



Changes in C:N ratios and chemical structures of estuarine humic substances during aging

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Abstract

Humic substances were isolated from the Satilla (GA), Altamaha (GA), and York (VA) rivers via the commonly used XAD-8 extraction technique. Humics were also formed in the laboratory by allowing *Spartina alterniflora* plants to humify under controlled laboratory conditions over the course of 1 year; the *S. alterniflora* had been grown with ^{15}N -enriched ammonium (NH_4^+) in the sediment such that the humics produced were enriched with ^{15}N . The chemical characteristics of the natural and laboratory-produced humic substances were evaluated in a number of ways.

Chemical shifts in the atomic carbon: nitrogen (C:N) ratio were monitored after exposing natural humic substances to environmentally relevant NH_4^+ concentrations. Structural characteristics of natural humics and laboratory-produced humics were determined using Fourier Transform Infrared (FTIR) spectroscopy, molecular weight (MW) size fractionation, and humic ^{15}N isotope enrichment. Results indicate that first, humic substances become more fulvic-like in character as aging time increased in the laboratory. Second, as humic aging time increased (from 1 week to 6 months), the humic-N becomes more enriched with ^{15}N . Third, the C:N ratio in the low MW size fraction of the humics decreased by more than half during the year of sampling (from 37 to 16). These results suggest that the structure and chemical composition of humic compounds change significantly during formation. Lastly, exposure of humic substances to environmentally relevant levels of NH_4^+ caused a significant decrease in humic C:N ratios. This result suggests that loss of ammonium during extraction using XAD can cause an underestimation of humic-N content such that humic substances in the environment are likely more N-rich than previously thought.

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1. Introduction

Humic substances can comprise 10–75% of the dissolved organic carbon (DOC) and 40–80% of the dissolved organic nitrogen (DON) in seawater (Beck et al., 1974; Thurman and Malcolm, 1983; Thurman,

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1985; Alberts and Takács, 1999; reviewed in Bronk, 2002), and as much as 95% of the natural organic matter transported to coastal marshes by rivers (Alberts and Filip, 1994). Humics are a heterogeneous mixture of organic molecules ranging in size from 0.5 to 1000 kDa that does not conform to any unique chemical structure (Thurman, 1985; Wershaw and Aiken, 1985). Rather humic compounds are defined as dissolved organic matter (DOM) that is retained by macroporous resins (e.g., XAD-8, more recently Supelco DAX-8) under acidic conditions (Thurman, 1985). Following extraction onto these resins, humic substances can be subdivided into three categories based upon their solubility in water: fulvic acids, humic acids, and humin. Fulvic acids tend to be smaller in molecular weight (MW), ranging from 0.5 to 2 kDa, and are soluble in water at all pHs. Humic acids are larger and often colloidal, typically ranging in size from 2 to 5 kDa or larger and precipitate from solution at pHs lower than two (Thurman et al., 1982). Humins are insoluble in water at any pH.

Natural humic substances, isolated with XAD extraction, contain 0.5–6% N (Rashid, 1985; Thurman, 1985; Hedges and Hare, 1987). This N can further be categorized as more N incorporated into humic acids, which are approximately 2–6% N, and fulvic acids, which are N poor at <1–3% 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 remainder 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 for humic acids and 45–55 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 down river 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 riverine and estuarine waters are also able to adsorb NH_4^+ that can be re-released into the surrounding water as salinity increases (See, 2003). In the laboratory, it has been observed that the traditional humic isolation technique is capable of stripping adsorbed NH_4^+ from the humic structure following a brief saturation of XAD extracted humic material with NH_4^+ (Fig. 1). Based on these observations, it was hypothesized that the atomic C:N ratio of humics in the natural environment, prior to XAD isolation, is likely more N-rich than currently thought (See, 2003). To test this hypothesis, this study (1) quantified changes in the atomic 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 kDa; intermediate MW, 3–10 kDa; HMW, >10 kDa) the NH_4^+ adsorbs; and (3) quantified structural changes that occur within a humic molecule as aging time in the laboratory increased utilizing Fourier Transform Infrared (FTIR) spectroscopy, wet chemical, and stable isotope techniques.

