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Improved speciation of dissolved organic nitrogen in natural waters: amide hydrolysis with fluorescence derivatization

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Abstract

The objective of this study was to improve primary-amine nitrogen (1°-N) quantification in dissolved organic matter (DOM) originating from natural waters where inorganic forms of N, which may cause analytical interference, are commonly encountered. Efforts were targeted at elucidating organic-N structural criteria influencing the response of organic amines to known colorimetric and fluorescent reagents and exploring the use of divalent metal-assisted amide hydrolysis in combination with fluorescence analyses. We found that reaction of *o*-phthaldialdehyde (OPA) with primary amines is significantly influenced by steric factors, whereas fluorescamine (FLU) lacks sensitivity to steric factors and allows for the detection of a larger suite of organic amines, including di- and tri-peptides and sterically hindered 1°-N. Due to the near quantitative recovery of dissolved peptides with the FLU reagent and lack of analytical response to inorganic nitrogen, we proposed that FLU be utilized for the quantification of primary amine nitrogen. In exploring the application of divalent metal promoted peptide hydrolysis to the analysis of organic forms of nitrogen in DOM, we found that Zn(II) reaction increased the total fraction of organic-N detectable by both OPA and FLU reagents. Zn-hydrolysis improved recovery of organic-N in natural waters from < 5% to 35%. The above method, coupled with standard inorganic-N analyses, allows for enhanced resolution of dissolved organic nitrogen (DON) speciation in natural waters.

Key words: dissolved organic matter/nitrogen (DOM/DON); *o*-phthaldialdehyde; fluorescamine; ninhydrin; amino acids; amide hydrolysis; peptides

Introduction

Methods for molecular characterization of dissolved organic nitrogen (DON) in environmental samples are rapidly developing due to interest in bioavailability and retention of organic-N in aquatic environments and in the connections between the carbon and nitrogen biogeochemical cycles (Stevenson and Cheng, 1970; Clements and Hilbish, 1991; Fisher *et al*., 2001; Jones *et al*., 2002; Yu *et al*., 2002). Successful spectroscopic approaches have used derivatization techniques to identify DON compounds by chemical modification of amine groups to form either a chromophore, as in the case of the ninhydrin method, or a fluorophore as with the *o*-phthaldialdehyde (OPA) and fluorescamine (FLU) reagents (Roth, 1971; Roth and Hampaï, 1973; Zhu and Kok, 1998). In combination with chromatographic separations, these reagents have facilitated the measurement of individual amino-compounds within complex environmental samples (Zhu and Kok, 1998; Jones *et al*., 2002; Yu *et al*., 2002; Nishibori *et al*., 2003; Yamashita and Tanoue, 2003). In contrast to chromatographic techniques, bulk measurements, which refer to the simultaneous measurement of individual classes of organic-N compounds, such as amide-N, 1°-N, or heterocyclic-N, are useful for the rapid and inexpensive investigation of molecular scale dynamics of nitrogenous organic compounds, including but not limited to amino acids (Fisher *et al*., 2001; Jones *et al*., 2002).

The ninhydrin reagent, first developed in 1948, has been extensively evaluated for use in biological and forensic samples and has come to be used widely in the analysis of amino acids in environmental samples (Stevenson and Cheng, 1970; Benson and Hare, 1975; Stevenson, 1983; Curotto and Aros, 1993; Sheng *et al*., 1993; Freidman, 2004). Reaction between ninhydrin and reactive nitrogen produces a chromophore useful for colorimetric determination of solution concentration. Furthermore, ninhydrin is known to be reactive with ammonium and can overestimate solution α-amino acid concentration unless additional measures, such as pre-treatment with strong base, are taken.

OPA and FLU, developed in the 1970's, offer improved sensitivity and selectivity over the ninhydrin method (Roth, 1971; Udenfriend *et al*., 1972) via formation of fluorescent adducts with 1°-N (Table 1). Additionally, OPA is known to be insensitive to inorganic ammonium, although

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Table 1 Comparative reactivity of *o*-phthaldialdehyde (OPA), fluorescamine (FLU), and ninhydrin reagent to 19 α-amino acids

	Reactivity (%)		
	OPA method[a]	FLU method[b]	Ninhydrin method[c]
Aliphatic			
Glycine	85.0	56.0	90.0
Alanine	79.0	59.2	89.0
Valine	72.0	78.4	91.0
Leucine	86.0	87.8	89.0
Isoleucine	85.0	84.1	89.0
Aromatic			
Phenylalanine	85.0	82.8	78.0
Tyrosine	64.0	92.0	79.0
Tryptophan	75.0	12.3	64.0
Acidic			
Aspartatic acid	90.0	74.8	78.0
Glutamatic acid	89.0	74.2	94.0
S-containing			
Cysteine	5.0	83.4	48.0
Methionine	90.0	94.5	89.0
Alchohols			
Serine	100.0	73.5	84.0
Threonine	88.0	89.6	82.0
N-containing			
Glutamine	86.0	78.6	88.0
Asparagine	88.0	88.0	84.0
Lysine	76.0	99.0	100.0
Arginine	85.0	78.5	89.0
Histidine	80.0	89.8	92.0

