DISSOLVED ORGANIC NITROGEN FRACTIONATION

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ABSTRACT

A preparative dissolved organic fractionation method was developed to concentrate isolated fractions in organic nitrogen for nitrogenous disinfection by-product research. The method was developed and tested on dissolved organic matter (DOM) derived from an algal culture, a bacterial culture, a mesotrophic lake during the beginning of an algae bloom and an effluent from a wastewater-treatment plant. The method was based on the assumption that most organic nitrogen in DOM is in proteins and amino acids. Proteins, peptides, and certain amino acids are retained on the macroporous styrene/ divinylbenzene resins XAD-1 and XAD-4 at pH > 10, whereas the macroporous methylmethacrylate resin XAD-8 used in standard DOM fractionation did not adsorb these compounds at pH > 10. Nitrogen-rich colloids were first isolated by dialysis of a roto-evaporated concentrate. The dialysis permeate was passed through a 1 liter (L) column of XAD-1, a 0.5 L column of XAD-4, and a 1 L column of MSC-1H cation-exchange resin in series. Hydrophobic acids were desorbed from the XAD-1 resin with 1 L of 0.1 M NaOH and amphiphilic acids were desorbed from the XAD-4 resin with 0.5 L of 0.1 M NaOH. After desorption of the acids, the XAD-1 and XAD-4 resins were immediately acidified with a 0.01 M HCl rinse, and the protein rich hydrophobic neutral fraction (XAD-1), and amphiphilic neutral (peptide and amino acid) fractions were desorbed with 75% acetonitrile/25% water. After evaporation and freeze drying, the hydrophobic neutral fraction was extracted with 50 milliliters (mL) of ethyl acetate to remove non-nitrogenous acids. The fractions were characterrized by elemental and spectral analyses. The bacterial and algal samples had large percentages of nitrogen-rich colloid and base and neutral fractions, and the lake sample and wastewater treatment plant effluent had large percentages of nitrogen-poor acids.

Keywords: Dissolved organic nitrogen, organic matter fractionation, infrared spectrometry, ^{13}C-nuclear magnetic resonance spectrometry, elemental analyses.

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1. INTRODUCTION

A preparative dissolved organic fractionation method was specifically developed to enrich isolated fractions in organic nitrogen for nitrogenous disinfection by-product research. Dissolved organic nitrogen (DON) is a growing area of research, especially in drinking water supplies [1]. A more fundamental question is whether organic nitrogen is incorporated into dissolved organic matter (DOM) as a mixture that can be separated by a fractionation scheme, or is a molecular conjugate of DOM constituents that cannot be enriched in organic nitrogen by fractionation.

Dissolved organic nitrogen (DON) is primarily composed of degraded amino sugars, peptides and porphyrins [2]. The amino sugar component of DON is considerably larger in molar mass than aquatic fulvic acids, so membrane dialysis can be used to isolate amino sugars [3]. Peptides and amino acids have both amphoteric and amphiphilic properties. These properties cause proteins, peptides and certain amino acids to be retained on the macroporous styrene/divinylbenzene resins XAD-2 and XAD-4 at pH < 3 and pH > 10, whereas the macroporous methylmethacrylate resin XAD-7 did not retain these compound classes at pH > 10 [4]. Hydrophilic amino acids and weakly basic degraded prophyrins not retained on the XAD resins are retained on hydrogen-form cation-exchange resins and can be recovered as hydrophilic bases [1].

Based on the likely chemical and physical properties of DON compared to non-nitrogeneous dissolved organic matter, the major objective of this study was to design a DON fractionation analytical scheme that selectively concentrates certain fractions in organic nitrogen. This DON fractionation scheme was applied to and tested with waters derived from an algal culture, a bacterial culture, a mesotrophic lake (Saguaro Lake) and an effluent from a wastewater-treatment plant in Colorado.

2. MATERIALS AND METHODS

2.1. Water Samples.

Two samples were obtained from the field in October 2005 and two separate samples from laboratory cultures. Water was collected at 1-m depth from Saguaro Lake, Arizona, a water-supply reservoir for central Arizona, and filtered (Whatman† GF/F); a detailed description of the reservoir is given elsewhere [5]. The second field sample was collected at a wastewater-treatment plant (WWTP) in Colorado. The WWTP employs trickling filter technology and achieves partial nitrification.

Algae and wastewater bacteria were cultured separately in laboratory reactors. Algae photobio-reactors were used based on designs described elsewhere [6]. Briefly, the reactor consisted of glass culture tubes [4.8 centimeters (cm) ID x 60 cm long] suspended in a constant temperature bath (20˚C). Fluorescence lamps provided 90 ± 5 moles of photons per square meter per second of illumination. Humidified and filtered air was bubbled through the reactor to keep the algae suspended in solution. An inorganic growth medium [6] that was used to grow a blue-green alga (*Oscillatoria prolifera*) was obtained from the University of Texas (UTEX No. 1270). After 10 minutes of settling, 75% of the reactor volume was removed and filtered (GF/F) to represent algal soluble microbial products (SMPs).

Wastewater bacteria were cultured in a 40 L aerated sequencing batch reactor. Activated sludge biomass was collected from an activated sludge process WWTP in Arizona, rinsed, and used as a seed for the bacterial reactor. The initial total and volatile suspended solids concentrations were 13.2 g/L and 10.5 g/L, respectively, and were maintained at similar levels through the production of wastewater bacterial SMPs. An inorganic growth medium, in addition to 250 mg/L of glucose, was added to support biological growth. The reactor was operated in 3 to 5 day cycles, after which solids were settled and the supernatant decanted. The system was operated for three cycles to achieve steady state and flush out any residual wastewater organic material before collecting water for isolation in this study [7]. Samples were filtered (Whatman GF/F) and stored at 4˚C.