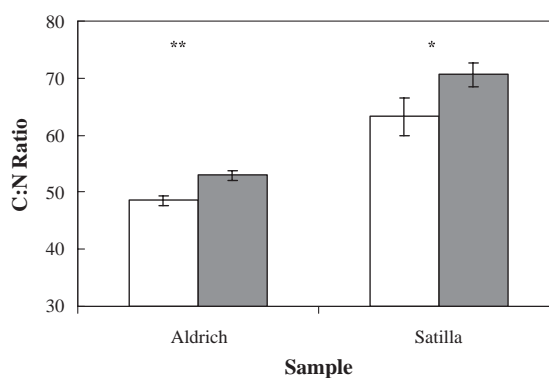


Fig. 1. The C:N ratio of humic substances enriched at $0.048 \mu\text{mol N}/\mu\text{mol humic C}$ for Aldrich humic acids and Satilla River humic substances before (□) and after (■) extraction onto DAX-8 resin. *Represents a significant difference in C:N ratio at the $p < 0.05$ level, and **represents significance at the $p < 0.01$ level. Error bars represent ± 1 standard deviation. Figure is taken from See (2003).

2. Materials and methods

2.1. Sample sites

Natural aquatic humic substances were collected from upriver stations on the Satilla River (GA) on four cruises, from the Altamaha River (GA) on a single research cruise, and from the York River (VA). 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 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 of >55% forested, >25% agricultural, and <5% urban (T. Dai, personal communication), 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 Satilla, Altamaha, and York Rivers respectively (Alberts and Takács, 1999; Raymond and Bauer, 2001).

2.2. Humic isolation

Humic substances were extracted on Supelco DAX-8 resin, as previously described for Amberlite XAD-8 resin (Aiken, 1985). 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. Any remaining salts were removed from the resin by rinsing the column with deionized water (DIW) until the eluate reached a pH > 5. Following the rinse with DIW, the column was back-flushed with two bed volumes of 0.2 N sodium hydroxide (NaOH) to elute the bound humic substances from the resin.

XAD-8 resins can bleed small organic molecules with the eluate (Aiken, 1988). Therefore, the DAX-8 resin was cleaned using a Soxhlet extraction procedure (solvents include ether, acetonitrile, and metha-

nol) followed by extensive rinses of HCl, NaOH, and DIW (Thurman and Malcolm, 1981; Aiken, 1985) prior to sample extraction. DIW water was then acidified, eluted through the column, and neutralized with HCl as sample and background levels of DON, NH_4^+ , and nitrate (NO_3^-) as well as DOC were determined to correct for compounds that may have leached from the resin (Parsons et al., 1984; Peltzer et al., 1996; Hansen and Koroleff, 1999; Bronk et al., 2000).

2.3. 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). 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 at the University of Georgia. The plants were watered regularly with a ^{15}N -labeled ammonium chloride (NH_4Cl) solution ($4.0 \text{ mmol N L}^{-1}$; ^{15}N 98+%). The plants were harvested by cutting off the leaves at soil level, dried in an oven for 1 week at 40°C , and shredded in a Wiley mill (60 mesh). The shredded *S. alterniflora* (42 g) was then divided equally into six 1-L 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 gently stirred with a magnetic stir bar. The coastal inoculum was created by concentrating 6 L of coastal water collected from SkIO over a $0.2 \mu\text{m}$ filter to a final volume of 60 mL. Each flask containing the shredded *S. alterniflora* received 10 mL of the coastal inoculum prior to incubation.

One-liter samples were removed from the dark over the period of 1 year with time points of 1 week, 2 weeks, 1 month, 3 months, 6 months, and 1 year. At each time point, the samples were filtered through a $0.2 \mu\text{m}$ filter, to remove particulate matter including bacteria, and the humic substances were isolated onto DAX-8, as previously described, neutralized with HCl, and frozen for future experiments.

In comparing changes over time, 1 week was chosen as a baseline because a true time zero concentration was problematic as the starting material was a solid *S. alterniflora* shoot.

