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Spectral Deconvolution and Quantification of Natural Organic Material and Fluorescent Tracer Dyes

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Abstract

Fluorescent dyes have become an integral part of the study and management of ground water in karst environments. Researchers have striven to reduce detection limits and analyze multiple dyes in a single sample while minimizing dye concentrations for environmental, aesthetic and health reasons. The unambiguous separation from background, identification and quantification of fluorescent tracer dyes has increasingly taken on legal implications. Synchronous fluorescence spectroscopy and curve fitting software represent a major advances in the quantitative analysis of low levels of tracer dyes against naturally occurring background fluorescence.

Determination of levels of detection (LOD) and levels of quantification (LOQ) are an important part of dye trace design and implementation. Factors that impact LOD and LOQ include levels of natural fluorescent compounds, absolute fluorescence of the specific dyes, the presence of multiple dyes with overlapping peaks and instrumental noise. Characterization of the spectral shapes and concentration dependences of the natural fluorescence background and applied tracer dyes are important to the determination of a positive dye trace result. Rather than representing noise, the natural fluorophores contain information about the flow environment. Spectral deconvolution with curve fitting software is an important tool in the karst researcher's toolbox.

Introduction

The following paper illustrates the process of separating and quantifying xanthene dyes like fluorescein, eosin and Rhodamine WT from naturally occurring fluorescent organics using PeakFit™ non-linear curve fitting software (SeaSolve, 2003). The examples presented analyze direct water samples but similar procedures can be applied to elutants from activated carbon detectors.

Previous efforts (Tucker and Crawford, 1999) have treated the natural background as something to be subtracted. The natural background fluorescence is representative of local environmental conditions but analytical efforts have not utilized spectral deconvolution (Kalbitz etal, 1999, Miano and Senesi, 1992 and Artinger etal, 1992). Subhash and Mohanan (1997) used an earlier version of PeakFit™ to model chlorophyll samples. Characterization of the natural background allows the quantification and detection limits of fluorescent tracers to be reduced to levels comparable to the instrumental noise. The introduction of man-made organics unrelated to the applied tracer dyes is also a significant concern (Smart and Karunsaratne, 2001).

Methods

Direct water samples collected as part of the Hiawatha (Hwy 55) / Crosstown (Hwy 62) dye trace to Camp Coldwater Spring in the summer of 2001 in Minneapolis, Minnesota. The dye trace was

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conducted through the relatively flat-lying Paleozoic Platteville formation composed of shaly limestones and dolomites. Examples were selected to illustrate the analysis of waters with and without tracer dyes present. All spectra presented are synchronous scans run on a Shimadzu RF-5000 scanning spectrofluorophotometer using a borosilicate cuvette with a 13 mm light path. The resulting spectra are emission referenced with $\Delta\lambda$ of 15 nm, 5 nm bandwidths, a scan rate of 30 nm/sec and 0.02 sec response time. All spectra were fitted using PeakFit™ version 4.0 non-linear curve fitting software (SeaSolve, 2003).

Background Fluorescence

Many naturally occurring substances are fluorescent. Natural Organic Materials (NOM) including chlorophyll, humic acids and fulvic acids are commonly found in significant concentrations and are relatively fluorescent (Leenheer and Croué, 2003) although only a small fraction of the dissolved organic material is responsible for the fluorescence (Miano and Senesi, 1992). These fluorescent organics are collectively referred to as fluorophores. While NOMs are extremely complex mixtures of large, charged organic ions the fluorophores are likely products of distinct structures within the molecules (Stenson et al, 2002). Distinct classes of fluorophores form broad, overlapping peaks with relatively constant peak centers and widths. Fulvic acids are dominated by emission peaks centered around 390, 440 and 470 nm, at $\Delta\lambda$ = 15 nm, with Full Width at Half Maximums (FWHM) of 30 to 50 nm. Humic acids form emission peaks centered near 470, 490 and 510 nm with slightly wider FWHM of 40 to 60 nm (Miano and Senesi, 1992). Chlorophylls produce two main peaks at 640 and 680 nm with FWHM of 25 and 60 nm, as correlated from emission spectra of Subhash and Mohanan (1997). All of these NOM have significantly wider FWHM than the roughly 20 nm FWHM of the commonly used xanthene dyes (Käss, 1998).