2.2. Laboratory Equipment

Glass chromatography columns with Teflon end caps were obtained. Column dimensions for 1-L resin bed-volume were 5 cm ID X 60 cm long; 500-mL resin-bed volume were 5 cm ID X 30 cm long; 80-mL resin-bed volumes were 1.5 cm ID X 30 cm long; and 20-mL resin-bed volumes were 1 cm ID X 28.5 cm long. Excess volume in each column was left vacant to allow for resin bed expansion during different chemical treatments. A reciprocating, ceramic piston pump was used to pump water and reagents through the 1 L and 500 mL resin-bed columns connected with 3 mm ID, 6 mm OD fluorinated ethylene propylene (FEP) Teflon† tubing at a flow rate of 250 mL/minute (min). A smaller pump (FMI Lab Pump Model RP-SY) was used to pump water and reagents through the 80-mL resin-bed column at a flow rate of 20 mL/min and through the 20 mL resin-bed column at a flow rate of 10 mL/min. These smaller columns were connected to the pump with 1.5 millimeter (mm) OD, 3 mm ID FEP Teflon† tubing. Selection, preparation, regeneration, capacities, and packing of resin adsorbents have been discussed previously [8, 9].

Spectra/Por-3 regenerated cellulose dialysis membrane with a molar mass cutoff of 3,500 Daltons (Da) was used. Prior to use, an appropriate length of membrane was cut to accommodate the volume to be dialyzed, and the membrane was washed by soaking it in 4 L of deionized water overnight. For dialysis, the bottom of the dialysis tubing was closed with a plastic snap closure, the solution was poured into the resulting bag using a funnel, and the top of the bag was closed with another closure. Dialysis was then conducted in 4 L Teflon beakers and the solution was stirred with a magnetic stirrer.

For evaporations, one freeze-dryer and two vacuum rotary evaporators were used. The large Buchi Rotavapor 150 had 10 L evaporation and condens-ation flasks. Its evaporation rate for water was about 2 L per hour for a water-bath temperature of 60^{o}C at 750 mm mercury vacuum. A small Buchi Rotavapor R had 1 L evaporation and condensation flasks, and its evaporation rate for water was about 0.5 L per hour for a water-bath temperature of 60^{o}C at 750 mm mercury vacuum. The freeze dryer was a Labconco Lyph-Lock 6 Model.

For centrifuge separations used in the isolation procedure [3] for the hydrophilic acid plus neutral fraction, a Sorvall Superspeed RC-2B refrigerated centrifuge was used with a fixed-angle head. Centri-fuge bottles were 250 mL high-density polyethylene that were spun at 700 revolutions per minute for 30 min.

† Any use of trade, form, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

2.3. DON Fractionation Scheme

2.3.1 Evaporation, Dialysis, and Adsorption/ Desorption Sequence

The sequence of steps for the DON fractionation scheme is shown in Figure 1. Membrane dialysis and the adsorption sequence were performed at pH 1 to better disrupt natural organic matter (NOM)/metal aggregates than previous fractionations [3] that were conducted at pH 2 or greater. The colloid fraction was isolated by successive dialysis against 0.2 M HF (to remove silica), deionized water, and freeze-dried as reported previously [3]. The permeate from the membrane dialysis was pumped through the three columns in series as shown in Figure 1.

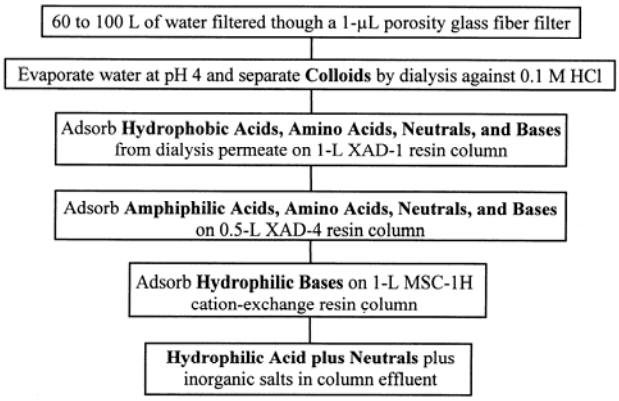

Figure 1 Dissolved organic nitrogen fractionation scheme (evaporation, dialysis, and adsorption sequence)

The XAD-1 resin was selected to adsorb hydrophobic DON because of its styrene/divinylbenzene matrix that retained proteinaceous DON at low and high pH values, and because of its large pore size (200 Å) that could accommodate large DON molecules [9]. The XAD-4 resin, which also has a styrene/ divinylbenzene matrix, was selected to adsorb amphiphilic DON because its smaller pore size (50 Å) resulted in a high surface area that resulted in large capacity factors for amphiphilic molecules [10].

2.3.2 Column Rinse, Desorption and Fraction Isolation Sequences

Approximately 4 L of a 0.01 M HCl solution (pH 2) rinse was pumped through the three columns until the effluent conductance was the same as the influent rinse conductance. The rinse had sufficiently low pH to retain the acid fractions on the column, yet it was sufficiently dilute that the HCl could be removed by evaporation and freeze-drying. The columns were disconnected for desorption of each isolated fraction.

Hydrophobic acids (HPO-A) were desorbed from the XAD-1 column with 1 L of 0.1 M NaOH. A 0.5-L column of MSC-1H resin was connected in series to the XAD-1 column to remove the NaOH. The XAD-1 and MSC-1H columns were rinsed with 2 L of 0.01 M HCl. The column effluent (including rinse) was vacuum evaporated and freeze dried. Because HCl forms a negative azeotrope with water, the hydrophobic acids isolate as a sticky mass that is difficult to remove from the freeze-dry flask. This sticky mass was redissolved in a minimum volume of 75% acetonitrile/25% water, diluted 10-fold with water, and freeze-dried a second time to remove the remaining HCl and produce a powdered isolate that is suitable for quantitative removal and weighing.

Hydrophobic base/neutrals (HPO-B/N) were desorbed from the XAD-1 column with 800 mL of 75% acetonitrile/25% water followed by 1 L of deionized water rinse. The acetonitrile plus water rinse were vacuum evaporated and freeze-dried twice as with the hydrophobic acids described above.

Amphiphilic acids (AMP-A) were desorbed from the XAD-4 column with 0.5 L of 0.1 M NaOH. The same MSC-1H column used previously (without desorption and regeneration) for the hydrophobic acid fraction was connected in series to remove the NaOH. The XAD-4 and MSC-1H columns were rinsed with 2 L of 0.01 M HCl. The column effluent (including rinse) was vacuum evaporated and freeze-dried twice.

Amphiphilic base/neutrals (AMP-B/N) were desorbed from the XAD-4 column with 400 mL of 75% acetonitrile/25% water followed by 600 mL of deionized water rinse. The column effluent (including rinse) was vacuum evaporated and freeze-dried twice.

