Progress Report for New York State Water Resources Institute

# Profiling Labile Amino Acids in Aquatic Dissolved Organic Matter by High Resolution Liquid Chromatography-Mass Spectrometry

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**Summary.** Labile amino acids (AAs) represent an important source of nutrients to aquatic biota, serve as precursors to transformation products in water treatment systems, and contribute to the fluxes of carbon and nitrogen in a watershed. Therefore, the quantitation of AAs has been a long-standing focus in the characterization of dissolved organic matter (DOM) in natural and engineered water systems. Here we present a new method for profiling 23 AAs in aquatic DOM, including the 20 proteinogenic AAs, oxidized cysteine dimer (cystine), and two urea cycle-linked AAs (ornithine and citrulline). In addition to providing this comprehensive AA profiling, this method affords three notable advantages to current methods: (i) the exclusion of a derivatization step to simplify sample preparation, (ii) no need for a two-step tandem mass spectrometry by coupling high-resolution liquid chromatography with a single-step high-accurate orbitrap mass spectrometry, and (iii) direct quantitation of AAs using an isotope ratio-based approach. Following optimization of AA detection and quantitation, we applied this method to obtain the first AA profiling of the Suwannee river natural organic matter (NOM) reference sample and compare it to its fulvic acid (FA) isolate, a widely-used proxy for aquatic DOM. We found that the Suwannee river FA had up to 2-fold higher content of labile AA than the Suwannee NOM but the relative distribution of the detected AAs was remarkably similar. We have also initiated preliminary application of our method to characterize AAs in engineered water systems. Specifically, we sought to determine the storage and pre-treatment needed for the samples before LC-MS analysis. We compared frozen and non-frozen samples both with and without pH adjustments. Discrepancies in the AA profiles of these different samples highlighted profile AAs after different stages of the drinking water treatment plant of the City of Newburgh. Discrepant results between the analytes of the different treatment conditions highlight the need for further method development. However, the results did point out that AA levels were elevated for several AAs following the two chlorination/fluorination stages, thus implying that these

stages triggered AA production from DOM in the solution. However, the subsequent mixing or clearing stage led to removal of the AAs from solution.

## **1.** INTRODUCTION

Dissolved organic matter (DOM), a remnant of organic inputs from plant and microbial activities, is composed of a complex assemblage of biomolecule-derived organics in terrestrial and marine aquatic environments. Fluxes of DOM through a watershed captures the dynamics of both sinks and sources of organic nutrients. Notable amongst these nutrients are free amino acids (AAs), which represent an important source of carbon and nitrogen for metabolism and energy in micro and macro biota in natural surface waters aquatic biota (Flynn and Butler, 1986; Coffin, 1989; Leenheer 2003, Munster 1998). The AA distribution has been considered as a proxy for microbial cycling of nitrogen with respect to protein biosynthesis (Kirchman et al 1985). In addition, the presence of amino nitrogen typical in AAs is implicated in the transformation pathways of organic in natural waters and engineered water treatments (Dotson et al., 2009; Chen et al., 2008; Liu et al, 2014; Du et al., 2017). Therefore, due to the important of AAs in biological and chemical processes in both natured and engineered waters, development of analytical methods for AA profiling has been a long-standing focus in water research.

The AAs commonly profiled in DOM are the 20 proteinogenic AAs, which can be divided into four categories based on the chemistry of their side chains (Table 1): nonpolar AAs [glycine (Gly), alanine (Ala), proline (Pro), valine (Val), isoleucine (Ile), leucine (Leu), methionine (Met)], uncharged polar AAs [(Serine, Ser), threonine (Thr), asparagine (Asn), glutamine (Gln), cysteine (Cys)], charged polar AAs [aspartate (Asp), lysine (Lys), glutamate (Glu), arginine (Arg)], and aromatic AAs [histidine (His), phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp)]. In addition, of important interest are also citrulline and ornithine, which are