2.4. Determination of aged humic ^{15}N enrichment

Humic-N isotopic enrichment was measured as atom percent excess (e.g., the ^{15}N enrichment above standard N_2 gas). Five milliliters of each of the aged humic substance samples was dried onto a pre-combusted GF/F filter (500 °C for 2 h) in an oven at 40 °C. The filters were then wrapped in tin discs and the atom percent excess was determined by analysis on an isotope ratio mass spectrometer (Europa Geo 20/20 with ANCA sample preparation unit).

2.5. Enrichment of humics with NH_4^+

Humic substances were enriched with NH_4^+ by swirling humic samples (100 mL, 833 μM humic C; $n=3$) for 16–18 h with low or high concentrations of NH_4Cl to give final ratios of 0, 0.048, and 0.48 $\mu\text{mol N}:\mu\text{mol humic C}$. Unfortunately, humic concentrations are rarely measured in a typical suite of environmental parameters, and when quantified, they are usually reported in units of C. In addition, humic and NH_4^+ concentrations are rarely measured together making it difficult to obtain a robust estimate of environmentally realistic $\text{NH}_4^+:\text{humic C}$ ratios. Therefore, 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 $\text{NH}_4^+:\text{DOC}$ in aquatic systems (Table 1).

Following enrichment, the sample pH was increased to 10 with 1.0 N NaOH and degassed to remove residual unbound NH_4^+ (as NH_3) by spinning in a SpeedVac concentrator and applying a strong vacuum (1 Torr) to the headspace over the sample for 7–8 h (See, 2003); the sample was kept below room temperature (20 °C) throughout this procedure. Each sample was then allowed to warm to room temperature, reneutralized with 5% HCl, and reconstituted to 100 mL with DIW. Final concentrations of NH_4^+ in samples run through the procedure were determined manually via the phenol-hypochlorite

Table 1
Ratio of $\mu\text{mol NH}_4^+$ to $\mu\text{mol C L}^{-1}$ DOC in coastal estuaries

Location	NH_4^+ (μM)	DOC (μM)	$\text{NH}_4^+:\text{DOC}$ ($\mu\text{mol N}:\mu\text{mol C}$)
<i>Streams</i>			
Highland, Scotland ^a	1.1–1.2	500–517	0.002
Cairngorm, Scotland ^a	0.6–2.1	450–675	0.001–0.005
Southwest Scotland ^a	2.4	533–933	0.003–0.005
Tweed, Scotland ^a	0.8–1.3	400–483	0.002–0.003
Dog Creek ^b			
High flow	6.2 ± 0.7	1325 ± 83	0.005
Low flow	5.5 ± 0.4	933 ± 167	0.006
Oyster Creek ^b			
High flow	7.0 ± 0.5	2142 ± 250	0.003
Low flow	15.6 ± 0.8	2208 ± 333	0.007
			Range=0.001 to 0.007
<i>Rivers</i>			
Altamaha River, GA ^c	0.4–4.5	300–475	0.001–0.015
Changjiang, China ^{d,e}	14.6	142–400	0.037–0.103
Huanghe, China ^{d,f}	5.3	167–333	0.016–0.032
Lena River, Siberia ^g	0.01–0.3	309–1042	0.000–0.001
Mura River, Slovenia ^h	6.4–85.7	150–708	0.009–0.571
Ogeechee River, GA ^c	0.3–1.02	267–525	0.001–0.004
Satilla River, GA ^c	0.2–8.3	525–3133	0.000–0.016
Savannah River, GA ^c	0.7–7.0	225–417	0.002–0.031
Zhujiang, China	11.5	142	0.081
Sapelo Island, GA ^{i,j}			
Summer	0.4–40.6	34182	0.004–4.872
Winter	1.8–73.4	50–292	0.006–1.468
			Range=0.000–4.872
<i>Mangroves</i>			
Coral Creek ^k	0.1–0.8	92–125	0.001–0.009
Taylor River ^l	0.6–4.5	708–1533	0.000–0.006
Caeté Estuary, Brazil ^m			
Dry season	2.5–14.0	292–400	0.006–0.048
Rainy season	3.0–19.5	283–558	0.005–0.069
			Range=0.000 to 0.069

Where available, ranges are reported. Data for Dog Creek and Oyster Creek are reported as mean ± standard error.