Amino acids (AA) that desorb from the XAD-1 and XAD-4 resins with 0.1M NaOH are adsorbed on the MSC-1H resin column used to remove the NaOH. The MSC-1H resin column was rinsed with deionized water until the effluent conductivity was < 50 microsiemens (µS). The MSC-1H column was eluted with 0.5 L of 3 M aqueous ammonia and rinsed with deionized water until the effluent conductivity was < 100 µS. The aqueous ammonia eluent plus rinse was evaporated and freeze-dried.

Hydrophilic bases (HPI-B) adsorbed on the MSC-1H column used in the adsorption sequence were first rinsed with deionized water until the effluent conductivity was < 50 µS. The column was then desorbed with 1 L of 3 M aqueous ammonia and rinsed with deionized water until the effluent

conductivity was < 100 μS. The aqueous ammonia eluent plus rinse was evaporated and freeze-dried.

Hydrophilic acids plus neutrals (HPI-A+N) were desalted and purified by a combination of zeotrophic distillation with acetic acid to remove sodium chloride, selective precipitations to remove phosphate, silica, and sulfate, and selective evaporations with methanol to remove borate. These detailed procedures, which also involved ion exchange on small columns and centrifugation to remove precipitates, are reported previously [3].

The HPO-B/N fraction contains both lipid-derived organic matter (depleted in DON) and proteinaceous organic matter (rich in DON). The lipid-derived organic matter was extracted by slurrying the hydrophobic base/neutral fraction with 50 mL of ethyl acetate and stirring for 24 hours. The suspension was filtered through a 1 μM glass fiber filter, and the ethyl acetate filtrate was evaporated and freeze-dried to isolate the hydrophobic base/neutral ethyl acetate subfraction (HPO-B/N, (EtAc)). Material retained on the filter was dissolved by slowly passing 50 mL of 75% acetonitrile/25% water through the filter. This filtrate was evaporated and freeze-dried to isolate the hydrophobic base/neutral acetonitrile subfraction (HPO-B/N, (ACN/H_2O)).

2.4. Fourier Transform-Infrared (FT-IR) Spectrometry

FT-IR spectra were collected using 2 to 5 mg of NOM fraction isolates in KBr pellets. The Perkin Elmer System 2000 FT-IR used a pulsed laser source and a deuterated triglycine sulfate detector The instrument was set up to scan from 4,000 to 400 cm^{-1} averaging 10 scans at 1.0 cm^{-1} intervals with a resolution of 4.0 cm^{-1}. All spectra were normalized after acquisition to a maximum absorbance of 1.0 for comparative purposes.

Comprehensive interpretation of FT-IR spectra of specific compounds is difficult because so many absorption bands are generated; however, the complex mixture properties of fractionated NOM simplifies interpretation of the spectra because only the strongest bands can be identified and associated with the predominant structures. For interpretation of the FT-IR spectra of specific compounds, the reader is referred to Pouchert [11], and for the analyses of complex biomolecular structures and humic substances, to Bellamy [12] and Stevenson [13], respectively. Table 1 lists characteristic FT-IR frequency bands for structures found in organic matter isolates for this study.

Table 1 FT-IR frequency bands for various structures in organic matter isolates [11-13] [a]

Compound Class	Frequencies (cm^{-1}) and Structure
Carbohydrates	3400-3300 (O-H), 1100-1000 (C-O)
Lignin	1510 (C=C), 1460 (CH_2), 1420 (C=C), 1268 (ϕ-O), 1225 (C-O), 1127 (OCH_3), 1030 (ϕ-H)
Condensed Tannins	1610 (C=C), 1515 (C=C), 1280-1230 (ϕ-O), 1180-1140 (C-O), 1020, (ϕ-H)
Fulvic and Humic Acids	3400-3300 (O-H), 2700-2500 (COOH),1760 (COOR), 1720 (COOH), 1660-1630 (ϕ-C=O), 1630-1600 (H-bonded enols and quinones, C=C), 1280-1150 (ϕ-O, COOH)
Hydrocarbons	2960 (CH_3), 2940 (CH_2), 1460 (CH_2), 1380 (CH_3)
Proteins	1660 (Amide I band, N-C=O), 1540 (Amide II band, N=C-O)
N-Acetyl Amino Sugars	1660 (Amide I band, N-C=O), 1555 (Amide II band, N=C-O), 1380 (CH_3)
Aromatic Sulfonic Acids	1180, 1125, 1040 (ϕ-SO_3H), 1010 (ϕ-H)

[a] ϕ = aromatic carbon

2.5. Solid-State Cross Polarization Magic Angle Spinning (CPMAS) ^{13}C-Nuclear Magnetic Resonance (NMR) Spectrometry

Solid-state ^{13}C-NMR spectra were obtained on a Varian Inova 400 megahertz (MHz) spectrometer operating at 100.58 MHz. The sample was spun at 10 kilohertz (kHz) using a 4 mm probe employing cross-polarization magic angle spinning. Scans were accumulated for 12-24 hours. Each scan had a sweep width of 50 kHz, 3 s pulse delay, and a 3 millisecond (ms) contact time. If a 5 ms contact time had been used, the spectral response between non-protonated carbons and protonated carbons would have been more equivalent [14], but the loss in sensitivity with 5-ms contact time given the small fraction masses made the 3-ms contact time a suitable compromise between sensitivity and quantitative accuracy. Validation of this solid-state method used by the investigators is provided elsewhere [15]. Table 2 gives organic structure assignments for the various regions of ^{13}C-NMR spectra [16, 17].