[a] Fisher *et al*. (2001), normalized to Serine; [b] Bantan-Polak *et al*. (2001), normalized to Lysine; [c] Moore and Stein (1948, 1954), normalized to Lysine.

the response of FLU to ammonium is not as definitive (Fisher *et al*., 2001). With the exception of Clements and Hilbish (1991), who found significant cross-reaction with NH_4^+, other studies indicate negligible formation of fluorescent products upon reaction with ammonium (Udenfriend *et al*., 1972; Weigele *et al*., 1972; O'Reilly and Lanza, 1995; Bantan-Polak *et al*., 2001). Both OPA and FLU form fluorescent adducts with simple 1° amine compounds such as most amino acids (excluding praline, a cyclic secondary amine), simple aliphatic amines and small peptides (Udenfriend *et al*., 1972; Benson and Hare, 1975; Lindroth and Mopper, 1979; Yu *et al*., 2002). The chemical structure of adducts for FLU and OPA reagents identified by ^{1}H NMR techniques illustrate that both reagents form fluorescent products with the 1°-N of small compounds, including α-amino acids, irrespective of the nature of the side-chain functional group (Weigele *et al*., 1972; Simons and Johnson, 1976). Additionally, the side-chain functional group attached to the 1°-N remains covalently attached, rendering the fluorescent adducts of different organic amines structurally distinct. Thus there exist inherent compound-specific reactivity towards both OPA and FLU reagents dependent upon: (1) the electron density about the reactive nitrogen with decreased electron density negatively affecting adduct formation (electronic effects), and (2) physical barriers impeding reactivity of the reactive nitrogen with large and bulky functional groups in proximity to the reactive nitrogen negatively affecting adduct formation (steric effects).

Relative reactivities of α-amino acids to OPA, FLU, and Ninhydrin have been previously explored, although the relative fluorescent response of other common N containing compounds is still unknown (Moore and Stein, 1954; Weigele *et al*., 1972; O'Reilly and Lanza, 1995; Bantan-Polak *et al*., 2001; Fisher *et al*., 2001). Not all amino acids display equal reactivity across the three reagents as differences in molecular structure contribute to the differential response of amine compounds to each reagent. For bulk measurements it is important to choose a standard with an analytical response that is representative of the entire compound class. The lack of a uniform standard can complicate cross-laboratory comparisons.

Technology for the detection of amino acids has clearly advanced, however, determination of organic nitrogen contained within amino acid polymers, such as polypeptides and other amides, has received considerably less attention. Compelling evidence of metal-assisted peptide hydrolysis indicates that certain divalent metal cations facilitate peptide degradation by promoting hydrolysis of the amide bond between specific amino acid sequences in proteins and polypeptides (Zhu and Kostic, 1992; Hegg and Burstyn, 1995; Ueda *et al*., 1996; Bal *et al*., 1998; Zhu and Kok, 1998;). Grant and coworkers have developed a protocol using fluorescence detection of bulk amino acids derivatized with FLU subsequent to Zn(II) promoted peptide hydrolysis, in controlled biochemical systems (Grant and Pattabhi, 2001; Bantan-Polak and Grant, 2002). Here we apply the results from chemical and biochemical exploration of metal assisted peptide hydrolysis towards the analysis of oligopeptides and other amides in soil solution. The hydrolysis of oligopeptides, potentially derived from decomposing proteins, would convert amide nitrogen into 1°-N, which is reactive to both OPA and FLU. To our knowledge, this is the first application of metal assisted peptide hydrolysis with fluorescence derivatization to the study of dissolved organic matter in natural water samples.

The goal of this investigation is to evaluate the inherent selectivity of OPA and FLU reagents along with metal-assisted peptide hydrolysis to develop a protocol for the efficient and inexpensive determination of bulk-solution 1°-N without the expense or time requirements of HPLC separations, especially in studies where the precise amino acid composition is not required. This investigation includes three specific objectives: (1) quantify the reactivity and relative response of various forms of organic (peptides, aliphatic and aromatic amines) and inorganic nitrogen to ninhydrin, OPA and FLU reagents, and elucidate potential steric and electronic factors influencing response; (2) use Zn(II)-promoted amide hydrolysis to determine bulk concentrations of dissolved amino acids and hydrolyzable amide-N in soil solution, (3) apply the results from probe compound reactivity and Zn(II) digestion towards improving experimental determination of dissolved organic nitrogen speciation in natural water samples and natural organic matter isolates.