Background fluorescence spectra are relatively stable in particular locations while runoff conditions remain the same, so that fluorescent spectra can be used to characterize and identify source areas for a range of marine, fresh waters (Coble, 1996), wastewaters (Hsu and Lo, 1999, Galapate et al, 1998 and Provenzano et al, 2001) and landfill leachate contamination (Christensen et al, 1998). In a time series of samples from one location, organic acids may be preserved in lake sediments (Wolfe et al, 2002) or speleothems (Baker et al, 1996). As sources and ages of NOM vary at a particular point, amplitudes of the component peaks vary producing shifts in the overall fluorescence spectra (Miano and Senesi, 1992). On shorter time scales individual spectra can vary dramatically (Smart and Karunaratne, 2002).

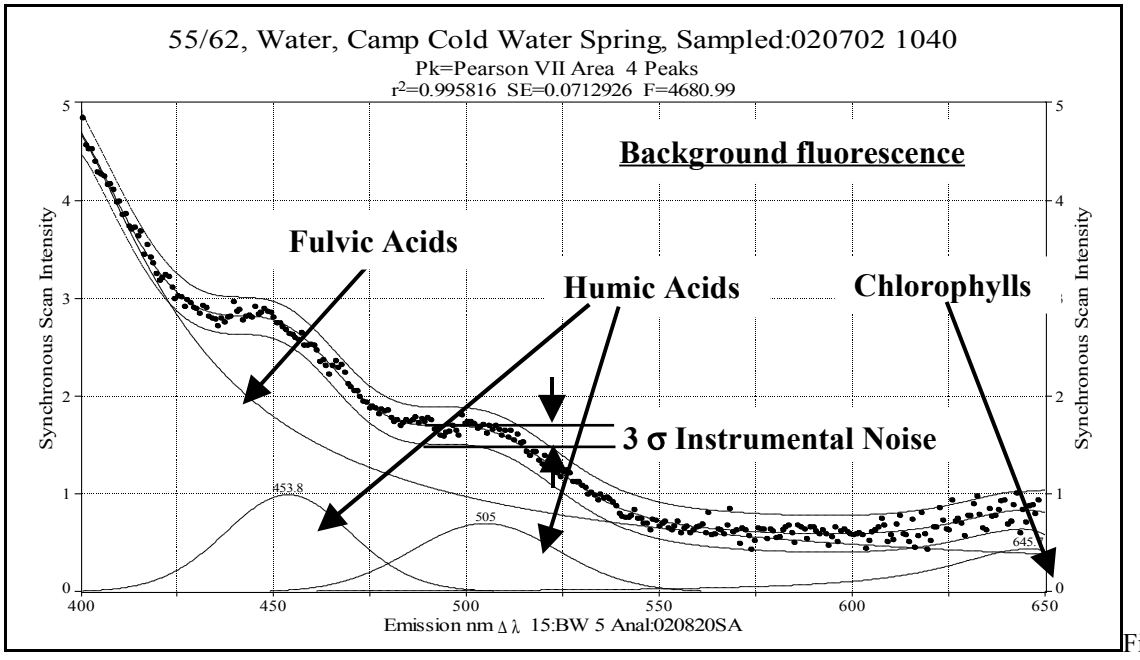

Figure 1. Annotated example of background fluorescence spectrum from Camp Coldwater Spring.

Figure 1 shows a high-resolution background spectrum prior to the introduction of any dyes from the Camp Coldwater dye trace. This spectrum contains 312 data points, with one point every 0.8 nm. This density of data allows detailed resolution and separation of the background peaks and any dye peaks. The spectrum has a generally concave upward form through the area of interest for the dyes fluorescein, eosin and Rhodamine WT that is characteristic of naturally occurring fluorophores. Applying peak fitting software the spectrum can be divided into its constituent components. In this example there is a large, fulvic-like peak dominating the short-wavelength (left) side of the spectrum. Superimposed on this large peak are two smaller humic-like peaks at 454 and 505 nm and a small chlorophyll peak at 645 nm. The individual data points are plotted as "+" symbols that scatter across the modelled spectrum line (solid) and are enclosed by the 3 sigma error estimate line (dashed). Instrumental noise arises primarily from thermal instability in the photodetector and from suspended particles along the optical path (Guilbault, 1990).

Following the methodology of Subhash and Monahan (1997) the peaks are fitted using Pearson Type VII functions. The Pearson VII function produces a more realistic model of the underlying fluorescence by accounting for both Doppler and collisional broadening of the individual peaks (Jansson, 1997). A simple gaussian peak shape does not account for secondary effects on the sides of each peak (Tucker and Crawford, 1999); an error that is compounded by straight-line base-line subtraction leading to poor fits at low dye concentrations.