Table 2 Structural assignments for ^{13}C-NMR spectra [15, 16]

Chemical Linkage	Compound Type	Chemical Shift Range (ppm)
C-H	Aliphatic Hydrocarbon	0 - 55
C-N	Amines, Amides, Proteins	40-60
O-CH_3	Methoxy Groups in Tannins and Lignins	55-60
C-O	Aliphatic Alcohols, Ethers, and Esters	60-90
O-C-O	Anomeric Carbon in Carbohydrates, Lactols	90-110
ϕ	Aromatic Carbon	95-165
ϕ-O	Aromatic Esters, Ethers, and Phenols	135-165
ϕ-SO_3H	Aromatic Sulfonic Acids	140-145
O=C-O, O=C-N	Carboxylic Acids, Esters, Amides	160-190
O=C-C=C	Flavones, Quinones	170-200
O=C-C	Aliphatic and Aromatic Ketones	190-220

2.6. Carbon, Hydrogen, Nitrogen Analyses

Approximately 2 mg of sample is measured into a tin capsule (Elementar Americas, D1034) using a Sartorius M2P microbalance (readability: 0.001 mg, range: 0.1 mg - 55 g). The tin capsule is then carefully folded into a cube approximately 2-3 mm in width with forceps on a pane of glass. The capsule was then loaded into a Perkins Elmer 2400 Series II CHNS/O analyzer for analysis. This analyzer operates by flash combusting the sample encapsulated in a tin cup at 1760 °C. The resulting gases are chemically scrubbed of the halogens and sulfur followed by separation in a gas chromatographic column prior to detection by a thermal conductivity detector.

3. RESULTS AND DISCUSSION

3.1. DON Fractionation

Bar diagrams presenting fraction mass concentrations (isolated mass/sample volume) for the four samples are presented in Figures 2-5. The fractionation pattern for the algal (Fig. 2) and bacterial (Fig 3) samples are similar except the algal sample has a greater colloid concentration. Hydrophobic, amphiphilic, and hydrophilic acid fraction concentrations for both algae and bacterial samples are very low when compared to acid fractions from a natural water such as Saguaro Lake (Fig. 4). The sewage effluent sample (Fig. 5) had a similar relative fraction concentrations to the Saguaro Lake sample except that fraction concentrations of the effluent sample were greater and the colloid fraction was greater. The hydrophobic neutral fraction from the algae and bacterial samples was not sub-fractioned because the FT-IR spectra did not show any evidence of lipid-derived organic matter.

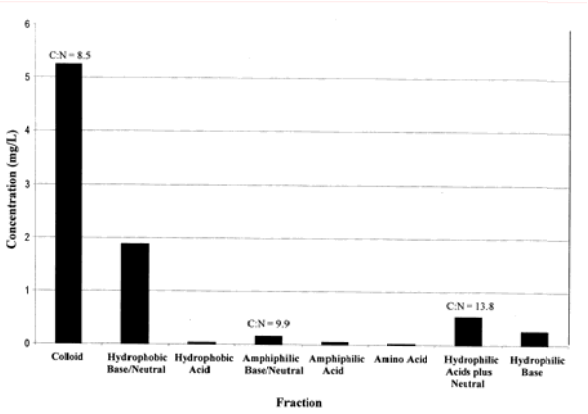

Figure 2 Concentration bar diagram presenting dissolved organic-matter fractionation of water derived from an algal culture.

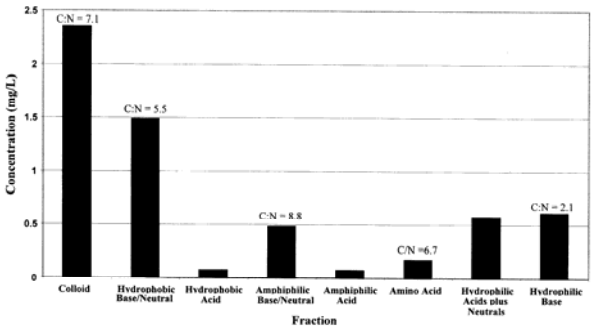

Figure 3 Concentration bar diagram presenting dissolved organic-matter fractionation of water derived from a bacterial culture.

The XAD-1 resin did not appear (based on color) to irreversibly adsorb DOM for the bacterial, algal, and Saguaro Lake samples; however, the blue dye Erioglaucine commonly found in wastewaters [18], and a brown color remained on the resin for the

WWTP sample. In an attempt to elute these colors, the resin was sequentially eluted with methanol, glacial acetic acid, N,N-dimethylformamide, pyridine, benzene, and acetonitrile. Only glacial acetic acid eluted the brown color whose FT-IR spectrum indicated organic phosphate ester acids. The blue dye still remained on the resin. This irreversible adsorption suggests aromatic π-π electron interactions of the blue dye with the styrene/divinyl benzene resin matrix.

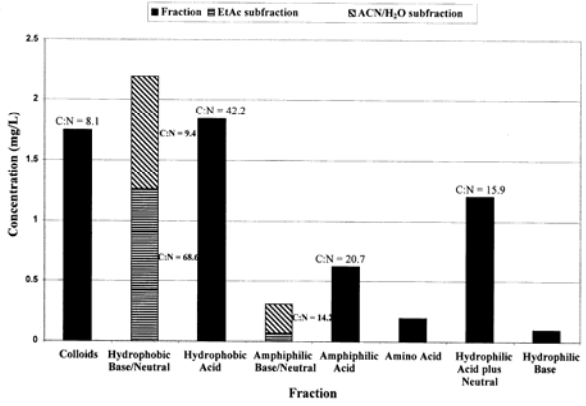

Figure 4 Concentration bar diagram presenting dissolved organic-matter fractionation of water from Saguaro Lake.

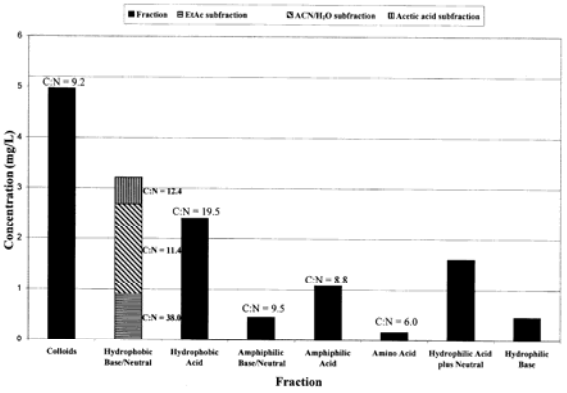

Figure 5 Concentration bar diagram presenting dissolved organic-matter fractionation of wastewater treatment plant effluent.

3.2. Carbon, Hydrogen, and Nitrogen Analysis

Carbon, hydrogen, and nitrogen analyses are presented in Table 3. Atomic C:N ratios are also presented in the bar diagrams of Figures 2-5. Fractions which had insufficient mass namely, 1) the hydrophilic base fraction, which contained ammonium ion; 2) certain hydrophilic acid fractions, which contained nitric acid; and 3) fractions which were sticky and hydroscopic were generally not analysed. For example, the low C:N ratio of 2.1 for the hydrophilic base fraction isolated from the bacterial culture is biased by the presence of ammonium ion.

