

Criteria for selecting fluorescent dye tracers for soil hydrological applications using Uranine as an example

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Abstract: Calibrating and verifying 2-D and 3-D vadose zone flow and transport models requires detailed information on water and solute redistribution. Among the different water flow and mass transfer determination methods, staining tracers have the best spatial resolution allowing visualization and quantification of fluid flow including preferential flow paths. Staining techniques have been used successfully for several decades; however, the hydrological community is still searching for an “ideal” vadose zone tracer regarding flow path visualization. To date, most research using staining dyes is carried out with Brilliant Blue FCF. Fluorescent dyes such as Uranine, however, have significant advantages over non-fluorescents which makes them a promising alternative. This paper presents the first analysis of key properties any fluorescent substance must possess to qualify as a staining fluorescent tracer in vadose zone hydrological applications. First, we summarize the main physico-chemical properties of Uranine and evaluate its staining tracer potential with conventional suitability indicators and visibility testing in a soil profile. Based on numerical analysis using the theory of fluorescence, we show that a low molar absorption coefficient is a crucial parameter to quantify concentration accurately. In addition, excitation of a tracer on wavelengths different from the maximum excitation wavelength can extend the linear range of the concentration-fluorescence relationship significantly. Finally, we develop criteria for evaluating the suitability of any potential fluorescent soil staining compound for soil hydrological applications: 1) high quantum yield, 2) low molar absorption coefficient, 3) fluorescence independent of temperature, 4) low photodecomposition rates, and 5) fluorescence stable across a wide range of pH values.

Keywords: Staining; Preferential flow paths visualization; Molar extinction coefficient; Tracer concentration quantification; Vadose zone hydrology.

INTRODUCTION

Accurately determining water fluxes and mass transfer in natural soils remains an important hydrological issue. The presence of preferential flow paths (PFP) affects soil water redistribution and results in quick catchment stormflow response and rapid transport of pollutants through the vadose zone (Alaoui et al., 2011; Flury et al., 1994; Sidle et al., 2000). Field investigations and modeling of water and tracer movement are important in studying numerous hydrological problems, including water resource contamination (Gödeke et al., 2006; Sudicky and Illman, 2011; Zheng et al., 2011), flood mitigation (Baker, 1987; Gubareva and Gartsman, 2010; Wood et al., 1990), and landslide initiation due to excess pore pressure generation (Iverson, 2000; Wilson et al., 2012). Usually the influence of PFP on mass transfer is determined experimentally by measuring water or tracer outflow from soil columns, lysimeters or in natural catchments (Everts and Kanwar, 1989; Ghodrati and Jury, 1992; Kung et al., 2000; Mallants et al., 1996; Tsuboyama et al., 1994; Vanderborght et al., 2000). Experimental data collected this way simplifies often tortuous flow pathways to a quasi-1-D process. To calibrate and verify 2-D and 3-D flow and transport models, however, detailed distributions of measured water content, pressure head or tracer concentration data are needed in multiple dimensions. Among various water flow and mass transfer determination methods, staining tracers have the best spatial resolution (Allaire et al., 2009), allowing the visualization and quantification of fluid flow and PFP at different scales. Although staining techniques have been used for several decades, the hydrological communi-

ty is still searching for an ideal vadose zone tracer (Flury and Wai, 2003). In the purest sense, such a tracer does not exist except for the water molecule itself (Käss, 1994). For tracers to be useful for investigations of hydrological pathways they must be selected based on soil type, scale, methodology, and other considerations.

Examples of successful implementation of staining techniques in soil hydrology include identifying and describing different filtration regimes (Weiler and Flühler, 2004); indicating the influence of PFPs on water flow (Alaoui et al., 2011; Flury et al., 1994; Tsuboyama et al., 1994); determining the hydrodynamic dispersion coefficients (Persson et al., 2005; Sidle et al., 1977); and evaluating the impact of preferential flow on chemistry (Bogner et al., 2012) and microbial activity (Bundt et al., 2001). One of the most popular staining dyes used in vadose zone hydrology is Brilliant Blue FCF. Evaluations of Brilliant Blue (BB) dye concentrations in a natural soil profile enabled quantification of the subsurface flux (Aeby et al., 1997; Forrer et al., 2000), in addition to more traditional assessments of stained and unstained portions of soil profiles. In the past decade fluorescent dyes have been proposed as staining tracers while methods for quantifying tracer concentrations were developed concurrently (Aeby et al., 2001; Duwig et al., 2008; Vanderborght et al., 2002). Compared to common dyes, fluorescent substances have several distinct advantages: (1) they are visible in soils of different colors, even very dark soils (Duwig et al., 2008; Hangen et al., 2004); (2) two or more fluorescent dyes with separated excitation and emission wavelengths can be used simultaneously (Aeby et al., 2001; Vanderborght et al., 2002); and (3) they allow examination of soil profiles at any

wavelength different from the excitation range of the staining dye being employed. Different fluorescent dyes have been used as staining substances, including Pyranine (Duwig et al., 2008), Rhodamines (McNeil et al., 2006), Sulforhodamine B, Brilliant Sulfoflavine, and Oxazine 170 (Aeby et al., 2001; Vanderborght et al., 2002). To the best of our knowledge, however, no criteria have been established for selecting fluorescent tracers for soil staining, and no comparison of dye properties that are significant for soil staining has been reported. Physico-chemical dye properties related to soil staining have been established for only a few dyes, including BB FCF (Flury and Flühler, 1995; German-Heins and Flury, 2000; Kasteel et al., 2002), while other fluorescent dye properties were mostly investigated in groundwater conditions such as karst zones (Käss, 1998).