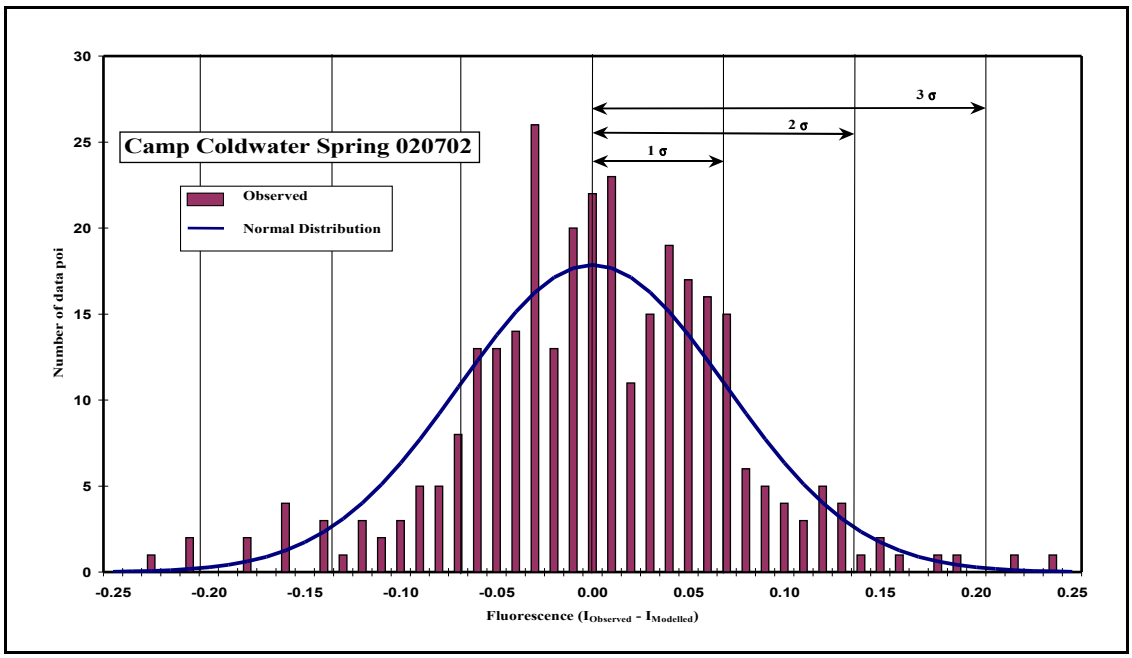

Figure 2. Distribution of instrumental background noise for Camp Coldwater Spring compared to theoretical normal distribution.

Instrumental noise is described by the departures from the smooth, fitted spectrum; assuming that the fitted curves fully describe the fluorescent components of the sample. In Figure 2, the residuals are plotted as a histogram of the difference in fluorescence intensity between the observed data points and the modelled spectrum from Figure 1. The residuals, plotted as bars, have a nearly normal distribution with a three standard deviation (3σ) of plus or minus 0.21 intensity units. For comparison the solid line is the calculated normal distribution for n = 312, mean = 0 and $3\sigma = 0.21$. The instrumental noise is distinct from variations in the background fluorophores. By fitting each spectrum individually temporal variations in the component NOM can be modelled sample to sample. The normal distribution of errors suggests that there is no systematic bias, or "hidden peaks", in the modelled spectra.

Fluorescent Dye Tracers

For the Camp Coldwater dye trace eosin (CAS# 17372-87-1) and fluorescein (CAS 518-47-8) were the introduced tracers. Another commonly used dye tracer, Rhodamine WT (CAS# 37299-86-8), was not used, but a series of dye standards was created to define calibration curves for each. All dye standards were serially diluted from stock dye solutions. Carbonate-saturated groundwater of pre-industrial age (Alexander and Alexander, 1989) was used to provide contaminant-free pH buffering, especially at low dye concentrations. All dilutant was sterilized by filtration through 0.2μm filters. Standards with a roughly logarithmic distribution were produced to define a linear calibration curve in log-log concentration versus peak area space.