For the fractions analyzed, the bacterial culture fractions had lower C:N ratios than the analogous algal culture fractions. The range in C:N atomic ratios varied from 5.5 for the HPO-B/N fraction from the bacterial culture to 68.6 for the HPO B/N (EtAc) subfraction for the Saguaro Lake sample. The general trend in C:N ratios for the Saguaro Lake and WWTP samples was amino acids < colloids ~ HPO-B/N (ACN/H_2O) ~ AMP-B/N ~ AMP-A < HPO-A < HPO-B/N (EtAc). The subfractionation of the HPO-B/N fractions of the Saguaro Lake and WWTP effluent samples was very effective in concentrating nitrogen for the acetonitrile/water extract and depleting nitrogen for the ethyl acetate extract. Neither acetonitrile nor ethylacetate was detected in these subfractions by specific and sharp peaks in the ^{13}C-NMR spectra. The exceptionally low C:N ratio of 5.5 for the HPO-B/N fraction of the bacterial culture indicates that this fraction must be almost pure protein, and the low C:N ratio of 6.0 for the amino acid fraction of the WWTP effluent indicates this fraction must be a mixture of amino acids and peptides.

Carbon and nitrogen species measurements and calculated recoveries of organic carbon and nitrogen are presented in Table 4. Carbon recoveries of 78% and 86% for Saguaro Lake and WWTP, respectively, were similar to previous recoveries on natural waters by similar NOM isolation techniques [3]. The laboratory cultured isolates from algae and bacteria did not provide such high quality results; carbon recoveries of greater than 100% are suspect because of the inability to accurately measue DOC in the bulk water samples due to a large percentage (40–60% of isolated mass) of colloidal NOM present. Colloidal DOM is suspected to be poorly oxidized during dissolved organic carbon analysis. Poor calculated recoveries of nitrogen are suspected to be due to the inherent interferences associated with calculating DON without dialysis [19].

3.3. FT-IR Spectra

FT-IR spectra of DOM fractions from the bacterial culture, Saguaro Lake, and the WWTP effluent are shown in Figures 6, 7 and 8, respectively. The FT-IR spectrum of the algal culture sample is not shown as it was very similar to the sample from the bacterial culture.

The FT-IR spectra of the bacterial sample indicated that the colloids consisted of peptidoglycans

from bacterial cell walls [3]. The hydrophobic base/neutral fraction is mostly protein (amide I band at 1660 cm^{-1} and amide II band at 1540 cm^{-1}) with only a trace of carboxylic acids near 1720 cm^{-1}. The amphiphilic neutral fraction and amino acid fraction also has amide groups indicative of peptides, but the acid carboxyl groups near 1720 cm^{-1} are substantial in these two fractions. The composition of the hydrophilic base fraction is difficult to determine as it was isolated in ammonium salt form that interferes with the differentiation of the carboxyl and amide groups. However, its base properties indicate that it is enriched in basic amino groups. Therefore, the fractions in the top five spectra in Figure 6 are all rich in DON. The hydrophobic acid, amphiphilic acid, and hydrophilic acid/neutral fractions in Figure 6 are all fulvic acid fractions that have major carboxyl group peaks near 1720 cm^{-1} that become increasingly polar as the hydroxyl-group content increases in going from hydrophobic to hydrophilic characteristics. These fractions are depleted in DON functional groups. The sharp peak at 1385 cm^{-1} in the hydrophilic acid plus neutral fraction is caused by nitric acid that was not completely removed by the isolation procedure.

Table 3 Elemental analyses of isolated organic fractions (NA = not analyzed; SF = sub-fractioned, WWTP = wastewater-treatment plant; Fraction acronyms are defined in the Materials and Methods section).

Source	Fraction	Isolated Mass (mg)	Percent Carbon	Percent Hydrogen	Percent Nitrogen	Atomic C:N Ratio
Algae	Colloids	334.8	45.09	7.12	6.2	8.5
Algae	HPO-B/N	119.4	NA	NA	NA	NA
Algae	HPO-A	2.0	NA	NA	NA	NA
Algae	AMP-B/N	10.1	38.43	5.10	4.52	9.9
Algae	AMP-A	2.5	NA	NA	NA	NA
Algae	Amino Acid	1.0	NA	NA	NA	NA
Algae	HPI-A+N	67.9	11.10	3.19	0.94	13.8
Algae	HPI-B	16.2	NA	NA	NA	NA
Bacteria	Colloids	142.7	41.59	6.53	6.79	7.1
Bacteria	HPO-B/N	90.1	45.56	6.61	9.72	5.5
Bacteria	HPO-A	4.5	NA	NA	NA	NA
Bacteria	AMP-B/N	29.2	10.52	1.19	1.40	8.8
Bacteria	AMP-A	4.4	NA	NA	NA	NA
Bacteria	Amino Acid	10.8	44.76	5.35	7.74	6.7
Bacteria	HPI-A+N	35.0	NA	NA	NA	NA
Bacteria	HPI-B	37.3	25.6	5.11	14.12	2.1
Saguaro L	Colloids	123.4	44.76	6.64	6.43	8.1
Saguaro L	HPO-B/N	182.7	SF	SF	SF	SF
Saguaro L	HPO-B/N, EtAc	89.1	58.28	6.65	0.99	68.6
Saguaro L	HPO-B/N, ACN/H_2O	66.1	47.76	6.31	5.94	9.4
Saguaro L	HPO-A	130.2	49.53	5.04	1.37	42.2
Saguaro L	AMP/B/N	28.4	SF	SF	SF	SF
Saguaro L	AMP/B/N, EtAc	4.7	NA	NA	NA	NA
Saguaro L	AMP/B/N, ACN/H_2O	17.0	43.88	5.09	3.61	14.2
Saguaro L	AMP-A	44.3	44.69	4.84	2.16	20.7
Saguaro L	Amino Acid	13.6	NA	NA	NA	NA
Saguaro L	HPI-A+N	85.4	31.68	4.01	2.32	15.9
Saguaro L	HPI-B	6.8	NA	NA	NA	NA
WWTP	Colloids	467	45.16	7.01	5.74	9.2
WWTP	HPO-B/N, ACN/H_2O	167.2	51.71	6.40	5.29	11.4
WWTP	HPO-B/N, Acetic Acid	49.2	41.83	4.98	3.92	12.4
WWTP	HPO-A	226.2	47.08	5.09	2.81	19.5
WWTP	AMP-B/N	43.0	42.53	5.20	5.20	9.5
WWTP	AMP-A	101.6	39.82	4.56	5.25	8.8
WWTP	Amino Acid	16.0	46.17	5.62	9.04	6.0
WWTP	HPI-A+N	151.4	NA	NA	NA	NA
WWTP	HPI-B	43.4	NA	NA	NA	NA