1 Materials and methods

All standards and reagents were used as received without further purification. Fluorescamine 98%,

o-phthaldialdehyde 97%, zinc chloride 98%, 3-mercaptopropionic acid 99+%, and all organic amines (aniline 99.5+%, 2-ethylaniline 98%, 3-ethylaniline 98%, 4-ethylaniline 98%, anthranilic acid 98+%, 3-aminobenzoic acid 98%, 4-aminobenzoic acid 99%, methylamine (40 wt.%), ethanolamine 99+%, isopropylamine 99.5%, *t*-butylamine 98%, and aminodiphenylmethane 97%) were purchased from Aldrich Chemicals (USA). Peptide standards (Gly-Leu, Leu-Gly, Val-Gly, Gly-Glu, Lys-Leu, and Leu-Gly-Gly), 2-methoxyethanol 99.3+%, tin chloride dihydrate 98%, and sodium cacodylate trihydrate 98% were purchased from Sigma Chemicals (USA). Sodium carbonate, acetone 99.7%, sodium acetate anhydrous 99+%, and boric acid 99.9% were obtained from Mallinckrodt (USA). Amino acid standards (20 standards, 99+%) were purchased from Fluka (USA). Ammonium nitrate 99.9+% and ninhydrin 98% were both acquired from JT Baker (USA).

1.1 Selection and analysis of probe compounds

A series of aliphatic and aromatic amine probe compounds were selected to investigate steric and electronic factors influencing the formation of fluorescent product in the FLU and OPA methods, in addition to the analysis of the 20 common amino acids. Six aliphatic amines were selected to represent a spectrum of sterically hindered 1^o amines, from methylamine with no steric interferences to the sterically encumbered aminodiphenylmethane (Table 2). Seven aromatic 1° amines (aniline derivatives) were chosen to represent a set of electron-donating amines (ethylanilines) and three electron-withdrawing amines (aminobenzoic acids), with each functional group, which were quantitatively indexed using Hammett Constants (σ_p-ethyl = –0.13, σ_p-carboxyl = +0.44). Peptide probes were selected based upon commercial availability. For each probe compound, the colorimetric (ninhydrin) and fluorescent (FLU and OPA) response was measured at four solution concentrations, 0.25–1.5 mmol/L for ninhydrin and 5–100 mol/L for OPA and FLU, in triplicate measurements to obtain a four point standard/calibration curve (ninhydrin: R^2 from 0.981 to 0.999; OPA and FLU: R^2 from 0.959 to 0.999). A linear regression was computed for each standard curve and the slope of the regression line was used to denote "reactivity". Reactivity percentage was determined relative to the reactivity of *L*-leucine, our analytical benchmark, which was also measured in triplicate for each concentration standard. *L*-leucine was selected based on the recommendation of Methods of Soil Analysis (Stevenson, 1996) and due to its comparable reactivity to ninhydrin, OPA and FLU (Table 1). Results from our selection of aliphatic, aromatic and peptide probe compounds are reported in Table 2, as complementary data to the amino acid results (Table 1) and are intended to shed light on the fundamental chemistry which governs differences in analytical response.

Table 2 Reactivity of peptides, organic amines and inorganic nitrogen to the OPA, FLU, and ninhydrin reagents using *L*-leucine response as 100% reactivity

Standard	Reactivity (%)		
	OPA	FLU	Ninhydrin
Leucine	100	100	100
Aliphatic amines			
Methylamine	100	91	102
Ethanolamine	101	71	103
Isopropylamine	69	85	> 0.1
t-Butylamine	26	80	> 0.1
Aminodiphenylmethanea	9	109	55
Glucosamine	63	77	87
Aromatic amines			
Aniline	14	112	> 0.1
ortho-2-Ethylaniline	10	40	> 0.1
meta-3-Ethylaniline	27	110	> 0.1
para-4-Ethylaniline	45	52	> 0.1
o-2-Aminobenzoic acid	6	2	> 0.1
m-3-Aminobenzoic acid	4	119	> 0.1
p-4-Aminobenzoic acid	7	51	> 0.1
Peptides			
Gly-Leu	36	115	95
Gly-Glu	52	114	96
Leu-Gly	21	100	87
Val-Gly	17	119	86
Lys-Leu	39	114	66
Leu-Gly-Gly	9	108	61
Inorganic-N			
NH_4NO_3	2.2	0.70	95

For this study, aminodiphenylmethane is treated as an aliphatic amine due to the separation of the reactive amine from the conjugated aromatic π-system by an sp^3-carbon spacer. Thus, the nitrogen atom is not truly in resonance with either aromatic ring.