two amino acids that are often overlooked but are essential to the urea-cycle nitrogen metabolism (Table 1). Due to facile oxidation of Cys to the dimer Cys-Cys by the joining of the two disulfide bonds, both Cys and Cys-Cys are often monitored in oxic waters (Table 1). Analytical methods to isolate these AAs in solutions have employed a derivatization method followed by liquid chromatography (LC) detection (Lindroth and Mopper, 1979; Fujii et al., 1997) or gas chromatography (GC) (Mawhinney etal., 1986) without or with mass spectrometry (MS). The LC method with fluorescence detections has been applied to profile AAs in boreal freshwater samples (Münster, 1999), in secretions by marine phytoplankton (Andersson et al., 1985; Furhman and Fergusson, 1986; Furhman, 1987; Hama et al., 1987), in humic substances (Aiken et al., 1985), in growth medium for marine microalgae (Flynn and Butler, 1986), estuarine free and peptidic AAs (Coffin, 1989).

A major limitation of these methods is the lack of comprehensive isolation of the 23 AAs described above. In this study, we sought to develop an analytical method that can provide this comprehensive AA profiling and omit the use of a derivatization step, which uses undesirable toxic chemicals. Here we couple LC with single step high-resolution orbitrap MS to obtain a robust method that can achieve high-resolution profiling of the 23 AAs simultaneously in solution without derivatization. We applied our method to obtain the AA characterization of reference samples of Suwanee River natural organic matter (SRNOM) and Suwanee River humic acid (SRHA). Subsequently, we started to evaluate the performance of our method to profile AA at different stages of the water treatment plant at the City of Newburgh, NY.

## **2.** MATERIALS AND METHODS

### *2.1 Materials*

Suwannee River fulvic acid standard II (SRFA), and Suwannee River Aquatic NOM II (SRNOM) were purchased from the International Humic Substances Society (IHSS) (St. Paul, MN). SRHA, SRFA, and SRNOM stock solutions were prepared by mixing a known amount of dry sample with ultrapure water produced by a Milli-Q water filtration system (Millipore Billerica, MA) or with a water/methanol (Optima$^{TM}$ LC/MS) solution. Istopically-labeled AAs were obtained from Cambridge isotopes.

*2.2. Method Development*

Amino acid standards were prepared various times during method development. Stock solutions were diluted to a 60 nM concentration with LC/MS water and subsequently transferred into LC/MS vials for analysis. Extracts were then analyzed by high-performance liquid chromatography (HPLC) mass spectrometry (MS). Data was collected on negative mode as two mobile phases were injected, in varying amounts, into the LC column at a flow rate of 0.180 mL/min. Several methods were developed and tested until all 23 target amino acids were successfully detected (Table 1). Method details for the final amino acid method are outlined (Figure 1). The final amino acid method was then used to analyze subsequent samples produced with the SRHA, SRFA, and SRNOM stock solutions.

*3.3 Sample Preparation and Analysis*

Test tubes containing 10 mL of 1 g/L SRNOM or SRFA were shaken at 150 RPM, 25 °C. Solutions were made with LCMS water solution and neutralized with 5 M KOH from pH = 3 to pH = 7.5. Sodium Azide (50 nM) was added to the 10mL tubes until the concentration of $NaN_3$ reached 1mM. Samples were prepared in triplicate and sampled at 24, 48, and 72 hours. At each time point, 500 µL of sample was collected, centrifuged for 5 minutes at 15,000 RPM in 0.22-µm centrifuge filters and then diluted 10 times.

To quantify the AA concentration in the samples, 250 µL of sample was spiked with 250 µL of a solution containing a cocktail of the labeled AAs. The isotopically-labeled cocktail was prepared with 100 nM or 250 nM AA concentration. After mixing with the sample, the AA concentration in the final solution was either 50 nM or 125 nM. Control experiments were also conducted without the reference DOM samples. The measured unlabeled fraction ($U_{f-AA}$) of each AA in solution is defined as

$$U_{f-AA} = \frac{X_{AA}}{X_{AA}+I} \qquad (1)$$

wherein $X_{AA}$ is the unknown concentration of AA in solution and $I$ is the known concentration of the isotopically-labeled AA added to the solution. Equation 1 was re-arranged to calculate the unknown AA concentration.

