentrations of NH_4^+ were chosen for the humic substance enrichment. They were chosen to represent moderate (4 μM) and high (40 μM) levels of enrichment that would likely be observed in nature. The humic substances adsorbed the free NH_4^+ such that half of the enriched Satilla River seasonal samples exhibited a statistically significant decrease in the C:N ratio ($p < 0.05$; Fig. 2A), consistent with observations that humic substances efficiently bind NH_4^+ (Rosenfeld 1979, Gardner et al. 1991). The nature of this association was not investigated in this experiment, but is believed to be weak, as re-extraction with XAD will again remove this NH_4^+. These data imply that isolation with XAD-8 (or DAX-8) resin underestimates the N content of humics in the environment by stripping loosely bound N from the humic structure prior to analysis, and thus skewing the C:N ratio upward (Fig. 1). This finding is consistent with a number of previous observations. First, as much as 75% of the N bound to humics is loosely associated and can be removed during passage through a cation exchange column (Roulet et al. 1963 cited in Schnitzer, 1985, Sowden and Schnitzer 1967, Khan and Sowden 1972). Second, elution of humic substances from XAD columns with ammonium hydroxide (NH_4OH) approximately

doubles the N content of the extracted humic material, indicating a binding of the amino N to the humic structure (Thurman 1985). Third, DOM isolated via ultrafiltration (UDOM, > 1kD) from marine systems has a C:N ratio two to three times lower than humic substances extracted via XAD techniques (Benner 2002). Although McCarthy et al. (1996) have suggested that UDOM is chemically distinct from humic substances, this assumption is based largely on the elevated C:N ratios reported for humics extracted via XAD resins. An additional contributing factor to the apparent difference in the C:N ratios of humics and UDOM may arise from the fact that ultrafiltration does not strip NH_4^+ from organic matter but XAD resin does, thereby isolating HMW humics from solution with a lower C:N ratio.

All experiments were run at the same concentrations of humic-C, in fresh water, at the same temperature, and at the same pH; thus the different adsorption patterns observed suggest that the structural composition of the humic compounds varies seasonally. This may be due to the fact that *S. alterniflora*, the dominant plant found within the marsh ecosystem of the southeastern United States (Pomeroy et al. 1981, Alberts and Filip 1994), is also the primary source for refractory carbonaceous compounds, including humic substances, in salt marshes along the Atlantic and Gulf coasts (Filip and Alberts 1988). Because a majority of *S. alterniflora* biomass is formed during the growing season in spring and summer, then die and be subjected to physical and bacterial breakdown, the overall structure of the DOM would be expected to change throughout the course of the year due to degradation by bacteria, changes in light levels and photochemical effects, changing redox conditions, and other chemical processes. Values for NH_4^+ adsorption could also vary from year to

year because these environmental conditions affect the production of *S. alterniflora* and/or delivery of NH_4^+ within the marsh ecosystem.

Another potential explanation for the seasonal changes could be related to the humic source. Although the determination of a humic source pool during each season was beyond the scope of this study, some reasonable assumptions can be made. In the late winter and spring, much of the humics are likely from terrestrial sources, entering the river and estuary during the spring freshet and periods of high water flow. However, during the summer and winter, periods with low water flow, the majority if the humics are likely formed *in situ*, and could be considered more of a true aquatic humic.

In the Satilla River, NH_4^+ adsorption was observed in the autumn, winter, and spring samples, while the C:N ratio of the summer sample did not change significantly with added NH_4^+ (Fig 2A). The lack of a decrease in the C:N ratio in the summer may be related to low discharge and relatively high flushing times of the Satilla River (Alber and Sheldon 1999) combined with high temperatures and primary productivity during the summer months. Under these conditions, free NH_4^+ concentrations are likely quite low, reducing the likelihood of adsorption. During the summer months, humic substances are also degraded such that their capability to adsorb NH_4^+ may be decreased. In the Altamaha River, the C:N ratio of the autumn humic substances also decreased after enrichment with NH_4^+, however an exposure to increased concentrations of NH_4^+ (0.48 μmol NH_4^+:μmol humic-C) resulted in higher C:N ratio in the humics than those exposed to 0.048 μmol NH_4^+:μmol humic-C, a result which we cannot explain. It is unknown if this is a general trend in this river,

or an isolated occurrence. In general, the C:N ratio decreased in all York River samples, similar to that observed in the Satilla River.

The binding and/or adsorption of NH_4^+ to humic substances is environmentally significant for a number of reasons. First, NH_4^+ is a ubiquitous compound readily found in aquatic ecosystems, and can reach high concentrations in areas of humic formation (e.g. Haines, 1979, Chambers, 1997). Though highly variable (see Table 3), the C:N ratio of humic substances is often reported as 40 (e.g. Thurman 1985, Hedges et al. 1992, Alberts and Filip 1994). Data from this study suggest that the actual value is 12% lower than the previously reported value of 40, such that an overall mean value of 35, with a range of 27-40, may be more appropriate for bulk humic substances (Table 4). While this shift in C:N may appear to be small relative to the C:N ratio from previous reports, it can have substantial effects on environmental N budgets, as humic substances often comprise a majority of the DOM in aquatic systems. For example, in the Satilla River humic-C concentrations often exceed 1250 μM humic-C (Georgia Rivers Land Margins Ecosystem Research (GARLMER) project, unpubl. data). By using a C:N value of 40, rather than the corrected 35, humic associated N is underestimated by 4 μM, or 12.5%. This is the equivalent of more than $60*10^6$ g N per year in the Satilla River, assuming a discharge rate of 34 m^3 sec^{-1} (Alber and Sheldon 1999). Thus, it is imperative that the C:N ratio of humic substances in the aquatic environment be determined more accurately.

Second, phytoplankton and bacteria readily take up NH_4^+ as a N source, and any process that affects free NH_4^+ concentration could impact primary and secondary

Table 3. C:N ratio of humic substances isolated
from aquatic sources. Where available,
ranges are reported.

Location	Atomic C:N
humic acids	
Amazon River[1]	14.9 - 58.1
Bogs/Marshes[2]	91
Ogeechee Stream[3]	44
Suwanee River[4]	16.7
Streams and Rivers[2]	18
Groundwaters[4]	11 - 25
Mangrove Swamp[7]	22.3
Marine Surface Sediment[8]	11.1 - 13.3
Phytoplankton (Diatoms, Dinoflagellates, Coccoliths)[8,9]	10.3 - 15.4
fulvic acids	
Amazon River[1]	43.2 - 85.0
Bogs/Marshes[2]	69
Ogeechee Stream[3]	62.7
Suwanee River[4,5]	100
Streams and Rivers[2]	47
Groundwaters[4,6]	33 - 1000
Lakes, Rivers, and Ponds[10]	14 - 96
Sargasso Sea[11,12]	7.8
Pacific Ocean[10]	37
humic substances	
Humic-Rich Stream (UK)[13]	62.5 - 66.7

[1]Ertel et al. (1986); [2]Thurman (1985); [3]Malcolm (1985);
[4]Thurman and Malcolm (1981); [5]Thurman and Malcolm (1983);
[6]Steelnik (1985); [7]Moran and Hodson (1994);
[8]Vandenbroucke et al. (1985); [9]Pelet (1983);
[10]McKnight and Aiken (1998); [11]Stuermer and Harvey (1974);
[12]Harvey and Boran (1985); [13]Hunt et al. (2000).

Table 4. C:N ratio of humic substances saturated in the laboratory at increasing NH_4^+:humic C. Data for the 0:1 spring York River saturation are not included due to contamination, and 40:1 autumn Yor River sample was lost due to instrument error. N/A = not available. * indicates significance at $p < 0.05$.

River	Season	Saturation (μmol NH_4^+:μmol humic C)	Atomic C:N		std. dev	% Decrease from 0:1 Saturation
Satilla	Spring	0:1	99.11	±	8.86	
		0.048:1	69.87	±	1.89	29.5*
		0.48:1	67.18	±	3.78	32.3*
	Summer	0:1	44.85	±	2.59	
		0.048:1	44.39	±	1.25	1.0
		0.48:1	44.80	±	1.15	0.1
	Autumn	0:1	58.24	±	2.87	
		0.048:1	52.02	±	2.25	10.7
		0.48:1	52.83	±	2.36	9.3
	Winter	0:1	60.54	±	1.81	
		0.048:1	56.02	±	1.75	7.5
		0.48:1	54.23	±	1.46	10.4*
Altamaha	Autumn	0:1	51.75	±	1.44	
		0.048:1	37.57	±	1.23	27.4*
		0.48:1	43.93	±	0.36	15.1*
York	Spring	0:1	N/A			
		0.048:1	15.13	±	0.26	
		0.48:1	14.68	±	0.002	
	Autumn	0:1	13.76	±	0.30	
		0.048:1	12.57	±	1.04	8.7
		0.48:1	N/A			
	Winter	0:1	14.47	±	0.24	
		0.048:1	13.40	±	0.21	7.4*
		0.48:1	13.19	±	0.12	8.8*
				Mean		12.2 ± 10.9

production. Ammonium appears to make up a large portion of the N within humic compounds, with as much as 50% of the N in humic substances in the amine form (Schnitzer 1985). If the adsorbed NH_4^+ is no longer available to phytoplankton, humic concentrations could affect NH_4^+ bioavailibility. In contrast, if the phytoplankton community is able to access this N, humic substances could provide an important transport mechanism for moving labile N down river. Previous studies suggest that humic-N can be utilized either directly or indirectly by phytoplankton as a N source in coastal marine systems (Carlsson and Granéli 1993, Carlsson et al. 1993, Carlsson et al. 1995). A study of the uptake of ^{15}N-labeled humic substances by coastal phytoplankton and suggests that humic-N may be directly available to a suite of phytoplankton groups including diatoms, dinoflagellates, and chlorophytes (see Section IV, this volume). The humic-N may be potentially accessed by phytoplankton during the photochemically mediated release of NH_4^+, via cell surface enzymes, or during the pinocytosis of dissolved humics (Palenik and Morel 1991, Pantoja et al. 1993, Bushaw et al. 1996, Legrand and Carlsson 1998).

Third, these findings suggest that photochemical ammonification studies using isolated humics should be reevaluated. A number of studies have been performed showing a release of NH_4^+ and other bioavailable N compounds from humic material when exposed to sunlight or simulated sunlight (for example Bushaw et al. 1996, Bushaw-Newton and Moran 1999). However, the majority of these experiments have used humic substances isolated via the XAD extraction technique, potentially stripping loosely associated NH_4^+ from the humic structure during isolation. If this

loosely bound N is also photoreactive, previously reported values of photochemical N release have likely been underestimated.

Changes in chemical characteristics as humic substances age

While the geochemistry of humic substances has been an active area of research for decades, particularly in the soil sciences, the mechanisms responsible for the formation of humic substances remain a mystery. Indeed, "the formation of humic substances is one of the least understood aspects of humus chemistry..." (Stevenson 1994). Several theories exist as to the pathway of humic formation, including lignin degradation (Waksman 1932), polyphenol and quinone condensation (Flaig 1966, Flaig et al. 1975), sugar amine condensation (Hedges 1978), and lipid autoxidative cross-linking (Harvey et al. 1984). While a complete scheme for the formation and appearance of humic and fulvic acids has not yet been established, and a combination of mechanisms is most likely responsible, many researchers currently favor the polyphenol condensation pathway (Stevenson 1985, 1994, McKnight and Aiken 1998). The true age of riverine humics is unknown and likely will vary between locations. Hedges et al. (1986) estimated Amazon river humics to be relatively young (< 30 years) based on radiocarbon dating. Thurman (1985) and Malcolm (1985) have dated humic substances in groundwater and the Pacific Ocean to be between 660–4000 years old. Fulvic acid isolated from surface waters has also been shown to be much younger than those obtained from groundwaters and the deep ocean (Malcolm 1985). Thus, following the changes in the chemical makeup and structure of humic substances over the course of one year allowed for a better

understanding of humic formation and alteration in surface waters and the estuarine environment and are described below.

Changes in the C and N compositions of the LMW and HMW fractions observed in this study support the idea that smaller fulvic molecules polymerize and combine to form larger humic and humin molecules (Flaig 1966, Flaig et al. 1975). The observed net decrease in the C content of the LMW size fraction was from 65% to approximately 48% C throughout a year of formation with most of the changes occurring in the first 3 months (Fig. 4A). However while a net decrease was observed, there was a slow but significant ($p < 0.005$) increase in the LMW fraction after 3 months. This suggests that higher MW compounds are broken down into smaller more fulvic-like molecules. Support for this pathway is provided by Ertel et al. (1984) who demonstrates that the lignin signatures analyzed from humic acids are transformed into those from fulvic acids via demethylation and oxidation reactions, but fulvic acid lignin signatures are not transformed into those from humic acids. Most likely, a combination of pathways occurs in which the highly refractory C polymerizes and forms larger and larger compounds, while that which is more labile is slowly hydrolyzed into smaller molecules. The net increase in % N for both the LMW and HMW size fractions most likely occurs as the building blocks of humic substances, lignin, bacterial quinones, and lignin quinones, polymerize and combine with dissolved inorganic nitrogen (DIN), including NH_4^+, and DON, such as dissolved primary amines, in the water, increasing the N content of the humics (Stevenson 1994).

Changes in structures with age

FTIR spectroscopy is a powerful tool that can be used in the identification of complex compounds. The first set of humic substances analyzed was isolated seasonally in the Satilla River, GA. Seasonal spectra showed little, if any difference in the structure of the riverine humics. The overall lack of differences between spectra does not imply that there are no structural differences between the humic substances, however, as humic compounds can best be described as a consortium of compounds, any differences in the structures may be obscured by the larger, more similar fractions such as lignin backbones, common among estuarine humic substances.

The second set of FTIR samples was analyzed to monitor changes in the laboratory-formed humics produced by degrading *S. alterniflora* in coastal seawater. While the spectra appeared to be relatively similar, several differences arose in the FTIR structures as humic substances aged. The increase in aliphatic C, a decrease in aromatic chains, and an increase in O-H bonds suggest that more fulvic acid residues were present in the overall humic substances isolated (Thurman 1985, Stevenson 1994). The strong absorption present in the humic substances at about 1635 cm^{-1} is probably due to peptide carbonyls, and the decrease in the absorption of the small peak at about 835 cm^{-1} is difficult to explain, but may be due to a decrease in hydrocarbon ring structure chains (Don England pers. comm.).

These observations support the hypothesis that as humic substances age, their major components (including lignin) are broken down into smaller, more fulvic-like

subunits by bacterial enzymes, demethylation, and oxidation reactions (Waksman 1932, Flaig 1966, Flaig et al. 1975, Ertel et al. 1984).

CONCLUSIONS

The objectives of the experiments reported here were threefold. The first was to determine how the C:N ratio of humic materials changed with NH_4^+ enrichment. The results indicate that NH_4^+ can bind to humic substances during exposure at environmentally relevant concentrations, and implies that the C:N ratio of humic substances in natural waters is lower than traditional XAD isolation and analysis indicate. Thurman's 1985 value of 40 is often reported as the mean C:N ratio of aquatic humic substances isolated with XAD resin. The current study suggests that humic substances as they exist in riverine/estuarine waters have a mean C:N ratio that is 12% lower, or closer to 35 (27-40), depending on the humic source and sampling season. As the composition of humic substances varies significantly among different ecosystems, it may be more informative to develop ratios indicative of specific environments (riverine, estuarine, open ocean, etc.) rather than compile a bulk ratio for all humic substances. The second objective was to identify the MW fraction with which NH_4^+ binds on the humic structure. Results indicate that NH_4^+ is adsorbed to both the HMW and LMW fractions of the isolated humic substances, apparently at the expense of the intermediate MW fraction. The third objective was to quantify the changes in the humic structure as they aged. The FTIR structural data suggests an increase in fulvic acid residues as humics formed in the lab aged.

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SECTION III:

EXAMINATION OF SALINITY-REACTIVE HUMIC NITROGEN EXPOSED TO SIMULATED SUNLIGHT

60

ABSTRACT

Humic substances comprise a large percentage of dissolved organic nitrogen (DON) in riverine and estuarine environments. These compounds have been shown to release loosely bound ammonium (NH_4^+) when they are: 1) exposed to high salinities, 2) extracted onto XAD resins, and 3) exposed to sunlight. Humic substances extracted from three rivers and commercially available humic acids were examined to determine how the salinity-reactive and photo-reactive NH_4^+ pools interact in a riverine/estuarine environment, as this interaction could change the conventional views regarding the rates of photochemical NH_4^+ production. All samples released NH_4^+ when exposed to increased salinities, while only the Satilla and York River humics displayed a light-mediated release of NH_4^+. It was concluded that as humic substances pass through the estuary, changes in salinity and irradiance can remove or prevent the adsorption of NH_4^+ on the humic molecule, and literature values of N photoproduction from humic substances likely represent good estimates of rates occurring in the coastal ocean.

INTRODUCTION

Dissolved organic matter (DOM) comprises a large percentage of the total dissolved nitrogen (N) found within estuarine and marine environments (Thurman 1985). Humic substances, operationally defined as the compounds that can be isolated onto XAD-8 resin, often make up a majority of this DOM pool within an

estuary (Thurman 1985, Alberts and Filip 1994, Alberts and Takács 1999). While

several studies suggest that phytoplankton can use dissolved organic N (DON; i.e.

(Flynn and Butler 1986, Bronk and Glibert 1991, Bronk 2002), the prevailing thought

is that bulk DOM must first be broken down into more labile components prior to its

utilization by phytoplankton. Environmental mechanisms responsible for producing a

more labile substrate from DOM include bacterial breakdown, extracellular enzymes,

and photochemical cleavage (reviewed in Carlson 2002).

It also appears that a fraction of loosely bound ammonium (NH_4^+) on the

humic structure can be replaced by other cations when the surrounding ions reach

high enough concentrations. These observations are consistent with other studies that

show an increase in salinity can force a release of NH_4^+ ions from humic compounds

as salt ions replace the NH_4^+ on organic matter cation binding sites in aquatic

sediments and the water column (Rashid 1969, Rosenfeld 1979, Gardner et al. 1991).

Furthermore, a recent study suggests that much of the loosely associated NH_4^+ is

removed as the humics flow along an estuarine salinity gradient towards the sea (see

Section VI, this volume).

In a similar vein, N can also be removed from humics during their isolation

due to the high ionic strength of solutions used for extraction. The currently accepted

method for the isolation of humic substances from the aquatic environment is

extraction onto XAD-8 resin (Aiken 1985). Laboratory results suggest that the

loosely bound NH_4^+ on the humic molecule may be replaced with hydrogen ions

during extraction (see Section II, this volume). These observations are in agreement

with previous work that has shown that during the passage of humics through a cation

exchange resin as much as 75% of the N loosely bound to humics may be removed (Roulet et al. 1963 cited in Khan and Sowden, 1972, Sowden and Schnitzer 1967, Schnitzer 1985). As a result of N loss during isolation, literature values for the N content of humic substances in aquatic systems may underestimate actual concentrations by more than 12% (see Section II, this volume).