Table 4 Carbon and nitrogen species measurements and calculated recoveries (TN, total nitrogen; DON, dissolved organic nitrogen)

Measurement	Algae	Bacteria	Saguaro Lake	WWTP
Dissolved organic carbon (mg/L)	2.1	1.3	4.8	6.6
TN (mg/L)	2.5	10.8	0.41	23.1
Nitrite (mg/L)	0.30	0.33	0	0.37
Nitrate (mg/L)	0.25	0.28	0.22	19.6
Ammonia (mg/L)	0.08	8.67	0.05	1.25
DON (mg/L)	1.9	1.5	0.14	1.9
Calculated organic carbon and organic nitrogen concentrations from elemental analyses of isolated fractions				
Organic carbon (mg/L)	2.5	1.9	3.7	5.7
Organic nitrogen (mg/L)	0.3	0.4	0.3	0.6
Calculated Recoveries				
Organic carbon recovery	119%	151%	78%	86%
Organic nitrogen recovery	16%	26%	214%	31%

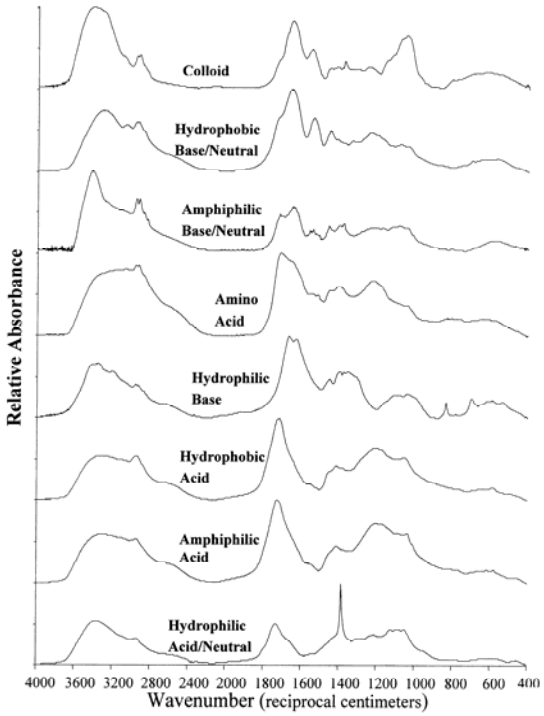

Figure 6 FT-IR spectra of dissolved organic fractions from bacterial culture sample.

The FT-IR spectra the materials isolated from Saguaro Lake (Fig.7) show similarities and differences as compared to the algal and bacterial samples. The spectra of the colloid, hydrophobic acid, amphiphilic acid and hydrophilic acid/neutral fraction are similar to these fractions in the algal and bacterial samples. However, the hydrophobic base/neutral fraction from Saguaro Lake was quite different in that it had a significant carboxylic acid component. The solvent extraction sub-fractionation of the hydrophobic neutral fraction separated a protein-rich component (the ACN/H_2O extract) and a hydrocarbon acid component (the EtAc extract). The amphiphilic base/neutral and amino acid fractions from Saguaro Lake also had greater carboxylic acid content than was found for these fractions in the bacterial and algal samples. Inputs from degraded terrestrial plant material such as terpenoids, tannins and lignins likely contributed to the greater carboxylic acid group content of several fractions from Saguaro Lake as compared to the algal and bacterial cultures that only went through one growth and decay cycle. The FT-IR spectra of Figure 7 indicate that amide and amino functional groups rich in organic nitrogen were present in the top five spectra, whereas organic nitrogen is likely depleted in the bottom four spectra of the acid fractions.

The FT-IR spectra of the WWTP effluent (Fig. 8) are very similar to the Saguaro Lake spectra (Fig. 7) with the exception that the hydrophobic base/neutral, hydrophobic acid, amphiphilic base/neutral and amphiphilic acid fractions contain aromatic sulfonate bands at 1010 and 1040 cm^{-1}. These aromatic sulfonates are well known as degradation products of various sulfonate detergents [20]. The HPO-B/N acetic acid subfraction contains phosphate bands near 1000 and 490 cm; these phosphate groups are likely conjugated with this fraction as phosphate esters because free phosphoric acid should be removed by the previous rinse and column elution procedures.

3.4. ^{13}C-NMR spectra

^{13}C-NMR spectra of selected organic fractions from

Saguaro Lake, the WWTP effluent, HPO-N sub-fractions, and colloids are shown in Figures 9-12. The C-N linkage of the N-acetyl amino sugars is seen as a shoulder near 55 parts per million for the colloid fractions, and this C-N peptide linkage also occurs as a distinct peak at 55 ppm for the proteins in the HPO-N (ACN/H_2O) sub-fractions. The colloid and hydrophilic acid plus neutral fractions have a distinct peak near 100 ppm (Fig. 9), which is indicative of the anomeric carbon in carbohydrates. This anomeric carbon peak is small to absent in all of the other fractions. The broad aliphatic carbon peaks from 0-50 ppm (Figs. 9 and 10) for the HPO-N (EtAc) sub-fraction, and HPO-A and AMP-A fractions are indicative of aquatic fulvic acid derived from terpenoid precursors [21].