^a Chapman et al. (2001).

^b Wahl et al. (1997).

^c This paper.

^d Cauwet and Mackenzie (1993).

^e Zhang cited in Zhang et al. (1999).

^f Zhang (1996).

^g Lara et al. (1998).

^h Brodnjak-Vončina et al. (2002).

ⁱ Haines (1979).

^j Alberts and Takács (1999).

^k Boto and Wellington (1988).

^l Davis et al. (2001).

^m Dittmar and Lara (2001).

technique modified for colored/turbid water to ensure that the NH_4^+ had been removed (Koroleff, 1983).

2.6. 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 CO₂, which was measured via IR fluorescence. Nitrogen was converted to nitric oxide (NO) during combustion and measured via chemofluorescence after mixing with ozone gas.

2.7. Molecular weight determination

To obtain MW information, as well as to determine if NH_4^+ binds preferentially to humics of differing MW, triplicate samples of DAX-8 extracted humic substances were separated by size using Amicon Centriprep centrifugal filter devices (Millipore). These devices 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 kDa and <10 kDa MW compounds, respectively. As these membranes contain trace amounts of glycerin as a preservative, 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 min 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. To ensure that MW samples in this experiment were not separated using columns that had been damaged during the cleaning procedure, triplicate columns were used and the resulting values compared for

consistency in DOC and TDN values. Blanks for DOC MW samples contained $63 \pm 21 \mu\text{M C}$; TDN blanks were below detection limits.

2.8. Structural determination

Samples isolated from the Satilla River and humic substances formed in the laboratory by *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 were aged in the laboratory. Analyzing aquatic humics in their aqueous state is advantageous because drying 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 in a SpeedVac concentrator (Thermo Savant) to enhance resolution, and to assure that additional variables would not result in any observed differences, all experiments were run at the same concentrations of humic C, in fresh water, at the same temperature, and at the same pH. 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).

2.9. Data analysis

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

3. Results

3.1. C:N ratio of enriched humic substances

In general, there was a decrease in C:N ratio with increasing saturation with NH_4^+ . This decrease in C:N was attributed to an increase of N on the humic

structure, rather than C removed from the humic structure, because there was no decrease in DOC values for the samples with increased exposure to NH_4^+ (data not shown).

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 (Fig. 2A). The decrease in the C:N ratio between the field sample extracted by DAX-8 and the sample enriched with $0.048 \mu\text{mol N}:\mu\text{mol humic C}$ was significant in both the spring and winter samples (Fig. 2A). There was also a decrease in the C:N ratio between the

$0.048 \mu\text{mol}$ and $0.48 \mu\text{mol N}:\mu\text{mol humic C}$ in autumn and summer, but this decrease was not statistically significant.

The bulk C:N ratio decreased with increasing enrichment in the Altamaha and York River samples (Fig. 2B and C). The C:N ratio of the Altamaha River decreased approximately 27% after enriching with $0.048 \mu\text{mol N}:\mu\text{mol humic C}$ (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 $0.048 \mu\text{mol N}:\mu\text{mol humic C}$ enrichment (Fig. 2C).