The release of N from humics during exposure to high salinities in the field or during isolation in the laboratory has significant implications for our understanding of photochemical NH_4^+ release. Should this loosely bound NH_4^+ also be photochemically active, reported values for the rates of *in situ* photochemical NH_4^+ production from humic substances, and likely DOM, may have been underestimated.

Light energy, primarily in the ultraviolet (UV) spectrum, can break the chemical bonds of refractory organic molecules and result in the release of labile compounds into the surrounding water. Numerous studies have demonstrated a release of bioavailable nitrogenous compounds from DOM, including NH_4^+, amino acids, and nitrite (NO_2^-) after exposure to natural or simulated sunlight (Bushaw et al. 1996, Gardner et al. 1998, Bushaw-Newton and Moran 1999, Kieber 2000, reviewed in Bronk 2002). Much of the research on N photoproduction to date has focused on the release of NH_4^+ from isolated humic and fulvic acids rather than total DOM (Bushaw et al. 1996, Bushaw-Newton and Moran 1999, Wang et al. 2000) due the difficulty of isolating bulk DOM from solution (Bronk 2002) and the abundance of humic substances within the DOM pool (Thurman 1985). However, the release of N from DOM is not a universal occurrence, as a number of studies have shown either a loss or no change in the ambient NH_4^+ concentration after exposure of DOM to

sunlight and/or UV light (Kieber et al. 1997, Jørgensen et al. 1998, Koopmans and Bronk 2002, McCallister 2002). Current estimates of N delivery to the coastal ocean via photochemical release are dependant on rates of photoproduction measured using freshwater or riverine samples (i.e. Bushaw et al. 1996) assuming that photochemically active N reaches the coastal ocean where it is then released. However, if this loosely bound NH_4^+ can be removed by exposure to both light and increased salinity, it is possible that a majority of the photochemically reactive NH_4^+ will be dissociated within rivers and estuaries prior to reaching the coastal ocean, resulting in an overestimation of N photoproduction in the coastal zone.

The first step to resolving this possibility is to determine how loosely bound NH_4^+ on the humic molecule responds to changes in salinity and solar irradiance. Two pools of exchangeable NH_4^+ are recognized in this study. The first pool, that fraction of the NH_4^+ associated with humics that will dissociate at the salinities encountered within estuaries, is defined as the "salinity-reactive" pool of NH_4^+. The second, defined here as the "photo-reactive" pool of NH_4^+, is that fraction of the NH_4^+ associated with humics released during exposure to solar radiation. Humic substances extracted from three rivers, as well as commercially available humic acids, were exposed to simulated sunlight and increased salinity to imitate the changes in salinity and/or irradiance within an estuary. Overall net release of NH_4^+ was low, but exposure to UV light prevented the readsorption of NH_4^+ to the humic structure. It was concluded that within a natural estuary, NH_4^+ is either desorbed from the humic molecule with increasing salinity, or prevented from adsorbing to the humic molecule under increased irradiance prior to reaching the coastal ocean. Therefore, little to

none of loosely bound NH_4^+ remains on the molecule to be cleaved by photochemical reactions in the coastal ocean, suggesting that previously published rates of photochemical release of NH_4^+ from humic substances isolated in the coastal ocean have not been underestimated.

MATERIALS AND METHODS

Sample sites

Humic substances were extracted for analysis from upriver stations in the Satilla and Altamaha Rivers, GA and the York River, VA. The Satilla River is considered to be a blackwater river and contains high DOM concentrations, with humic concentrations often exceeding 15 mg C L^{-1} (Alberts and Takács 1999); Georgia Rivers Land Margin Ecosystem Research project, unpubl. data). The Altamaha River has the second largest watershed in the United States and DOM concentrations are moderate (6-8 mg humic C L^{-1}). The York River is relatively clear compared to the other rivers examined and has the lowest DOM concentrations. All three rivers tested have similar land usage and are considered relatively pristine with urban areas comprising less than 5% and forested areas more than 55% of the land use for each watershed (T. Dai, pers. comm.).

Along with the riverine humic samples, humic acids purchased from Sigma-Aldrich, Inc. were also obtained and analyzed. These humics are isolated from the soil in Germany, and are referred to as Aldrich humics throughout the manuscript.

Sample isolation

For the river samples, humic substances were extracted onto Supelco DAX-8 resin as previously described for XAD-8 resin by Aiken (1985). DAX-8 and XAD-8 resins have been shown to isolate similar bulk humic solutes from aquatic sources, producing mixtures with similar chemical compositions (Peuravuori et al. 2002) As organic acids are adsorbed to the resin in the protonated form, each sample was first acidified to a pH of 1.8 with 6 N hydrochloric acid (HCl) and passed through a glass column packed with acidified DAX-8 resin. The column was then rinsed with deionized water (DIW) until the eluate reached a pH in excess of five to remove any remaining salts from the resin. Finally, the column was backflushed with two bed volumes of 0.2 N sodium hydroxide (NaOH) to remove bound humic substances from the resin, and the eluate collected. Following extraction, the sample was immediately neutralized with 6 N HCl and frozen until use.

These resins have been shown to bleed small amounts of organic molecules with the eluate (Aiken 1988). Therefore, prior to extraction of humic substances, the DAX-8 resin was cleaned via an extensive Soxhlet extraction procedure (solvents include ether, acetonitrile, and methanol) followed by numerous rinses of HCl, NaOH, and DIW (Thurman and Malcolm 1981, Aiken 1985). Prior to sample extraction, DIW water was passed through the resin to establish background levels of DON, NH_4^+, and NO_3^- as well as DOC that may leach from the DAX-8 resin (Parsons et al. 1984, Peltzer et al. 1996, Hansen and Koroleff 1999, Bronk et al. 2000).

Sample enrichment

In an attempt to recreate humic substances as they occur in the aquatic environment, the humic samples were enriched with NH_4^+ (Section II, this volume). Aquatic samples were first diluted to a concentration of 20 mg humic C L^{-1} with DIW. Ammonium chloride was then added to achieve a final concentration of 0.048 μmol NH_4^+:μmol humic-C. This enrichment level was chosen because at this ratio humic substances appear to have reached full saturation, and this ratio is environmentally relevant in riverine ecosystems (Section II, this volume). Following the addition of NH_4^+, the humic samples were shaken gently overnight on a shaker table. The pH of each sample was then raised to 10 with 1.0 N NaOH to convert the free, unbound NH_4^+ to ammonia (NH_3) gas, and the entire solution was degassed under strong vacuum for 1 hour (hr) to remove the excess, unbound ammonia gas from solution. The samples were then neutralized with 5% HCl and reconstituted to their original volume with DIW.

Removal of free NH_4^+ via vacuum

Raising the pH of the samples and applying a vacuum appeared to be an effective method of removing the excess NH_4^+ from solution. On average, 91 ± 4% of the initial NH_4^+ was removed from the samples. The greatest removal of NH_4^+ occurred in the York River sample (94%), and the least in the Altamaha River sample (85%). It was also found that removal was the most efficient when the vacuum pressure was removed after 30 minutes, the sample stirred vigorously for several

minutes to allow gases back into the solution, and the vacuum reapplied for an additional 30 minutes to remove remaining NH_3.

Release experiments

Following enrichment, each 20 mg humic C L^{-1} sample was divided into two 500 mL fractions. To one of the fractions, an additional 500 mL of DIW was added to produce one liter of freshwater humic substances at 10 mg humic C L^{-1}. The other 500 mL fraction received an additional 500 mL of 70‰ laboratory formed artificial seawater to create one liter of 10 mg humic C L^{-1} at 35‰ salinity.

Sixty-four quartz vials (30 mL capacity) were then overfilled with either the fresh or the saline sample (32 vials for each treatment) and sealed with a Teflon liner to exclude air from the vials. Half of the vials for each salinity were then wrapped in aluminum foil to exclude light. This resulted in a total of four treatments: 0‰ dark, 0‰ light, 35‰ dark, and 35‰ light. All vials were then exposed to simulated solar light by placing them under a light table.

The light table contained six 48-inch fluorescent bulbs positioned above an adjustable shelf. Three bulbs emitted light in the UV-A spectrum (295-365 nm, Q-Panel UVA-340). The remaining bulbs emitted light in the visible spectrum, mimicking the visible solar spectrum (400-750 nm, GE Full Spectrum F40T12/SR). For this experiment, samples were placed 10 inches below the lamps which replicated approximately 0.5 times the intensity of the noon sun for April at the Virginia Institute of Marine Science, Gloucester Point, VA as measured by a light meter and the photobleaching of Aldrich humics (data not shown). Samples placed under the

lamps were kept cool by submersing them underwater (20 °C) and by circulating the air under the lamps with a fan. Water was added as necessary to keep all vials submersed. Both light and dark samples were incubated under identical conditions.

Duplicate vials from each of the four final treatments were sacrificed at predetermined time points throughout the 72 hr experiment. Samples were mixed well, and NH_4^+ concentrations were immediately analyzed using the phenol/hypochlorite method adjusted for colored/turbid waters (Hansen and Koroleff 1999).

Statistical analysis

Differences in data points were considered significant at the $p < 0.05$ level, unless otherwise noted, and calculated via either ANOVA or t-tests using Excel and SAS statistical software packages.

RESULTS

Response of humics to increased light and salinity

In general, samples from all riverine systems showed three similar responses. First, increasing the salinity resulted in a release of NH_4^+ from the humic material. Second, exposure to simulated sunlight generally resulted in higher free NH_4^+ concentrations than those treatments that were kept in the dark. Third, the 0‰ dark treatment readsorbed free NH_4^+ from the surrounding water in all the samples tested

following a lag time of approximately 12 to 24 hrs. These responses are described in further detail for each sample below.

In the York sample, increasing the salinity resulted in a significant increase of 0.3 μM in free NH_4^+ (Fig. 1). This increase reflects the initial loss of NH_4^+ from the humic structure due to the higher salinity. Concentrations of NH_4^+ were higher in the 35‰ light treatment than in the 35‰ dark treatment, but the difference was not statistically significant (Fig. 1B). There was also a significant decrease of free NH_4^+ in the 0‰ dark treatment over time (p < 0.01) after a lag of 12 hrs; concentrations of free NH_4^+ fell from 2.3 to 0.1 μM. The 0‰ light treatment exhibited a significant increase in free NH_4^+ concentrations at T_{72} when compared to T_0.

In the Altamaha River the 35‰ dark and 35‰ light treatments showed a net release of NH_4^+ into the surrounding water (0.6 and 0.8 μM respectively), and this release was statistically different from T_0 (Fig. 2B). There were no significant changes in free NH_4^+ concentration in the 0‰ light sample. In the 0‰ dark treatment the concentration of NH_4^+ did not change for the first 24 hrs, but there was a statistically significant decline of 2.5 μM NH_4^+ by the end of the experiment (p < 0.01, Fig. 2A).

In the Satilla River the free NH_4^+ concentration increased in both the 35‰ dark and 35‰ light treatments (1.0 and 1.2 μM respectively, Fig. 3B). The free NH_4^+ in the 35‰ light treatment at T_{72} was significantly greater than T_0. In the 0‰ light treatment, the exposure to light resulted in a steady release of NH_4^+ after 4 hrs

Fig. 1. Concentration of free NH_4^+ in the York River, VA humic samples during the 72 hr exposure to simulated sunlight. (A) 0‰ water (B) 35‰ water. Error bars are equal to ± 1 standard deviation. The dashed line (- - -) indicates the free NH_4^+ concentration at T_0. Values above the dashed line signify release; values below the dashed line signify adsorption.
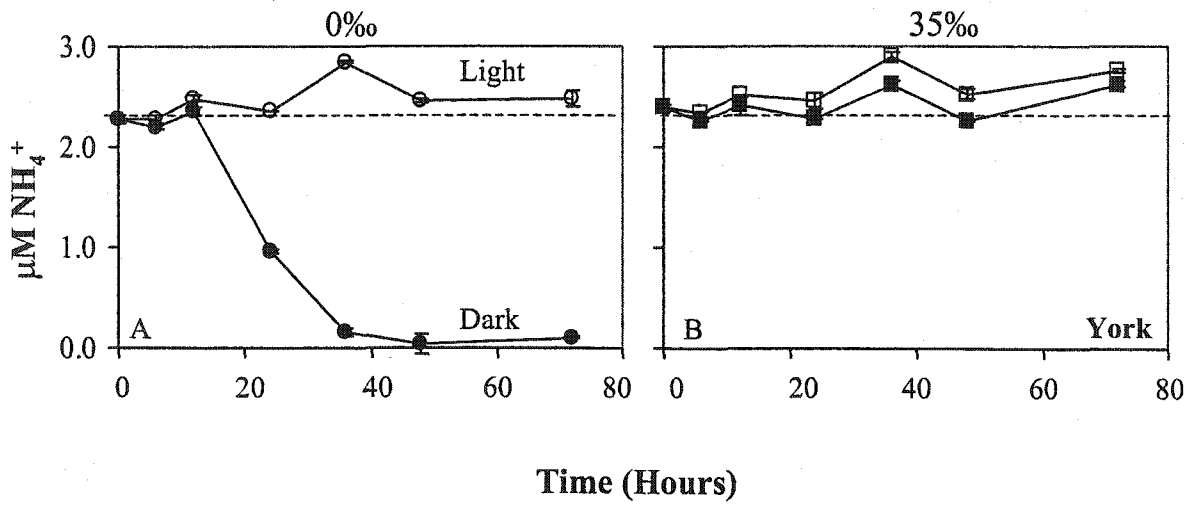
Fig 2. Concentration of free NH_4^+ in the Altamaha River, GA humic samples during

the 72 hr exposure to simulated sunlight. (A) 0‰ water for the Altamaha

River. (B) 35‰ water for the Altamaha River. Error bars are equal to ± 1

standard deviation. The dashed line (- - -) indicates the free NH_4^+

concentration at T_0. Values above the dashed line signify release; values

below the dashed line signify adsorption.

Time (Hours)
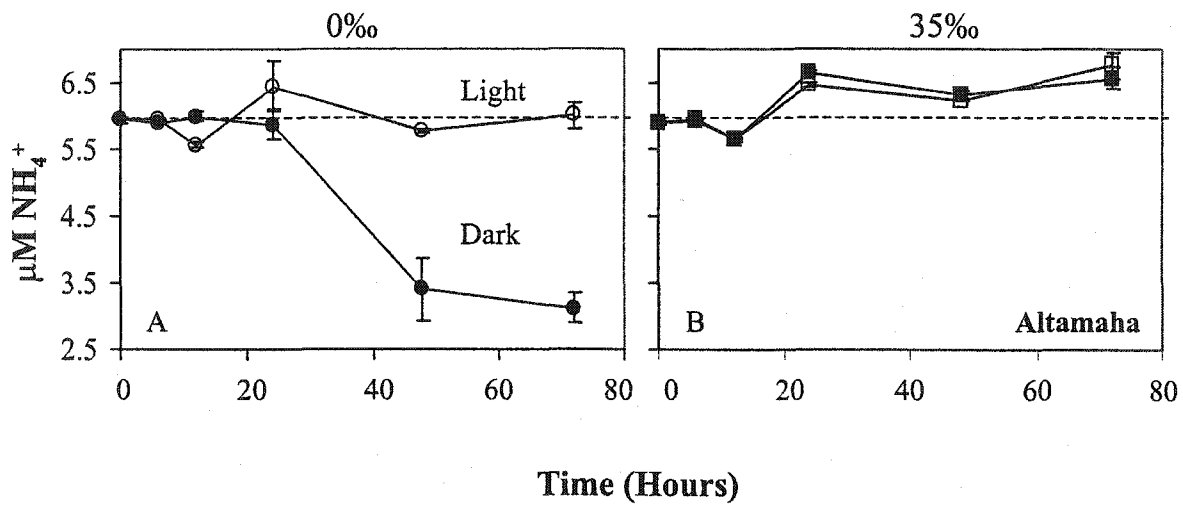
Fig. 3. Concentration of free NH_4^+ in the Satilla River, GA humic sample during the 72 hr exposure to simulated sunlight. (A) 0‰ treatments. (B) 35‰ treatments. Error bars are equal to $\pm$ 1 standard deviation. The dashed line (- - -) indicates the free NH_4^+ concentration at T_0. Values above the dashed line signify release; values below the dashed line signify adsorption.

0‰   35‰

Time (Hours)
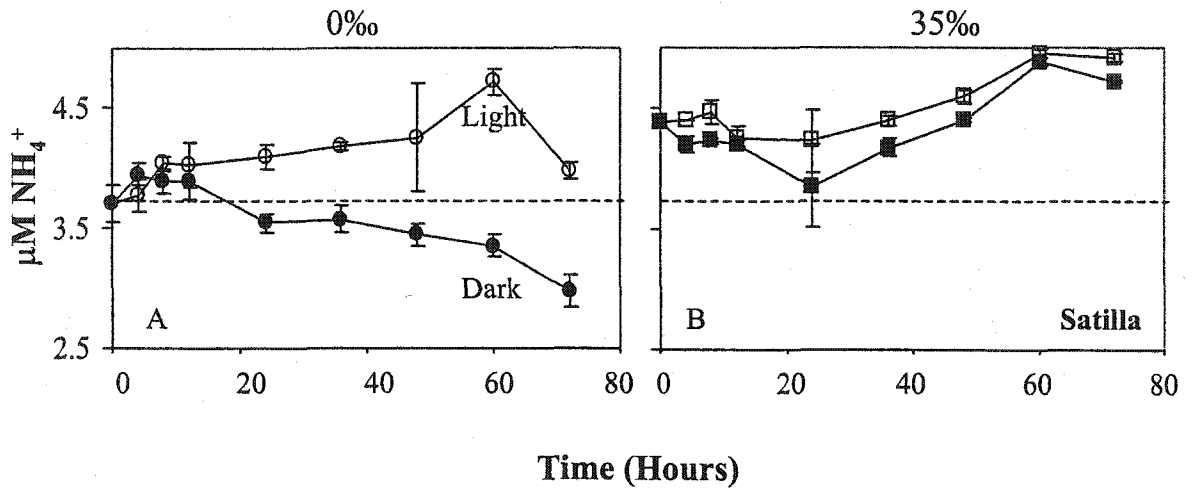
(0.007 μmol N L^{-1} h^{-1}; Table 1) from the humic material. There was also a decline in the free NH_4^+ concentration in the 0‰ dark treatment. In the Aldrich humic acids there was an immediate increase (0.3 μM) in NH_4^+ concentration in the high salinity treatments (35‰ dark and 35‰ light, Fig. 4B). Exposure to light resulted in significantly higher free NH_4^+ concentrations in the 35‰ light than the 35‰ dark treatment at T_{72} ($p < 0.01$), and there was a significant decrease in the concentration of free NH_4^+ after T_{12} ($p < 0.01$, Fig. 4A) in the 0‰ dark treatment. In contrast to the other samples, there was a steady decline in free NH_4^+ concentration in the 0‰ light treatment. Concentrations of free NH_4^+ in the 0‰ light treatment were significantly lower than T_0 NH_4^+ concentrations ($p < 0.01$) but significantly higher than free NH_4^+ at T_{72} in the 0‰ dark treatment.