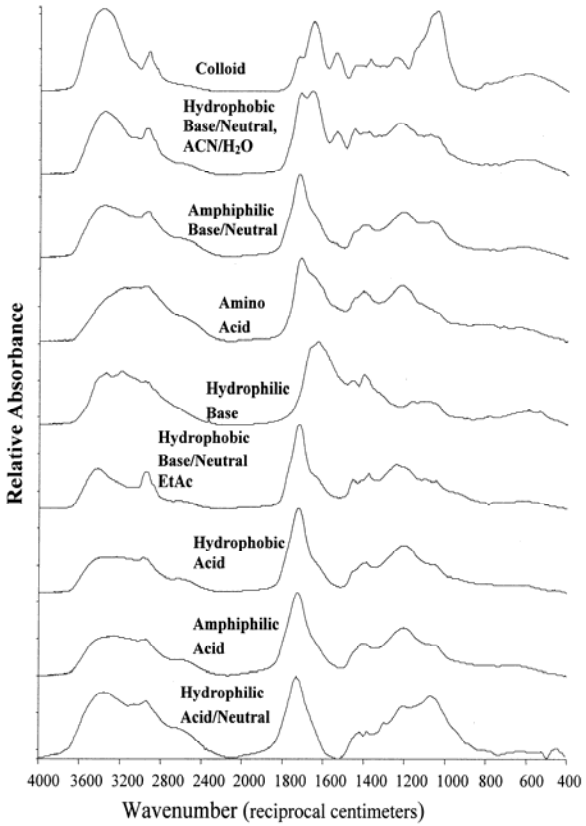

Figure 7 FT-IR spectra of dissolved organic fractions from Saguaro Lake.

This broad peak results from aliphatic alicyclic structures in terpenoids. The absence of an aromatic C-O peak from 145-160 ppm indicates that lignin and tannins are not significant precursors for the fulvic acid fractions isolated in this study. The peaks near 30 and 40 ppm in the HPO-N (ACN/H_2O) sub-fractions (Fig. 11) result from chain structures in proteins. The WWTP effluent sample (Fig. 10) has aromatic sulfonic acid bands at 145 ppm for all fractions (colloids excepted) that are not seen in the fractions isolated from Saguaro Lake (Fig. 9). These aromatic sulfonic acids, which lack organic nitrogen, are especially concentrated in the HPO-N (ACN/H_2O) sub-fraction from the WWTP-effluent (Fig. 11), and their occurrence explains the greater C:N ratio (11.4) of this sub-fraction in the WWTP effluent as compared to the C:N ratio (9.4) of this sub-faction in the Saguaro Lake sample.

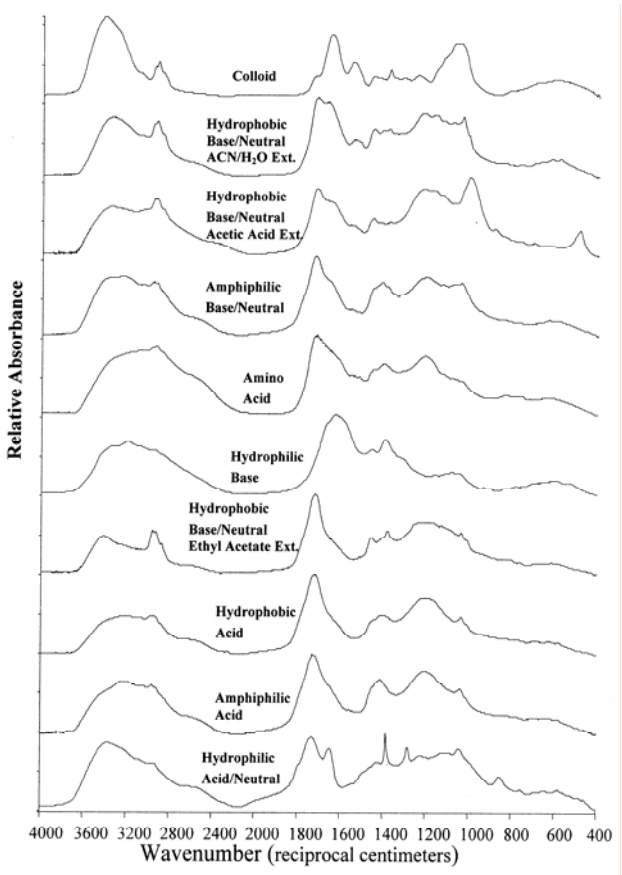

Figure 8 FT-IR spectra of dissolved organic fractions from the wastewater treatment plant effluent

The ^{13}C-NMR spectra of the four colloid fractions are compared in Figure 12. All four samples have generally similar spectra except that the algae colloids have a greater hydrocarbon peak at 18 ppm which is indicative of methyl groups (Table 1). These methyl groups may be attached to peptides and lactic acid units in peptidoglycan structures, which are present in greater abundance in the algal sample.

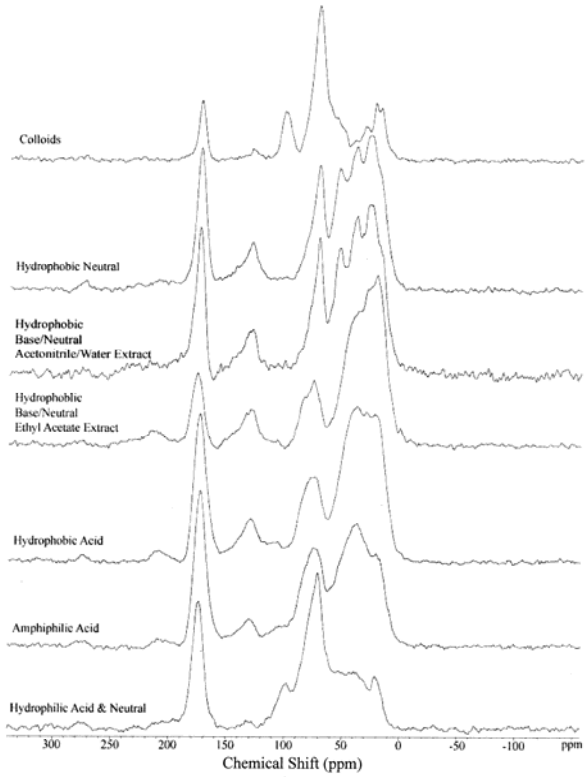

Figure 9 ^{13}C-NMR spectra of selected organic fractions from Saguaro Lake.

