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Fluorescence Inner-Filtering Correction for Determining the Humification Index of Dissolved Organic Matter

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The use of fluorescence spectrometry has been suggested as a simple method to determine the extent of natural organic matter humification by quantifying the red-shifting of fluorescence emission that occurs with increasing humification. Humification indices are calculated by dividing fluorescence intensity at longer wavelengths by intensity at shorter wavelengths. These indices calculated without any specific efforts to standardize dissolved organic matter (DOM) concentration will result in index values that vary with DOM concentration due to fluorescence inner-filtering effects. This study critically evaluated the effect of DOM concentration on humification index determination using organic matter isolated from field corn extract, soil: water extract, and soil fulvic acid. The results show that humification index values are sensitive to DOM concentration of the solution and are linear with respect to transmittance of the solution at the 254 nm used as the excitation wavelength. An approximate correction for DOM is to exploit the linear nature of the regression fit and to determine index values at the extrapolated 100% transmittance value. An exact correction using explicit correction factors for both primary and secondary inner-filtration effects was shown to give humification index values that are concentration invariant when absorbance of the solution at 254 nm was less than approximately 0.3 unit. Defining the humification index as the fluorescence intensity in the 300→345 nm region divided by the sum of intensity in the 300→345 nm and 435→480 nm regions was statistically advantageous. This study suggests that for quantitative results which can be used to compare humification of natural organic matter across different studies, correction of the fluorescence emission spectra for inner-filtration effects is needed.

Introduction

The chemical nature of natural organic matter (NOM) has been investigated intensively due to its involvement in environmental processes such as the complexation of trace metals (*1*), binding of organic contaminants (*2*), and C cycling in the ecosystem (*3*). NOM is formed through a humification process that converts specific, lower molecular weight organic compounds derived from plant and animal products to more condensed, higher molecular weight polymers primarily by microbial synthesis (*4*). Soil organic matter has become recognized for its critical role in environmental issues related to long-term sustainability of agroecosystems (*5*) and the terrestrial sequestration of C (*6*).

Although organic matter is present as a continuum, it may be conceptualized as being composed of at least three pools at different stages of humification: active, labile pool with turnover time <1 yr; intermediate pool with turnover times from years to centuries; and passive, stable pool persisting in soils for thousands of years (*7*). As humification increases, chemical properties such as total exchangeable acidity, carboxyl group content, and N-containing functional group content increase (*8*). This increase in functional group content would be expected to enhance DOM interaction with both ionic constituents in solution and mineral surfaces through mechanisms such as cation and anion exchange, ion-pair formation, protonation, ligand exchange, and cation bridging (*9*). With increased awareness of the role of NOM in the global carbon cycle, tools are needed to evaluate the effects of environmental and soil management perturbations on the distribution of organic matter pools in terrestrial and aquatic ecosystem.

Fluorescence spectroscopy has been utilized to probe the chemical structure of NOM because of its ability to distinguish different classes of organic matter (*10*). Recently, fluorescence spectroscopy methods have been proposed to determine the extent of humification by quantifying the extent of shifting of the emission spectra toward longer wavelengths with increasing humification. Zsolnay and co-workers calculated the humification index (HIX) by dividing the emission intensity in the 435→480 nm region by intensity in the 300→345 nm (*11, 12*). Kalbitz et al. used 390 nm/355 nm, 400 nm/360 nm, 470 nm/360 nm, and 470 nm/400 nm intensity ratios of synchronous fluorescence spectra with a wavelength offset of 18 nm to calculate HIX (*13*).

Fluorescence intensity measurements are subject to attenuation by the solution itself prior to detection. Primary inner-filtration refers to the absorption of the excitation beam prior to reaching the interrogation zone, and secondary inner-filtration refers to the absorption of the emitted fluorescence photons (*14*). The use of ratios for determining HIX allows ignoring the primary inner-filtration effect since intensities of emission at all wavelengths are similarly affected (*14*). However, the presence of secondary inner-filtration may require the correction of HIX values since organic matter absorbs UV light in the regions where fluorescence is being monitored. Inner-filtering correction was used in a simpler fluorescence emission intensity index (450 nm/500 nm with excitation at 370 nm) to distinguish between precursor organic sources for aquatic fulvic acids (*15*).