*3.4 Total Organic Nitrogen Quantitation.*

To measure the nitrogen content for each DOM reference, we make use of the Simplified TKN (s-TKN™) TNTplus Vial Test (range 0-16 mg/L N) in addition to the Ammonia TNTplus Vial Test, ULR (range 0.015 - 2.00 mg/L $NH_3$-N), which were both purchased from Hach Company (Loveland, CO) to quantify total organic nitrogen in SRNOM and SRHA samples. Neutralized samples were prepared with ultrapure Milli-Q water at 1 g/L concentration in triplicate and digested using procedures outlined in the Simplified TKN TNTplus as well as the Ammonia TNT plus vial tests. Total organic nitrogen (TON) was then calculated using the following formula:

$$TON = TN - (NO_3+NO_2+NH_3) \qquad (2)$$

Where TN is total N and the sum of nitrate ($NO_3$), nitrite ($NO_2$), and ammonia ($NH_4$) is total inorganic N concentration in the solution. To determine the fractional amount of the total organic N as labile AA-associated N in SRFA and SRNOM samples:

$$\text{Total AA (\%)} : \sum\left(\frac{X_{AA}}{Organic\ N}\right) * 100$$

*3.5. Profiling AA in Newburgh Water Treatment Plant*

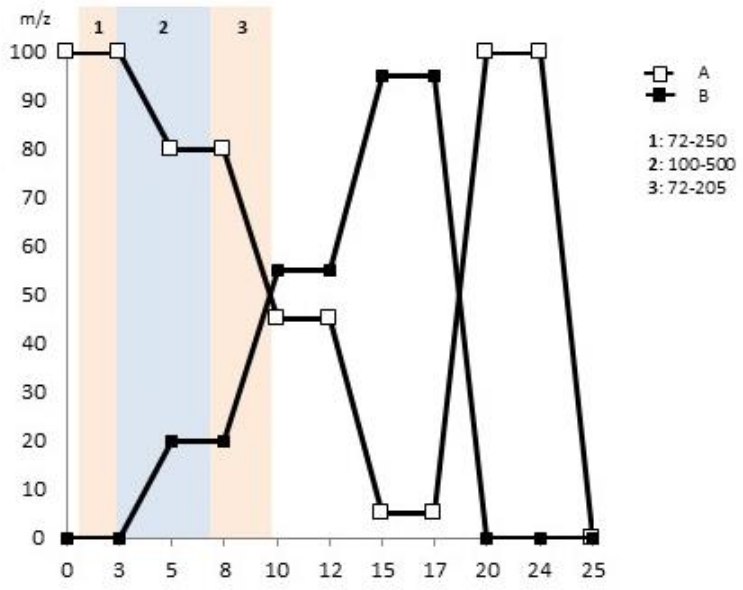
The Water Treatment Plant from the City of Newburgh provided us with three sets of samples obtained during three different times through the day: Morning (9-10 AM), Afternoon (5-7 PM), and Night (10-11PM). Upon arrival to our laboratory, samples were filtered (0.2-µm nylon) and split and stored in two conditions: Frozen (in a freezer) and Non-frozen (in a refrigerator). Four replicates from each were analyzed in LCMS for each site specified in Appendix A. For each replicate, 200 µL of each sample was concentrated 5 times by drying the sample under $N_2$ and then suspending in 40 µL LCMS pure water.