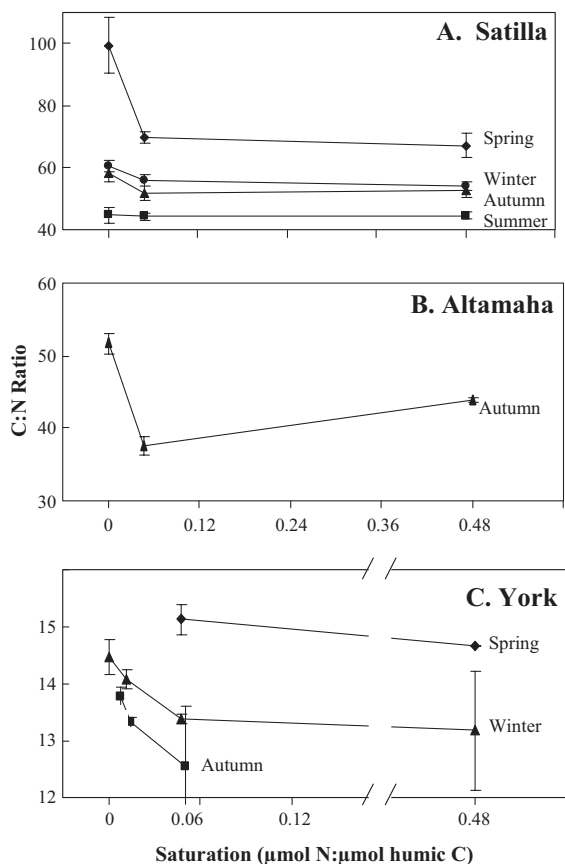


Fig. 2. The atomic C:N ratio of humic substances after enrichment with increasing concentrations of NH_4Cl . Humics were extracted from (A) the Satilla River, GA, during spring, summer, fall, and winter, (B) the Altamaha River, GA during autumn, and (C) the York River, VA during spring, autumn, and winter. Data for $0.48 \mu\text{mol N}:\mu\text{mol humic C}$ for the autumn York River sample were lost due to instrument error, and the York River $0 \mu\text{mol N}$ enrichment spring sample was lost due to contamination. Error bars represent ± 1 standard deviation.

3.2. 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 is consistent with previous studies that have identified a decrease in the C:N ratios of organic matter as it ages (e.g., Aluwihare and Repeta, 1999). The decrease seen for the humic substances is due to a significant increase in the concentration of DON from 17.9 to $34.3 \mu\text{M N}$ over this time period while DOC concentrations were constant (data not shown). After week 2 of aging, additional aging time appeared to have little influence on the C:N ratio of the bulk humics, and samples taken from these time periods were not significantly different (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 over time. 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 MW distribution of the humic substances also changed considerably with aging time in the laboratory (Fig. 4). 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 ($r^2 = 0.83$,

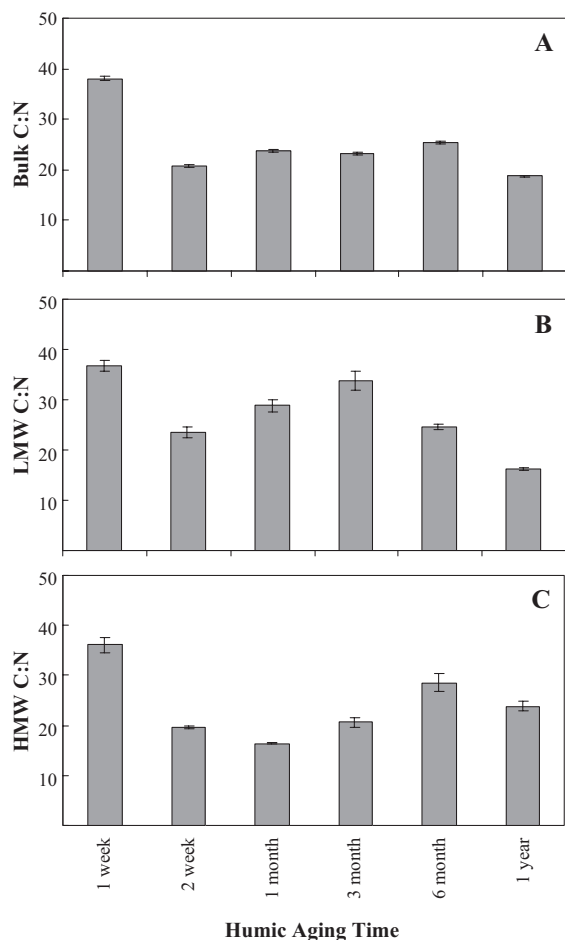


Fig. 3. Atomic C:N ratios of laboratory humic substances with relationship to humic aging time isolated from degraded *S. alterniflora*: (A) the bulk sample, (B) the LMW (<3 kDa) size fraction, and (C) the HMW (>10 kDa) size fraction. Error bars represent ± 1 standard deviation.