DISCUSSION AND CONCLUSIONS

Here the release and readsorption of free NH_4^+ to humic substances with respect to light and salinity, the differences observed between the riverine systems examined, and the implications of these findings are discussed in further detail.

Adsorption of NH_4^+ to humic substances

In all four samples free NH_4^+ concentrations decreased in the 0‰ dark treatment over the 72 hr time period, following a lag period ranging from 12 to 24 hrs. The underlying reasons for the readsorption of NH_4^+ in the 0‰ dark

Fig. 4. Concentration of free NH_4^+ in the commercially available Aldrich humic

acids sample during the 72 hr exposure to simulated sunlight. (A) 0‰

treatments. (B) 35‰ treatments. Error bars are equal to ± 1 standard

deviation. The dashed line (- - -) indicates the free NH_4^+ concentration at T_0.

Values above the dashed line signify release; values below the dashed line

signify adsorption.

Time (Hours)
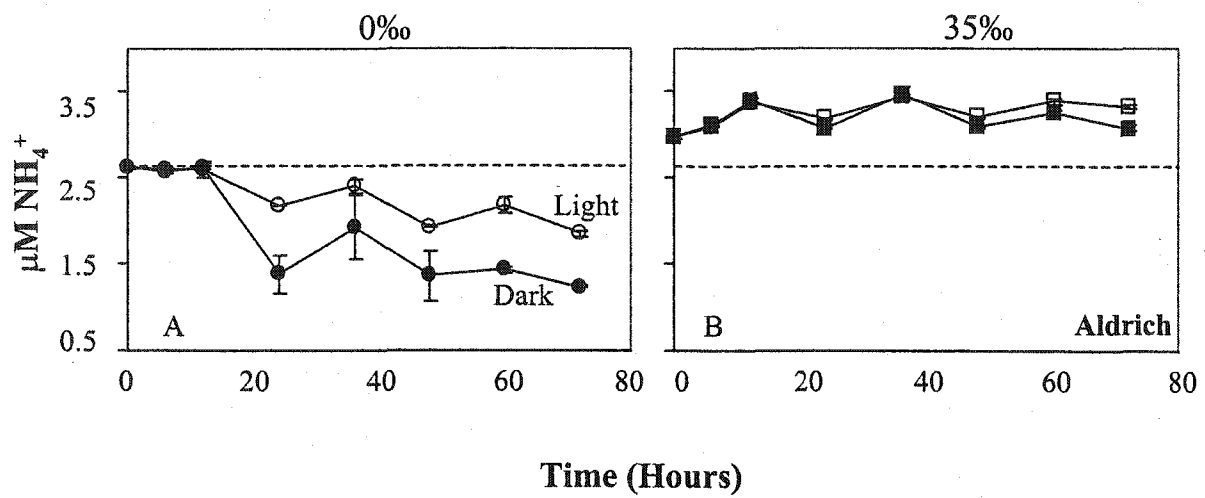
Table 1. Calculated rates of the photochemical release of NH_4^+ from humic substances from the York River, VA, Altamaha and Satilla Rivers, GA, and Aldrich humic acids. Rates were calculated by subtracting the NH_4^+ concentration in the 0‰ treatment at T_0 from the 0‰ light treatment at T_X, and dividing by the time. Positive values correlate to a release of NH_4^+ from the humic material, while negative values correspond to an uptake of free NH_4^+ from solution onto the humic structure. No sample was taken at timepoints labeled N.S. Only the York and Satilla River samples are significantly greater than zero at T_{72}.

Photochemical Production rate μM NH_4^+ hr^{-1}

	York River	Altamaha River	Satilla River	Aldrich Humic Acids
T_4	N.S.	N.S.	0.018 ± 0.052	N.S.
T_6	0.000 ± 0.010	0.002 ± 0.009	N.S.	-0.003 ± 0.004
T_8	N.S.	N.S.	0.043 ± 0.021	N.S.
T_{12}	0.017 ± 0.005	-0.031 ± 0.006	0.027 ± 0.021	-0.002 ± 0.008
T_{24}	0.003 ± 0.002	0.021 ± 0.016	0.017 ± 0.008	-0.018 ± 0.000
T_{36}	0.016 ± 0.001	N.S.	0.013 ± 0.004	-0.006 ± 0.002
T_{48}	0.004 ± 0.001	-0.004 ± 0.001	0.012 ± 0.010	-0.014 ± 0.000
T_{60}	N.S.	N.S.	0.017 ± 0.003	-0.007 ± 0.002
T_{72}	0.003 ± 0.001	0.001 ± 0.003	0.004 ± 0.002	-0.011 ± 0.000

treatments for all samples are unclear, but may be related to the technique used to remove the excess NH_4^+ from solution. When the pH is raised to remove the excess free NH_4^+, base hydrolysis reactions might occur that remove a fraction of the exchangeable NH_4^+ from the humic structure. After the NH_3 was removed via vacuum degassing and subsequent neutralization of the solution, release experiments may have started too soon (within 2-3 hrs) to allow NH_4^+ to re-equilibrate on the humic molecule. However, the observation that free NH_4^+ concentration did not begin to decline until 12 hrs (Aldrich humic acids, York River, and Satilla River) or 24 hrs (Altamaha River) after the experiment had begun argues against this possible artifact. Another potential explanation is that bacteria within the quartz vials utilized the free NH_4^+, thus lowering the measured concentration. While bacterial counts were not performed, samples were prefiltered through 0.22 μm cellulose acetate filters prior to NH_4^+ saturation. Also, no decline was seen in the free NH_4^+ concentrations for the riverine samples exposed to light. Furthermore, if bacterial utilization was responsible for the decline in NH_4^+ concentration, a more gradual decline in the free NH_4^+ would be expected throughout the 72 hr experiment, and a decline would be expected in the 35‰ dark treatments as well. None of these conditions were observed in the samples examined, thus arguing against bacterial utilization as a cause for the decrease of free NH_4^+ concentrations.

In all cases, however, the addition of salt (35‰) and exposure to light prevented readsorption of NH_4^+ to the humic molecule as observed in the dark treatments. It is possible that the exposure to UV light or the increased concentrations of salts alters the conformation of the humic material in solution

making the cation binding sites inaccessible to the free NH_4^+ in solution. Another possibility is that NH_4^+ may be simultaneously adsorbed and released from the humic molecule until some equilibrium is reached when exposed to UV light. An isotope dilution experiment in which $^{15}NH_4^+$ is utilized to monitor the isotopic signature of humic-N and the free NH_4^+ would help quantify the rates of each process.

Salinity-mediated release

In all four samples the increase in salinity not only prevented the readsorption of NH_4^+, but also forced a statistically significant release of NH_4^+ from the humic substances (Fig. 1-4). This release was statistically significant in all four samples examined. These results are consistent with previous results that suggest humic substances are capable of adsorbing NH_4^+ in the environment and re-releasing it into surrounding waters (Rosenfeld 1979, Gardner et al. 1991), Section VI, this volume). At the end of the experiment, the largest salinity-mediated release when compared to T_0 was observed in the Satilla River (1.0 ± 0.3 μM) followed by the Altamaha River (0.6 ± 0.5 μM), Aldrich humic acids (0.5 ± 0.03 μM), and York River (0.3 ± 0.1 μM). These data suggest a possible continuum with rivers containing high humic concentrations and high terrestrial input of humic material (blackwater rivers like the Satilla) being the most capable of releasing free NH_4^+ when they encounter high salinities, followed by terrestrial humic substances (i.e. Aldrich humic acids), and finally rivers with lower humic concentrations.

Light-mediated release

The Satilla and York Rivers were the only samples to exhibit a light-mediated release of NH_4^+ in freshwater treatments (Fig. 1 and 3A). Bushaw et al. (1996) measured NH_4^+ photoproduction rates from Satilla River fulvic acids and found a rate of 0.050 ± 0.015 μM h^{-1} over 8 hrs, consistent with that observed for the Satilla in this study (0.043 μM h^{-1}, Table 1). For the Altamaha River, NH_4^+ concentrations were also higher at T_{72} than at T_0; however, the difference was not significant, nor was a trend of continual increase observed over the course of the experiment. For the Aldrich humic acids general trends were the same as those seen in the rivers examined, except free NH_4^+ concentrations decreased in the presence of light at 0‰.

One possible explanation for the general lack of a light-mediated release may be due to the source of light used. UV-B light (280-315 nm) has been shown to be more efficient in the formation of labile photoproducts than photons in the near visible range by at least an order of magnitude (Miller 1999, Moran et al. 2000, Miller et al. 2002). The light table in this experiment used the UV-A and visible spectra. Addition of UV-B wavelengths, while only a minor percentage of the solar spectrum, might have increased the rate of photoproduction of NH_4^+ in the samples from this experiment.

Photochemical implications

What are the implications of these observations to the understanding of N photoproduction in natural waters? Experiments previously performed with humic

substances isolated with XAD or DAX resins use humic substances that have potentially been stripped of much of their bound NH_4^+ (see Section II, this volume). As a result, potential release rates are likely underestimated because humics in nature likely have higher amounts of bound N susceptible to release. For example, Satilla River humic substances, as they exist in nature, more likely resemble the 0‰ dark treatment at T_{72}, in which the humics have been allowed to readsorb NH_4^+ at ambient river concentrations, than those simply extracted by the DAX-8 resin. If these humics were exposed to UV or sunlight, NH_4^+ could potentially be released at rates many times higher (Fig. 3A) than humics that were resaturated with NH_4^+ and maintained in the light (0‰ light) or humics that were extracted and not resaturated with NH_4^+. In this sense, previously published rates of photochemically released NH_4^+ from humic substances likely underestimate the potential of humics to release NH_4^+.

The adsorption of NH_4^+ to humic substances was not expected. Therefore, the photoreactivity of the NH_4^+ adsorbed onto the humics in the 0‰ dark treatments was tested using a small amount of leftover river and Aldrich samples. Though no photochemical NH_4^+ release was observed in any of the four samples, this result should not be considered conclusive due to experimental constraints, as samples were exposed to natural sunlight for a period of only four hours.

Environmental implications

What then are the implications of these observations to the understanding of how humic substances and free NH_4^+ interact in natural waters with respect to salinity

and solar irradiation? To illustrate the potential implications, two example riverine/estuarine environments are discussed in further detail: one that is turbid with low light penetration and one that is a shallow and/or well mixed system. In more turbid systems, including blackwater rivers and the heads of estuaries, exposure to high levels of UV is likely to be small due to the strong attenuation of light at the water's surface. Under these conditions, humic substances would likely be capable of binding considerable quantities of NH_4^+ from the surrounding waters, as seen in the latter part of the 0‰ dark treatments examined in this paper (Fig. 1-4). As these "N-loaded" humics pass down the river, or move through the estuary, increasing salinities would cause a release of the loosely bound NH_4^+ from the humic structure (Rosenfeld 1979, Gardner et al. 1991), see Section VI, this volume). As a result, when these humic substances reach the lower estuary or coastal ocean, where UV penetration tends to be more extensive, or where the mixing regime is such that the humics are more readily exposed to surface irradiance, only a small amount of additional NH_4^+ will likely be available for photochemical release into the surrounding waters because the NH_4^+ was released further upriver (i.e. differences between 35‰ dark and 35‰ light treatments, Fig. 1-4).

Alternatively, in shallow and/or well-mixed rivers or estuaries humic substances may be exposed to higher levels of UV radiation on a more regular basis. Under these conditions, readsorption of NH_4^+ to the humic structure is likely to be low as seen in the 0‰ light treatments (Fig. 1-4). As these humics pass down the river, or through the estuary, less NH_4^+ will be loosely bound to the humic substances and available for release by increased salinities. Furthermore, only a small amount of

additional NH_4^+ will likely be available for photochemical release into the surrounding waters when these humic substances reach the lower estuary or coast.

Laboratory procedures used to extract isolate humic substances from riverine sources expose humic substances to low pH, and thus large quantities of hydrogen ions (H^+). The exposure to large cation concentrations mimics the exposure of aquatic humic substances to salt cations along an estuarine salinity gradient in a more turbid river system. Excess NH_4^+ likely does not bind to humic substances in more well mixed systems where humic substances are exposed to surface irradiation, and thus is not available for removal during extraction onto XAD resins. Based upon these environmental parameters, natural humic substances likely do not contain large amounts of loosely bound NH_4^+ when they reach the coastal ocean and its deeper UV penetration. Therefore, literature values of N photoproduction from humic substances, although potentially stripping the loosely bound N and lowering the overall N content of riverine humics during extraction, are likely accurate representations of photochemical rates of NH_4^+ production from humic substances occurring in the coastal ocean.

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SECTION IV

DIRECT UTILIZATION OF *SPARTINA*-DERIVED HUMIC NITROGEN BY

ESTUARINE PHYTOPLANKTON[†]

[†]In preparation for Limnology and Oceanography

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ABSTRACT

Humic substances are a collection of colored organic acids characterized by high

molecular weight (HMW) and low nitrogen (N) content. These compounds, which

comprise a large percentage of the dissolved organic matter (DOM) in riverine and

estuarine environments, have historically been thought to be biologically recalcitrant,

and utilization of humic-N was only possible following breakdown of the molecule

by bacterial enzymes or photooxidation. However, recent studies have begun to

demonstrate the ability of phytoplankton cells to take up HMW DOM. In this study,

a wide suite of estuarine phytoplankton was examined to determine their capability

for taking up humic-N. ^{15}N-labeled humic substances were formed in the laboratory

and added to cultures as the sole N source. All estuarine isolates examined were

capable of taking up humic-N under N-limited conditions. The ability of

phytoplankton to utilize humic substances as a N source could provide a sink for

terrestrial DOM as it approaches the coastal ocean and could have implications on

environmental N loading budgets.

INTRODUCTION

Humic substances are a collection of colored organic acids characterized by

large size and high molecular weight (HMW), ranging from 500 to 10^6 Daltons

(Thurman 1985, Wershaw and Aiken 1985). These compounds can comprise 10-75%

of the total dissolved organic carbon (DOC) pool in seawater (Thurman 1985, Alberts

and Takács 1999), and can constitute as much as 95% of the natural organic matter transported to coastal marshes by rivers (Alberts and Filip 1994).

In general, humic substances contain little nitrogen (N; 0.5-6%; Rashid 1985, Thurman 1985, Hedges and Hare 1987). Amino acids, amino sugars, ammonium (NH_4^+), and nucleic acids comprise 46-53% of the N in humic acids and 45-59% of fulvic acid N (Schnitzer 1985). The remaining N associated with humic substances, approximately 50% of the total humic-bound N, remains unidentified (Carlsson and Granéli 1993). Early works largely suggest that HMW organics, such as humic substances, are highly refractory, and therefore do not play a significant role as either an energy or N source for organisms (Fenchel and Blackburn 1979) as compounds within this group are up to 3000 times the size of compounds considered more labile, such as amino acids and simple sugars (Moran and Hodson 1994b). Previous studies have also indicated that humic compounds are not as refractory as believed (Amon and Benner 1994). Bacteria are capable of utilizing C from humic and humic-like substances for growth (Tranvik 1988, Moran and Hodson 1990, 1994a, b, Hunt et al. 2000). Additions of humic substances isolated from rivers, as well as commercially available humic acid salts, appear to increase growth, chlorophyll concentrations, and rates of primary production of natural plankton assemblages (Carlsson and Granéli 1993, Carlsson et al. 1993, Carlsson et al. 1995, Carlsson et al. 1998, Carlsson et al. 1999) and phytoplankton cultures (Prakash and Rashid 1968, Prakash et al. 1973). Much of this stimulating effect is attributed to the capability of humic material to alter the chemistry of the local environment either by the addition of nutrients or the removal of potential toxic materials. Humic compounds can also undergo

photochemical oxidation into small more readily assimilable compounds (Bushaw et al. 1996, Bushaw-Newton and Moran 1999, Kieber 2000). Preliminary work has also demonstrated the direct uptake of ^{15}N-labeled humic substances by the planktonic community (> 0.7 μm size fraction) in both riverine and coastal waters (Bronk unpubl. data). It is unknown whether this uptake was due to phytoplankton or bacterial utilization and/or recycling of humic-N.

The objective of the current study was to determine if a suite of estuarine phytoplankton strains were capable of utilizing humic compounds as a N source. Humic substances, labeled with ^{15}N, were formed in the laboratory and added to phytoplankton isolates as the sole N source. It was found that all estuarine phytoplankton strains tested were capable of taking up humic-N from solution.

MATERIALS AND METHODS

Culture selection

A survey approach was employed to assay which of a suite of phytoplankton isolates are able to access humic-N as a N source. Seventeen isolates from estuarine environments and one polar clone (*Phaeocystis* cf. *antarctica*) were tested (Table 1). The cultures were not axenic (bacterial biomass approximately 5% of the total biomass in culture as determined by the method of (Porter and Feig 1980)), and were maintained in 0.2 μm filtered f/2-enriched Sargasso seawater (Guillard 1983), diluted with deionized water (DIW) to the appropriate salinity when necessary. All coastal

Table 1. Uptake rates of NO_3^- for a variety of coastal isolates grown on NO_3 media. Values are +/- 1 standard deviation. N/A represents samples that were lost and not available.