Previous research on suitability of dye tracers for vadose zone applications identified the following critical properties that any tracer substance must possess (no criteria were established specific for staining with fluorescent dyes) (Flury and Wai, 2003; Käss, 1994): (1) high water solubility; (2) conservative and stable, i.e. tracer moves in a manner similar to water, without sorption, degradation and interaction with other soil substances; (3) low background concentration, i.e. is highly visible and easily discernible; (4) insensitive to pH and changes in ionic strength; (5) low toxicity and (6) inexpensive and easy to apply. The primary aim of this study was to develop criteria to evaluate fluorescent substances as potential staining tracers in vadose zone hydrological research, using the fluorescent dye Uranine as an example. Our specific objectives were to: (1) provide a summary of available literature data on Uranine's main physical and chemical properties; (2) evaluate other properties of this fluorescent dye that are important in view of its application as a staining agent in natural soils; (3) determine the extent to which Uranine satisfies the six conventional critical properties mentioned above; (4) elucidate the influence of excitation wavelength on these properties, and (5) to test if Uranine is qualified as a useful dye for vadose zone applications using additional fluorescent staining dye criteria developed in this study.

REVIEW OF PHYSICO-CHEMICAL PROPERTIES OF URANINE

As an alternative to previously used fluorescent dyes, an inexpensive and non-hazardous fluorescent dye Uranine (fluorescein sodium salt) has been proposed for quantitative staining in

soils (Gerke et al., 2008). In their exploratory experiments on natural forested hillslopes, Uranine was found to be a promising soil staining tracer and in concentrations of approximately 2 g L^{-1} was easily discernible in dark forest soils with the naked eye (Fig. 1). However, several reported Uranine properties differ significantly and are not established for excitation in the UV range, making evaluation for soil staining impossible without further studies. In this review section we summarize all known important physico-chemical properties of Uranine published in the open literature.

Uranine or sodium fluorescein is a well known dye with applications in medicine, karst hydrology, groundwater assessments, and environmental studies in other media. It emits a bright yellowish-green fluorescence color under blue light (480 nm) and a summary of its main chemical and physical properties is available in Table 1. Furthermore, Uranine molecules are known to be hygroscopic. Following purification of commercially available Uranine by dissolution in ethanol and drying in a vacuum oven, infrared spectral analysis revealed insignificant impurities, mostly due to the low water content (Mota et al., 1991). Uranine is also known to have a low toxicity to humans and other mammals; no adverse effects from inhalation have been reported (CRI, 2004).

Käss (1998) reports Uranine to be the most intensive fluorescent substance known; modern spectrofluorometers can detect concentrations as low as $0.002 \mu\text{g L}^{-1}$. However, comparing the fluorescence intensity of various hydrological tracers provided by Käss is different from data of other researchers (e.g., Lyons, 1993; Smart and Laidlaw, 1977). Lyons (1993) and Smart and Laidlaw (1977) used fluorimeters with filters to characterize dyes but did not specify concentrations in their comparisons; Käss (1998) obtained more reliable tracer data using a modern spectrofluorometer. Regardless of differences in reported fluorescence intensity, it is undoubtedly one of the dyes with the most intense fluorescence, as evidenced by its wide usage as a tracer and its extremely high quantum yield value of 0.85–0.94 (Heller et al., 1974; Schmidt, 2005).

Fluorescent properties of Uranine depend on many factors such as pH, temperature, sorption onto soil minerals and organic matter, concentration, energy of the incident light, and microbial decomposition. Understanding these factors is important when designing dye tracer tests under field conditions, where all factors may exhibit a wide range in values and may interact with the applied dye.

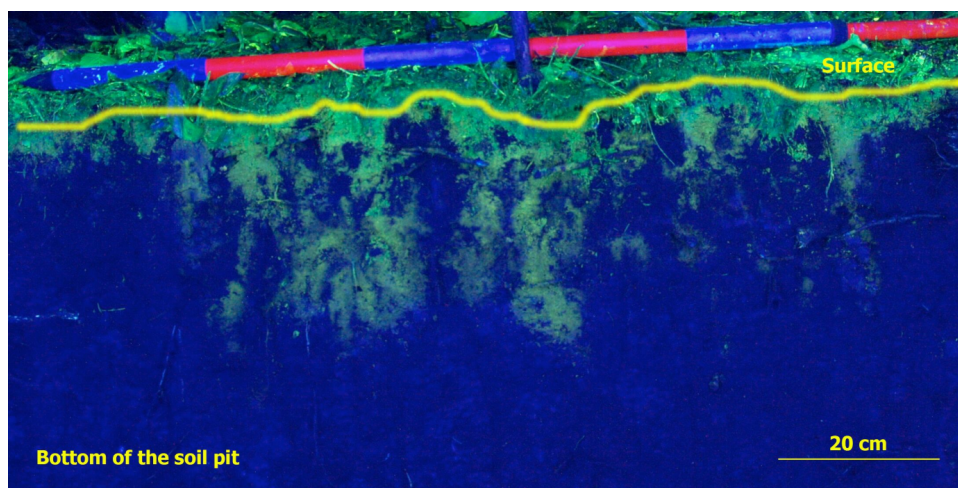


Fig. 1. Soil profile stained with 2 g L^{-1} Uranine solution (raw image). Picture was taken in UV light. One can observe a nearly similar image with the naked eye.

Table 1. Main chemical and physical properties of Uranine.