$p < 0.005$). The %N in the LMW fraction increased significantly through time ($r^2 = 0.77$, $p < 0.0001$). 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.

3.3. 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} (Fig. 5A, B). 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 laboratory by degrading *S. alterniflora* were also examined via FTIR to determine changes in the structure as the aging time increased (Fig. 5B). These data indicate an increase in either O–H or N–H bonds (3400 cm^{-1}) over time.

3.4. Changes in the ^{15}N -humic enrichment

As the humic substances aged in the laboratory, the atom percent enrichment of the humic compounds

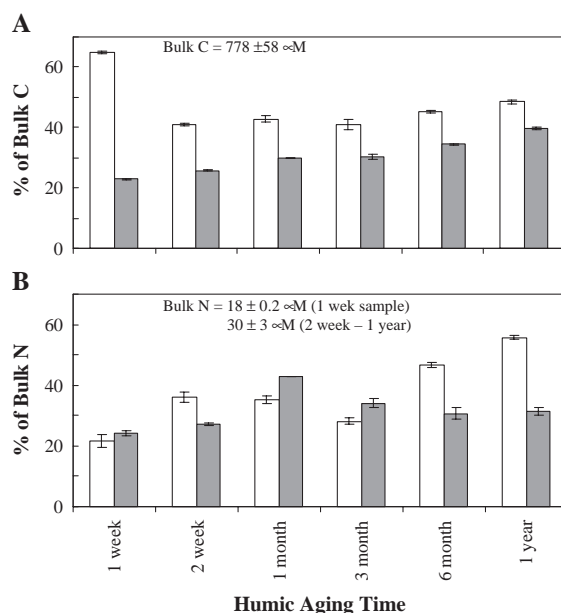


Fig. 4. Mean percent of the bulk (A) C and (B) N in the <3 kDa size fraction (\square) and the >10 kDa size fraction (\blacksquare) by weight for humics with increased humic aging time. Error bars represent ± 1 standard deviation.

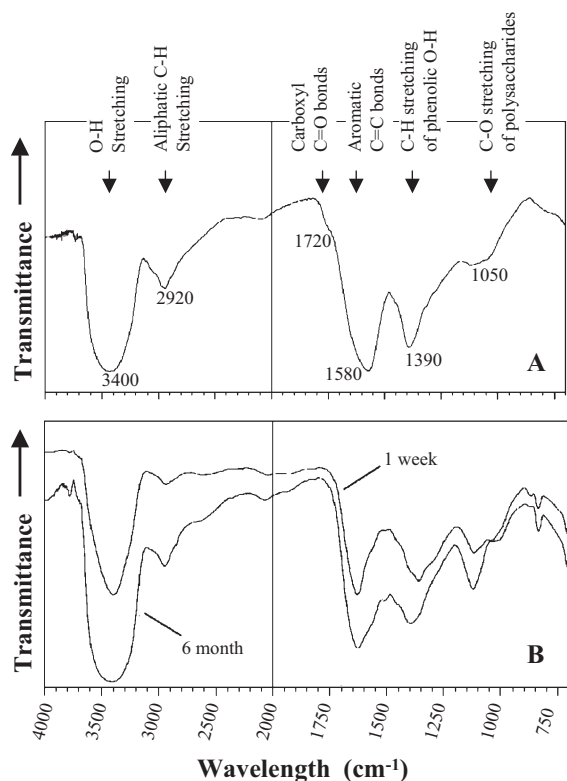


Fig. 5. (A) Sample FTIR spectrum of humics isolated from the Satilla River, GA in April 2001. (B) Changes in the FTIR spectrum of *S. alterniflora* derived humics produced in the laboratory as aging time increased from 1 week to 6 months.

increased from the 1 week sample to the 6 month sample, approaching a maximum value of 10.0 (Fig. 6). However, from 6 months to 1 year, the atom percent enrichment decreased to 6.0, a value below the initial enrichment.