Taxa	Strain	Source of Origin	Culture Salinity	nmol NO_3^- μg chl^{-1} hr^{-1}	fmol NO_3^- $cell^{-1}$ hr^{-1}
Chlorophyceae					
Ankistrodesmus sp.	HP9101	Choptank River (estuary), MD	16‰	1.78 ± 0.48	1.90 ± 0.03
Selenastrum sp.	SCAEL010524-NF	Kiawah Island (brackish pond), SC	5‰	0.87 ± 0.02	0.28 ± 0.01
Chrysophyceae					
Ochromonas sp.	SCAEL970626	North Inlet (estuary), SC	30‰	2.09 ± 0.08	0.09 ± 0.00
Cryptophyceae					
Storeatula major	HP9001	Choptank River (estuary), MD	16‰	10.61 ± 0.74	28.70 ± 2.00
Cyanophyceae					
Anabaenopsis elenkini	SCAEL010524-1C3	Kiawah Island (brackish pond), SC	5‰	10.68 ± 4.58	0.02 ± 0.01
Limnothrix sp.	HP9101	Choptank River (estuary), MD	16‰	9.93 ± 0.96	0.79 ± 0.08
Synechococcus sp.	HP9101	Choptank River (estuary), MD	16‰	9.13 ± 0.09	N/A
Bacillariophyceae					
Nitzschia sp.	SCAEL940210	North Inlet (estuary), SC	30‰	0.59 ± 0.10	0.43 ± 0.07
Phaeodactylum sp.	HP9101	Choptank River (estuary), MD	16‰	58.28 ± 2.21	N/A
Thalassiosira cf. *miniscula*	HP9101	Choptank River (estuary), MD	16‰	5.27 ± 0.20	4.15 ± 0.16
Dinophyceae					
Amphidinium carterae	CCMP1314	Falmouth Great Pond (brackish), MA	30‰	0.62 ± 0.48	0.24 ± 0.19
Prorocentrum minimum	SCAEL010403-1A3	Murrells Inlet (estuary), SC	30‰	4.37 ± 0.07	35.86 ± 0.54

Table 1 cont.

Haptophyceae/Prymnesiophyceae

Phaeocystis cf. *antarctica*	CCMP1871	Bellingshausen Sea, Antarctica	36‰	0.76 ± 0.03	1.49 ± 0.30
Prymnesium parvum	SCAEL010524-1B2	Kiawah Island (brackish pond), SC	5‰	1.74 ± 0.13	2.85 ± 0.21
Pavlova sp.	HP9101	Choptank River (estuary), MD	16‰	2.37 ± 0.06	1.25 ± 0.03

Raphidophyceae

Chattonella subsalsa	CAAE1662X	Kiawah Island (brackish pond), SC	20‰	4.12 ± 0.23	7.81 ± 0.43
Fibrocapsa japonica	CAAE1661X	Kiawah Island (brackish pond), SC	20‰	8.06 ± 0.21	2.12 ± 0.06
Heterosigma akashiwo	CAAE1665X	Neuse River (estuary), NC	20‰	6.97 ± 1.09	22.99 ± 3.60

cultures were grown on a 12 hr: 12 hr light: dark cycle under fluorescent light at 20 °C, and the polar strain (*P. antarctica*) was grown under constant light at −1.5 °C.

Preparation of ^{15}N-labeled Spartina alterniflora *humics*

Estuarine humic substances labeled with ^{15}N were produced in the laboratory by growing *S. alterniflora* plants in buckets with ^{15}N-labeled NH_4Cl added to the surrounding sediment. *Spartina alterniflora* was chosen as a source for humic formation because it is the dominant primary producer within most marsh ecosystems of the southeastern United States (Pomeroy et al. 1981, Alberts and Filip 1994). It is also the primary source for refractory carbonaceous compounds, including humic substances, in salt marshes along the Atlantic and Gulf coasts (Filip and Alberts 1988). Small *S. alterniflora* plants were collected at the Skidaway Institute of Oceanography (SkIO) and transferred into buckets where they were grown over a three month period (April – June) while being watered with a 4.0 mM $^{15}NH_4Cl$ solution (Cambridge Isotope Laboratories; ^{15}N, 98+%). The ^{15}N-label was added to the plants approximately every third day. After three months, the *S. alterniflora* shoots were harvested, dried in an oven, and shredded in a Wiley mill (60 mesh). Shredded *S. alterniflora* (8 grams) was added to 1 L coastal seawater collected at SkIO (Skidaway River) from which the humic material had been extracted, and the mixture stirred (magnetic stir bar) in the dark for two weeks. Humic substances were isolated onto DAX-8 resin (see below) to produce "fresh" humic material, which was then neutralized and frozen until use.

Isolation of humic substances

Humic substances were extracted onto Supelite DAX-8 resin as previously described by Aiken (1985) for Amberlite XAD-8. DAX-8 is an acrylic ester resin, and both Supelite DAX-8 and Amberlite XAD-8 resins have been shown to isolate comparable bulk humic solutes from aquatic sources, producing mixtures with similar chemical compositions (Peuravuori et al. 2002). Because humic substances adsorb to DAX-8 resin in the protonated form, each sample was acidified with 6 N hydrochloric acid (HCl, pH < 2) and passed through a glass column (2.5 cm X 50 cm) packed with acidified DAX-8 resin. The resin was then rinsed with DIW until the eluate reached a pH > 5 to remove any remaining salts from the resin. Following the rinse, the column was backflushed with two bed volumes of 0.2 N sodium hydroxide (NaOH) to elute the bound humic substances from the resin.

XAD-8 resins have been shown to bleed small amounts of organic molecules with the eluate (Aiken 1988). Therefore, prior to the extraction of humics, the DAX-8 resin was cleaned over several days via a Soxhlet extraction procedure (solvents include ether, acetonitrile, and methanol) followed by extensive rinses of HCl, NaOH, and DIW (Aiken 1985, Thurman 1985). Prior to sample extractions, DIW was extracted as sample to establish baseline levels of dissolved organic N (DON), NH_4^+, and nitrate (NO_3^-) as well as DOC that may leach from the XAD-8 resin (Moran and Hodson 1994b).

Uptake experiments

Prior to incubation of estuarine strains with the laboratory formed ^{15}N-labeled humics, each isolate was transferred a minimum of two times into amended f/2-enriched seawater containing commercial humic acid salts (Aldrich humic acids) at a concentration of 10 mg humic-C L^{-1}. The concentration of N in the media was also reduced to ensure that N limiting conditions would occur (10:1 N:P). Culture growth was monitored daily by *in vivo* chlorophyll (chl) *a* fluorescence on a Turner 10AU Fluorometer, calibrated with a solid standard, and used to estimate actual chl *a* concentration. Uptake experiments were initiated only after it had been determined that each individual culture had depleted NO$_3^-$ to concentrations less than 1 μM (Parsons et al. 1984).

To measure uptake, 15 mL of each isolate was dispensed into six 25-mL culture tubes. Two culture tubes received 0.5 mL of the ^{15}N-labeled humic substances (8.07 at%; final concentration of 10.0 μM humic-N). Two culture tubes received a 15 μl aliquot of mercuric chloride (HgCl), were mixed by hand, and then allowed to sit for 5 min prior to the addition of humic label (0.5 mL). These poisoned tubes were used as killed controls to correct for adsorption of the humic label to phytoplankton cells and filter (see below). The remaining two culture tubes received a 0.13 μM addition of K^{15}NO$_3$ (^{15}N, 98+%) and were used to ensure that cultures were active when the uptake experiments were initialized.

To ensure any uptake of the humic label would be detected, samples were incubated for 3 hrs in a Percival Scientific incubator under fluorescent light at the culture maintenance light and temperature levels (11.6 μE m^{-2} sec^{-1} and 20 °C for

coastal isolates and 56.5 $\mu E\ m^{-2}\ sec^{-1}$ and $-1.5\ °C$ for the polar strain). Following

incubation, phytoplankton cells were collected via filtration onto a 25-mm Whatman

GF/F filter (0.7 μm nominal pore size) and frozen until analysis.

Determination of uptake rate

For analysis, sample filters were dried at 50°C and wrapped in tin discs for

analysis on an isotope ratio mass spectrometer (Europa Geo 20/20 with ANCA

sample preparation unit). The uptake rates of humic-N and NO_3^- were calculated

using the equations of Dugdale and Goering (1967). Adsorption of the humic label to

phytoplankton cells and filter was corrected at the at% level prior to calculation of the

uptake rate with the killed control samples using the following equation:

$$Atom\%xs_{Corrected} = \left(\frac{\dfrac{(PN_{Live})(Atom\%xs_{Live})}{100} - \dfrac{(PN_{Killed})(Atom\%xs_{Killed})}{100}}{PN_{Live}} \right) * 100$$

where $Atom\%xs_{Corrected}$ is the atom% excess value (atom% value over natural

abundance) used for calculating uptake, PN_{Live} is the μmol particulate N L^{-1} in the

live samples, $Atom\%xs_{Live}$ is the atom% excess for the live sample, PN_{Killed} is the

μmol particulate N L^{-1} for the poisoned samples, and $Atom\%xs_{Killed}$ is the atom%

excess for the poisoned samples. Addition of the humic label raised NH_4^+

concentrations by 0.9 μM in the culture tubes. The contaminant NH_4^+ in the humic

label was isolated via solid phase extraction (Cochlan and Bronk 2001), and was

determined to have an isotopic ratio similar to the humic label (8.15 at%). Uptake rates were also corrected for the uptake of NH_4^+ by subtracting the maximum potential uptake rate of NH_4^+ from the calculated uptake rate.

To calculate actual uptake rates, ambient concentrations of each substrate in solution are necessary. For NO_3^-, ambient concentrations were determined on an Alpkem autoanalyzer (Parsons et al. 1984). However, due to a lack of culture volume, ambient concentrations of humic-N were not quantified prior to incubation. Although grown in media amended with humic acids, it was assumed that the concentration of bioavailable humic-N remaining in the cultures at the time of the uptake experiments was negligible. This assumption is based on time course experiments with labeled humic substances which showed that within 3-12 hours, most, if not all of the bioavailable humic-N had been used (see Section V, this volume). This resulted in a higher estimate for the atom% enrichment of the humic substrate and a more conservative estimate of uptake.

Cell Counts and Statistics

Cells were fixed in formalin (final concentration of 5%) and counted under a microscope using either a Bright Line or Nageotte counting chamber (Hausser Scientific). For each sample, a minimum of 200 cells was counted. Bacterial cells were counted using the method of Porter and Feig (1980). Humic-N and NO_3^- uptake rates were compared via one-way analysis of variance (ANOVA) using SAS statistical analysis software.

RESULTS

All cultures took up NO_3^-, indicating they were active at the time of the experiments (Table 1). In addition, all estuarine isolates were capable of taking up the ^{15}N-label associated with the humic compounds added as tracer, as all uptake rates were significantly greater than zero ($p < 0.05$; Fig 1). The potential uptake from contaminant NH_4^+ in the labeled humics could not account for the observed uptake for any of the strains. In contrast, *P. antarctica* did not take up the humic-N.

When normalized to chl *a*, the humic-N uptake rates for *Synechococcus* sp. (329.9 ± 14.6 nmol humic-N μg chl^{-1} h^{-1}) and *Phaeodactylum* sp. (445.1 ± 222.0 nmol humic-N μg chl^{-1} h^{-1}) were significantly greater than all other isolates ($p < 0.05$; Fig. 1). Normalizing to cell counts, *Prorocentrum minimum* and *Storeatula major* had the highest uptake rates at 153.9 ± 4.1 and 111.6 ± 5.1 fmol humic-N cell^{-1} hr^{-1}, respectively (Fig. 1). Note that cell counts were not available for *Pheaodactylum* sp. and *Synechococcus* sp.

DISCUSSION

The results from this study suggest that the ability to take up humic-N is widespread in estuarine phytoplankton. It is interesting that all estuarine clones exhibited this ability while the polar isolate, *P. antarctica*, did not. These data provide an impetus for follow-up tests of differences in humic-N uptake potential between estuarine, coastal marine, and open ocean phytoplankton communities.

Fig. 1. Humic-N uptake rates A) normalized to µg chl *a* (uptake by *Synechococcus*

sp. and *Phaodactylum* were significantly higher than all other isolates and

were excluded in the determination of significant difference between the

remaining cultures), and B) normalized to cell abundance. Cell counts for

Synechococcus sp. and *Phaodactylum sp.* were not available. Error bars

represent 1 standard deviation. B.D. = below detection. Values identified by

the same letter were not significantly different at $p < 0.05$.

Uptake Rate

nmol humic-N μg chl^{-1} h^{-1}　　　　fmol humic-N cell^{-1} h^{-1}
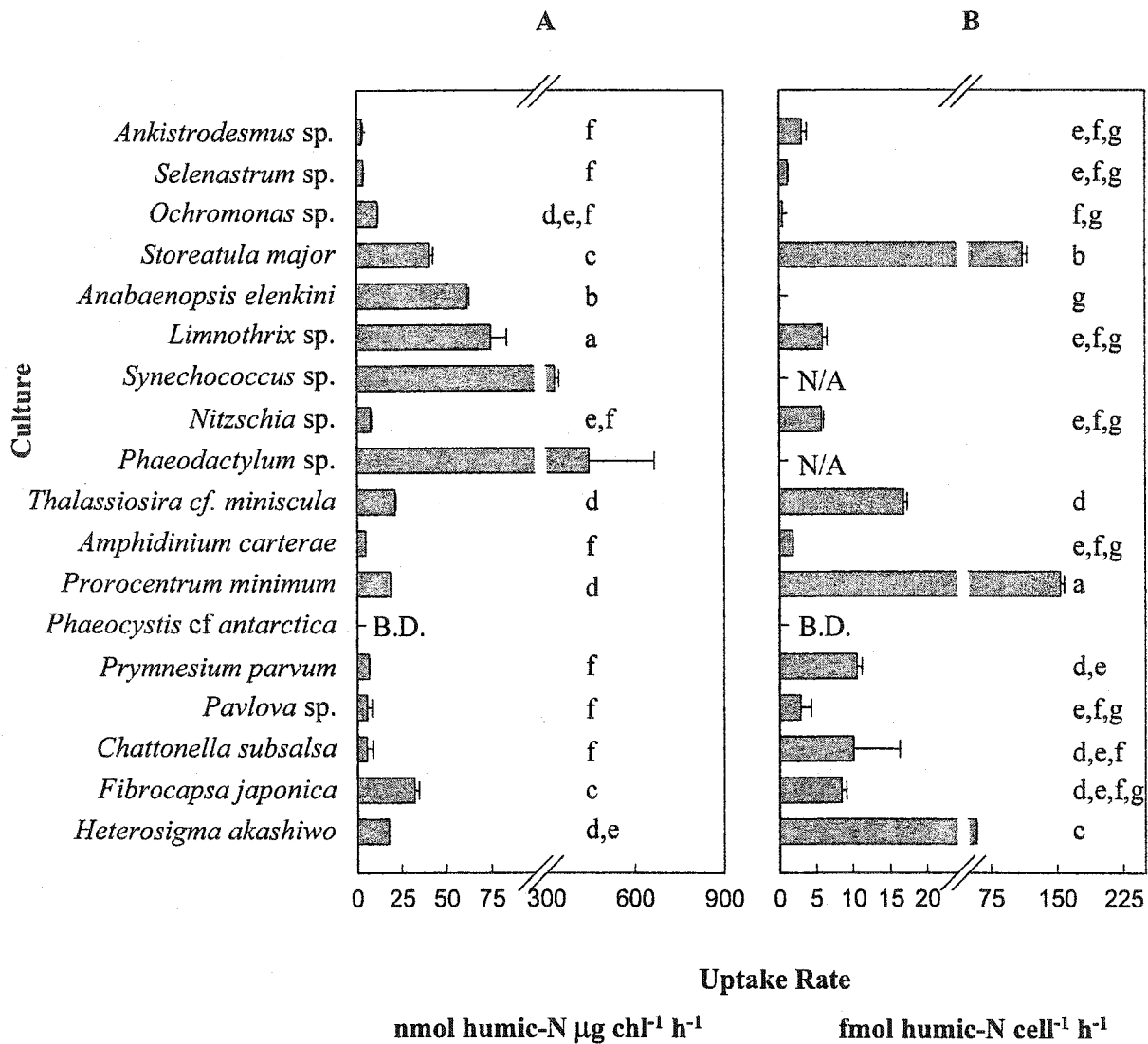
Potential mechanisms of humic utilization

Phytoplankton may potentially access the N associated with humic substances in a number of ways including direct uptake via pinocytosis, uptake of released N after extracellular hydrolysis (phytoplankton or bacterial), photochemical breakdown, or bacterial remineralization. It was beyond the scope of this study to determine which of the potential mechanisms for humic-N uptake was prevalent in the experiments, and this speculation is confounded by the taxonomic diversity of phytoplankton in this experiment; e.g. pinocytosis may be prevalent in some cultures (e.g. *A. carterae* and *P. minimum*) and cell surface enzymes in others (e.g. *Synechococcus* sp. and *Ankistrodesmus* sp.). However, based on the experimental conditions and incubation times used, it is likely that indirect uptake by bacterial or photochemical degradation or salinity release of NH_4^+ is less likely than direct uptake or enzymatic cleavage.

Bacterial breakdown of humic substances followed by uptake of released N has been argued as a mechanism for the phytoplankton uptake of humic-N (Carlsson et al. 1993). However, in the data presented, bacterial-N was found to be on average only $5.2 \pm 5.8\%$ of the total N biomass in fixed samples and never exceeded 14% of the total particulate N (data not shown) when previously reported N cell^{-1} values are used (Verity et al. 1992, Montagnes et al. 1994, Thompson et al. 1994, Fukuda et al. 1998, Meyer et al. 2002). Furthermore, on average < 50% of bacterial cells are retained onto GF/F filters during filtration (Lee et al. 1995). These data argue against either the bacterial remineralization and phytoplankton uptake of liberated humic-N or measurement of direct uptake by bacteria retained on the filter.

When exposed to ultraviolet light, humic materials can release inorganic N (Bushaw et al. 1996, Bushaw-Newton and Moran 1999, Kieber 2000) as well as small assimilable organic molecules including amino acids and urea (Amador et al. 1989, Jørgensen et al. 1998, Bushaw-Newton and Moran 1999) into the surrounding environment. However, it is unlikely that the isolates tested obtained N released from humic substances via photooxidation reactions, because the experiments were performed under fluorescent light in Pyrex test tubes.

Humic substances have also been shown to release NH_4^+ into surrounding waters when exposed to higher salinity waters (see Sections II and VI, this volume). However, this release has only been shown to occur with humic substances exposed to NH_4^+ following XAD extraction (see Section II, this volume). Experiments for this study were performed using humic substances that were not exposed to NH_4^+ following extraction onto XAD resin. Furthermore, should a salinity mediated release of NH_4^+ from the added humic label be responsible for the observed uptake, an uptake should also have been observed in the polar *P. antarctica* strain. The lack of an uptake signal in this strain argues against a release and subsequent uptake of NH_4^+ in the cultures.

It has been demonstrated that heterotrophic flagellates are capable of the direct uptake of HMW fluorescent dextrans (Sherr 1988). Legrand and Carlsson (1998) have also shown similar capabilities by the dinoflagellate *Alexandrium catenella* to take up HMW dextrans directly, most likely by pinocytosis. This approach may be another mechanism for the utilization of additional large organic molecules, including humic substances, by specific phytoplankton strains.