## **3.** PRELIMINARY PREESENTATION OF FINDINGS

**Table 1.** Molecular formula, high-resolution chromatographic retention time, and mass accuracy ($\delta_M$) of the targeted amino acids.

| Amino Acids | Molecular Formula | Retention Time[1] (min) | Theoretical[2] m/z | Measured[2] m/z | $\delta_M$[3] (ppm) |
|---|---|---|---|---|---|
| Proteinogenic AAs | | | | | |
| **Nonpolar** | | | | | |
| Glycine (Gly) | $C_2H_5O_2N$ | 1.34 | 74.0242 | 74.0233 | 12.2 |
| Alanine (Ala) | $C_3H_7O_2N$ | 1.34 | 88.0399 | 88.0389 | 11.4 |
| Proline (Pro) | $C_5H_9O_2N$ | 1.43 | 114.0555 | 114.0547 | 7.0 |
| Valine (Val) | $C_5H_{11}O_2N$ | 1.58 | 116.0712 | 116.0705 | 6.0 |
| Isoleucine (Ile) | $C_6H_{13}O_2N$ | 2.17 | 130.0868 | 130.0861 | 5.4 |
| Leucine (Leu) | $C_6H_{13}O2N$ | 2.34 | 130.0868 | 130.0861 | 5.4 |
| Methionine (Met) | $C_5H_{11}O_2NS$ | 1.90 | 148.0432 | 148.0426 | 4.0 |
| **Uncharged Polar** | | | | | |
| Serine (Ser) | $C_3H_7O_3N$ | 1.34 | 104.0348 | 104.0340 | 7.7 |
| Threonine (Thr) | $C_4H_9O_3N$ | 1.38 | 118.0504 | 118.0497 | 5.9 |
| Asparagine (Asn) | $C_4H_8O_3N_2$ | 1.33 | 131.0457 | 131.0450 | 5.3 |
| Glutamine (Gln) | $C_5H_{10}O_3N_2$ | 1.34 | 145.0613 | 145.0606 | 4.8 |
| Cysteine (Cys) | $C_3H_7O_2NS$ | 1.35 | 120.0119 | 120.0111 | 6.7 |
| Cystine (Cys-Cys) | $C_6H_{12}O_4N_2S_2$ | 1.34 | 239.0160 | 239.0160 | 0.0 |
| **Charged Polar** | | | | | |
| Aspartate (Asp) | $C_4H_7O_4N$ | 4.99 | 132.0297 | 132.0290 | 5.3 |
| Lysine (Lys) | $C_6H_{14}O_2N_2$ | 1.02 | 145.0977 | 145.0970 | 4.8 |
| Glutamate (Glu) | $C_5H_9O_4N$ | 4.62 | 146.0453 | 146.0446 | 4.8 |
| Arginine (Arg) | $C_6H_{13}O_3N_4$ | 1.02 | 173.1039 | 173.1034 | 2.9 |
| **Aromatic** | | | | | |
| Histidine (His) | $C_6H_9O_2N_3$ | 1.08 | 154.0617 | 154.0611 | 3.9 |
| Phenylalanine (Phe) | $C_6H_{11}O_2N$ | 4.36 | 164.0712 | 164.0706 | 3.7 |
| Tyrosine (Tyr) | $C_9H_8O_3N$ | 2.48 | 180.0661 | 180.0657 | 2.2 |
| Tryptophan (Trp) | $C_{11}H_{12}O_2N_2$ | 8.18 | 203.0821 | 203.0818 | 1.5 |
| Urea Cycle AAs | | | | | |
| Ornithine (Orn) | $C_5H_{12}O_2N_2$ | 1.02 | 131.0821 | 131.0814 | 5.3 |
| Citrulline (Cit) | $C_6H_{13}O_3N_3$ | 1.38 | 174.0879 | 174.0874 | 2.9 |

[1]Retention time using high-performance liquid chromatography (HPLC)
[2]mass-over-charge = *m*/*z*. The compounds were identified by following the HPLC-column with electrospray ionization and orbitrap mass spectrometry in negative mode.
[3]Mass accuracy ($\delta_M$) is the absolute value of 0.0001% offset of the theoretical *m*/*z* from the measured *m*/*z*.




| MS Method | |
|---|---|
| Time (min) | m/z scan range |
| 0.7-3 | 72 to 250 |
| 3-7.8 | 100 to 500 |
| 7.8-9.6 | 72 to 205 |

**Figure 1.** Final amino acid LC-MS method retention times and m/z scan ranges. Mobile phases A and B were utilized for this method. Mobile Phase A: water-methanol mixture supplemented with an ion-pairing agent (tributylamine). Mobile Phase B: 100% methanol.