4. Discussion

4.1. Underestimation of C:N ratios of natural humic substances

Two concentrations of NH_4^+ were chosen for humic substance enrichment to represent moderate (4 μM) and high (40 μM) levels of enrichment, similar to those observed in nature. The humic substances adsorbed the NH_4^+ such that half of the enriched Satilla River samples exhibited a statistically significant decrease in their atomic C:N ratio (Fig. 2A),

consistent with observations that humic substances efficiently adsorb 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 DAX-8 resin will remove this NH_4^+ (see Fig. 1). These data imply that isolation with DAX-8 (or XAD-8) resin can underestimate the N content of humics, either nitrogen bound to or associated with the humic compound, by stripping loosely associated N from the humic structure prior to analysis. This finding is consistent with a number of previous observations (Roulet et al., 1963; Sowden and Schnitzer, 1967; Khan and Sowden, 1972; Thurman, 1985).

It has also been demonstrated that DOM isolated via ultrafiltration (UDOM, >1kDa) from marine systems has a C:N ratio two to three times lower than humic substances extracted via XAD techniques (Benner, 2002). A 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, the way that XAD resin does, and therefore the isolated HMW humics have a lower C:N ratio.

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 pro-

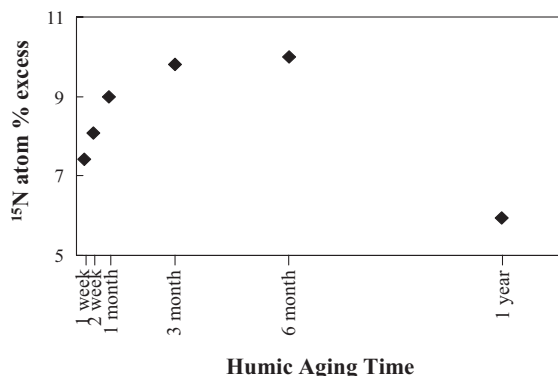


Fig. 6. The change in ^{15}N -humic atom percent excess (e.g., the ^{15}N enrichment above standard N_2 gas) as *S. alterniflora* derived humic substances are formed in the laboratory.

ductivity during the summer months. Under these conditions, humic substances may be degraded such that their capability to adsorb NH_4^+ is decreased. In the Altamaha River, the C:N ratio of the humic substances in autumn also decreased after enrichment with NH_4^+ . However, an exposure to increased concentrations of NH_4^+ (0.48 $\mu\text{mol N}:\mu\text{mol humic C}$) resulted in higher humic C:N ratios than those exposed to 0.048 $\mu\text{N}:\mu\text{mol humic C}$, a result that 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 adsorption of NH_4^+ to humic substances is environmentally significant for a number of reasons. First, NH_4^+ is a ubiquitous compound found in aquatic ecosystems and can reach high concentrations in areas of humic formation such as marshes, wetlands, and swamps (e.g., Haines, 1979; Chambers, 1997). Data

from this study suggest that the C:N ratio of humic substances may be as much as 12% lower than those previously reported (Table 2). 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, decreasing the C:N ratio by 12% for humic compounds in the Satilla River results in an underestimation of humic associated N of $60 \times 10^6 \text{ g N year}^{-1}$ (Alber and Sheldon, 1999).

Second, phytoplankton and bacteria readily take up NH_4^+ as a N source, and any process that affects the free NH_4^+ concentration could impact primary and secondary production. If the NH_4^+ adsorbed to humic compounds is no longer available to phytoplankton for uptake, changes in aquatic humic concentration could affect NH_4^+ bioavailability, and humic substances could provide an important transport mecha-

Table 2
C:N ratio of humic substances saturated in the laboratory at increasing NH_4^+ :humic C