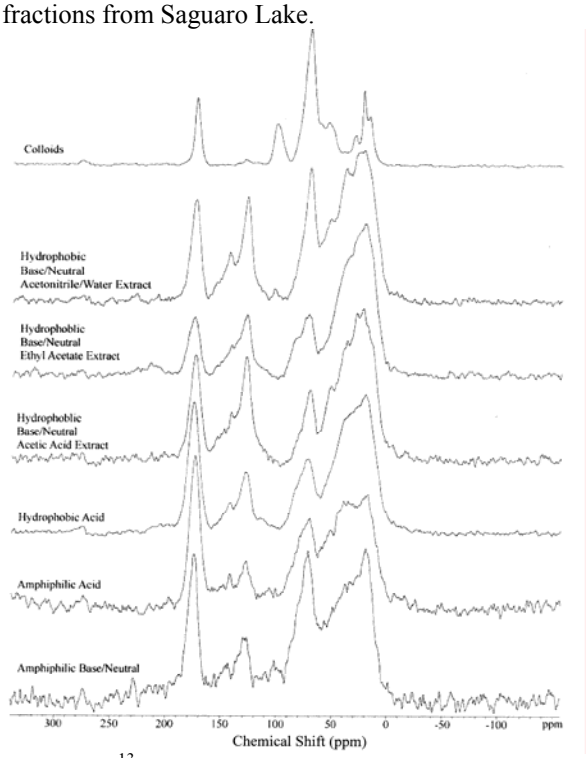

Figure 10 ^{13}C-NMR spectra of selected organic fractions from wastewater-treatment plant effluent.

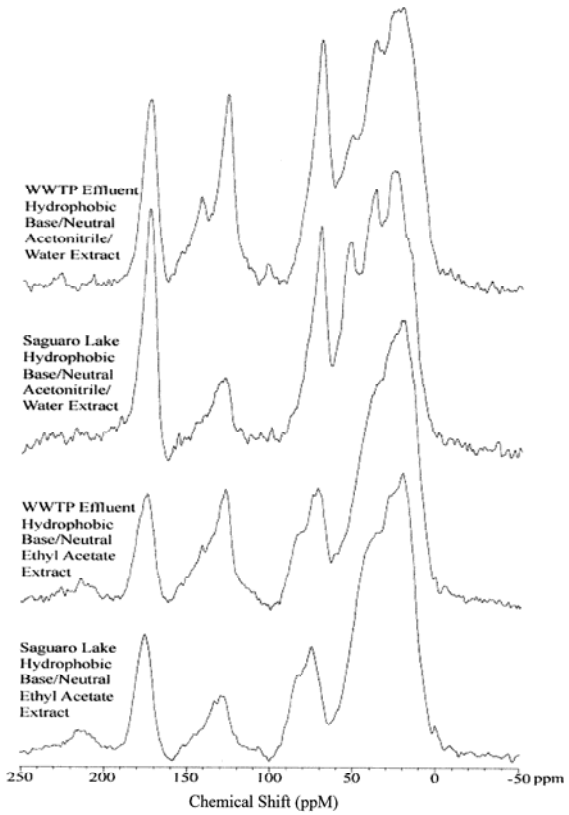

Figure 11 ^{13}C-NMR spectra of hydrophobic base/neutral subfractions from Saguaro Lake and wastewater-treatment plant (WWTP) effluent.

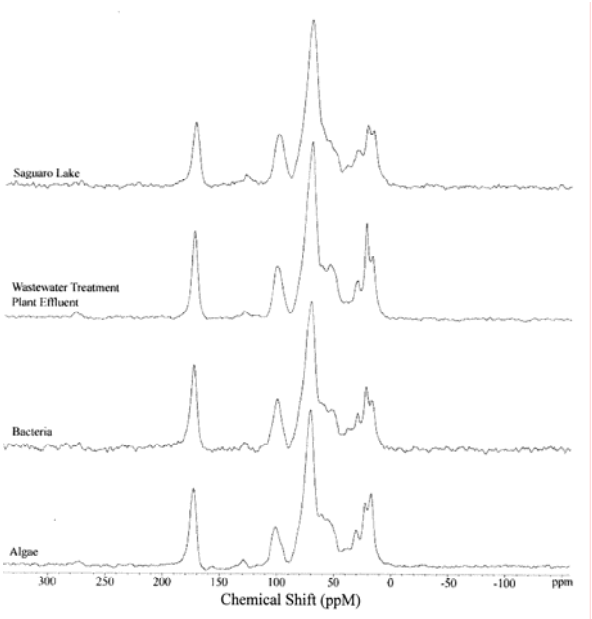

Figure 12 ^{13}C-NMR spectra of colloid fractions from the four source waters of this study.

4. CONCLUSIONS

The organic nitrogen fractionation procedure of this report concentrated the amino acid fraction, amphiphilic acid, amphiphilic neutral, colloid fraction, and the hydrophobic base/neutral (ACN/H_20) sub-fraction with organic nitrogen. The bulk of the dissolved organic nitrogen in an algal culture, a bacterial culture, a sample from Saguaro Lake and a sample from a wastewater-treatment plant effluent was in the colloid fraction (consisting of algal and bacterial cell-wall peptidoglycans) and in the hydrophobic base/neutral (ACN/H_20) sub-fraction (consisting of proteins). The hydrophobic acid and hydrophilic acid plus neutral fractions, and hydrophobic base/neutral (EtAc) sub-fraction were depleted in organic nitrogen. The bacterial and algal culture samples had large percentages of nitrogen-rich colloid, base and neutral fractions, and the lake sample and wastewater-treatment plant effluent had large percentages of nitrogen-poor acids. The most important conclusion of this study is that much of DON can be separated and is not chemically conjugated with bulk DOM.

A secondary conclusion is that the DON fractionation method should be modified to prevent irreversible adsorption of certain constituents, such as the irreversible adsorption of the blue dye Erioglaucine on XAD-1 resin. The modified scheme would replace the XAD-1 with XAD-8 resin in the adsorption sequence in Figure 1. Desorption of the XAD-8 resin with 0.1 M NaOH would leave strongly adsorbed HPO-N constituents such as Erioglaucine on the XAD-8 resin, from which it can be desorbed with 75% acetonitrile/ 25% water [18]. Nitrogen-rich amphoteric HPO-B desorbed with 0.1 M NaOH can then be re-adsorbed on XAD-1 or XAD-4 resin columns and separated from the nitrogen-depleted HPO-A fraction that does not adsorb the XAD resins at high pH. The remainder of the DON fractionation scheme after this modification remains the same.

5. REFERENCES

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