<i>Property</i>	<i>Value</i>	<i>Reference</i>
Chemical formulae	$C_{20}H_{10}Na_2O_5$	–
CAS #	518-47-8	–
Color Index	C.I. 45 350, Acid Yellow 73	–
Molar mass	378.28	–
Quantum yield	0.85–0.94	(Heller et al., 1974; Schmidt, 2005)
Extinction maxima	491 nm (main absorption) 322 nm (secondary maximum)	(Käss, 1998) (Käss, 1998)
Fluorescence maxima	512 nm	(Käss, 1998)
Molar extinction at 491 nm	$84000 \text{ L cm}^{-1} \text{ mol}^{-1}$	(Käss, 1998)
Molar absorption coefficient (pH = 8)	$67700\text{--}79000 \text{ M}^{-1} \text{ cm}^{-1}$	(Mota et al., 1991)
Solubility	in water $> 600 \text{ g L}^{-1}$, insoluble in benzene, hardly soluble in propanols, acetone; 10 g L^{-1} in ethanol	(Käss, 1998)
Diffusion constant in water	$1.9 \cdot 10^{-5} \text{ cm}^2 \text{ s}^{-1}$	(Kozlov and Sarzhevskii, 1975)
Dissociation constants	$P_{k1} = 1.95, P_{k2} = 5.05, P_{k3} = 7.00$ $P_{k1} = 2.27, P_{k2} = 4.32, P_{k3} = 6.50$	(Zanker and Peter, 1958) (Mchedlov-Petrosyan, 1979)
Temperature dependency coefficient ($^{\circ}\text{C}^{-1}$)	–0.0036 –0.0041	(Smart and Laidlaw, 1977) (Stampfli, 1983)
Freundlich isotherm parameters for forest soils:		
$K (\text{L kg}^{-1})$	0.09–154.1 (in alcalic-acidic solutions)	(Gerke et al., 2008)
n (dimensionless)	1–2.26	(Gerke et al., 2008)
Photodecomposition decay coefficient k	$4.5 \times 10^{-2} - 3.9 \times 10^{-1}$	(Smart and Laidlaw, 1977, Lyons, 1993)

In solid form, Uranine forms dark red crystals which are not fluorescent. Very concentrated solutions ($> 10 \text{ g L}^{-1}$) are also dark red and do not or only slightly fluoresce. In aqueous solutions, Uranine dissociates into sodium cations and fluorescein anions with the latter exhibiting fluorescence. Uranine anions can exist in four prototropic forms (cation, neutral molecule, univalent anion, bivalent anion) with equilibrium constants shown in Table 1. A detailed description of each form can be found in Förster (1951). Mota et al. (1991) estimated the fraction of each form as a function of pH using equilibrium constants obtained by Mchedlov-Petrosyan (1979). Because of pH dependency of the dissociation and different prototropic forms in solution, the fluorescence intensity of Uranine is pH dependent. Also, changes in the charge of the Uranine molecule affect its sorption characteristics including the kinetics (Gerke et al., 2008). The conversion of Uranine's anionic to the cationic form is reversible and after pH rises to values above approximately 7, fluorescence is restored to its original intensity. Different pH dependencies of fluorescence are reported in the literature (Hiramoto et al., 1964; Käss, 1998; Lyons, 1993; Romanchuk and Kenneth, 1982; Rozwadowski, 1961; Smart and Laidlaw,

1977) and differences are especially large for low (< 3) and high (> 8) pH values.

The pH dependency of fluorescence is important in developing soil staining procedures. Previously mentioned studies investigated different fluorescent properties of fluorescent compounds on the maximum extinction and excitation wavelengths. However, due to the particular reflecting properties of soil profiles, Uranine should be excited at a wavelength of 350 nm (Gerke et al., unpublished data). Thus, to correctly assess pH dependency of fluorescence, as well as dependency with different excitation wavelengths that quantify Uranine concentrations in natural soil profiles, an independent study was conducted herein and results were compared to other studies.

Temperature is another important factor to consider during measurements of fluorescence intensity because fluorescence intensity varies inversely with the heat energy of the solution due to the collisional quenching effect (i.e., decrease in fluorescence intensity) attributed to faster movement of molecules at higher temperatures. The half-width value of the fluorescence spectrum increases with increasing temperature, so lower temperatures produce more distinct fluorescence peaks (Käss,

1998). Experimentally determined fluorescence at different temperatures has been described previously by an exponential function (Smart and Laidlaw, 1977); the exponential coefficients for temperature dependency of Uranine are presented in Table 1. Temperature changes can affect the measurements performed by fluorometers (Dunn and Vaupel, 1965), but Uranine exhibits negligible changes ($< 0.5\%$) in fluorescence intensity within the temperature range of this study (from $+5^{\circ}\text{C}$ to $+25^{\circ}\text{C}$), so temperature corrections have been omitted. Adams and Davis (1991) showed that Uranine can be a relatively conservative tracer (i.e., no decomposition) in reservoirs with temperatures $< 210^{\circ}\text{C}$ for test durations < 1 month; thus, for most applications in soil hydrology temperature-driven decomposition is not an issue.

Field application of fluorescent dyes entails several challenges. For instance, there are several reasons why pore-water concentrations of fluorescent dyes in field measurements might be lower than the applied solution concentration, but the main one is the adsorption of dye molecules on the mineral surfaces and organic matter of soils (German-Heins and Flury, 2000; Sidle et al., 1977; van Genuchten et al., 1974). Comparatively few studies about the sorption characteristics of Uranine in field soils are available, but one detailed study of sorption of Uranine on forest soils showed that adsorption isotherms are comparable to isotherms for BB FCF dye on agricultural soils (Table 1; Gerke et al., 2008). Directly comparing the effect of soil type on sorption characteristics is difficult since dyes were never tested on the same soils; nevertheless, adsorption properties for Uranine on forest soil can be considered to be of the same order of magnitude as BB FCF on agricultural soil.

Another factor to consider when applying fluorescent dyes is their photochemical decomposition. When a fluorescent dye absorbs light, molecules are excited to higher energy states, and subsequently fluorescence is emitted when the molecules revert back to their ground states. High energy states more readily take part in chemical reactions compared to the ground state; thus, as the substance fluoresces, it often decomposes due to oxidization or other chemical reactions. Because decay rates depend on the energy of the incident light, photochemical decomposition (or photolysis) is dependent on both light intensity and wavelength. Decomposition is stronger at the excitation maxima wavelength (Käss, 1998) and increases with temperature (Behrens and Demuth, 1992), however, ultraviolet light is known to cause more rapid photodecomposition than longer wavelengths (Smart and Laidlaw, 1977).