Previous work has shown that correction for both primary and secondary inner-filtration effects is essential for the accurate representation and comparison of fluorescence spectra of humic acid (*16*). The HIX procedure used by Cox et al. states that dissolved organic matter (DOM) concentration effects were avoided by diluting the samples to an absorbance value of $<0.1\ cm^{-1}$ at the excitation wavelength of 254 nm and further that corrections were not necessary because the HIX ratio is an "internal" parameter (*11*). Kalbitz et al. standardized organic matter concentration at 10 mg of C L^{-1} (*13*). These conditions may lessen the influence of primary inner-filtering effects on the calculated HIX values. However, the presence of secondary inner-filtering effects may require correction to obtain accurate HIX values.

The objective of this study was to critically evaluate the effect of the concentration of DOM on the fluorescence-based HIX and to determine a method of obtaining an index

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corrected for DOM concentration effects. Three sources of organic matter were examined: an aqueous extract of field corn residue, 1:1 (m:v) soil:water extract, and purified fulvic acid (FA) extracted from the same soil.

Experimental Section

The surface horizon of a Nicholville (coarse-silty, mixed, frigid, Aquic Haplorthod) soil was sampled from the University of Maine Sustainable Agriculture Research Farm, Stillwater, ME. The soil was passed through a 4-mm sieve to remove coarse fragments and root debris and then air-dried. The pH (1:1 soil:H_2O) was 6.1, and total C content of 21.3 g kg^{-1} was determined using a Leco CN-2000 analyzer. Particle-size distribution was 57% sand, 32% silt, and 11% clay. Field corn residue was obtained from same field where the soil was sampled, dried at 60 °C, and ground to pass a 1-mm sieve.

Soil DOM extract was obtained by adding 50 mL of deionized−distilled water (DI-H_2O) to 50 g of soil in a plastic bottle and shaking for 15 min on an orbital shaker. The bottle was then centrifuged at 900*g* for 30 min, and the supernatant was vacuum-filtered through a 0.4-μm filter. The filtrate was diluted volumetrically by bringing 1.56, 2.50, 3.57, 5.00, 8.33, and 12.50 mL up to 25 mL with DI-H_2O. The corn residue was extracted by adding 80 mL of cold DI-H_2O to 2.00 g of corn residue. The bottle stood in a refrigerator for 18 h after briefly being stirred and was then centrifuged and filtered as above. The filtrate was diluted by bringing 1, 2.5, 10, 15, and 20 mL up to 500 mL with DI-H_2O. All dilutions were adjusted to pH 6.0.

The FA was extracted using the International Humic Substance Society method (*17*) with some modifications. Twenty grams of soil was extracted with 200 mL of 0.1 N NaOH under N_2 by shaking on an orbital shaker for 24 h in a centrifuge bottle. The bottle was then centrifuged at 900*g* for 30 min, and the supernatant was vacuum-filtered through fiber glass filters. The filtrate was acidified to pH 1.5 by the addition of 6 N HCl and allowed to stand for 24 h to precipitate the humic acid. The solution was centrifuged and filtered again through fiber glass filters to separate the FA from the precipitated humic acid. The filtrate containing the FA was purified by passing it through a 25-mL column of purified XAD-7 resin. The resin was purified by five batch extractions with 0.1 N NaOH stirred for 1 h, followed by three repeated Soxhlet extractions with methanol for 4 h. The resin was packed in a column and eluted with 5 column volumes each of 0.1 N NaOH, 0.1 N HCl, and DI-H_2O. The absorbance at 225 nm was measured for the 0.1 N NaOH elutent, and it was <0.008 absorbance unit, indicating effective purification of the resin. The sorbed FA was desorbed in batch mode by raising the pH of solution to 7 (*18*). The solution was decanted, passed through a H^+-saturated cation exchange column to protonate the FA, and then freeze-dried. Fulvic acid solution was prepared by dissolving 50.0 mg of the material in 100 mL of DI-H_2O. Dilutions were made by bringing 1, 2, 4, 8, 12, and 20 mL to 200 mL of DI-H_2O after adjustment to pH 6.