1.2 Ninhydrin-N

A variation of the classic ninhydrin method was used in this study (Moore and Stein, 1954; Stevenson, 1996). Ninhydrin reagent was prepared by dissolving 2.0 g of ninhydrin in a solution of 50 ml of 2-methoxyethanol (methyl cellusolve), 25 ml of 5.4 mol/L acetate buffer (pH 5), and 25 ml of DI-water followed by the addition of 80 mg of $SnCl_2$(S). Ninhydrin reactive nitrogen in each sample was determined by the addition of 0.5 ml of sample or standard to 0.5 ml of a 0.4 mol/L sodium citrate solution and 2 ml of the ninhydrin reagent. This mixture was then heated at 85°C for 20 min and allowed to cool to room temperature before measuring the solution absorbance at 570 nm (BioSpec-1601 Spectrophotometer, Shimadzu, Japan).

1.3 OPA-N

The method described by Fisher *et al*. (2001) was modified as follows. The OPA reagent was prepared by dissolving 100 mg of OPA in a solution of 10 ml of methanol, 0.1 ml of 3-mercaptopropionic acid (3MPA), and 200 ml of a 20 mmol/L borate buffer (pH 9.5). We selected 3MPA over β-mercaptoethanol due to the increased stability of the final fluorescent adduct and its lower vapor pressure. The OPA-reactive nitrogen in each sample was determined by adding 3 ml of OPA reagent to 0.1 ml of sample. Preliminary studies indicated that the sample-reagent solution mixture required approximately 15 min for the formation of the fluorescent product prior to analysis. A 200-μl sub-sample was transferred to a Costar 96-well round-bottom polystyrene assay plate for fluorescence analysis (Perkin Elmer HTS 7000 BioAssay

Plate reader, HTSoft Windows-based software, USA). The plate reader was fitted with 350 nm excitation and 460 nm emission filters (35 nm bandwidth), selected from excitation and emission spectra of individual amino acids for our specific experimental conditions (Agilent Technologies Fluorometer model 1100, USA). The optimal wavelengths for fluorescence excitation and emission changed minimally (±10 nm) with choice of reducing agent (β-mercaptoethanol vs. 3MPA).

1.4 Fluorescamine-N

Fluorescamine-reactive nitrogen was determined following a procedure modified from Grant and co-workers (Grant and Pattabhi, 2001; Bantan-Polak and Grant, 2002). The fluorescamine reagent was prepared by dissolving 1 mg of fluorescamine in 1 ml of acetone (0.1%, wt./*V*). The 195 μl of sample or standard were transferred to a Costar 96-well round-bottom polystyrene assay plate followed by 15 μl of a 1 mol/L sodium carbonate buffer (pH 9.9) and 90 μl of the fluorescamine-acetone solution. The assay plate was allowed to react at room temperature for approximately 5 min prior to analysis by fluorescence using the BioAssay plate reader fitted with 365 nm excitation filter and 505 nm emission filter (35 nm bandwidth). Native chromophores and fluorophores present within DOM do not interfere with this analysis due to intrinsic fluorescence at this excitation/emission wavelengths or the absorption of photons emitted by the fluorophore produced by reaction of primary amines with the FLU reagent.

1.5 Inorganic-N

Nitrate and ammonium ions were measured using standard methods with TRAACS 8000 auto-analyzer (Norderstedt, Germany). Briefly, ammonium was measured using sodium phenoxide and sodium hypochlorite oxidation followed by sodium nitroprusside addition for colorimetric determination at 660 nm. Total dissolved nitrogen (NO_3^- + NH_3 + DON) was determined colorimetrically by hydrazine reduction subsequent to oxidative digestion using potassium persulfate, where all sample nitrogen is recovered as nitrate. Nitrite measurements were attempted but were below detection limits for all soil solutions.

1.6 Field samples: natural water collection

Two soil water collection systems were installed in the Duke Forest (Durham, NC, USA); one within a stand of loblolly pine (*Pinus taeda*) overlying an Appling soil series (Ultisol), the second within a forest dominated by white oak (*Quercus alba*) overlying a Georgeville soil series (Ultisol). Each set of collectors consisted of two tension-free 0-horizon water collectors and parallel set at 15 cm. Samples were not composited and were analyzed separately, except for the Georgeville 15 cm samples (Oak) that were composited due to limited sample volume. Samples were filtered to 0.4 μm (polycarbonate membrane filters), acidified to pH 2 with HCl, and stored in the dark at 4°C until analysis. Polycarbonate filters were washed with 0.1 mol/L HCl prior to sample processing. This pretreatment was determined to result in no leaching of carbon (determined by TOC analysis, Shimadzu TOC 5000A, USA) or nitrogen (organic or inorganic forms). Samples were analyzed within 3 weeks of collection, and analyzed in triplicate (coefficient percentage of variation < 5%).