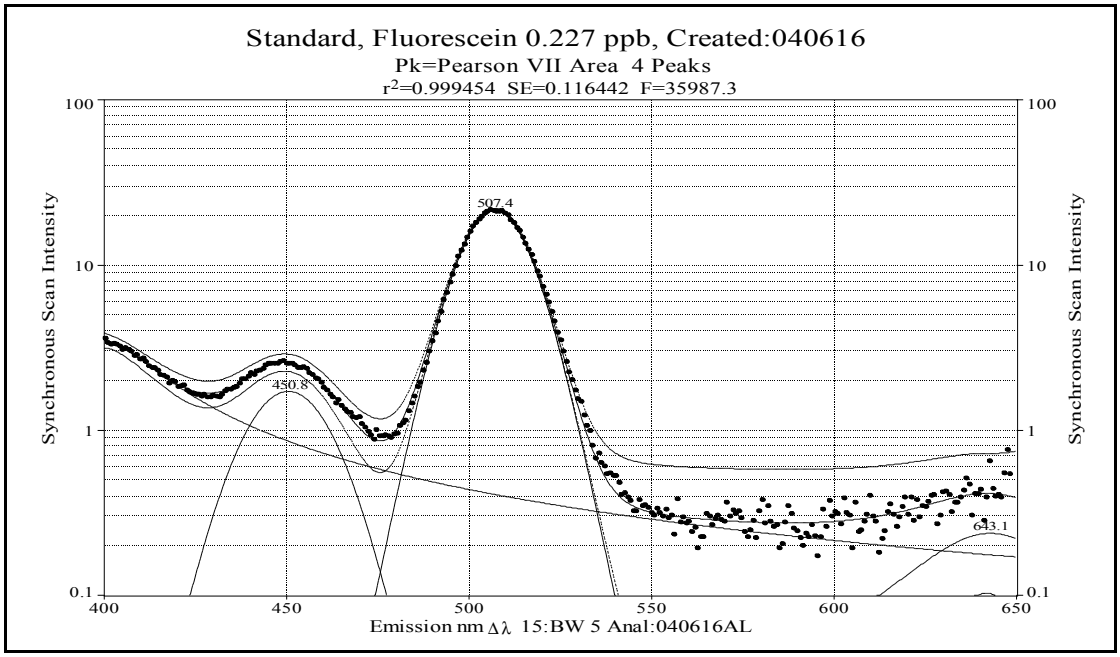

Figure 3. Fluorescein dye standard (0.227 ppb) in carbonate-saturated groundwater.

Figure 3 shows the addition of 0.227 ppb fluorescein to filtered pre-industrial groundwater. The natural groundwater used to dilute the fluorescein contains some NOM. Figure 3 is plotted on a semi-log scale to illustrate the peak fit analysis of NOM and tracer dye. The fluorescein forms a large peak centered at 507.4 nm with a FWHM of 19.9 both of which are within the statistical variation observed for fluorescein as found in Table 1. Table 1 represents dye standards mixed and analyzed at the University of Minnesota between 1993 and 2005.

Table 1. Commonly used tracer dye peak centers and FWHM (U of M Geology data).

Dye	CAS #	# of standards	peak center	peak FWHM
fluorescein	518-47-8	32	506.3 ± 1.2	20.2 ± 1.2
eosin	17372-87-1	30	532.7 ± 0.9	21.3 ± 0.9
Rhodamine WT	37299-86-8	42	573.5 ± 0.5	21.3 ± 0.5

The limits of detection and quantification are well-developed terms routinely used by analytical chemists. The International Union of Pure and Applied Chemistry (IUPAC) has adopted standard definitions of these terms as outlined by Long and Winefordner (1983) and Keith et al (1983). The regions of analytical certainty can be divided into three areas: 1) No Detection, or the analyte is not present at levels statistically different from a blank, 2) a Region of Detection, where the analyte is present a levels significantly above a blank but is not yet quantifiable and 3) a Region of Quantification, where the analyte is present at quantifiable levels.

The Limit of Detection (LOD) as defined by the IUPAC is:

$$\mathbf{LOD = 3\ \sigma_{background}.}$$

This assumes that any additional data points have a 99% probability of falling within three σ of the modelled background. For the example shown in Figure 2 three σ is 0.21 intensity units. The detection limit defines the presence or absence of a given fluorescent compound to a 99% certainty level. The level of quantification (LOQ) as defined by the IUPAC is:

$$\mathbf{LOQ = 10\ \sigma_{background}.}$$

The region of quantification should be significantly above the limit of detection. The IUPAC recommends a minimum of 10 σ. Detection and quantification of a given analyte at low levels is often one of the most difficult decisions for the analyst. At low levels, knowledge of the selectivity of the method and possible interferents is very important. As concentrations rise far above the LOQ measurement and identification become much simpler (Keith et al, 1983).