Fluorescence measurements were obtained using a Hitachi F-4500 spectrofluorimeter. Instrumental parameters were excitation (EX) and emission (EM) slits, 5 nm; response time, 8 s; and scan speed, 240 nm min^{-1}. The EM spectra were obtained by using 254 nm for EX and EM recorded from 280 to 500 nm. Fluorescence intensity values are relative to the instrument conditions at the time of measurement and are a function of source intensity, optical efficiency, and detector efficiency. The sensitivity and stability of the instrument was measured using the Raman band signal intensity (EX, 350 nm; EM, 397 nm). The Raman band intensity was determined prior to each sample, and the fluorescence intensities were divided by the Raman intensity to correct for any fluctuations in instrumental conditions.

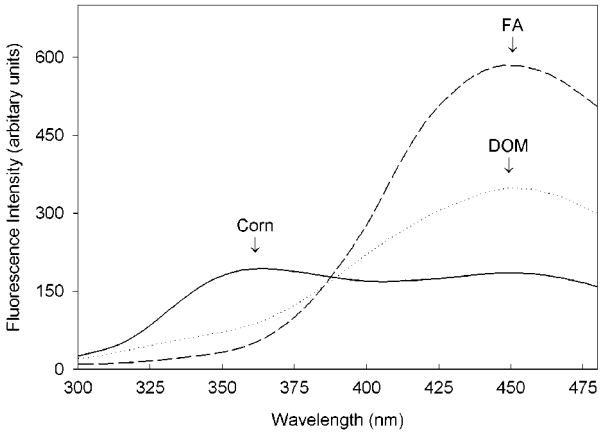

FIGURE 1. Fluorescence emission spectra of field corn residue extract, soil:water extract, and soil fulvic acid. Excitation wavelength was 254 nm.

Ultraviolet−visible absorption spectra from 250 to 500 nm were obtained using an Agilent 8453 diode array spectrophotometer. The HIX value was calculated as

$$\mathrm{HIX} = (\sum I_{435\rightarrow480})/(\sum I_{300\rightarrow345})$$

where *I* is the fluorescence intensity at each wavelength (*11*).

Results and Discussion

Spectral Characterization of Organic Matter. The three types of organic matter used in this study were selected to represent materials along the NOM continuum. Plant residue is a major source of carbon-rich, nonhumified, highly water-soluble material in soils, representing the initial reactants from which organic matter is formed by microbial synthesis and chemical reactions catalyzed by metal oxides (*8*). Fulvic acids are the refractory end products of the humification process (*19*). Water-soluble DOM is the most labile of the soil organic matter fractions and is intermediate between the starting biotic residue and the humic substance end products (*20*).

The fluorescence emission spectra from the three sources of organic matter at dilutions where UV absorbance at the EX wavelength of 254 nm was equal to 0.48 ± 0.01 are shown in Figure 1. The red-shifting of the emission spectra in the corn → DOM → FA sequence is clearly observed. The HIX as calculated by Zsolnay and co-workers (*11*, *12*) was selected for use in this study because it utilizes emission intensity at a range of wavelength values rather than an arbitrarily selected wavelengths for the index. Because of the broad nature of the fluorescence emission spectra of organic matter (Figure 1), the use of an integrated area of fluorescence intensity may be more representative of the spectral red-shifting than the use of two individual wavelength emission values for HIX calculation. The HIX parameter values calculated from the spectra shown in Figure 1 are corn, 2.12; DOM, 6.54; and FA, 28.5 indicating the expected increase in the HIX value with greater humification of the source material.