River	Season	Saturation ($\mu\text{mol NH}_4^+:\mu\text{mol humic C}$)	Atomic C:N		SD	% Decrease from 0:1 Saturation
Satilla	Spring	0:1	99	±	9	
		0.048:1	70	±	2	29.5*
		0.48:1	67	±	4	32.3*
	Summer	0:1	45	±	3	
		0.048:1	44	±	1	1.0
		0.48:1	45	±	1	0.1
	Autumn	0:1	58	±	3	
		0.048:1	52	±	2	10.7
		0.48:1	53	±	2	9.3
Winter	0:1	61	±	2		
	0.048:1	56	±	2	7.5	
	0.48:1	54	±	1	10.4*	
Altamaha	Autumn	0:1	52	±	1	
		0.048:1	38	±	1	27.4*
		0.48:1	44	±	0	15.1*
York	Spring	0:1		N/A		
		0.048:1	15	±	0	
		0.48:1	15	±	0	
	Autumn	0:1	14	±	0	
		0.048:1	13	±	1	8.7
		0.48:1		N/A		
	Winter	0:1	14	±	0	
		0.048:1	13	±	0	7.4*
		0.48:1	13	±	0	8.8*
					Mean	12 ± 11

Data for 0:1 spring York River saturation are not included due to contamination, and 0.48:1 autumn York River sample was lost due to instrument error. N/A—not available.

* Indicates significance at $p < 0.05$.

nism for moving a labile N source down river (as much as 77×10^6 g N year⁻¹, See, 2003).

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 NH₄⁺ 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 NH₄⁺ 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.

4.2. Changes in chemical characteristics with increased humic substances aging time

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% of the total 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 increase 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 fulvic-like molecules. The net increase in %N for both the LMW and HMW size fractions of the XAD extracted humics likely occurs as the building blocks of humic substances (lignin, bacterial quinones, and lignin quinones), polymerize

and combine with NH₄⁺ and DON, such as dissolved primary amines, released from the breakdown of *S. alterniflora* biomass into the water resulting in an increase in the N content of the humics (Stevenson, 1994).

Changes in the ¹⁵N enrichment of humic-N also suggest transformations in the chemical composition with increased humic aging time. As *S. alterniflora* humifies, a greater percentage of ¹⁵N is incorporated into the humic structure (Fig. 6). In this study, the highest humic ¹⁵N enrichment was found in the 6-month-old sample suggesting that the coastal bacteria used to inoculate the initial *S. alterniflora* mixture preferentially utilized and assimilated the light ¹⁴N, leaving the heavier ¹⁵N to be incorporated onto the humic compound. However, after 6 months, the ¹⁵N enrichment of the humic substances decreased, either by incorporation of additional ¹⁴N onto, or by the removal of ¹⁵N, from the humic structure.

4.3. Changes in structures with aging time

FTIR spectroscopy is a powerful tool that can be used in the identification of complex compounds. The isolated Satilla River seasonal spectra showed little, if any difference, in the structure of the riverine humics over time. The overall lack of differences between spectra does not imply that there are no structural differences between the humic substances; rather, any differences in the structures may be obscured by the larger, more similar fractions such as lignin backbones, common among estuarine humic substances.

Samples analyzed to monitor changes in the laboratory-formed humics produced by degrading *S. alterniflora* in coastal seawater appeared to be similar. However, several unique differences arose in the FTIR structures as the aging time of the humic substances increased including an increase in aliphatic C, a decrease in aromatic chains, and an increase in O–H bonds suggesting that more fulvic acid residues were present in the older humic substances (Thurman, 1985; Stevenson, 1994). The strong absorption present in the humic substances at about 1635 cm⁻¹ can be linked to peptide carbonyls, and the decrease in the absorption of the small peak 835 cm⁻¹ may be due to a decrease in hydrocarbon ring structure chains. These observations support the hypothesis that as humic

substances age in the environment, 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).

5. Conclusions

The results of this study indicate that as humic aging time increased humic-N became more enriched with ^{15}N , indicative of a preferential breakdown and removal of the lighter isotope from the structure, and that the atomic C:N ratio in the low MW size fraction of the humics decreased by more than half. These results suggest that the structure and chemical composition of humic compounds change significantly during formation. Furthermore, it was shown that NH_4^+ can bind to humic substances during exposure at environmentally relevant NH_4^+ concentrations. This suggests that the C:N ratio of humic substances, as they exist in natural waters, is lower than traditional XAD isolation and analysis indicate, which may have implications for the calculation of N loading budgets to coastal waters, our understanding of the transport and bioavailability of humic-N, and the interpretation of photoproduction studies that used XAD isolated humics.

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