1.7 Zinc-promoted amide hydrolysis

Metal-promoted hydrolysis experiments were conducted to explore the efficacy of Zn(II) facilitated hydrolysis of different forms of amide-N to 1°-N. Zinc was selected from the spectrum of metals available for peptide hydrolysis (e.g., Pd(II), Ni(II), Cu(II)) due to the adaptability of the procedure described by Grant and Pattabhi (2001). Briefly, 1 ml aliquots of a 0.2 mmol/L leucine solution, 0.2 mmol/L peptide solutions (Gly-Leu, Val-Gly, Gly-Glu, Leu-Gly, and Leu-Gly-Gly), filtered soil-solutions were individually added to 1.7 ml micro-centrifuge tubes containing 0.2 ml of 10 mmol/L $ZnCl_2$ in 50 mmol/L sodium cacodylate buffer (pH 7.5). All Zn-digestions were performed at 70°C for 20 h. Sample volatilization within the small headspace is considered to be minimal, provided that leucine controls were quantitatively recovered (Fig.3). Digested samples were analyzed using OPA and FLU according to methods described above for soil solutions.

2 Results

2.1 Organic amine reactivity

Reaction of aliphatic amines with OPA displayed the following reactivity percentage trend relative to *L*-leuciene (Table 2): methylamine – ethanolamine > isopropylamine > *t*-butylamine > aminodiphenylmethane. This trend suggests that OPA was generally reactive to small amine-containing compounds, with displayed sensitivity to the steric environment surrounding the reactive amine functional group. Smaller amines, with the amine functional group adjacent to a primary carbon (methyl- and ethanol amine) were nearly 100% reactive, whereas compounds with amine groups adjacent to secondary or tertiary carbon (isopropylamine, *t*-butylamine, aminodiphenylmethane) were not fully reactive compared to *L*-leucine. The presence of secondary and tertiary carbons appears to cause structural interference to the interaction between the reactive nitrogen and the OPA reagent. Although FLU did appear to display moderate differential reactivity with aliphatic amines (71%–109%), the trend is not distinctly suggestive of steric sensitivities. Ninhydrin was nearly 100% reactive with amine groups adjacent to a primary carbon, moderately reactive to compounds with amine groups adjacent to secondary carbons, and unresponsive to amine groups adjacent to tertiary carbon. Considered together, these data emphasize that the FLU reagent is relatively unaffected by the presence of bulky groups that could sterically interfere with the reaction between the reagent and the primary amine.

Ethyl and carboxylic acid aniline derivatives were selected based on their contrasting electron donat-

ing/withdrawing properties. OPA displayed < 50% reactivity across all aniline compounds tested. However, reactivity was greater with the ethylaniline (electron donating) derivatives than the carboxylic acid (electron withdrawing) compounds (⩾ 10% vs. ⩽ 7%, Table 2). Among the different ethylaniline derivatives we found the reactivity trend with OPA was 4-ethylaniline > 3-ethylaniline > aniline > 2-ethylaniline. The response relative to aniline was enhanced with an electron donating substituent in the *meta* and *para* positions and diminished with a substituent in the *ortho* position, likely due to the neighboring ethyl group interfering with reagent interaction with 1°-N. Reactivity towards aromatic amines was significantly suppressed by the presence of electron withdrawing carboxylic acid functional groups as indicated by the very low fluorescence response to carboxylic acid aniline derivatives (4%–7% vs. *L*-leucine).

The relative responses of ethyl aniline derivatives with FLU indicated the *meta* isomer has the greatest fluorescence response whereas the *ortho* and *para* isomers only showed 40% and 52% reactivity, respectively. Similarly, response with carboxylic acid derivatives demonstrated a greater reactivity of the *meta* isomer (119%) and diminished reactivity for the *ortho* and *para* isomers. Reaction with *o*-aminobenzoic acid was probably hindered by intramolecular hydrogen bonding interactions between the *ortho* amino and carboxyl groups (Table 2), whereas the electron withdrawing (through resonance) by the carboxylic acid group at the *para* position diminished the response of the *para* isomer. With the exception of *o*-aminobenzoic acid, FLU displayed a comparatively greater reactivity to aromatic amines than OPA.

Even though polypeptides contain greater quantities of organic nitrogen per mole of compound, the overall fluorescent response to OPA was significantly reduced (< 51%) compared to *L*-leucine. However, this reduction was not as extreme with ninhydrin. Notably, FLU showed greater than 100% reactivity to peptides. Differences in sensitivity towards the steric environment of the reactive nitrogen between the OPA and FLU reagents were also manifest in their reactivity towards polypeptides.