The use of cell surface or extracellular deaminases is also a method that may be employed by specific phytoplankton classes to access up humic-N, as half of the N associated with humic substances can exist in the form of amino acids, amino sugars, NH_4^+, and nucleic acid bases (Schnitzer 1985). These deaminases allow a cell to cleave N bound to DON and take up the released N while leaving the remainder of the compound external to the cell (Palenik and Morel 1990a, Palenik and Morel 1990b, Pantoja et al. 1993, Pantoja and Lee 1994). Amino acid oxidases are one type of deaminase that may be used as humic-associated amino acids can make up > 96% of total dissolved amino acids in riverine systems (Lytle and Perdue 1981). Several classes of phytoplankton, including dinoflagellates, chlorophytes, and prymnesiophytes have previously been shown to possess extracellular deaminases (e.g. Palenik and Morel 1990a, Palenik and Morel 1990b).

Ecological significance

Rivers have the potential to be an important source of material to the DOM pool of the global ocean (Hedges et al. 1992). However, marine DOM lacks a strong terrestrial signal (Williams and Gordon 1970, Eadie et al. 1978). The mechanisms responsible for the removal of the terrestrial DOM, as it enters the coastal ocean, are unknown. One possible removal mechanism is the photochemical breakdown of terrestrial DOM when it reaches the coastal ocean (Amon and Benner 1996, Mopper and Kieber 2002). The present findings that all estuarine isolates tested were capable of humic-N uptake stresses the need to consider phytoplankton as an additional sink

for terrestrial DOM in estuaries and the coastal ocean. Although humic compounds are considered to be largely refractory, approximately 50% of the N contained within the humic structure is in the amine form (Schnitzer 1985). This is a highly reduced, potentially usable N pool that may be more accessible to phytoplankton than bacteria, given the high capacity for DOM ingestion by pinocytosis (or even phagocytosis; e.g. Kivic and Vesk 1974, Cucci et al. 1989, Legrand and Carlsson 1998). These results support previous findings that some fraction of the N associated with humics can be a labile source of N to phytoplankton in the estuarine environment (e.g. Bronk et al. unpubl. data, Carlsson et al. 1999) and emphasize the need for research into the mechanisms of uptake, the ecological relevance of the process to phytoplankton community dynamics and DON flux, and the regulation of this process by environmental conditions and humic chemistry.

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SECTION V

UPTAKE OF *SPARTINA*-DERIVED HUMIC-NITROGEN AND CARBON BY

THREE COASTAL PHYTOPLANKTON STRAINS[†]

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ABSTRACT

Coastal phytoplankton have previously been shown to take up nitrogen (N) from laboratory-formed humic substances in the absence of additional N sources. The objective of this study was to further investigate this observation and to determine how changes in the age of the humics (1 week to 1 year) alter uptake rates. The relative incorporation of N versus carbon (C) was used to infer potential mechanisms employed by phytoplankton groups to utilize this N bound to humics. Humic substances were formed in the lab, labeled with ^{15}N, and aged for up to one year to alter their chemical structure. Additional humic substances were labeled with both ^{15}N and ^{13}C. Humic uptake rates were measured in three phytoplankton strains shown capable of taking up humic-N at high rates (*Synechococcus sp.*, *Amphidinium carterae*, and *Thalassiosira* cf. *miniscula)*, and then compared to uptake rates of ammonium and nitrate measured in parallel. Chemical characterization of the material found that younger, fresher humic compounds were taken up at a higher rate than older, more fulvic-like compounds. Younger humic substances were also taken up at rates higher than inorganic N during short-term incubations, though the high rates of humic-N uptake were not sustained but dropped substantially after the first few hours of incubation. None of the isolates exhibited an uptake of humic-C. The observation that humic-N can be utilized rapidly by phytoplankton suggests that the importance of humic-N to coastal phytoplankton should be reevaluated, and should be considered as a potential sink for terrestrial-N prior to reaching the ocean.

INTRODUCTION

Humic substances can be described as high molecular weight (HMW), carbon (C)-rich compounds. Due to their size and chemical composition, they have historically been perceived as biologically recalcitrant. More recently, the refractory nature of humic substances has been challenged, suggesting that humics are more biologically labile than previously believed. For example, bacteria have the ability to utilize humic substances as a C source (Tranvik 1988, Moran and Hodson 1990, 1994a, b, Hunt et al. 2000). With respect to phytoplankton, uptake of the humic structure by the harmful algal bloom species *Karenia brevis* has been observed using ^{125}I-labeled humic substances (Heil et. al, unpubl. data).

The ability to directly utilize humic substances as a nitrogen (N) source has not been thoroughly investigated. This is in part due to the fact that, on average, N is only 0.5-6% (Rashid 1985, Thurman 1985, Hedges and Hare 1987) of the humic molecule, and only 50% of this N is in the amine form and likely to be labile (Schnitzer 1985). However, evidence is accumulating that suggests both bacteria and phytoplankton in the coastal environment are able to take up humic-N. A direct utilization and remineralization of humic-N by bacteria followed by utilization of this N by coastal phytoplankton has been hypothesized (Carlsson et al. 1995, Carlsson et al. 1998, Carlsson et al. 1999). Bronk et al. (unpubl. data) showed the uptake of laboratory produced ^{15}N-labeled humic compounds into the planktonic (> 0.7 μm) size fraction in both riverine and coastal ecosystems. Most recently uptake of ^{15}N-labeled laboratory produced humic substances has been demonstrated in a wide

suite of coastal phytoplankton strains including representatives of chlorophytes, chrysophytes, cryptophytes, cyanobacteria, diatoms, dinoflagellates, haptophytes, prymnesiophytes, and raphidiophytes under nutrient deplete conditions (see Section IV, this volume).

With the increasing evidence that phytoplankton are capable of directly utilizing humic substances as a N source, the objective of this work was to determine how the uptake of humic-N compares to that of dissolved inorganic N (DIN) and how changes in age and the humic structure can affect the uptake of humic-N. Representative phytoplankton isolates previously shown capable of utilizing humic-N (see Section IV, this volume) were examined to determine how changes in humic age would affect N uptake rates. *Synechococcus sp., Amphidinium carterae*, and *Thalassiosira* cf. *miniscula* were examined as representative phytoplankton for cyanobacteria, dinoflagellates, and diatoms, respectively. *Synechococcus sp.* was selected because its small size restricts likely uptake mechanisms to extracellular or cell-surface enzymes. *A. carterae* was chosen because some dinoflagellates can engulf HMW particles via pinocytosis (Legrand and Carlsson 1998). Finally, *Thalassiosira* cf. *miniscula* was selected because no diatom has been shown to possess cell-surface amino oxidases (Palenik and Morel 1990b), potentially used to cleave ammonium (NH_4^+) from humic molecules. However, some diatoms do possess a high affinity for organic molecules (Lewin and Lewin 1960, 1967). Laboratory formed ^{15}N-labeled humic substances aged in the laboratory, or ^{13}C, ^{15}N-dual-labeled humic substances, were added to the cultures in a time-course experiment and uptake rates of humic-N (and N and C) were compared to the uptake

rates obtained for NH_4^+ and nitrate (NO_3^-) measured in parallel. It was found that all

three strains tested took up humic-N, but not humic-C, and over short time scales

(1 hour) this N uptake was equal to, or greater than, rates obtained for the uptake of

equivalent concentrations of NH_4^+ and NO_3^- when relatively young humics (aged

1 month or less) were used.

MATERALS AND METHODS

Culture selection

Phytoplankton strains were isolated from coastal waters and maintained in

0.2 µm filtered f/2 enriched Sargasso seawater (Guillard 1983). Cultures were grown

on a 12 hr: 12 hr light: dark cycle under fluorescent light (227 $\mu E\ m^{-2}\ sec^{-1}$) at 20 °C

The isolates were not axenic, however, bacterial-N was found to be on average only

$5 \pm 6\%$ of the total biomass in fixed samples (see below). As the isolates were

obtained from coastal waters, seawater was diluted with deionized water (DIW) to the

required salinity prior to nutrient enrichment.

Preparation of ^{15}N-labeled humics from Spartina alterniflora

Humics labeled with ^{15}N were produced in the laboratory by growing

S. alterniflora plants with ^{15}N-labeled ammonium chloride (NH_4Cl) added to the

surrounding sediment. *Spartina alterniflora* was chosen as the source for humic

formation, because it is the dominant primary producer within most marsh

ecosystems of the southeastern United states and is responsible for 80% of marsh

primary production (Pomeroy et al. 1981, Alberts and Filip 1994). Small *S. alterniflora* plants were collected at the Skidaway Institute of Oceanography (SkIO), grown in buckets over a period of three months (April – June), and watered regularly with a 4.0 mM $^{15}NH_4Cl$ solution (Cambridge Isotope Laboratories; ^{15}N, 98+%). The ^{15}N label was added to the plants in a series of 32 treatments (approximately every third day). After the *S. alterniflora* plants were harvested, they were dried in an oven for one week at 40 °C, and shredded in a Wiley mill (60 mesh). The shredded *S. alterniflora* (8 g) was then added to coastal seawater collected from SkIO, from which the humic material had been extracted (see below), and spun in the dark with a magnetic stir bar. To determine the effects of aging on bioavailability, subsamples were removed from the dark at 1 week, 2 weeks, 1 month, 3 months, 6 months, and 1 year. At each time point, the humic substances were isolated onto DAX-8 resin (see below), neutralized, and frozen until use in the uptake experiments.

Preparation of dual-labeled (^{13}C and ^{15}N) humics

To prepare dual-labeled humic substances, *S. alterniflora* plants were grown under the same conditions described above for the ^{15}N-labeled humics with the following exceptions. In a series of three treatments, ^{13}C-labeled sodium bicarbonate (Isotec; ^{13}C, 98+%) was added to the surrounding atmosphere. Three grams of ^{13}C-labeled sodium bicarbonate was added to a beaker, and both the plant and beaker were enclosed in a large nylon bag (Reynolds Metals Company, Richmond, VA). Hydrochloric acid (HCl, 6 N) was then added to the beaker to release ^{13}C-labeled

carbon dioxide (CO_2) into the atmosphere. All dual-labeled humic substances were extracted after a 3 month period.

Isolation of humic substances

Humic substances were extracted onto Supelite DAX-8 resin as previously described by Aiken (1985) for Amberlite XAD-8. Supelite DAX-8 and Amberlite XAD-8 resins have been shown to isolate comparable bulk humic solutes from aquatic sources, producing mixtures with similar chemical compositions (Peuravuori et al. 2002). As humic substances adsorb to DAX-8 (and XAD-8) resin in the protonated form, each sample was acidified to a pH < 2 with 6 N HCl and passed through a glass column (2.5 cm X 50 cm) packed with acidified DAX-8 resin. The resin was rinsed with DIW to remove any remaining salts from the resin until the eluate reached a pH > 5. Following the rinse, the column was backflushed with two bed volumes of 0.2 N sodium hydroxide (NaOH) to elute bound humic substances from the resin.

Uptake experiments

All coastal isolates were maintained in f/2 media and grown on NO_3^-. Prior to the incubation of the coastal isolate cultures with ^{15}N-labeled humics, each culture was transferred a minimum of two times into amended f/2 enriched seawater containing commercial humic acid salts (Aldrich humic acids) at a concentration of 10 mg humic-C L^{-1}. The media N:P ratio was also reduced to 10:1 to ensure N-limiting conditions would occur. Culture growth was monitored via *in vivo*

fluorescence (excitation 460nm, emission 685 nm) and used to approximate actual chlorophyll a (chl) concentration using a solid chl standard for calibration. Uptake experiments were initiated only after it had been determined that each individual isolate had depleted NO_3^- concentrations within the culture to less than 1 μM (Parsons et al. 1984).

To measure the uptake rates, 10 mL of each isolate was dispensed into 25 mL culture tubes and the ^{15}N-labeled humic substances were added (Table 1). Parallel sets of tubes received either $^{15}NH_4Cl$ (Cambridge Isotopes 98+ atom%) or $K^{15}NO_3$ (98+ atom%). Volumes of each label were calculated to add 40 μM N to each culture tube. This concentration was used to ensure no label would be exhausted throughout the experiment. To half of the tubes receiving humic label, 10 μl mercuric chloride (HgCl) was added, mixed by hand, and allowed to sit for 5 min prior to the addition of humic label. These poisoned tubes were used as killed controls to correct for any abiotic adsorption of the humic label to phytoplankton cells and/or the filter (see below).

Samples were then incubated for a period of 24 hrs in an environmental chamber under fluorescent light on a 12 hr light/dark cycle. At 1, 3, 12, and 24 hrs duplicate samples of each treatment were sacrificed and filtered onto 25 mm Whatman GF/F filters (0.7 μm nominal pore size). A time-course experiment was used to determine if the uptake of humic-N was sustainable, or if only a small fraction of the bound-N was bioavailable. It also allowed for the monitoring of changes in the ratio of C uptake to N uptake for the dual-labeled humics.

Table 1. Humic label used for uptake experiments.

Age	Concentration mg humic-C L^{-1}	Atomic C:N Ratio	atom% enrichment
1week	129.7 ± 1.0	38.1	7.4
2 week	87.2 ± 2.2	20.7	8.1
1 month	77.8 ± 3.9	23.7	9.0
3 month	90.1 ± 6.4	23.2	9.8
6 month	127.3 ± 0.8	25.5	10.0
1 year	64.11 ± 0.6	18.8	6.0
dual-labeled	14.35 ± 0.2	14.0	C: 2.73 N: 17.96

Determination of uptake rate

Following incubation, sample filters were dried at 50°C and wrapped in tin discs for analysis on an isotope ratio mass spectrometer (Europa Geo 20/20 with ANCA sample preparation unit). The uptake rates of NH_4^+ and NO_3^- were calculated using the basic equations of Dugdale and Goering (1967). Prior to calculating the uptake rates of humic-N, the atom% of the cells was corrected for the atom% of the isolate at the start of the experiment, measured independently, and the atom% of the killed control (see Section IV, this volume).

To calculate actual uptake rates, ambient concentrations of each substrate in solution must be known. For NH_4^+ and NO_3^-, ambient concentrations were determined using the methods of Hansen and Koroleff (1999) and Parsons et al. (1984). NH_4^+ concentrations were below detection for all samples examined. Although grown in media amended with humic acids, bioavailable humic-N was considered to be negligible. This assumption is based on the fact that within 3-12 hrs, most, if not all of the bioavailable humic-N had been used (see below), and all uptake experiments began a minimum two weeks following a transfer into new media. This assumption resulted in a higher estimate for the atom% enrichment of the humic substrate and therefore a more conservative estimate of humic-N uptake.

Cell abundance

Cells were fixed with formalin (final concentration of 5%) and counted under a microscope using either a Bright Line or Nageotte counting chamber (Hausser Scientific). For each sample, a minimum of 200 cells was counted. Bacterial cells

were stained with 4', 6-Diamidino-2-phenylindole (DAPI) and counted using the

method of Porter and Feig (1980).

RESULTS

^{15}N-labeled humic substances

Uptake rates for each culture were normalized to both chl a concentrations

and cell abundance (Tables 2 and 3, respectively). In general, uptake rates in the

coastal isolates decreased both with increasing incubation time and humic age (Fig. 1

and 2).

Uptake of DIN

Uptake rates for the inorganic N species remained relatively constant

throughout the experiment (Tables 2 and 3). In no case was the total N added to the

incubations exhausted.

Dual-labeled humic substances

Uptake rates of humic-N followed trends similar to those observed with the

singly-labeled ^{15}N-labeled humic substances in that there were higher initial rates of

uptake followed by a rapid decline in uptake rate over the course of the experiment

(data not shown). Uptake of ^{13}C from the humic substances was either below

detection or not significantly different from zero ($p < 0.05$) for all cultures at all time

Table 2. Uptake rates of humic-N and inorganic N over time. Rates have been normalized to chl a.

$$\textbf{nmol humic-N mg chl}^{-1}\ \textbf{hr}^{-1}$$

Sample	Synechococcus	Amphidinium	Thalassiosira
1 week label			
T_1	928.24 ± 57.61	141.44 ± 0.53	42.94 ± 56.56
T_3	378.67 ± 40.73	44.27 ± 1.96	31.14 ± 1.16
T_{12}	80.04 ± 35.75	22.30 ± 2.65	9.83 ± 0.07
T_{24}	57.77 ± 1.68	6.90 ± 0.00	4.84 ± 0.02
2 week label			
T_1	1732.25 ± 0.64	138.52 ± 42.25	134.91 ± 0.82
T_3	317.88 ± 21.92	66.97 ± 11.46	60.74 ± 0.52
T_{12}	221.51 ± 45.53	42.94 ± 2.38	16.48 ± 0.19
T_{24}	139.80 ± 2.76	20.81 ± 1.77	9.80 ± 0.27
1 month label			
T_1	1090.78 ± 504.68	102.05 ± 20.12	63.84 ± 5.27
T_3	390.32 ± 22.96	46.71 ± 17.35	36.13 ± 0.64
T_{12}	77.02 ± 4.12	15.64 ± 2.38	9.50 ± 0.85
T_{24}	69.49 ± 3.49	7.61 ± 2.93	4.84 ± 1.42
3 month label			
T_1	284.37 ± 114.59	36.00 ± 17.88	57.65 ± 5.22
T_3	212.68 ± 21.05	76.57 ± 44.87	25.89 ± 7.99
T_{12}	114.48 ± 0.81	21.96 ± 1.88	9.09 ± 0.98
T_{24}	78.11 ± 9.29	15.18 ± 2.69	5.71 ± 1.17
6 month label			
T_1	12.31 ± 1.79	26.57 ± 3.07	23.44 ± 1.77
T_3	31.64 ± 0.84	12.30 ± 2.20	8.43 ± 0.65
T_{12}	-0.24 ± 4.92	4.69 ± 1.11	2.75 ± 0.25
T_{24}	10.70 ± 4.44	2.29 ± 0.84	1.66 ± 0.21
1 year label			
T_1	123.64 ± 98.89	18.65 ± 5.54	30.07 ± 1.58
T_3	108.03 ± 36.99	8.53 ± 0.01	10.83 ± 1.03
T_{12}	30.94 ± 9.37	4.94 ± 0.76	2.77 ± 0.01
T_{24}	3.12 ± 1.12	2.66 ± 0.41	1.64 ± 0.12
NH_4			
T_1	1102.01 ± 97.70	50.14 ± 8.28	35.13 ± 0.31
T_3	411.24 ± 24.61	28.25 ± 8.42	19.02 ± 1.96
T_{12}	341.15 ± 13.27	27.29 ± 6.54	8.64 ± 1.13
T_{24}	336.73 ± 5.59	15.27 ± 0.77	9.37 ± 0.24
NO_3			
T_1	516.23 ± 39.07	18.19 ± 0.87	10.79 ± 1.79
T_3	407.49 ± 10.69	19.30 ± 0.39	7.92 ± 0.38
T_{12}	262.98 ± 31.43	30.91 ± 0.86	8.13 ± 0.42
T_{24}	321.34 ± 58.14	20.88 ± 0.84	6.69 ± 0.68