The normalized UV−Vis absorption spectra with relative absorbance at 254 nm set to a value of 1 for the three organic materials are shown in Figure 2. It is interesting to note that both soil DOM and FA spectra shows a smooth decline in absorbance with increasing wavelength, while the corn extract shows structure in the spectra. This probably reflects the spectral characteristics of distinct classes of biochemicals that are found in aqueous plant extracts. With increased humification of these initial reactants, spectral structure is lost as the individual classes of biochemicals present in the

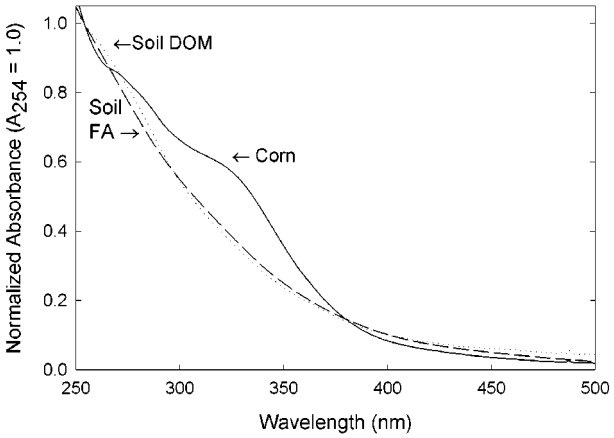

FIGURE 2. Normalized UV−Vis absorption spectra of field corn residue extract, soil:water extract, and soil fulvic acid. Spectra was normalized to a value of 1 at 254 nm.

plant residue become incorporated in the complex, random structure of soil organic matter.

Although the use of intensity ratios allows the correction for primary inner-filtering effect to be ignored in the HIX determination, the greater absorption in the 300−345 nm region as compared to the 435−480 nm region would suggest that secondary inner-filtering effects would be more prominent at the 300−345 nm wavelength region than at the higher wavelength region. This suggests that the HIX value would be concentration variant, with increased HIX values with greater DOM concentration when no correction for the secondary inner-filtering effect was used. The authors of the HIX methodology state that concentration effects were avoided by diluting the samples to absorbance values below 0.1 cm^{-1} (*11*). However, other works recommend that absorbance values be below 0.06 (*21*) or 0.01 cm^{-1} to avoid internal attenuation (*14*).

Organic Matter Concentration Effects on HIX. The concentration effects on HIX values are shown in Figure 3 with the HIX value on the *y*-axis and the concentration of the organic matter expressed as transmittance at 254 nm on the *x*-axis. The use of transmittance was found to be a useful transformation of the absorbance data to linearize the effect of concentration on the HIX values. For all three source materials, the apparent HIX value increased with increasing organic matter concentration. This increase resulted from secondary inner-filtration effects, which decreased the denominator of the index to a greater extent than the numerator.

The concentration variance shown in Figure 3 clearly demonstrates that the fluorescence-based HIX parameter is defined in part by the concentration of organic matter at which it is determined due to internal (secondary) attenuation of the fluorescence intensity. Without question, the HIX parameter is valuable in determining the relative positioning of DOM material along the NOM continuum. In general, organic materials become more reactive in environmentally relevant reactions due to increased functional group density with increased humification (*19*).

Methodological Implications. The determination of the HIX for unknown samples clearly requires the some set concentration criteria. One approximate approach would be to carefully dilute all samples to a fixed absorbance value (i.e., 0.1 at 254 nm). Another approximate approach that may be easier to implement is to exploit the linear nature of concentration effect and to use the linear regression value for the *y*-axis intercept as shown in Figure 3. This approach yields HIX values of corn, 1.23; DOM, 3.87; and FA,7.65. This would require the preparation of three or four diluted samples