Evaluating the photochemical decomposition rate is complicated in field conditions because it depends on factors such as dye concentration (e.g., fluorescent compounds in low concentration solutions will decompose faster than in high concentra-

tion solutions). Decay coefficients reported in the literature are usually in the exponential decay form. For Uranine, decay coefficients (Table 1) for day light conditions are approximately 5–8 times higher than for Rhodamine dyes (Smart and Laidlaw, 1977). Käss (1998), for example, exposed a $10\text{ }\mu\text{g L}^{-1}$ solution of Uranine to direct sunlight in clear and brown bottles; the brown bottle significantly reduced the photodecomposition. Rapid photodecomposition by excitation light during fluorometer measurements also can affect accuracy (Lakowitz, 1999).

Biodegradation can affect tracers in biologically active environments (Smart and Laidlaw, 1977). Uranine biodegraded within 6 weeks in natural river water, but no concentration change was observed in autoclaved river water (Käss, 1998). In soil applications, soil staining experiments usually take about 5–48 h (Aeby et al., 2001; Alaoui et al., 2011; Anderson et al., 2009; Duwig et al., 2008; Flury et al., 1994; Persson et al., 2005; Tsuboyama et al., 1994; Vanderborght et al. 2002; Weiler and Flühler, 2004) and biodegradation will be limited within the experimental timeframe. Hence biodegradation can generally be considered negligible.

Numerous studies on toxicity of hydrological tracers have been conducted (Aeby, 1998; Flury and Flühler, 1994). Compared to other dyes, Uranine has average to high mean lethal dose values for fish and rats (Käss, 1998). It is used in ophthalmology to color blood vessels and investigate disorders of the choroid, optical nerve, and retina. In these applications, about 10 mL of a 5% solution of Uranine typically is injected intravenously. Käss (1998) provides an excellent review on the toxicity of Uranine in medical applications and in experiments on animals. Overall, Uranine is known to have a low toxicity and no adverse effects from inhalation have been reported (CRI, 2004). Thus, there should be no adverse health effects associated with its application as a field dye tracer.

MATERIALS AND METHODS

To analyze fluorescent dye solutions, a fluorometer or fluorescence spectrophotometer was used (Fig. 2). Measurements involve illuminating a cuvette containing dye with incident light of a known wavelength (adjusted by a prism monochromator) to excite fluorescence in the sample, which was measured at the expected peak extinction (absorption) wavelength. Due to the Stokes shift effect, the emitted light has a longer wavelength than the absorbed light. For a new compound, excitation (illumination) and/or extinction maxima generally are unknown. The excitation maximum was determined by applying a spectrum of excitation wavelengths to a



Fig. 2. Fluorescence measuring procedure: a) the view of Hitachi spectrofluorometer and b) angular positioning of the cuvette.

series of fixed extinction wavelengths. If the excitation maximum could not be obtained in this way, the procedure was repeated with different fixed values of extinction wavelengths. Once the excitation maximum was found, the peak extinction wavelength was assessed in the same manner. When the extinction and excitation maxima were known, the fluorescence calibration curve (which provides the relationship between fluorescent compound concentration and fluorescence intensity) for a particular fluorescent compound was assessed by measuring the fluorescence of samples of different known concentrations.

Cuvettes can be configured differently relative to incident light and the excited fluorescence receiver. Uranine has a small Stokes shift and is particularly sensitive to concentration quenching. Inner filtering effects have also been observed during calibration. Here, angular configuration is preferable (Lakowitz, 1999). A disadvantage of this method is lower sensitivity which could affect measurements, including those discussed in this paper. However, because our concentrations of interest were higher and coincided with the beginning of the linear part of the calibration curve, this particular positioning of the cuvette was chosen (Fig. 2b).

We measured fluorescence intensity in a range of wavelengths including UV light using quartz cuvettes with 80% transmission capacity at 250 nm wavelength. Fluorescence intensities of Uranine solutions were measured with a Hitachi-850 spectrofluorometer (Fig. 2a). To obtain the calibration curve, 30 standard concentration samples were carefully prepared (dry Uranine weighed to ± 0.0001 g) with concentrations ranging from $0.00745 \mu\text{g L}^{-1}$ to 4 g L^{-1} . To eliminate possible errors due to the hygroscopic properties of Uranine (Kesavan and Doherty, 2001), three additional samples with concentrations of $10 \mu\text{g L}^{-1}$, 0.5 mg L^{-1} , and 2 mg L^{-1} (all within the linear range) were processed in the same way as the previous 30 samples, however, we used Uranine from a sealed container that was not previously used; hence, water uptake by Uranine from the atmosphere could not occur. Measured values indicate that the previously obtained calibration curve was sufficiently accurate and that hygroscopic effects were negligible (results not shown).

Measurements with a spectrofluorometer at a particular wavelength took less than 1 min, while a spectrum scan with a 200 nm wavelength range took about 20 min. As mentioned previously, photodecomposition can affect measurements both in laboratory and field conditions. To test whether excited fluorescence measurements are affected by photodecomposition, five Uranine solutions with concentrations of $1 \mu\text{g L}^{-1}$, $10 \mu\text{g L}^{-1}$, 1 mg L^{-1} , 10 mg L^{-1} , and 1 g L^{-1} (all at pH 10) were exposed to excitation light beams of five different wavelengths (300, 350, 400, 450 and 490 nm). Fluorescence intensities were measured before and 30 min after exposure. Further tests determined the rate of photodecomposition of Uranine due to the UV light. Solutions of low (1 mg L^{-1}) and high (1 g L^{-1}) concentration (pH adjusted to 10) were exposed to UV light for 1 h in quartz cuvettes that were closed to prevent evaporation. Spectrofluorometer measurements of the Uranine concentration were made every 10 min. In terms of excitation light source and distance between source and target Uranine compounds, test conditions were similar to those of a field experiment (see Fig. 1, four 40 Watt UV light bulbs with 350 nm wavelength peak separated 1.2 m from the soil profile).