Table 3. Uptake rates of humic-N and inorganic N over time. Rates have been normalized to cell number.

$$\text{fmol humic-N cell}^{-1}\text{ hr}^{-1}$$

Sample	Synechococcus	Amphidinium	Thalassiosira
1 week label			
T_1	29.95 ± 1.86	77.43 ± 0.29	37.91 ± 49.94
T_3	12.22 ± 1.31	24.24 ± 1.07	27.49 ± 1.02
T_{12}	2.58 ± 1.15	12.21 ± 1.45	8.68 ± 0.06
T_{24}	1.86 ± 0.05	3.78 ± 0.00	4.27 ± 0.02
2 week label			
T_1	55.90 ± 0.02	75.83 ± 23.13	119.11 ± 0.72
T_3	10.26 ± 0.71	36.66 ± 6.27	53.63 ± 0.46
T_{12}	7.15 ± 1.47	23.51 ± 1.30	14.55 ± 0.17
T_{24}	4.51 ± 0.09	11.39 ± 0.97	8.65 ± 0.24
1 month label			
T_1	35.20 ± 16.28	55.87 ± 11.01	56.37 ± 4.65
T_3	12.59 ± 0.74	25.57 ± 9.50	31.90 ± 0.56
T_{12}	2.49 ± 0.13	8.56 ± 1.30	8.39 ± 0.75
T_{24}	2.24 ± 0.11	4.16 ± 1.60	4.27 ± 1.26
3 month label			
T_1	9.18 ± 3.70	19.71 ± 9.79	50.90 ± 4.61
T_3	6.86 ± 0.68	41.92 ± 24.56	22.86 ± 7.05
T_{12}	3.69 ± 0.03	12.02 ± 1.03	8.02 ± 0.86
T_{24}	2.52 ± 0.30	8.31 ± 1.48	5.04 ± 1.03
6 month label			
T_1	0.40 ± 0.06	14.55 ± 1.68	20.69 ± 1.56
T_3	1.02 ± 0.03	6.73 ± 1.20	7.44 ± 0.57
T_{12}	-0.01 ± 0.16	2.57 ± 0.61	2.42 ± 0.22
T_{24}	0.35 ± 0.14	1.25 ± 0.46	1.47 ± 0.18
1 year label			
T_1	3.99 ± 3.19	10.21 ± 3.03	26.55 ± 1.39
T_3	3.49 ± 1.19	4.67 ± 0.00	9.56 ± 0.91
T_{12}	1.00 ± 0.30	2.70 ± 0.42	2.44 ± 0.01
T_{24}	0.10 ± 0.04	1.46 ± 0.22	1.45 ± 0.11
NH_4			
T_1	35.56 ± 3.15	27.45 ± 4.53	31.01 ± 0.27
T_3	13.27 ± 0.79	15.46 ± 4.61	16.79 ± 1.73
T_{12}	11.01 ± 0.43	14.94 ± 3.58	7.63 ± 1.00
T_{24}	10.87 ± 0.18	8.36 ± 0.42	8.27 ± 0.21
NO_3			
T_1	16.66 ± 1.26	9.96 ± 0.48	9.53 ± 1.58
T_3	13.15 ± 0.34	10.57 ± 0.21	6.99 ± 0.33
T_{12}	8.49 ± 1.01	16.92 ± 0.47	7.18 ± 0.37
T_{24}	10.37 ± 1.88	11.43 ± 0.46	5.90 ± 0.60

Fig 1. Uptake rates of humic-N with respect to age normalized to chl content.

Uptake is shown for A) *Synechococcus sp.*, B) *Amphidinium sp.*, and C)

Thalassiosira cf. *miniscula*. Error bars are not shown to increase clarity, but

can be found in Table 2.

A. *Synechococcus sp.*

B. *Amphidinium sp.*

C. *Thalassiosira cf miniscula*

Uptake (nmol humic-N µg chl^{-1} hr^{-1})

Time (hrs)

Legend:
- 1 week
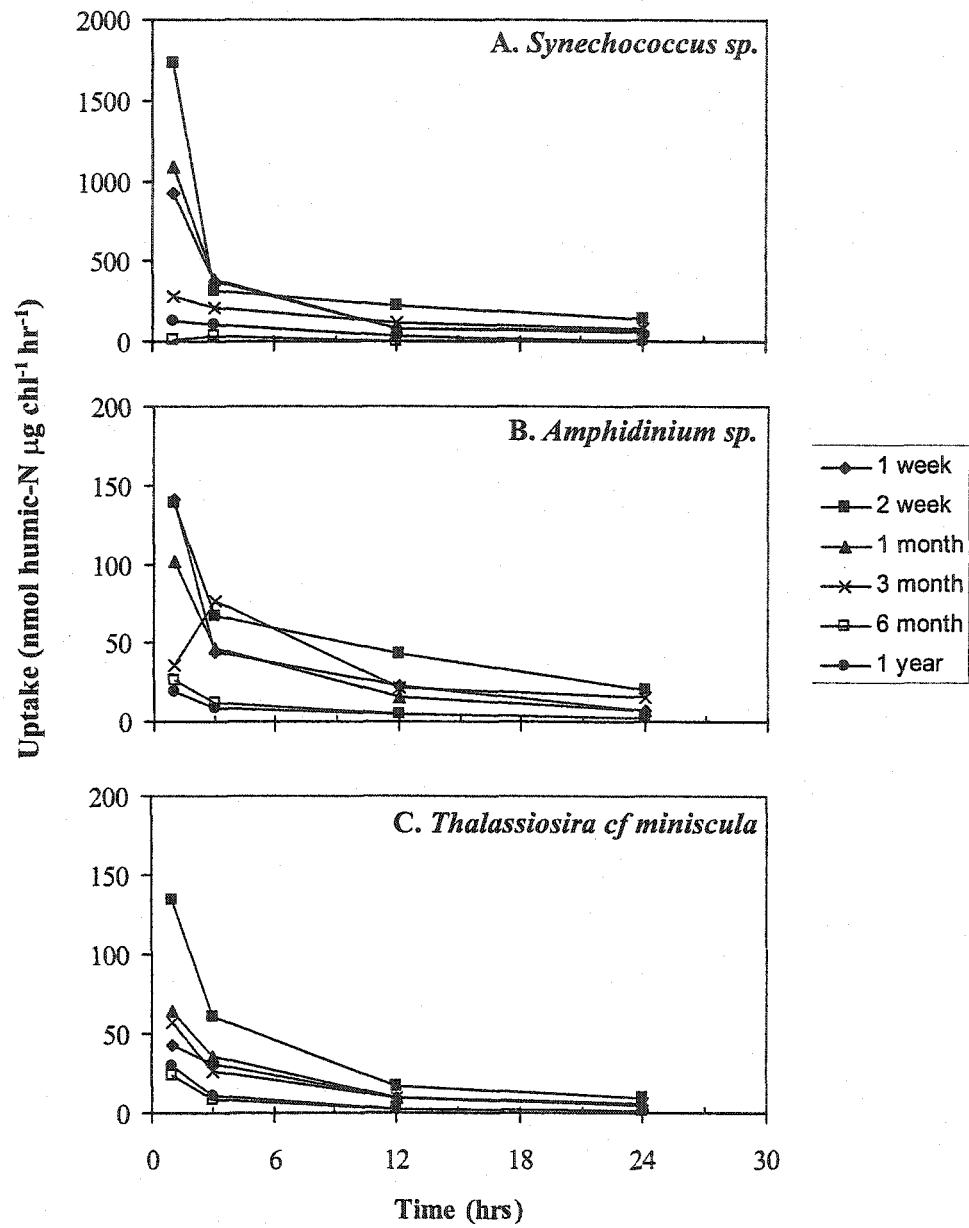- 2 week
- 1 month
- 3 month
- 6 month
- 1 year

Fig 2. Uptake rates of humic-N with respect to age normalized to cell number.

Uptake is shown for A) *Synechococcus sp.*, B) *Amphidinium sp.*, and C)

Thalassiosira cf. *miniscula*. Error bars are not shown to increase clarity, but
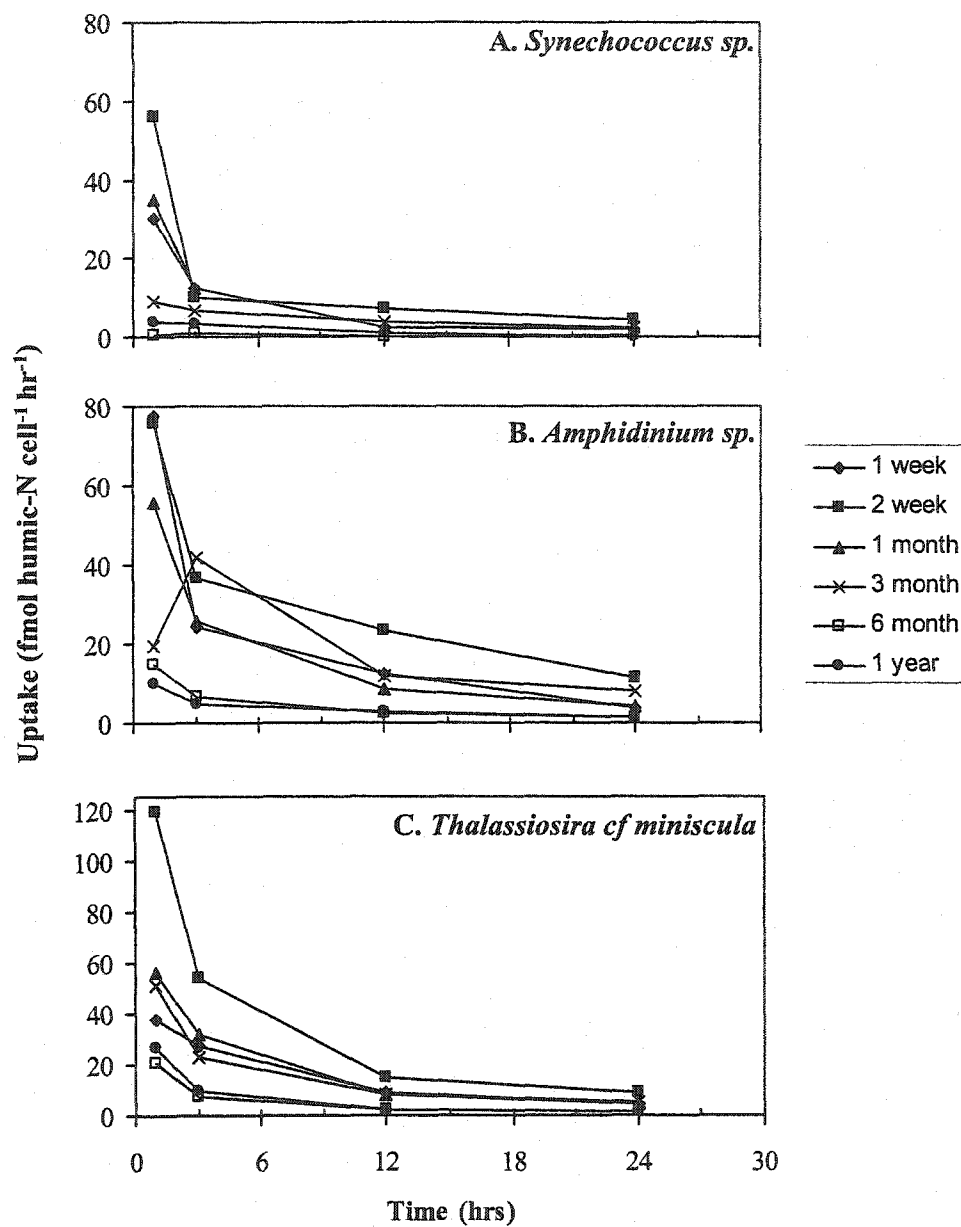
can be found in Table 3.

Y-axis (shared): Uptake (fmol humic-N cell^{-1} hr^{-1})

A. *Synechococcus sp.*

B. *Amphidinium sp.*

C. *Thalassiosira cf miniscula*

Legend:
- 1 week
- 2 week
- 1 month
- 3 month
- 6 month
- 1 year

Time (hrs)

points save one (*T. miniscula* at T_{12}, 0.16 ± 0.01 µmol humic-C L^{-1} hr^{-1}, data not shown).

DISCUSSION

In this section the uptake rates of humic-N are compared to the uptake rates of DIN for the isolates examined, how humic-N lability changes with increasing humic age is discussed, potential mechanisms for phytoplankton utilization of humic-N are inferred, and the environmental ramifications of humic-utilization by riverine, estuarine, and coastal phytoplankton are discussed.

Humic-N vs. inorganic-N

For the shorter time periods (T_1 for *Synechococcus* sp. and T_1 and T_3 for *A. carterae* and *T. miniscula*), uptake rates for humic-N exceeded those obtained for both NH_4^+ and NO_3^- in the incubations in which the younger humic substances (those aged 1 week, 2 weeks, and 1 month) were added. However, these high uptake rates were not sustainable, and rapidly dropped below the more sustained uptake rates calculated for both NH_4^+ and NO_3^- (Tables 2 and 3). The rapid decline in the uptake rate of humic-N suggests that the bioavailable N associated with humic substances is utilized quickly. However, the uptake rates in cultures given DIN remained relatively constant over the 24 hr experiment. For example, the atom% of samples given the 1 week old humic label remained constant over the 24 hr experiment, indicating that the majority of the uptake occurs in the first few hrs (Fig. 3). In contrast, the atom%

of cultures given NH_4^+ rises linearly suggesting that uptake of the inorganic label is sustained over time (Fig. 3).

Changes in lability as humic substances age

Humic substances are comprised of several subcategories, including humic and fulvic acids, based upon their solubility in water at varying pH. Humic acids are higher in molecular weight and more aromatic, while fulvic acids tend to be low molecular weight (LMW) and more aliphatic (Thurman et al. 1982, Thurman 1985). As humics age in the environment, they are degraded and converted into more fulvic-like compounds (Ertel et al. 1984, see Section II, this volume). It was initially hypothesized that the LMW, aliphatic compounds would be preferred for uptake. However, this does not appear to be the case as the younger "fresher" humic substances were taken up by the phytoplankton isolates at a higher rate than the older, presumably more degraded, humic substances (Fig. 1 and 2). One explanation may be related to the total N content of each fraction. Humic acids have been shown to contain, on average, 2-6% N, while the fulvic acid fraction contains only 1-3% N (Schnitzer 1976). Phytoplankton strains may have adapted to better breakdown and utilize the more aromatic humic acids, as they would receive a higher return of N for their energy investment. Furthermore, a size-reactivity continuum model has been developed that demonstrates that HMW DOM is more bioreactive than the LMW counterparts, because the HMW DOM is less diagenetically altered and closely resembles its source material (Amon and Benner 1996a, Benner 2002).

Fig 3. Atom% excess values (Atom% over natural abundance) for the uptake of the

1 week humic label and $^{15}NH_4^+$ for *Thalassiosira* cf. *miniscula*.
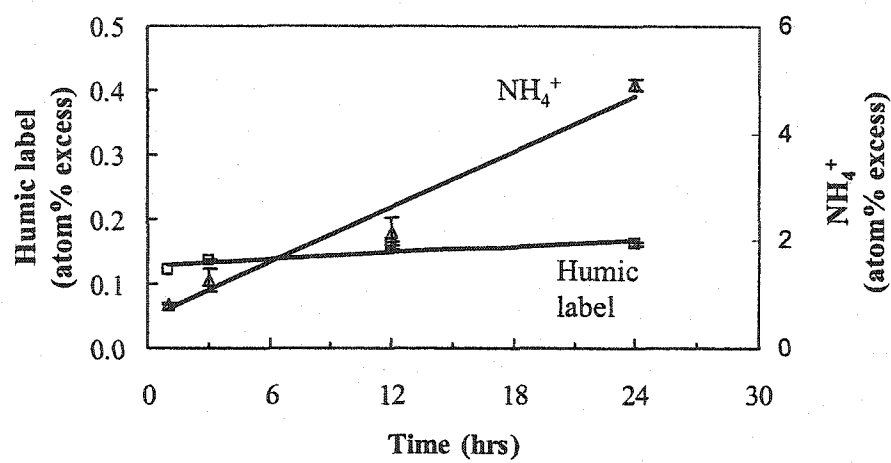
Potential mechanisms of humic-N uptake

Phytoplankton could access the N associated with humic substances in a number of ways including direct uptake via pinocytosis, or the uptake of released N after extracellular enzyme cleavage, bacterial remineralization, or photochemical breakdown. For the dual-labeled humic substances, rates of C and N uptake were determined and used to infer the potential mechanisms of the uptake of humic-N employed by the different strains. Based on the experimental conditions and incubation times used, it is believed that uptake by pinocytosis or the uptake of nitrogenous compounds released by cell surface enzymes are the mechanisms used by the isolates examined, as these mechanisms have been shown to be used by phytoplankton to access DOM (i.e. Palenik et al. 1988/1989, Palenik and Morel 1991, Pantoja et al. 1993, Legrand and Carlsson 1998). These mechanisms are described below in further detail.

It has been demonstrated that heterotrophic flagellates and the dinoflagellate *Alexandrium catenella* are capable of directly taking up HMW fluorescent dextrans, most likely by pinocytosis (Sherr 1988, Legrand and Carlsson 1998). If the isolates examined also use this approach, it would be expected that the uptake of labeled C and N would be in the same atomic ratio as the humic label added to the culture (14.0; Fig 4A). This was not seen in any of the cultures examined, as none demonstrated a sustained uptake of labeled C.

The use of cell surface or extracellular deaminases is one possible method that may be employed by phytoplankton to access up humic-N, because > 50% of the humic-associated N is in the form of amino acids, amino sugars, NH_4^+, and nucleic

Fig 4. Potential uptake mechanisms utilized by phytoplankton to access humic-N.