When tracer dyes are present in a sample, they are detectable when their peaks rise above the 3 sigma threshold of background, and quantifiable when they rise 10 sigma. In figure 4, Uranine and Eosine peaks have been extracted with peak heights of 1.5 and 2.25 units above background. The 3 sigma and 10 sigma limits are 0.25 and 0.75 units respectively in this example therefore both dyes are quantifiable.

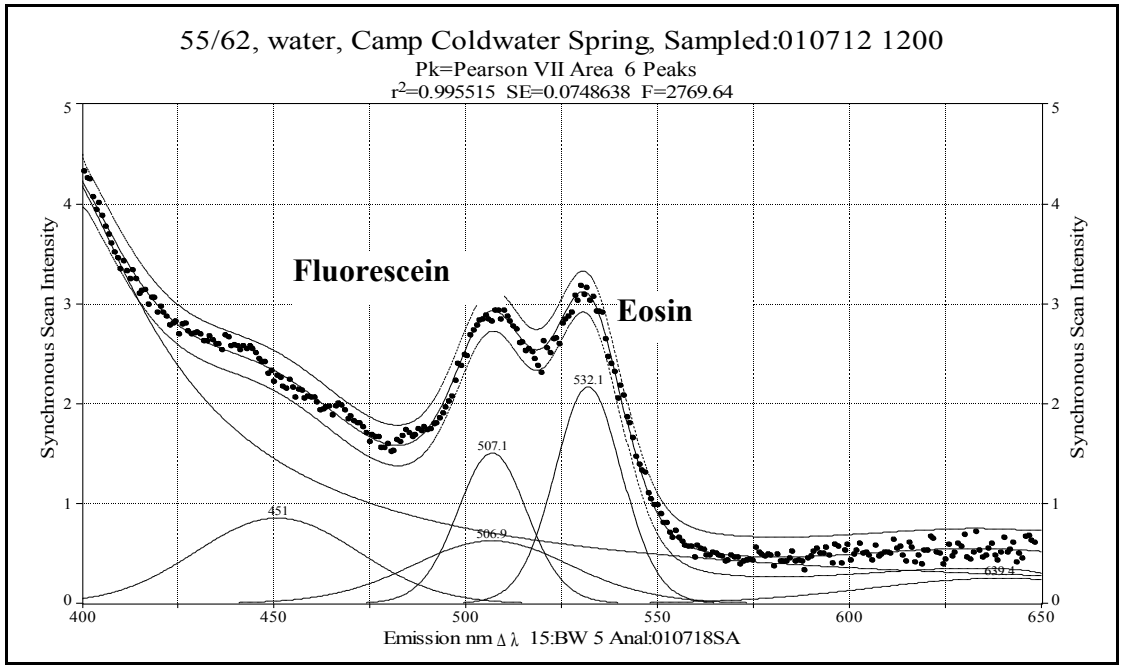

Figure 4. Quantifiable fluorescein and eosin peaks on a background spectrum. These peak areas correspond to concentrations of 0.016 ppb fluorescein and 0.176 ppb eosin.

By comparing the peak heights, or preferably peak areas, to gravimetrically prepared standards, dye concentrations can be calculated. Peak area is a much more robust estimator of dye concentration as it is defined, in this example, by more than 50 data points while peak height is determined from a single data point. Excessively wide, highly tailed or asymmetrical peaks would misrepresent the peak areas leading to anomalous dye concentrations.