To evaluate dependence of fluorescence intensity on pH, 16 Uranine solutions with concentrations of either 1 mg L^{-1} or 2 g L^{-1} were prepared; for each concentration, subsamples were adjusted to different pH levels (1, 2, 3, 4, 7, 9, 10 and 13) with

commercially available buffering solutions (pH 4, 7 and 10), NaOH and HCl. The solution pH was measured on a Horiba pH meter. First, the extinction maximum was determined by scanning the fluorescent intensities at an excitation wavelength of 350 nm in the range of 450 to 650 nm. After the peak extinction wavelength was determined, the excitation spectrum associated with the extinction maximum was scanned. Extinction maximum wavelengths may differ for samples with different pH, but for any excitation wavelength, the peak will be the same as for the extinction maximum. For instance, the emission spectra that were scanned for the 350 and 491 nm excitation peaks exhibited extinction maximum peaks at the same wavelength (513–514 nm), while fluorescence intensity differed (Fig. 3).

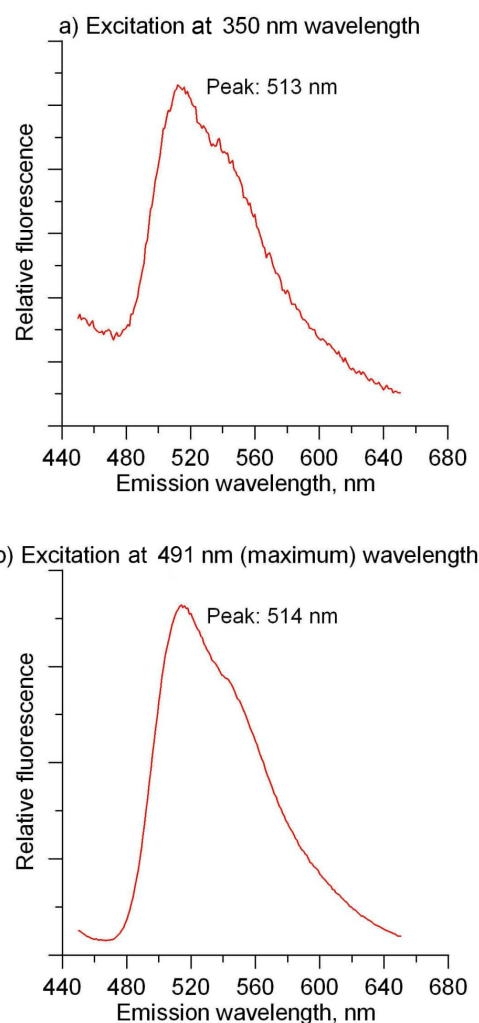


Fig. 3. Emission spectra for Uranine solution excited with (a) 350 nm and (b) 491 nm wavelengths.

RESULTS

The relationship between Uranine fluorescence intensity and Uranine solution concentration (for pH = 10) displays a complex behavior (Fig. 4a), which is linear in the concentration range from $1 \mu\text{g L}^{-1}$ to 2 mg L^{-1} (Fig. 4b). Käss (1998) reported the lower range of the linear portion of the calibration curve to be $10^{-12} \text{ g L}^{-1}$, which is much lower than in this study. Due to cuvette configuration or lower sensitivity of the spectrofluorometer used herein, the lower limit of the linear range was higher, but sufficient for this study, because concentrations of

interest were always above or within the linear range – from 0.0001 to 4 g L⁻¹ for adsorption studies (Gerke et al., 2008), and from 0.075 (detection limit specific to the test conditions similar to Fig. 1) to 2 g L⁻¹ for field experiments. At concentrations in excess of 2 mg L⁻¹, self-quenching occurred (Fig. 4a). Quenching indicates a process which reduces the fluorescence intensity of a particular compound; fluorescence self-quenching refers to quenching between identical molecules (Lakowitz, 1999). To measure fluorescence at concentrations higher than 2 mg L⁻¹, the solutions must be diluted to values within the linear range of the calibration curve. When the pH value of a solution was different from 10, it was diluted with buffering solution to ensure that solution's pH is similar to that of the calibration curve. It should be noted that the calibration curves obtained are valid only for the particular spectrofluorometer used in this study because every instrument usually has its own sensitivity, resulting in machine-specific calibrations (see Discussion).

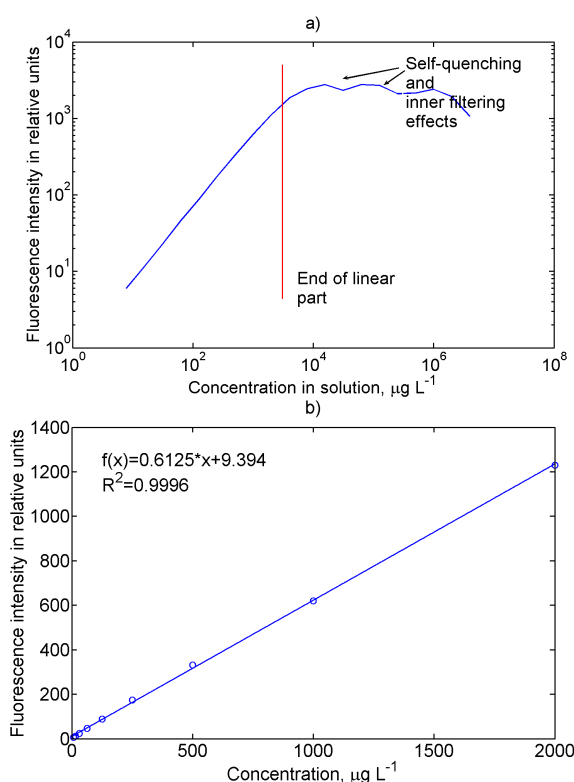


Fig. 4. a) Relationship between Uranine concentration and fluorescence and b) linear part of the calibration curve.