Our estimate of OPA response to ammonium (2.2%) closely matches that of Fisher and co-workers who recorded a 5% response to NH_4^+-N (Fisher *et al*., 2001). FLU analysis showed virtually no response to NH_4^+-N (0.07% vs. *L*-leucine), consistent with the findings of previous studies where essentially no cross-reaction with NH_4^+-N was observed (Udenfriend *et al*., 1972; Weigele *et al*., 1972; Grant and Pattabhi, 2001; Bantan-Polak and Grant, 2002). As previously stated, ninhydrin nearly quantitatively recovered NH_4^+-N (Stevenson, 1996). Ninhydrin is unresponsive to all aniline derivatives as well as isopropylamine and *t*-butylamine.

2.2 OPA and FLU determination of DON composition in soil-water samples

Concentrations of total nitrogen (TN) were approximately 2 mgN/L in 0-horizon (suficial horizon) solutions and decreased to 0.9–1.3 mgN/L at 15 cm depth (Table 3). Ammonium concentrations are higher in the 0-horizon, representing approximately 50% of the TN. Nitrate concentrations under pine (Appling samples) were 0.1–0.3 mgN/L in 0-horizon samples (App 0a and 0b) and decline to 0.01–0.04 mgN/L at 15 cm depth (App 15a and App 15b), whereas all Oak samples (Georgeville) were < 0.1 mgN/L as nitrate. Concentration of DON was nearly 1 mgN/L in all solutions collected, with the fraction of DON recovered by FLU being greater than that recovered by OPA in all samples, an outcome attributed to the complex nature of nitrogen within natural organic matter samples. The remainder of uncharacterized DON in our samples was > 90%.

2.3 Metal promoted amide hydrolysis

Five peptides, water samples were digested with $ZnCl_2$ to facilitate amide hydrolysis (Fig.1). Zinc digestion hydrolyzed 73% of amide bonds of the Val-Gly dipeptide, but this effect was essentially negligible with other dipeptides. That the Zn(II) reagent is unable to cleave all dipeptides with total efficiency is consistent with previous results in which this reagent was shown to be ineffective with Gly-Gly but cleaved around 90% of the dipeptide Gly-Ser (Bantan-Polak *et al*., 2001). Collectively, these results indicate that Zn(II)-promoted peptide hydrolysis requires specific amino acid side chain functional groups (Ueda *et al*., 1996; Grant and Pattabhi, 2001). However, structural details of the metal-peptide complex that render amide bonds susceptible to hydrolysis are still unknown.

DON in soil water was proved to be susceptible to peptide hydrolysis digestions with Zn(II). Fluorescent response of both OPA and FLU reagents before and after digestion with Zn(II) are depicted in Fig.2. Recovery of

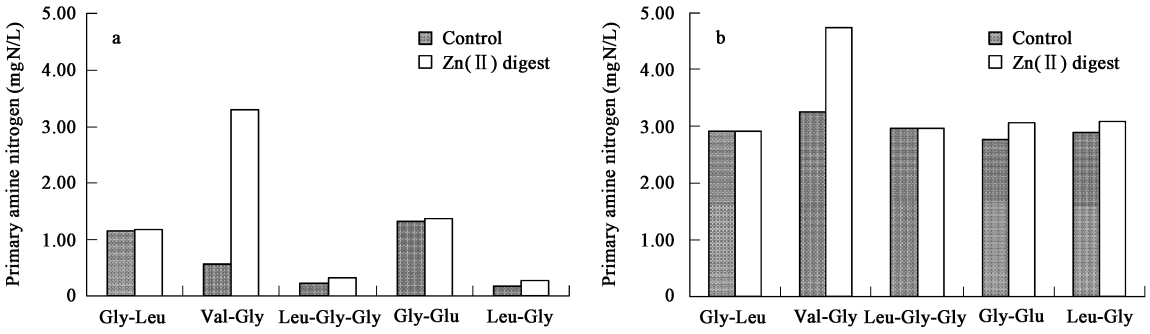

Fig. 1 Zn promoted hydrolysis of 5 peptides: (a) analysis with OPA; (b) analysis with FLU.

DON was increased by as much as 3.2 times (Tables 3 and 4), increasing organic-N quantification in DOM from < 10% to as much as 38%.

3 Discussion

3.1 Structural criteria influencing OPA, FLU, and ninhydrin reactivity

Both OPA and FLU reagents selectively react with 1°-N and do not suffer from cross-reaction with ammonium. The pattern of OPA reactivity across aliphatic amines indicates an intrinsic sensitivity to the steric milieu about the carbon adjacent to the reactive amine. Primary-C as in methylamine, is not sterically hindered, whereas tertiary-C as in isopropyl amine and quaternary-C as in *t*-butylamine are increasingly hindered (Tables 1 and 2). Structural criteria responsible for the trends among the amino acids (Table 1) are more difficult to discern due to the common degree of substitution of the adjacent carbon atom (tertiary, NH_2-CH(R)-CO_2H) across all amino acids. Nevertheless, reactivity differences between amino acids and peptides also support a steric effect for observed selectivity of the OPA reagent. The FLU reagent does not display the same degree of sensitivity to steric factors as the OPA reagent, demonstrated by the fact that FLU possesses > 71% response to all aliphatic amines tested and around 100% response with the N-terminus of small peptides, relative to *L*-leucine (Table 2).