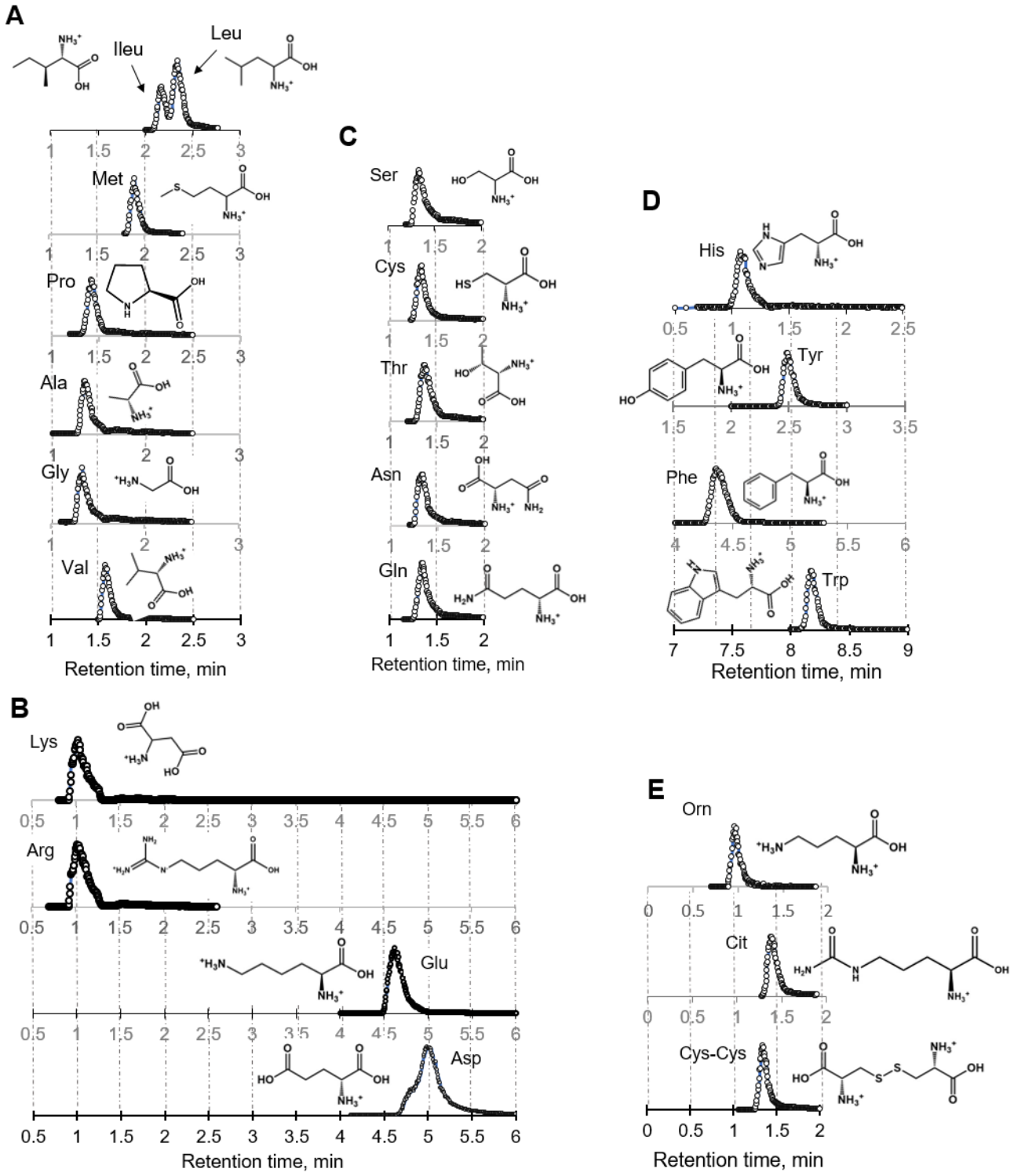



**Figure 2.** Extracted ion chromatograms at the m/z channel (± 20 ppm) corresponding to the targeted amino acids in a solution containing all 23 compounds. The specific retention time and mass accuracy for the m/z peak are listed in Table 1.