A) Direct uptake of the humic molecule via pinocytosis, and B) enzymatic

cleavage of the loosely bound humic-N followed by uptake of the released N

only. $\circ = {}^{13}C$, and $\bullet = {}^{15}N$ on the dual-labeled humic substances used for

uptake experiments.
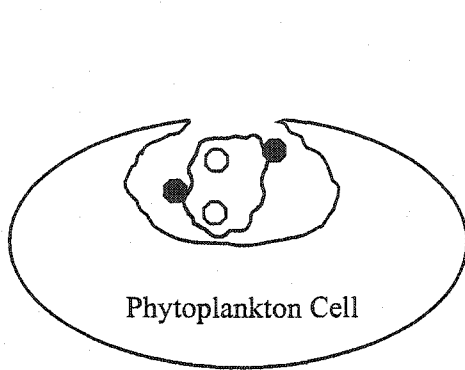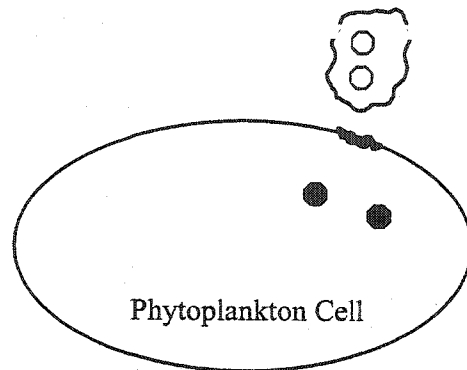
A. Direct Uptake **B. Enzymatic Cleavage**

acid bases (Schnitzer 1985). These deaminases allow cells to cleave N bound to DON followed by uptake of the released N while leaving the remainder of the compound external to the cell (Palenik and Morel 1990a, Palenik and Morel 1990b, Pantoja et al. 1993, Pantoja and Lee 1994). One type of deaminase that may be used is amino acid oxidases, as humic-associated amino acids can make up > 96% of total dissolved amino acids in riverine systems (Lytle and Perdue 1981). Several classes of phytoplankton, including dinoflagellates, chlorophytes, and prymnesiophytes have previously been shown to possess extracellular deaminases (e.g. Palenik and Morel 1990a, Palenik and Morel 1990b). Under this scenario, uptake of N should be detected, while the uptake of C would be negligible or nonexistent (Fig. 4B). This occurred in all of the cultures examined, suggesting that cell surface enzymes may be an important mechanism utilized by coastal phytoplankton to access humic bound N.

Bacterial breakdown of humic substances followed by uptake of released N also has been argued as a mechanism for the phytoplankton uptake of humic-N (e.g. Carlsson et al. 1993). However, in the data presented, bacterial-N was found to be on average only 5 ± 6% of the total N biomass in fixed samples (data not shown Verity et al. 1992, Montagnes et al. 1994, Fukuda et al. 1998, Menden-Deuer and Lessard 2000, Meyer et al. 2002). Furthermore, on average < 50% of bacterial cells are retained onto GF/F filters during filtration (Lee et al. 1995), arguing against the bacterial remineralization and subsequent uptake of liberated humic-N by phytoplankton and the measurement of direct uptake by bacteria retained on the filter.

Another potential method for the acquisition of N from humic substances is photooxidation. Humic materials can release inorganic N (Bushaw et al. 1996,

Bushaw-Newton and Moran 1999, Kieber 2000) as well as small labile organic molecules including amino acids and urea (Amador et al. 1989, Jørgensen et al. 1998, Bushaw-Newton and Moran 1999) into the surrounding environment when exposed to ultraviolet light. Due to their aromatic nature, humic substances are photoreactive to ultraviolet light. However, it is unlikely that the isolates examined obtained N released via photooxidation reactions, as experiments were performed under fluorescent light in Pyrex test tubes that do not allow ultraviolet light to penetrate.

Environmental ramifications

The observed uptake rates raise the question as to why phytoplankton cells would have evolved to utilize humic-N? Due to their high surface area to volume ratio, bacteria can often out compete larger phytoplankton cells for the energetically more favorable NH_4^+ at environmental concentrations. Based upon these observations, it would make ecological sense for phytoplankton cells to look elsewhere, including humic substances, for their N needs.

Furthermore, how do the rates measured in this experiment translate into the natural environment? *Spartina alterniflora* is the primary source for refractory carbonaceous compounds, including humic substances, in salt marshes along the Atlantic and Gulf coasts (Filip and Alberts 1988). The majority of *S. alterniflora* biomass is formed during the growing season in spring and summer, and then subjected to physical and bacterial breakdown during the remainder of the year. Thus, the overall structure of DOM, including humic substances, would be expected to change throughout the year due to degradation by bacteria, changes in light levels

and photochemical effects, changing redox conditions, and other chemical processes. Therefore, humics formed in the salt marsh and released into the surrounding waters during the growing season should be taken up and utilized at rates similar to those observed for the younger, newly produced, more labile humic substances, while humics released into the rivers and estuaries during the remainder of the year (autumn and winter months) would be taken up at rates more comparable to the older, less bioavailable humics utilized in this study.

On average, approximately 7.3×10^{12} g DON yr^{-1} is discharged into the coastal ocean annually (Meybeck 1982, Sarmiento and Sundquist 1992), with 40-80% of this N considered to be humic-N (Beck et al. 1974, Thurman and Malcolm 1983, Thurman 1985). Humic substances, and DON in general, have been largely ignored in current N-loading and eutrophication models. However, oceanic DOM lacks a strong terrestrial signal, suggesting this N is utilized or transformed prior to entering the bulk oceanic DOM pool. Currently, the photochemical breakdown of terrestrial DOM into more labile compounds is believed to be the major process responsible for the removal of this terrestrial signal (i.e. Miller and Zepp 1995, Amon and Benner 1996b, Mopper and Kieber 2002). Direct uptake of humic-N by phytoplankton is likely another vehicle for removing the terrestrial signal over short time scales (1 hr). The ability of phytoplankton to utilize humic-N, as well as other forms of DON, supports the theory that a large number of phytoplankton cells are capable of obtaining nutrition from sources other than inorganic N, and provides a need for the reevaluation of the importance of humic-N in ecosystem-wide nutrient budgets.

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SECTION VI:

HUMIC SUBSTANCES: A POTENTIAL SOURCE OF NITROGEN TO COASTAL

PHYTOPLANKTON AND THEIR ROLE AS A SHUTTLE FOR AMMONIUM IN

ESTUARINE SYSTEMS

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ABSTRACT

Humic substances comprise a large percentage of the dissolved organic matter (DOM) found in the riverine and estuarine environments. Evidence suggests that humic substances are capable of adsorbing ammonium (NH_4^+) from surrounding waters to cation binding sites located on the humic structure. The adsorption of NH_4^+ to humic substances makes them a potentially important shuttle for nitrogen produced upriver to the estuary and coastal ocean. As the humic materials move downriver, and encounter higher salinities, salt ions could displace the loosely-bound NH_4^+ ions on the humic structure, releasing them into the environment. The preliminary experiments described below were designed to determine the magnitude of release at different salinities and concentrations of sorbed NH_4^+ using humics collected from three rivers and a commercially available humic acid. In all cases, humic substances released NH_4^+ when the salinity of the surrounding water increased, and the release of NH_4^+ was rapid and reproducible. Results indicate that this "humic shuttle" is capable of releasing 77×10^6 g of NH_4^+ nitrogen into the estuary over the course of the year, or approximately 1 μmol of nitrogen per liter of water passing through the estuary. The release of NH_4^+ from aquatic humics along a salinity gradient is a previously unrecognized source of nitrogen to the mid-saline bloom regions (10-20‰) and has the potential to be an important source of NH_4^+ for organisms living in the higher salinity regions of estuaries or the coastal ocean.

INTRODUCTION

In a recent set of experiments humic substances isolated from east coast estuaries released ammonium (NH_4^+) when exposed to increased salinities (see Section III, this volume). This observation suggests that humic substances may be capable of releasing loosely bound NH_4^+ into the estuary as they are transported to the coastal ocean. There are a number of pieces of evidence that support this hypothesis.

First, previous studies have shown that NH_4^+ can be adsorbed to riverine and estuarine sediments. As higher saline water passes over these sediments, NH_4^+ can be desorbed from the sediment and released into the overlying waters as cation exchange sites on clay particles and organic matter in the sediment become occupied with seawater cations (Rosenfeld 1979, Gardner et al. 1991). Humic acids extracted from marine clay sediments can account for a large percentage of the cation exchange capabilities of marine sediments, and therefore probably play an important role in NH_4^+ adsorption (Rashid 1969).

Second, humic substances formed in upland riverine and marsh environments are often exposed to high concentrations of NH_4^+ and low salinity (see Section II, this volume). At these low salinities, aquatic humic substances will loosely adsorb NH_4^+ to the humic structure. As they then pass through the estuary, humics are exposed to higher salt concentrations in the water. The ability of humic substances to sequester and release NH_4^+ within marine sediments with changing salinities suggests that

perhaps aquatic humics also have the potential to adsorb, and subsequently release, NH_4^+ along an estuarine salinity gradient.

The preliminary experiments described below looked to determine the magnitude of release with varied concentrations of NH_4^+ adsorbed to humic substances and varied salinities. Data indicate that NH_4^+ will bind to humic substances and will subsequently be dissociated into the surrounding water in a direct relationship to the initial concentration of NH_4^+ adsorbed as the humics encounter more saline environments.

MATERIALS AND METHODS

Sample sites

Commercially available humic acids (Aldrich humic acid, sodium salt) and natural humic substances isolated from three rivers by XAD-8 extraction (Thurman 1985, Aiken 1988) were used to examine the ability of these compounds to release NH_4^+ into the surrounding waters. Samples were collected from upriver locations in the Satilla and Altamaha Rivers in coastal Georgia and the York River in Virginia (see Section II, this volume).

Extraction of humic substances

Humic substances were extracted on either Amberlite XAD-8 or Supelite DAX-8 resin as previously described. Resins were cleaned via a Soxhlet extraction

procedure (solvents include ether, acetonitrile, and methanol) followed by extensive rinses of hydrochloric acid, sodium hydroxide, and deionized water prior to extraction (Aiken 1985, Thurman 1985).

Sample enrichment

To quantify potential NH_4^+ release rates from humics, cation binding sites on isolated humic substances were first enriched with NH_4^+ by swirling concentrated samples (250 mL, 2500 μM humic-C) overnight with increasing levels of NH_4^+ to give final ratios of 0:1, 0.012:1, 0.024:1, and 0.048:1 μmol N: μmol humic-C. These concentrations reflect the levels of NH_4^+ humic substances would be exposed to in the salt marsh and riverine environment (see Section II, this volume). The sample pH was raised to 9.5, and the sample was degassed to remove residual unbound NH_3 in a SpeedVac centrifugal concentrator (1 torr) for 30 minutes. The sample was then reneutralized and reconstituted to its original volume with distilled water.

Salinity release

Enriched and reconstituted humic substances (25 mL) were added to 25 mL of an artificial saline matrix (muffled sodium chloride (NaCl) dissolved in distilled water) to achieve a final salinity of 0, 8, 15, and 35‰, representing the range of salinities humic substances encounter along an estuarine salinity gradient. Each sample was then swirled by hand (approximately 30 seconds) and immediately analyzed for the concentration of free NH_4^+ using the phenol/hypochlorite method for

colored or turbid waters (Hansen and Koroleff 1999). However, as the magnesium reagent used to precipitate the humic compounds from solution contains NaCl, it was the final reagent added to prevent the measurement of any excess ammonium released by these salts.

RESULTS AND DISCUSSION

In all cases, humic substances released NH_4^+ when the salinity of the surrounding water increased, and the release of NH_4^+ was rapid and reproducible (Table 1). The Aldrich humic acids showed the greatest release of NH_4^+, followed by the Satilla/Altamaha, and York River humics respectively (Fig. 1).

Samples enriched with higher concentrations of NH_4^+ released more NH_4^+ per μmol of humic-C. The higher the initial enrichment level of NH_4^+ (i.e. 0.048 μmol N:μmol humic-C), the higher the initial rates of NH_4^+ release (Fig. 2). Presumably, as concentrations of NH_4^+ in the surrounding waters increase, more NH_4^+ is adsorbed to the humic structure. Eventually, if NH_4^+ in the environment reached extremely high concentrations, all available cation-binding sites on the humic structure would be occupied with NH_4^+. At this point the humics would achieve a maximum level of enrichment, and no additional NH_4^+ could be adsorbed to the humic structure. It is believed that this concentration was approached during the 0.048 μmol NH_4^+:μmol humic-C, as previous experiments suggest higher enrichment

Table 1 NH_4^+ released by humic substances at differing saturatios for the Satilla, Altamaha, and York Rivers and Aldrich humic acids. Also shown is the percentage of the NH_4^+ released by 15‰ salinity

Sample	Date	Enrichment (µmol N:µmol C)	Total NH_4^+ Released (µM)	% released by 15‰
Satilla	Nov-00	0:1	0.0000	N/A
		0.012:1	0.6046	51.9
		0.024:1	0.6636	70.3
		0.048:1	1.0490	72.4
Satilla	Apr-01	0:1	0.0386	100.0
		0.012:1	0.2280	91.4
		0.024:1	0.5112	80.3
		0.048:1	1.2565	89.6
Satilla	Jun-01	0:1	0.0000	N/A
		0.012:1	0.2455	99.4
		0.024:1	0.5336	87.3
		0.048:1	0.6649	99.1
Satilla	Dec-01	0:1	0.0650	55.0
		0.012:1	0.2781	99.9
		0.024:1	0.7402	75.9
		0.048:1	1.7794	98.3
York	Jun-01	0.048:1	0.5492	78.0
York	Sep-01	0.048:1	0.1448	N/A
York	Jan-02	0.048:1	0.5050	95.6
York	Apr-02	0.048:1	0.4987	96.9
Altamaha	Nov-00	0:1	0.0000	N/A
		0.012:1	0.2044	92.5
		0.048:1	1.0477	86.8
Aldrich		0:1	0.0000	N/A
		0.012:1	1.0712	61.9
		0.024:1	2.4747	58.5

Figure 1. Release of NH_4^+ from humic substances in three rivers as well as

commercially available humic acids. Release is shown as nmol N:µmol

humic C at an initial enrichment of either 0.048 µmol:µmol humic-C (Satilla,

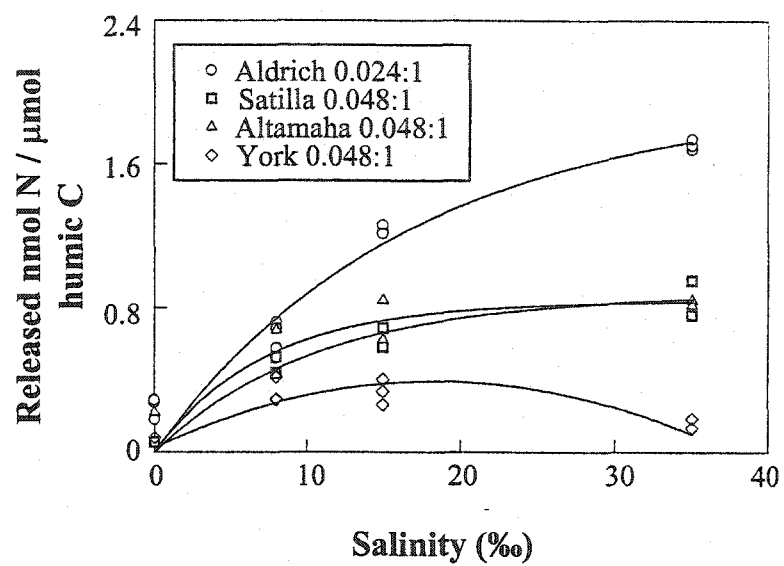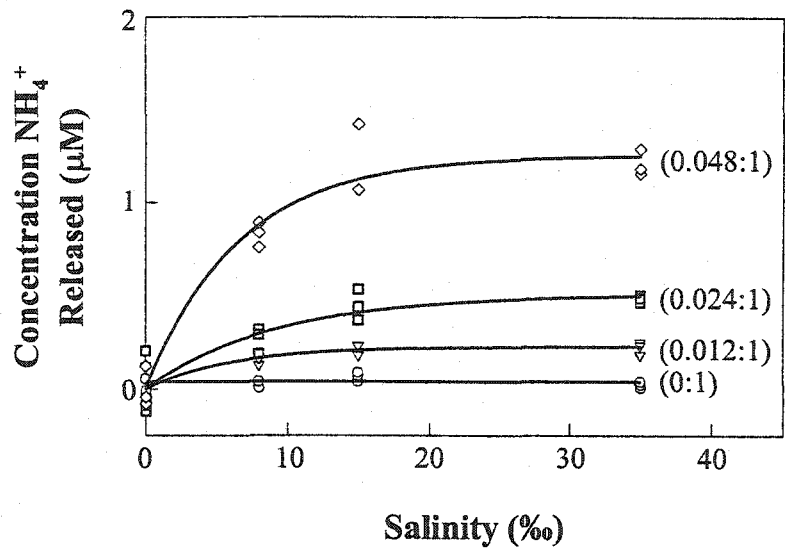Altamaha, and York Rivers) or 0.024 µmol:µmol humic-C (Aldrich humic

acids).

Figure 2. Response of Satilla River humic substances to increasing levels of salinity and enriching NH_4^+ concentrations (μmol NH_4^+:μmol humic-C). Higher levels of initial saturation resulted in both increased initial release rates and maximum concentrations released.

levels will not bind additional NH_4^+ to the humic molecule (see Section II, this volume).

In comparing the humic samples, Aldrich humic acids appeared capable of adsorbing the most NH_4^+. The reasons for this are unclear, however humic acids may contain a larger number of available cation binding sites per μmol of humic-C, allowing for a larger release of NH_4^+ from the humic structure when exposed to higher salinities. Thus, the observed differences in NH_4^+ adsorption may be a function of humic acids differing from bulk humic substances (humic acids, fulvic acids, and humin) in their ability to adsorb NH_4^+ from the environment. Isolated riverine humic substances may be 90% fulvic acids (Malcolm 1985), therefore any differences observed in the samples may reflect the differences in the binding and release affinities for humic and fulvic acids. Furthermore, humic acids tend to be more aromatic while fulvic acids more aliphatic (see Thurman 1985). As these compounds travel through the estuary, and the salinity increases, the conformation of humic molecules will also likely shift from more linear to more globular and begin to flocculate from solution (Sholkovitz 1976, Sholkovitz and Copland 1980). How these conformation shifts differ between humic substances from different sources is unknown, and could play a role in the availability of binding sites and the release of NH_4^+ from humic substances in solution.

A number of ancillary observations provide support for the measured salinity-induced release of NH_4^+. Previous work has shown that eluting humic materials from XAD resins with ammonium hydroxide decreases the C:N ratio of isolated humics (Stuermer and Harvey 1977, Thurman 1985), indicating the ability for a net uptake of

N by the humic substances. Moreover, as much as 75% of the N associated with humic and fulvic acids can be described as loosely bound and is easily removed via passage through cation exchange resins (Sowden and Schnitzer 1967, Khan and Sowden 1972, Schnitzer 1985). Carlsson et al. (1998) showed an apparent abiotic release of both amino acids and NH_4^+ from isolated riverine humic material into seawater solutions containing low ambient NH_4^+ concentrations (approximately 1 μM).