Susceptibility to electronic factors also differs between the OPA and FLU reagents. Although OPA shows a markedly low reactivity to aromatic amines (4%–45%), increased reactivity of the 4- and 3- ethylaniline isomers over the un-substituted aniline suggests that electron donation enhances the reactivity of amines to the OPA reagent. Steric interference from the adjacent ethyl group depresses the reactivity of 2-ethylaniline below even that of aniline, negating any enhancement via electron donation. Electron withdrawing groups, in particular carboxylic acids, can essentially de-activate the amine. FLU is not as sensitive to electronic perturbations from aryl substituents, although *ortho* and *para* substituents can render fluorescent reactivity only 40% of the un-substituted aniline, or even to 2% as with *o*-aminobenzoic acid.

The cause for the difference in amine reactivity to OPA and FLU is most likely influenced by the structure of the two reagents (Fig.3). The mechanism for each reaction is remarkably similar, with a nucleophilic addition of a

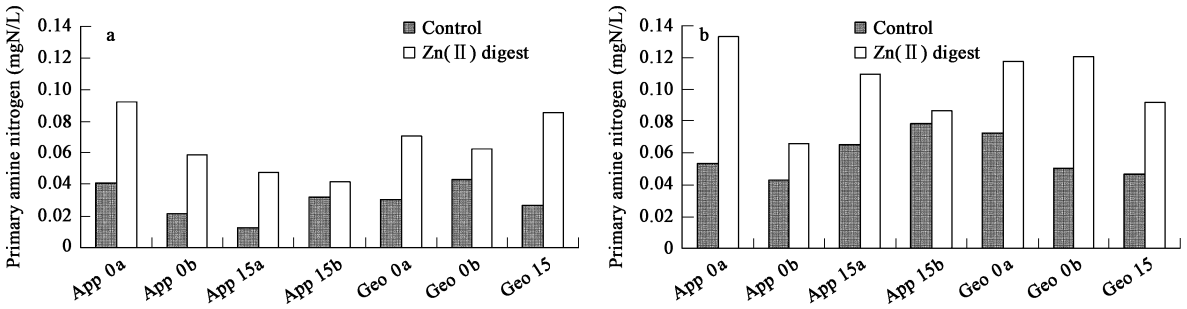

Fig. 2 Zn(II) promoted peptide hydrolysis of soil solution DON. (a) analysis with OPA; (b) analysis with FLU.

Table 3 Dissolved nitrogen analysis of Duke Forest soil, USA solution collection

Sample	TN (mgN/L)	NH_4^+-N (mgN/L)	NO_3^--N (mgN/L)	DON (mgN/L)	OPA-N (mgN/L)	FLU-N (mgN/L)	Nin-N (mgN/L)	Uncharacterized (%)
App 0a	2.21	1.31	0.280	0.62	0.041	0.053	0.087	91.4
App 0b	1.94	0.72	0.095	1.12	0.022	0.043	0.331	96.2
App 15a	1.33	0.06	0.012	1.26	0.012	0.065	0.062	94.8
App 15b	0.89	0.03	0.036	0.82	0.032	0.079	0.071	90.4
Geo 0a	1.60	0.56	0.037	1.00	0.031	0.073	0.484	92.7
Geo 0b	2.31	1.33	0.093	0.88	0.044	0.051	–0.320	94.3
Geo 15	1.29	0.06	0.076	1.16	0.027	0.047	0.033	96.0

App: appling (under pine); Geo: Georgeville (under oak); DON: TN – (NH_4^+-N + NO_3^--N); Nin-N: adjusted to account for 95% recovery of NH_4^+-N; [4] Uncharacterized = (DON – FLU-N)/DON × 100%; analytical error is < 5% for each sample.

Table 4 Dissolved organic nitrogen (DON) analysis of Duke Forest soil solution collection following Zn promoted peptide hydrolysis

Sample	DON (mgN/L)	OPA-N (mgN/L)	FLU-N (mgN/L)	FLU-OPA (mgN/L)	Peptide-N (mgN/L)	Uncharacterized (%)
App 0a	0.617	0.0924	0.133	0.041	0.103	61.7
App 0b	1.12	0.0585	0.066	0.008	0.020	92.3
App 15a	1.26	0.0478	0.110	0.062	0.155	79.0
App 15b	0.819	0.0418	0.0869	0.045	0.113	75.6
Geo 0a	0.998	0.0710	0.118	0.047	0.118	76.3
Geo 0b	0.881	0.0626	0.121	0.058	0.145	69.9
Geo 15	1.16	0.0859	0.0915	0.015	0.015	90.8

DON: see Table 2; peptide-N = 2.5 × (FLU-OPA); uncharacterized = (DON – FLU-N – peptide-N)/DON × 100%.