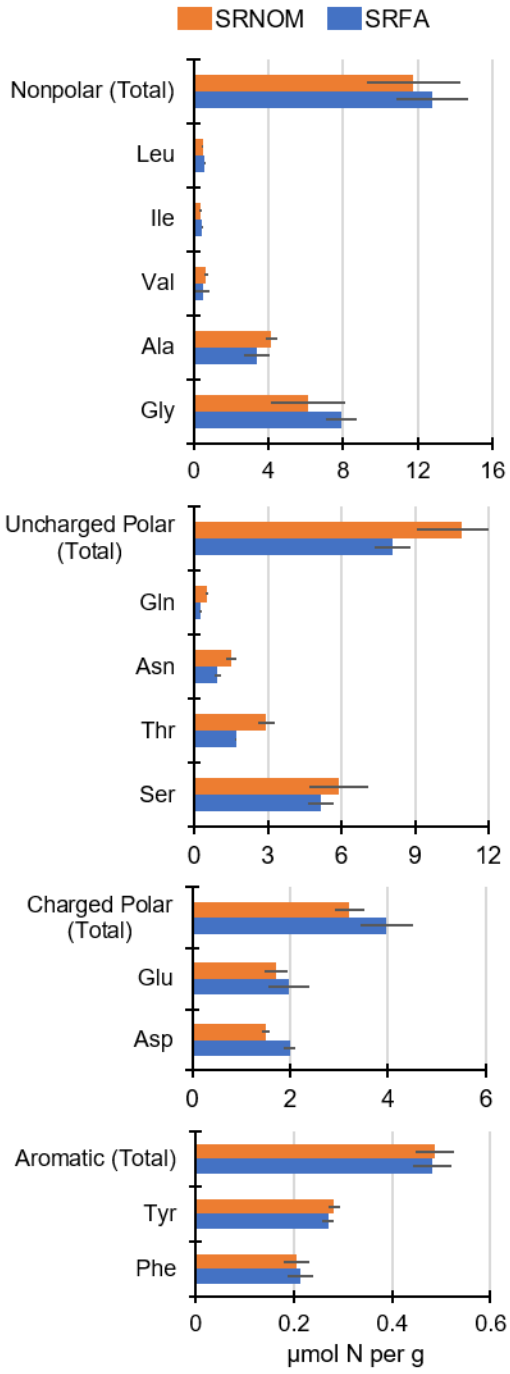



**Figure 3.** Distribution of amino acids (in µmol per g of HA or NOM) in each category (from top to bottom): Nonpolar, uncharged polar, charged polar, and aromatic.

**Table 2. Total amino acid (AA)-N accounting as a component of total organic N and total N**

| | Total AA | Total N | Total organic N | AA Fraction in total N | AA Fraction in organic N |
|---|---|---|---|---|---|
| | mg N $g^{-1}$ DOM | mg N $g^{-1}$ DOM | mg N $g^{-1}$ DOM | % | % |
| IHSS[1] | 0.457 | 6.7 | NR | 6.82 | NR |
| Suwannee river FA | 0.341 ± 0.056 | 6.41 ± 0.05 | 2.58 ± 0.05 | 5.32 ± 1.018 | 13.20 ± 2.53 |
| Suwannee river NOM | 0.357 ± 0.027 | 13.33 ± 0.99 | 5.81 ± 0.48 | 2.675 ± 0.319 | 6.14 ± 0.73 |

[1]Reported by the International Humic Substances Society (http://humic-substances.org)

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