On average, approximately 7.3 X 10^{12} g DON yr^{-1} is discharged into the coastal ocean annually (Meybeck 1982, Sarmiento and Sundquist 1992), with 40-80% of this N considered to be humic-N (Beck et al. 1974, Thurman and Malcolm 1983, Thurman 1985). Humic substances, and DON in general, have been largely ignored in current N-loading and eutrophication models. However, the findings presented here suggest that humics can serve as a shuttle transporting labile N from upriver areas - thus the term "humic shuttle." As a preliminary estimate of the significance of the humic shuttle to the transport of N through the estuary, the release of NH_4^+ in the Satilla and Altamaha River estuaries was calculated. Concentrations of NH_4^+ in water draining from a *Spartina alterniflora* dominated Georgia salt marsh during low tide have been measured and exceeded 73 μM (Haines 1979). Furthermore, the input of N from fertilizers and animal waste dominates the drainage basins of the Satilla and Altamaha Rivers (Asbury and Oaksford 1997), suggesting humic substances would be exposed to high concentrations of NH_4^+ en route to the estuary. Using estimates of humic concentrations in these systems as 1250 and 500 μM humic-C for

the Satilla and Altamaha respectively (Beck et al. 1974, Alberts and Takács 1999), μmol NH_4^+:μmol humic-C ratios greater than 0.048 (the highest ratio tested in this experiment) are readily encountered throughout the watersheds. As a first approximation, it is assumed that these salinity release estimates calculated in this experiment are constant over the course of a year. Applying this assumption to the annual water flow information in Alber and Sheldon (1999) for the Satilla and Altamaha Rivers results in an annual NH_4^+ input greater than 52.7×10^6 and 77.5×10^6 g N respectively, with ~49% of this N release occurring during the months of February, March, and April. These values are roughly equivalent to 47 and 17% of the NH_4^+ instream loads for the Satilla and Altamaha Rivers, respectively (Asbury and Oaksford 1997). However, it is important to note that the release of NH_4^+ from humic substances likely fluctuates throughout the year based upon source of the humic substances, watershed NH_4^+ concentrations, pH, and other physical and chemical properties of the water column.

The majority of the observed release occurred in the low to mid salinities (8-15‰). Sholkovitz (1976) and Sholkovitz and Copland (1980) have explored the effects of mixing riverine waters with seawater on the chemical characteristics of humic substances and found that riverine humic substances sequester metal ions and flocculate at high rates between 15 and 22‰ salinity. It is likely that humic-N also plays an important environmental role at these salinities. Franks (1992) and Franks and Anderson (1992) showed that toxic blooms of *Alexandrium tamarense* in the Gulf of Maine are consistently located in the coastal waters of river plumes. Jones et al. (1994) documented a toxic bloom of *Nodularia spumigena* occurring in a lagoon,

possibly due to high input from a local sewage treatment plant. Abiotic mechanisms may be present releasing nitrogenous compounds from humic substances. Nitrogen bound to the humics may also be more accessible at these salinities for enzymatic cleavage.

CONCLUSIONS

The abiotic release of NH_4^+ from humic substances during exposure to increased salinities is a novel and potentially significant pathway for labile N to be transported to estuaries and the coastal ocean. This source of N to the coastal zone has previously gone unrecognized, and therefore has not been included in ecosystem and nutrient cycling models. Preliminary calculations suggest that the "humic shuttle" may represent an important source of NH_4^+ for organisms within estuaries, and may be a particularly important source of N capable of fueling phytoplankton blooms in mid-salinity regions during periods of high water discharge in the spring.

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SECTION VII:

CONCLUSIONS

The research presented in this dissertation provides evidence that humic compounds are not refractory, biologically recalcitrant compounds as often reported and assumed. Rather, they are dynamic molecules, capable of altering the surrounding nutrient regime and supplying nitrogen (N) to phytoplankton in the environment.

Initial work for this dissertation focused on the chemical composition of humic substances in riverine environments. Humic substances were isolated from the Satilla and Altamaha Rivers, GA and the York River, VA via the commonly used XAD-8 extraction technique (see Section II, this volume). They were also formed in the laboratory by allowing *Spartina alterniflora* plants to humify under controlled laboratory conditions over the course of one year. The chemical characteristics of the natural and laboratory produced humic substances were evaluated in a number of ways. First, chemical shifts in the atomic carbon (C):N ratio were monitored after exposing natural humic substances to environmentally relevant ammonium (NH_4^+) concentrations. Second, structural characteristics of natural humics and changes as humics aged were determined via both Fourier Transform Infrared (FTIR) spectroscopy and molecular weight size fractionation. Results indicated that the exposure of humic substances to environmentally relevant levels of NH_4^+ caused a significant decrease of the C:N ratios in the bulk, low molecular weight (LMW; < 3 kD), and high molecular weight (HMW; > 10 kD) size fractions, but not the intermediate size fraction (3-10 kD), for a majority of the samples. These data indicate that extraction of humic substances from the aquatic environment via XAD can cause an underestimation of humic-N, suggesting that humic substances in the

environment are more N-rich than previously thought. Structurally, both LMW and HMW compounds were found to increase with increasing humic age. FTIR spectrums suggest that older humic substances contained a higher percentage of aliphatic-rich fulvic-like particles than younger humics.

The next section of this dissertation examined how an overestimation of humic C:N ratios affected the interpretation of literature values of photochemical production of NH_4^+ from DOM in rivers, estuaries, and the coastal ocean (see Section III, this volume). In this study humic substances extracted from three rivers as well as commercially available humic acids were examined to determine how the cation-reactive pool and photo-reactive NH_4^+ pool interacted within estuarine environments. All samples released NH_4^+ when exposed to increased salinities, while only the Satilla and York River humics displayed a light-mediated release of NH_4^+. It was concluded that due to physical and chemical processes occurring along the estuarine gradient (estuarine mixing and exposure to ultraviolet light), the values of photochemically released NH_4^+ from humic substances derived from this study are consistent with those that have been reported previously (i.e. Bushaw et al. 1996, Bushaw-Newton and Moran 1999, Kieber 2000, Wang et al. 2000).

Having demonstrated that humics in the environment are capable of being more N-rich than previously thought and investigating two mechanisms that could release N from humics (salinity and photochemistry), attention was then turned to the question of humic-N bioavailability (see Section IV, this volume). Seventeen coastal phytoplankton strains and one polar strain (used because it was not expected to have been exposed to salt marsh humic substances and therefore could serve as a control)

were examined for the ability to utilize humics as the sole source of N. ^{15}N-labeled humic substances were formed in the laboratory and added to the coastal isolates as the sole N source. All coastal isolates examined were capable of taking up humic-N under nutrient deplete conditions while the one polar isolate did not exhibit uptake. It was concluded that the ability of coastal phytoplankton to utilize humic substances as a N source could provide an important sink for terrestrial DOM as it approaches the coastal ocean and could have substantial implications on environmental N loading budgets.

After demonstrating the ability of coastal phytoplankton strains to take up laboratory-formed humic-N, a series of experiments were conducted to determine how changes in the chemical structure could alter this uptake (see Section V, this volume). Three of the coastal strains previously shown to take up humic-N at a high rate (*Synechococcus* sp., *Amphidinium carterae*, and *Thalassiosira* cf. *miniscula)* were used in uptake experiments with aged humics and humic compounds labeled with both ^{15}N and ^{13}C. It was found that younger, fresher humic compounds were taken up at higher rates than older more fulvic-like compounds, that uptake of labeled N, but not C, occurred, and that uptake rates dropped after the first few hours of incubation indicating that only a fraction of humic-N (approximately 33%) was available for phytoplankton uptake.

Scientific ramifications

The data presented in this dissertation suggest that isolation with XAD-8 (or DAX-8) resin can underestimate the N content of humics in the environment by

stripping loosely bound N from the humic structure prior to analysis, and thus skewing the C:N ratio upward (see Sections II and VI, this volume). This finding is consistent with a number of previous observations (i.e. Roulet et al. 1963 cited in Schnitzer 1985, Sowden and Schnitzer 1967, Khan and Sowden 1972, Thurman 1985). The ultrafiltration of HMW compounds (UDOM, > 1kD) appears to be a much more gentle form of isolation, as ultrafiltration does not strip NH_4^+ from organic matter, thereby isolating HMW humics from solution with a lower C:N ratio (Benner 2002). However, isolation of UDOM does not guarantee an isolation of true humic material, as it has been suggested that UDOM is chemically distinct from humic substances (McCarthy et al. 1996). A number of extraction techniques have been used in an attempt to isolate humic substances from aquatic sources including precipitation, freeze concentration, filtration, solvent extraction, and adsorption onto charcoal or resins (reviewed in Aiken 1985, Thurman 1985). Based on the data presented in this dissertation, it appears that the extraction of humic substances from natural waters again needs to be reevaluated such that either new extraction techniques are employed that do not alter the chemical makeup of natural humic substances, or the definition of humic substances is changed to one that is chemically, and not operationally, defined.

Conceptual diagram of humic compounds

As stated throughout this dissertation, humic compounds have largely been ignored in N-cycling and eutrophication modeling, despite their abundance, and information on the biological importance of these abundant organic compounds has

lagged far behind due to the perception that humic substances are refractory N-poor compounds that are largely biologically inert. While much still needs to be done, the research presented in this dissertation suggests the existence of four primary categories of humic-N: matrix N, cation-reactive N, photo-reactive N, and bioavailable N (Fig. 1). Matrix N is that N embedded into the humic structure and is not removed unless the entire molecule is oxidized. Cation-reactive N is loosely bound and removed via extraction onto XAD resins or exposure to increased salinities (see Sections II, III, and VI, this volume). Photo-reactive N can be removed from the humic structure by exposure to sunlight and ultraviolet radiation (see Section III, this volume), and bioavailable N can be utilized directly by the planktonic community in riverine, estuarine, and coastal systems (see Sections IV and V, this volume). The existence of these four distinct pools of humic-N (three of which are capable of providing N to the planktonic community) suggests that a shift is needed in the scientific community's perception that humic-N is unavailable for uptake and use by phytoplankton and bacteria. Rather, through a number of physical, chemical, and biological processes, humic substances have the potential to act as a source of labile N to phytoplankton communities in riverine, estuarine, and coastal ocean.

Environmental ramifications

To place these findings into a larger conceptual framework, the measured uptake rates and salinity release rates are combined with literature values to estimate the potential for humics to contribute labile-N to the global ocean. On average, approximately 7.3×10^{12} g dissolved organic N (DON) is estimated to be discharged

Figure 1. The hypothesized four major pools of N associated with humic compounds. Matrix N is that N that cannot be removed without complete oxidation of the molecule (Section I). Cation labile N is N that can be removed by either extraction onto XAD resins or by exposure to increased salinities (Sections II, III, and VI). Photo-labile N is cleaved from the humic structure by exposure to sunlight and ultraviolet radiation (Section III). Bioavailable N is accessible to phytoplankton and bacterial cells via pinocytosis, enzymatic cleavage, or some yet undiscovered mechanism (Sections IV and V). Percentages represent the relative contribution of each pool to the bulk humic-N.

Matrix N
21.0%

Bioavailable
32.9%

Salinity-Reactive
3.2%

Photo-Reactive
42.9%
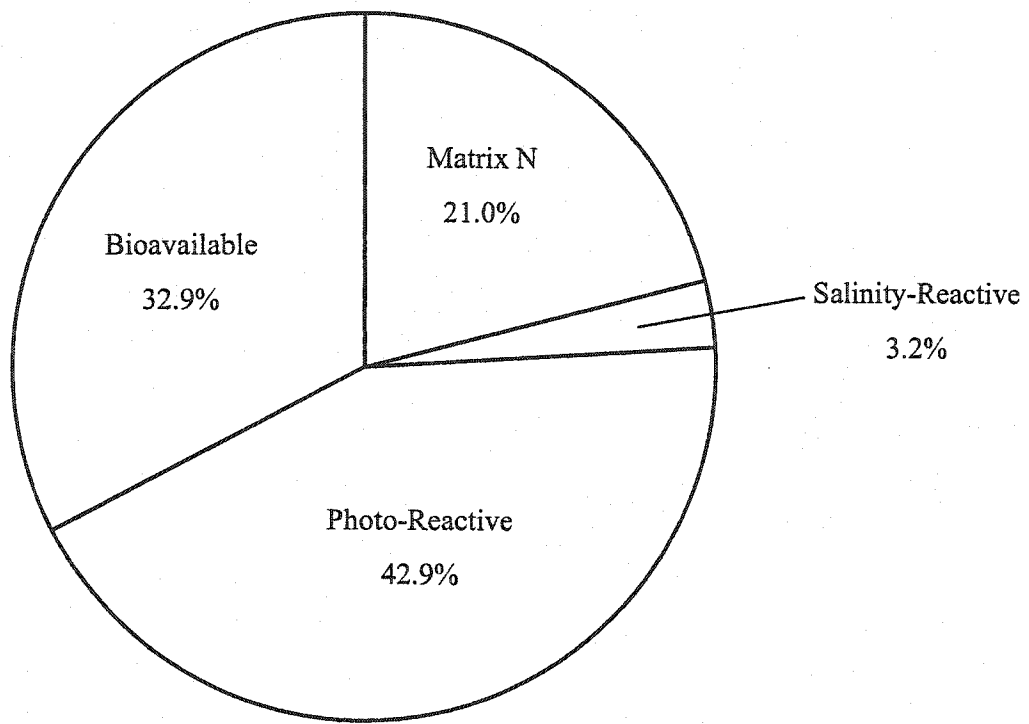
Humic-N

annually into the coastal ocean (Meybeck 1982, Sarmiento and Sundquist 1992). If it is assumed that 60% of this DON is humic in nature (40-80%; Beck et al. 1974, Thurman and Malcolm 1983, Thurman 1985), this would result in approximately 4.38×10^{12} g of humic-N discharged into the coastal ocean each year. However, as a fraction of this N is available to riverine, and estuarine phytoplankton via salinity release, photochemical reactions, or biological uptake, this number is an overestimate. The important question raised is how much of the N associated with humics is actually available to phytoplankton, either directly or indirectly, for uptake?

First, to estimate the potential contribution of salinity-reactive humic-N to the global ocean, preliminary data from the salinity-reactive experiments and the humic shuttle suggest that as much as 1 μM of the loosely associated NH_4^+ could be released into the surrounding waters per 0.015 g humic-C with increasing salinities (Thurman 1985, see Section VI, this volume). This estimation results in approximately 0.14×10^{12} g NH_4^+ released annually into the estuary for subsequent uptake by phytoplankton or bacteria (Table 1).

Second, the potential contribution of photo-reactive humic-N to the global ocean was estimated. Photochemical liberation of NH_4^+ from DON in the coastal ocean has been estimated at 6 μM NH_4^+, or 3.13×10^{12} g N discharged into the coastal ocean for the Southeastern United States (Bushaw et al. 1996). Again, assuming humic-N comprises 60% of the total DON discharged into the coastal ocean, and ultraviolet light reacts with and releases NH_4^+ from all DON equally (likely not true, as humic compounds are more photoreactive than other classes of marine DOM due to their high aromaticity and color (Bronk 2002)), 3.6 μM NH_4^+

Table 1. Calculation of the annual release each of the four humic-N pools into the coastal ocean. Matrix N was calculated by subtracting each of the other pools (salinity-reactive, photo-reactive, and bioavailable N) from bulk humic-N. Humic-N was calculated as 60% of total DON (Beck et al 1974, Thurman and Malcolm 1983, Thurman 1985).

Pool	Release (μmol L^{-1})	Global Discharge (X 10^{16} L yr^{-1}) [5]	Release μmol NH$_4^+$ (mg humic C)$^{-1}$	Atomic C:N [6]	Release μmol NH$_4^+$ (mg humic N)$^{-1}$	Total N (X 10^{12} g N yr^{-1})	% humic-N
DON[1]	–	3.74	–		–	7.3	–
Humic-N	–	3.74	–	40:1	–	4.38	–
Salinity-Reactive [2]	–	3.74	0.067	40:1	2.3	0.14	3.2
Photo-Reactive [3]	3.6	3.74	–	40:1	–	1.88	42.9
Bioavailable [4]	–	3.74	–	40:1	–	1.44	32.9
Matrix N	–	–	–	–	–	0.92	21.0
Total Available N						3.46	79.0

[1]Meybeck 1982, Sarmiento and Sundquist 1992; [2]Sections III and VI; [3]Bushaw et al. 1996; [4]Sections IV and V; [5]reviewed in Cauwet 2002; [6]Thurman 1985.

would be released for a total of 1.88×10^{12} g humic-N would be released annually

into the lower estuary and coastal ocean as NH_4^+ via photochemical oxidation

reactions (Table 1).

Third, the potential humic-N taken up by phytoplankton in the global coastal

ocean was estimated. Experiments in which laboratory formed ^{15}N-humic substances

were provided as the sole N source suggest that on average $33 \pm 14\%$ of humic-N can

be taken up by coastal phytoplankton (see Sections V and VI, this volume). Thus,

approximately 1.44×10^{12} g of the total humic-N discharged into the coastal ocean

would be considered bioavailable to coastal phytoplankton communities via

biological uptake mechanisms such as pinocytosis or extracellular enzymatic

cleavage (Table 1). Note that these experiments were conducted in cultures where

humic-N was provided as the only N source.

If the sum of these humic-N pools is taken, a maximum concentration of

humic-N available to phytoplankton communities, either directly or indirectly, is

obtained. The value calculated of available N, using the salinity-reactive, photo-

reactive, and bioavailable humic-N pools, is 3.46×10^{12} g N, or 79% of the bulk

humic-N estimated to enter into the coastal ocean annually (Fig. 1). It should be

noted that this is a maximum value, and it is likely that some fraction of this humic-N

can be categorized into more than one pool (i.e. N that is both bioavailable and photo-

reactive), which would lower the overall percentage of available humic-N.

Furthermore, the amount of humic-N entering the coastal ocean will vary with

environment. For example, along the east coast of the United States, humic

substances will travel through both river and estuary, allowing for maximum release

via interactions with salts and ultraviolet light prior to reaching the coastal ocean. However, along the western coast, humics will not pass through large estuaries en route to the ocean, perhaps preventing full release via interactions with light and salinity prior to reaching the coastal ocean. These assumptions require further investigation.

In conclusion, methods currently used to isolate humic compounds from aquatic sources creates an artifact that has skewed the thinking of the scientific community into believing that humics are carbon-rich, biologically unimportant compounds. However, the data presented in this dissertation challenges this notion. Through physical, chemical, and biological mechanisms, humic-N can provide an important source of N for riverine and estuarine communities. The scientific community needs to reevaluate the potential environmental and ecological role humic substances can play in riverine/estuarine environments rather than continue to portray humic compounds as the highly recalcitrant, biologically refractory compounds often reported. Humics should be recognized as biologically important compounds that play an integral role in the cycling of N in rivers, estuaries, and the coastal ocean and should be considered when modeling the eutrophication and N-loading of riverine/coastal environments.

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