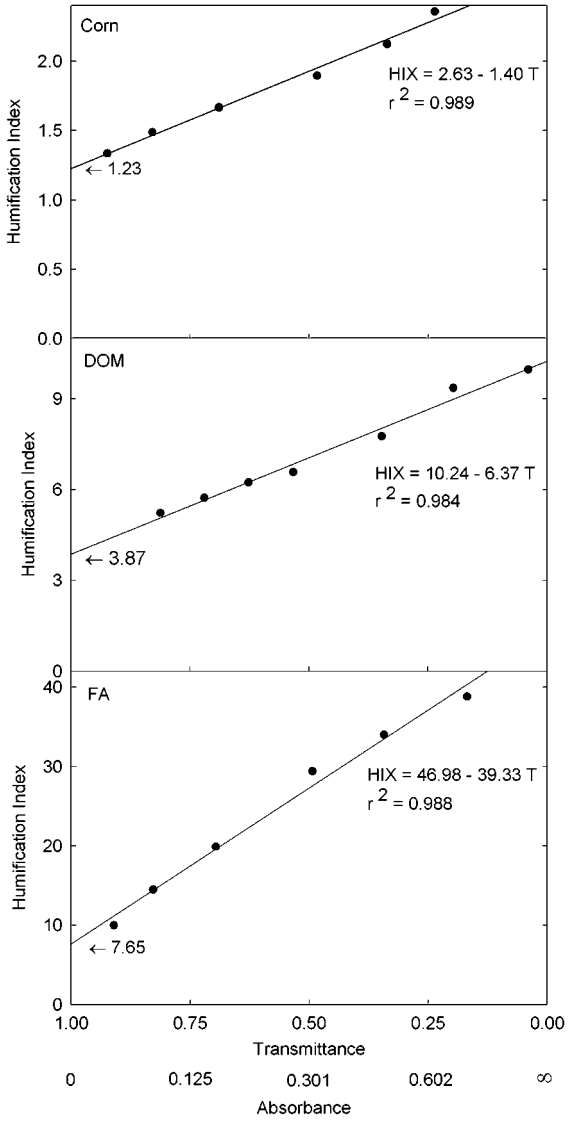

FIGURE 3. Effect of transmittance of field corn residue extract, soil:water extract, and soil fulvic acid solutions on the HIX value.

from the original sample and also requires absorbance values on each diluted solution. However, with appropriate manual or automated pipetting equipment, the dilution approach may be more time efficient than the preparation of diluted samples to a set absorbance value. These approaches are approximate in the sense that they do not explicitly correct for the secondary inner-filtering effect. They do standardize the process by which the HIX value is calculated to producing results that may be compared without a concentration bias.

An exact correction requires the correction for both primary and secondary inner-filtration effects at all the wavelengths utilized to calculate to HIX index. This requires absorbance measurement of the solution at all the wavelength used. The correction for both primary and secondary inner-filtering was calculated as (*18*)

$$I = I_0(10^{-b(A_{ex}+A_{em})})$$

where I = detected fluorescence intensity, I_0 = fluorescence in the absence of self-absorption, b = assumed path length of 0.5 for both excitation and emission beam, A_{ex} = absorbance at the EX wavelength, and A_{em} = absorbance at the EM wavelength. The data for the $\sum I_{435\rightarrow480}$ and $\sum I_{300\rightarrow345}$ and calculated HIX values with and without correction are shown

TABLE 1. Fluorescence Intensities for HIX Determination of DOM from Aqueous Extracts of Corn Residue at Multiple Concentrations of Organic Matter