After four samples with Uranine concentrations of 10 µg L⁻¹, 1 mg L⁻¹, 10 mg L⁻¹ and 1 g L⁻¹ were exposed for 30 min to excitation light beams with wavelengths of 300, 350, 400, 450 and 490 nm, only slight differences of measured fluorescence intensities were observed compared to their initial fluorescence intensities. For example, for the 10 µg L⁻¹ concentration sample, a change of < 1% was observed. We conclude that errors in fluorescence intensity measurements do not arise due to photodecomposition by the excitation beam during both point and spectra scan evaluations in our concentration ranges. In a related field and laboratory study of fluorescence detection in natural soils, vertical soil profiles containing fluorescent dye were exposed to UV light for 1–10 min; this exposure time is sufficient for soil profile preparation and digital image acquisition. In the laboratory study, Uranine samples with concentrations of 1 mg L⁻¹ and 2 g L⁻¹ showed no changes in fluorescent intensities after 30 min exposure to UV light with the same exposure

conditions (UV light intensity and distance to the UV lamps). Therefore, photodecomposition is considered negligible for all our fluorescence measurements, including both laboratory spectrofluorometer and field experimental studies.

Measured excitation and emission spectra for different pH values show that for a 1 mg L⁻¹ solution concentration, the emission maximum is almost stable around a wavelength of 512 nm (Fig. 5a). For the 2 g L⁻¹ concentration, emission peaks tended to be around 525 nm (Fig. 5b). Excitation maxima for the 1 mg L⁻¹ concentration solution are stable in the pH range 7 to 13 and equal at 491 nm (Fig. 6a); at acidic pH values (pH < 4), this maximum shifted to 438 nm. For the 2 g L⁻¹ Uranine concentration, the excitation peak was consistently around 509 nm in alkaline and neutral pH solutions, but more dispersed maxima at shorter wavelengths (436 to 480 nm) were observed in acidic solutions (pH < 4; Fig. 6b). Peak shifts should depend on form of the molecule. There are four monomeric forms in the pH range from 0 to 13, each with its own P_k value (see Table 1). As noted by Mota et al. (1991) for high concentrations dimmers and polymers may be formed, which in turn not only result in the decrease and quenching of fluorescence, but can also affect the relationship between fluorescence and pH.

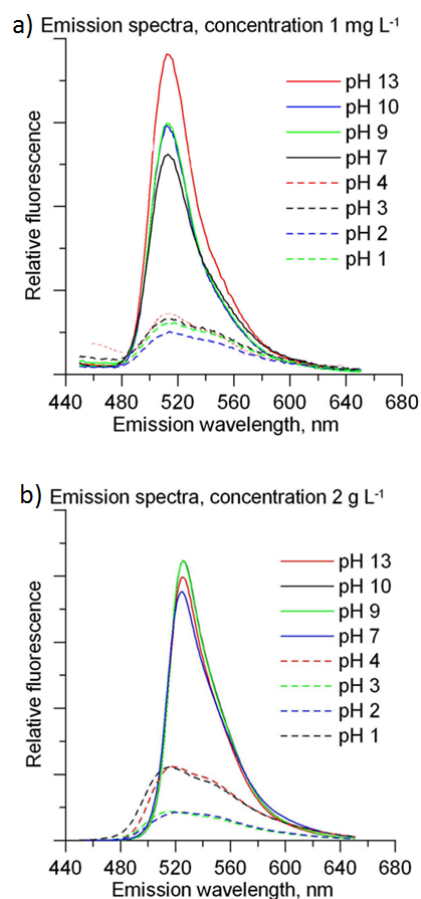


Fig. 5. Emission spectra of Uranine in solution measured on excitation wavelength of 350 nm with concentration of (a) 1 mg L⁻¹ and (b) 2 g L⁻¹.

Measurements of fluorescence intensity at different pH values for various excitation wavelengths including the excitation maxima (peak of the excitation spectrum, see Table 2) and the 350 and 491 nm wavelengths provide insight into pH dependence at low and high concentrations (Fig. 7). Considering the excitation wavelength of 491 nm, the pH dependence is similar

to that found by Käss (1998). For a correct representation of pH dependency of fluorescence, excitation and emission peak shifts must be considered (Fig. 7). The fluorescence-pH relationship curve presented by Lyons (1993) differs from ours. Lyons (1993) did not measure the shift in excitation/emission peaks due to pH changes, but instead used a filter fluorometer with a fixed bandwidth; as a result, this fluorescence-pH relationship curve would be different if shifted wavelengths for changes in pH were considered. Measuring the pH dependency at only a single (peak) excitation wavelength may lead to erroneous results in determining Uranine concentrations from fluorescence measurements. This is true not only for Uranine, but for other fluorescent dyes as well. Earlier findings, such as those by Smart and Laidlaw (1977), may therefore need to be reconsidered, especially if another excitation wavelength is to be used. Our analyses indicate that excitation at the 350 nm wavelength leads to a relatively small pH dependency in the pH range from 4 to 13 (Fig. 7a). All Uranine fluorescence data including excitation and emission peaks and relative fluorescence intensities measured at emission maximum for different excitation wavelengths at different pH values are presented in Table 2.