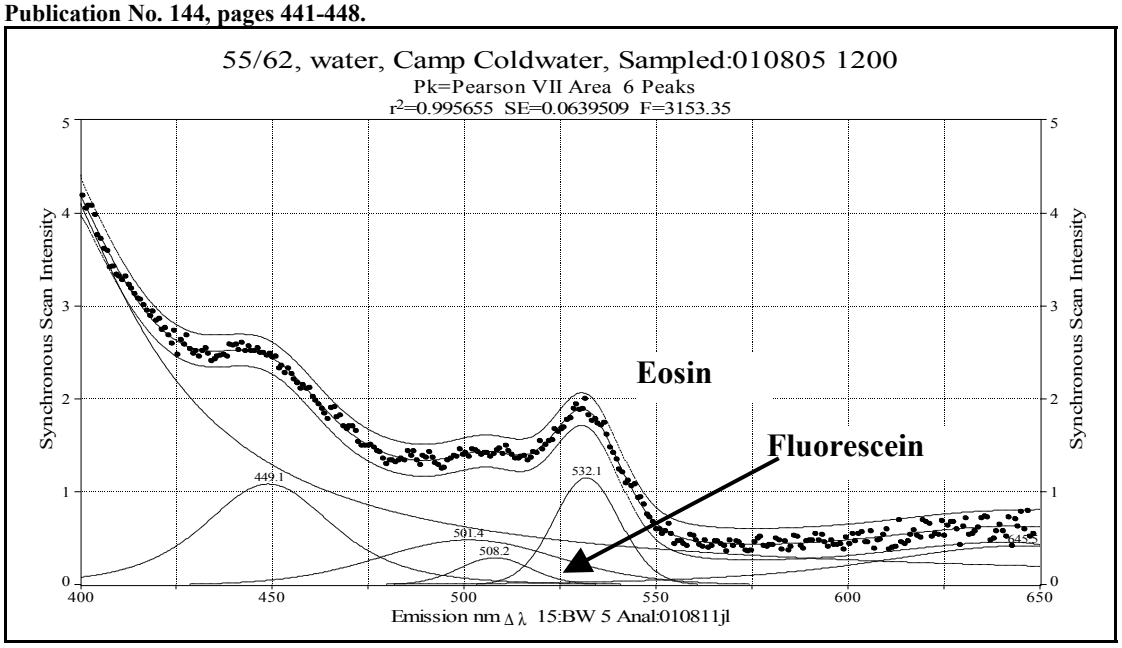

Figure 5. Quantifiable eosin and detectable fluorescein peaks (0.003ppb fluorescein and 0.089ppb eosin).

When dye concentrations are lower, the signal may drop below quantifiable levels, but remain detectable. In figure 5, eosin is still quantifiable with a peak height of 1⅛ intensity units, but fluorescein has fallen to near the three sigma probability level. If this were a single sample and analysis the fluorescein peak would be very tenuous, but in this case its occurrence in the tail of a well-documented tracer breakthrough strongly endorsed the identification.

The great power of dye tracing is to be found in a systematic breakthrough curve. This means that a typical positive dye trace is reproduced in a dozen if not hundreds or thousands of samples. A breakthrough curve creates an inherent reproducibility within a single dye trace. Where the background remains stable through the course of dye trace the quantification limit drops in proportion to the number samples represented on the breakthrough curve. Long and Winfordner (1983) demonstrate that the reduction in uncertainty follows the equation:

$$\sigma_{mean} = \sigma_{background} / (n_{samples})^{1/2}.$$

In the case of Camp Coldwater Spring the collection of NOM creating the background fluorescence has not significantly changed over the course of more than a month. This is not to say that the amplitude of the component peaks has not changed, but that the locations of the peak center and FWHM representing natural fluorophores have been stable. Samples were initially collected at a rate of sixteen per day and scaled back to four per day after two weeks. This means that the August 5th sample represents the 240th sample collected on the fluorescein and eosin breakthrough curve. The eosin peak is significantly above the 10 σ_{mean} quantification limit of 0.14 intensity units. Because of the reproducibility inherent in the repetitive samples fluorescein is still quantifiable for this example. The corresponding dye concentrations are: 0.003 ppb fluorescein and 0.089 ppb eosin. Combining flow volume measurements with dye concentrations to calculate dye recovery can produce another form of confirmation of positive dye trace results.

Conclusions

Scientifically, and legally, defensible dye trace results are the product of careful and appropriate planning and execution combined with well-designed analytical procedures. Sound analysis requires characterization of the natural background fluorescence through extensive sampling before and during the dye trace. Quantitative curve fitting software permits systematic analysis of fluorescence spectra providing

a base against which to assess putative dye-containing samples, including definition of detection and quantitation thresholds.

Characterization of natural fluorophores in each sample requires significant effort but produces maximum sensitivity to fluorescent tracers while minimizing potential false positives and helps ensure all introduced tracers are properly identified. The serial correlation of samples in a dye breakthrough curve and dye recovery analysis provide further assurances of positive dye trace results that would otherwise remain equivocal.

The NOM revealed as fluorophores in groundwater tracer studies represent a broad area of research in and of themselves. Rather than treating the background fluorescence as noise to be subtracted this fluorescence may contain valuable information about the study area that can augment dye trace results.

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