A_{254}	0.034	0.082	0.162	0.316	0.474	0.627	1.53
corrected							
$\sum I_{435\rightarrow480}$	148	300	609	1385	2515	3991	32785
$\sum I_{300\rightarrow345}$	116	222	443	1068	2101	3586	53672
HIX	*1.28*	*1.35*	*1.37*	*1.30*	1.20	1.12	0.61
	mean ± SD[a] = 1.33 ± 0.04						
	CV[a] = 3.0%						
	CI[a] = 1.26 → 1.39						
uncorrected							
$\sum I_{435\rightarrow480}$	137	247	415	656	822	906	863
$\sum I_{300\rightarrow345}$	102	166	249	346	387	383	214
HIX	*1.36*	*1.49*	*1.67*	*1.89*	2.12	2.36	4.03
	mean ± SD[a] = 1.60 ± 0.23						
	CV[a] = 14.6%						
	CI[a] = 1.24 → 1.97						
UnC/C[b]	1.06	1.10	1.22	1.45	1.97	2.11	6.61

[a] Mean, standard deviation, coefficient of variance, and 95% confidence interval of italicized HIX values. [b] Ratio of uncorrected HIX/corrected HIX.

TABLE 2. Fluorescence Intensities for HIX Determination of DOM from Aqueous Extracts of Soil at Multiple Concentrations of Organic Matter

A_{254}	0.091	0.142	0.202	0.281	0.460	0.704	1.41
corrected							
$\sum I_{435\rightarrow480}$	619	1000	1467	2156	4380	10117	44366
$\sum I_{300\rightarrow345}$	127	194	274	405	795	1828	13263
HIX	*4.89*	*5.15*	*5.36*	*5.32*	5.51	5.53	3.34
	mean ± SD[a] = 5.18 ± 0.22						
	CV[a] = 4.2%						
	CI[a] = 4.84 → 5.52						
uncorrected							
$\sum I_{435\rightarrow480}$	499	712	907	1104	1466	1885	1541
$\sum I_{300\rightarrow345}$	95	124	146	168	189	202	154
HIX	*5.22*	*5.73*	*6.23*	*6.58*	7.76	9.35	9.97
	mean ± SD[a] = 5.94 ± 0.59						
	CV[a] = 10.0%						
	CI[a] = 5.00 → 6.88						
UnC/C[b]	1.07	1.11	1.16	1.24	1.41	1.69	2.99

[a] Mean, standard deviation, coefficient of variance, and 95% confidence interval of italicized HIX values. [b] Ratio of uncorrected HIX/corrected HIX.

TABLE 3. Fluorescence Intensities for HIX Determination of Soil Fulvic Acid at Multiple Concentrations of Organic Matter

A_{254}	0.041	0.082	0.157	0.308	0.464	0.776
corrected						
$\sum I_{435\rightarrow480}$	415	839	1694	4176	7853	18661
$\sum I_{300\rightarrow345}$	44	62	96	180	330	874
HIX	*9.33*	*13.6*	*17.6*	*23.2*	23.8	21.4
	mean ± SD[a] = 15.9 ± 5.9					
	CV[a] = 37.1%					
	CI[a] = 6.5 → 25.3					
uncorrected						
$\sum I_{435\rightarrow480}$	375	686	1157	1992	2567	2886
$\sum I_{300\rightarrow345}$	39	47	58	68	75	72
HIX	9.*64*	*14.5*	*20.0*	*29.4*	34.0	38.8
	mean ± SD[a] = 18.4 ± 8.5					
	CV[a] = 46.1%					
	CI[a] = 4.9 → 31.9					
UnC/C[b]	1.03	1.07	1.14	1.27	1.43	1.81

[a] Mean, standard deviation, coefficient of variance, and 95% confidence interval of italicized HIX values. [b] Ratio of uncorrected HIX/corrected HIX.

for aqueous corn extract in Table 1, for 1:1 soil:water extract in Table 2, and for soil fulvic acid in Table 3.