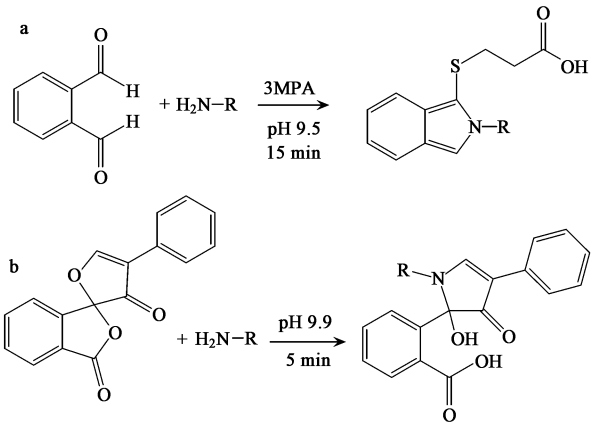

Fig. 3 Reaction mechanism of OPA (a) and FLU (b) reagents.

primary amine to a carbonyl carbon (direct nucleophilic addition with OPA and Michael addition to the α,β-unsaturated carbonyl with FLU). However, the carbonyl C is a benzaldehyde group for the OPA whereas the carbonyl C is part of a geometrically strained spiro acetal functional group for FLU. Spiro functional groups induce ring-strain into a molecule's skeleton, by causing geometrical distortions in bond angles. In the amine reaction with FLU, the spiro-ring system is opened upon Michael addition of the nucleophile to the α,β-unsaturated ketone. It appears that the energetic benefits involved in the relief of ring-strain must be sufficient to overcome the steric barriers that impede adduct formation.

Successful application of the ninhydrin reagent to analysis of our soil water samples requires an account of ammonia either by independent determination in each sample, as attempted here, or treatment with alkali and heat to volatilize ammonia. Based upon the lack of interference to ammonium, as well as greater analytic sensitivity, we advocate the use the fluorescent reagents OPA and FLU for the chemical characterization 1°-N in DON.

3.2 DON quantification based on metal-assisted amide hydrolysis

Forms of DON recovered by FLU include compounds such as small aliphatic 1° amines and amino acids, aniline derivatives, and the N-terminus of peptides. Small 1° amines and amino acids are also recovered by the OPA reagent, but aniline derivatives and peptides larger that di-peptides are not quantitatively recovered. Results of divalent metal-promoted peptide hydrolysis of soil solution indicate a substantial presence of dissolved poly-amide material hydrolyzable by the Zn(II) reagent, although the results of dipeptide digestions do not indicate an extensive degree of dipeptide hydrolysis. It has been previously observed that in wetland sediments (salt marshes) extracellular peptide hydrolysis efficiently degrades oligopeptides of 8 or more amino acid residues into smaller components; however dipeptides were not observed to be further degraded into amino acid monomeric units (Pantoja and Lee, 1999). Our results, within the context of these field-scale observations, indicated that divalent metal-promoted peptide hydrolysis may only affect the degradation of larger molecules with amide linkages. Furthermore, based upon the sensitivity of the OPA reagent to steric hindrances, particularly to the detection of dipeptides, and the observation that there may be considerable quantities of amines in the form of oligopeptides, the choice of the FLU reagent is preferable for the quantitative recovery of 1°-N in environmental aquatic samples.

4 Conclusions

Our attempts at metal promoted hydrolysis begin the effort of applying available biochemical methods to the analysis of peptides in soil solution. Our experiments using Zn(II)-promoted peptide hydrolysis were apparently successful at the conversion of molecularly uncharacterized organic nitrogen into forms of DON recoverable by OPA and FLU fluorescent techniques. Future work should be focused at the concomitant use of different divalent metals known to promote the cleavage of peptide bonds (e.g., Cu^{2+}, Ni^{2+}, Pt^{2+}, and Pd^{2+}) which may possess differential reactivity towards individual peptides (Zhu and Kostic, 1992; Hegg and Burstyn, 1995; Ueda *et al*., 1996; Bal *et al*., 1998; Grant and Pattabhi, 2001). Different divalent metal "cocktails" could theoretically facilitate a greater extent of peptide hydrolysis producing smaller amide and amine compounds reactive towards OPA and FLU reagents, improving the quantification of DON in forest ecosystems and decreasing the fraction that remains uncharacterized. Ultimately the results of this study demonstrated that the combination of metal assisted amide hydrolysis and FLU-derivatization can improve quantification of DON speciation in environmental samples.

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