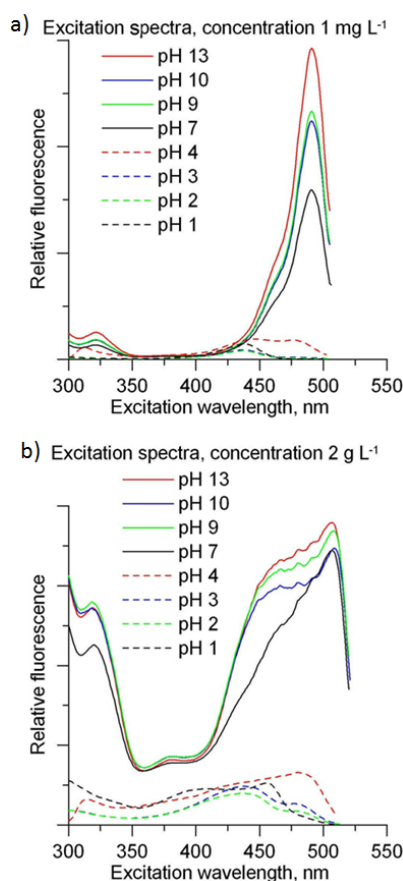


Fig. 6. Excitation spectra of Uranine in solution measured on emission maxima wavelengths for corresponding pH with concentration of (a) 1 mg L⁻¹ and (b) 2 g L⁻¹.

DISCUSSION

A particularly useful observation from this study relates to the dependency of excitation and emission peaks on pH and Uranine solution concentration. Excitation on wavelengths different from maximum excitation and shifts due to pH change can be used to separate different fluorescent tracers whose corresponding excitation and emission peaks are similar, more

efficiently. The nature of such shifts is a specific property for a given fluorescent compound and depends on molecule structure.

For a fluorescent dye dissolved in a non-absorbing and non-scattering medium, the fluorescence intensity F (W cm⁻²) is a function of incident light intensity (I) and the molar dye concentration (c):

$$F = k \times Q \times I (1 - e^{-2.303c \times m \times d}), \quad (1)$$

where Q is the dye's fluorescence quantum yield (ratio of number of photons emitted to number of photons absorbed) (–), I is the intensity of the incident light (W cm⁻²), c is molar concentration of the dye in solution (mol L⁻¹), m is molar absorption coefficient of the dye according to the Beer-Lambert law (mol⁻¹ L cm⁻¹), and d is the thickness of the absorbing layer (cm), (Rendell and Mowthorpe, 1987). The proportionality constant k (–) depends on instrument settings such as detector sensitivity, aperture, and exposure time. Clearly, higher quantum yield will result in higher visibility, lower exposure times and lower concentrations requirements for field applications. We establish high quantum yield as a first criterion to evaluate any potential fluorescent dye for soil staining.

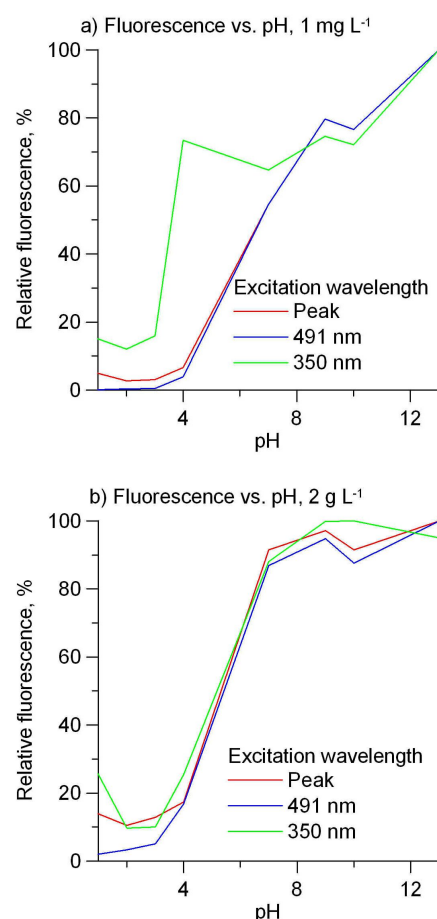


Fig. 7. pH dependency of fluorescence intensity of Uranine in solution measured on excitation maxima, 491 nm and 350 nm wavelengths with concentration of (a) 1 mg L⁻¹ and (b) 2 g L⁻¹.

Dye solutions for which the exponent $2.303c \times m \times d$ from Eq. (1) is less than 0.05 can be linearly approximated by (Aeby et al., 2001; Vanderborgh et al., 2002):

Table 2. Excitation and emission peaks, fluorescence intensity of Uranine.

pH	Peak (nm)		Relative fluorescence (%)**			Concentration
	Excitation	Emission	Peak	491 nm	350 nm	
13	491	512	100*	100*	100*	L ¹
10	491	512	76.67	76.67	72.14	L
9	491	512	79.72	79.72	74.56	L
7	491	513	54.48	54.48	64.75	L
4	448	511	6.58	3.94	73.41	L
3	438	512	3.05	0.48	15.99	L
2	438	514	2.77	0.31	12.05	L
1	438	518	4.97	0.08	15.08	L
13	507	525	100*	100*	95.07	H ²
10	509	526	91.51	87.59	100*	H
9	508	525	97.18	94.83	99.92	H
7	507	525	91.51	86.94	88.04	H
4	480	519	17.40	16.76	25.33	H
3	439	516	12.81	5.06	10.00	H
2	436	525	10.48	3.34	9.73	H
1	456	516	13.97	2.00	25.57	H

* Values taken as 100% for each column for each concentration, 1 – 1 mg L⁻¹ 2 – 2 g L⁻¹

**Relative fluorescence at: (i) peak excitation (values from column 2); (ii) excitation wavelength of 491 nm; and (iii) excitation wavelength of 350 nm.

Table 3. Different parameters for critical concentration calculations.