Correction for fluorescence inner-filtering has been reported to work well when the correction term is >0.33 (*14*). Inspection of the $\sum I_{435\rightarrow480}$ and $\sum I_{300\rightarrow345}$ data shown in Tables 1−3 shows that this corresponds to solutions having A_{254} of about 0.3 or less. The corrected mean ± standard deviation and 95% confidence intervals for the HIX values of 1.33 ± 0.04 and 1.26 → 1.39 for corn (Table 1) and 5.18 ± 0.22 and 4.84 → 5.52 for soil DOM (Table 2). The good relative variability of the HIX parameters shows that inner-filtering correction functions well within this constrained range of A_{254} for the aqueous plant and soil organic matter extracts (Tables 1 and 2). The correction for the soil FA worked less well than in the water extracts of corn residue and soil with mean HIX value of 15.9 ± 5.9 and a 95% confidence interval of 6.5 → 25.3 (Table 3). The coefficient of variation for the soil FA HIX value was 37% as compared to about 4% for corn and soil DOM. The greater variation in HIX for the soil FA sample probably reflects sensitivity of the ratio having a much smaller denominator term than in numerator (Table 3).

TABLE 4. HIX Values Using Inner-Filtering Corrected Fluorescence Intensity and the Equation: HIX = $\sum I_{435\rightarrow480}/(\sum I_{300\rightarrow345} + \sum I_{435\rightarrow480})$ for Corn, Soil DOM, and Soil FA Samples with Absorbance at 254 nm Less Than 0.32

	Corn			
A_{254}	0.034	0.082	0.162	0.316
HIX	0.561	0.576	0.579	0.565
	mean ± SD[a] = 0.570 ± 0.009			
	CV[a] = 1.5%			
	CI[a] = 0.557 → 0.584			
	Soil DOM			
A_{254}	0.091	0.142	0.202	0.281
HIX	0.830 0.838	0.843	0.843	0.842
	mean ± SD[a] = 0.838 ± 0.006			
	CV[a] = 0.7%			
	CI[a] = 0.829 → 0.848			
	Soil FA			
A_{254}	0.041	0.082	0.157	0.308
HIX	0.902	0.931	0.946	0.959
	mean ± SD[a] = 0.935 ± 0.025			
	CV[a] = 2.6%			
	CI[a] = 0.890 → 0.973			

[a] Mean, standard deviation, coefficient of variance, and 95% confidence interval of italicized HIX values.

An alternative expression of the degree of humification that avoids the sensitivity to the magnitude of the denominator is to calculate the index as

$$\text{HIX} = \sum I_{435\rightarrow480}/(\sum I_{300\rightarrow345} + \sum I_{435\rightarrow480})$$

The HIX value in this formula ranges from 0 to 1 with increasing degree of humification. The HIX values for the corn, soil DOM, and soil FA samples calculated this way are shown in Table 4. The coefficient of variation and 95% confidence intervals is improved for all three samples using this formula for determining HIX.

In summary, the use of HIX as a qualitative method to rank samples in order of humification does not favor any specific approach and does not require correction for inner-filtration effects as long as the absorbance values are kept within reasonable limits, perhaps 0.3 cm^{-1} or under. However, to use HIX in a quantitative mode does requires the careful consideration of the inner-filtration effects. This can be done approximately by diluting the solutions to a fixed absorbance value (i.e., 0.1 cm^{-1}) at the EX wavelength or by extrapolating to 100% transmittance. It has been shown that exact correction for primary and secondary inner-filtration works very well when the absorbance at the EX wavelength of 254 nm is below approximately 0.3 cm^{-1} or under. Expression of the extent of emission spectra red-shifting on a fractional basis by dividing the fluorescence intensity in the 435→480 nm region by the total intensities in the 300→345 + 435→480

nm regions rather than on a ratio basis reduced the HIX coefficient of variation for most of the highly humified sample, soil fulvic acid. With the use of inner-filtration correction, HIX values are concentration independent, which would allow easier comparison of results across different studies. With these advantages, exact correction is recommended using the following expression:

$$\mathrm{HIX} = \sum I_{435\rightarrow480} / \left(\sum I_{300\rightarrow345} + \sum I_{435\rightarrow480}\right)$$

With due consideration of these DOM concentration effects, the fluorescence-based HIX provides a robust tool for determining the extent of humification and thus the stability of the organic matter being investigated.

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