Group*	Quantum yield used for computations	m (l cm ⁻¹ mol ⁻¹) (reference)	c (mol l ⁻¹) (g l ⁻¹)	d (cm)	Indication of linear behaviour (Eq. (3))	Reference
1-U	0.85	$8.4 \cdot 10^3$ (Käss, 1998)	$2.26 \cdot 10^{-4} (0.075) \div 0.006(2)$	$6 \cdot 10^{-3}$	$c = 4.3 \cdot 10^{-4} (0.15 \text{ g l}^{-1})$	(Gerke, 2008; current study)
1-U350	0.85	$1 \cdot 10^3$ (Käss, 1998)	$2.26 \cdot 10^{-4} (0.075) \div 0.006(2)$		$c = 34 \cdot 10^{-4} (1.2 \text{ g l}^{-1})$	
2-BS	0.274	$16 \cdot 10^3$ (Aeby, 1998)	$1.2 \cdot 10^{-4} (5 \cdot 10^{-2}) \div 0.012(5)$		$c = 9 \cdot 10^{-5} (0.04 \text{ g l}^{-1})$	
2-SB	0.186	$84 \cdot 10^3$ (Aeby, 1998)	$1.7 \cdot 10^{-5} (10^{-2}) \div 3.4 \cdot 10^{-4} (0.2)$	$15 \cdot 10^{-3}$	$c = 1.7 \cdot 10^{-5} (0.001 \text{ g l}^{-1})$	Vanderborght et al. 2002)
3-RWT	0.65	$50 \cdot 10^3$ (Simon et al., 1988)	$8 \cdot 10^{-6} (5 \cdot 10^{-3}) \div 8 \cdot 10^{-4} (0.5)$	0.1^{-1}	$c = 4.3 \cdot 10^{-6} (2.5 \cdot 10^{-3} \text{ g l}^{-1})$ $c = 4.3 \cdot 10^{-7} (2.5 \cdot 10^{-4} \text{ g l}^{-1})$	(Schincariol et al., 1993; McNeil et al., 2006)
4-U	0.85	$8.4 \cdot 10^3$ (Käss, 1998)	$3 \cdot 10^{-5} (0.01) \div 4.8 \cdot 10^{-4} (0.16)$	$\approx 15 \cdot 10^{-3}$	$c = 1.7 \cdot 10^{-4} (0.06 \text{ g l}^{-1})$	(Garrido et al., 2000; Duwig et al., 2008)
4-P	0.65	$43 \cdot 10^3$ (Kotlyar et al., 1996)	$4.8 \cdot 10^{-4} (0.25) \div 3.8 \cdot 10^{-3} (2)$		$c = 3.4 \cdot 10^{-5} (0.017 \text{ g l}^{-1})$	

*1-U: Uranine excited at 491 nm wavelength; 1-U350: Uranine excited at 350 nm wavelength; 2-BS: Brilliant Suflavine; 2-SB: Sulforhodamine B; 3-RWT: Rhodamine WT; 4-U: Uranine; 4-P: Pyranine.

$$F = 2.303k \times Q \times I \times c \times m \times d. \quad (2)$$

Calibration curves for fluorescent tracer concentration quantification have been reported for: (1) simplified porous media like glass beads (McNeil et al., 2006; Schincariol et al., 1993); and (2) real soil using photodetectors (Aeby, 1998; Vanderborght et al., 2002) or fiber optic miniprbes (Garrido et al., 2000). Most published calibration curves have a linear component in the lower concentration range but become nonlinear at higher concentrations and typically have an exponential shape. For measurements involving fluorescent dye solutions, the nonlinear shape can be easily explained by fluorescence spectroscopy theory (Schmidt, 2005) and Eq. (1). As we demonstrated in Fig. 4 for Uranine, a linear calibration curve exists only for a very limited range of concentrations where curvature effects are negligible.

To define the limits of validity of Eq. (2), values or ranges of values for m , c and d listed in Table 3 were used to calculate

critical concentrations for which the inequality $2.303c \times m \times d < 0.05$, and, thus, the linearity between F and I no longer exists:

$$c_{\text{crit}} = \frac{0.02171}{m \times d}. \quad (3)$$

Data in Table 3 are divided into four groups that correspond to four research groups conducting tracer studies. Several publications report Uranine molar absorption coefficient values (m) that differ significantly from the values in Table 3. The difference in m values was investigated by an independent calculation that provides evidence for validity of the values determined by Käss (1998). The independent calculation involved estimating the m coefficient at the concentration for which the calibration curve becomes nonlinear. Substituting our previously obtained values of $c = 2 \text{ mg L}^{-1}$ and $d = 1 \text{ cm}$ (width of the cuvette) into the equation $2.303c \times m \times d = 0.05$ and solving for m gives $m = 3600 \text{ L cm}^{-1} \text{ mol}^{-1}$, which is in a relatively good agreement with the value of $8400 \text{ L cm}^{-1} \text{ mol}^{-1}$ (Käss, 1998).

(Table 3). However, the latter value is valid for only the maximum excitation wavelength. In the case of excitation with 350 nm, m is substantially smaller and can be estimated for subgroup #1-U350 (Table 3) using the difference in absorption between the maximum and 350 nm excitation wavelengths (Schmidt, 2005). For group #2 tracer studies, initial concentrations in solution were used (Vanderborcht et al., 2002). Group #3 represents Hele-Shaw cell experiments with Rhodamine WT plume evolution where the depth of the fluorescent layer d was estimated to be 0.1–1 cm. For group #4 studies, our calculations used an approximate value of $d = 15 \cdot 10^{-3}$ cm according to findings of Aeby (1998); the depth of the fluorescent layer d depends on excitation and emission wavelengths, soil profile optical properties, grain size (Gerke, 2008), and water content (Bänninger et al., 2006).

Eq. (1) was further used to calculate a theoretical calibration relationship. All model parameters (Q , m , d , and c), except the experimental setup dependent k and incident light intensity I , were obtained from published literature. A simple iterative technique based on linear programming determined k while all other parameters were fixed and equal to their experimentally

calibrated values ($Q = 0.85$ and $m = 8400 \text{ L cm}^{-1} \text{ mol}^{-1}$) for Uranine using values from Table 1. We used an arbitrary value of $I = 100 \text{ photons cm}^{-2} \text{ s}^{-1} \text{ nm}^{-1}$ for simplicity (note that I also depends on experimental setup, e.g., power of lamps, and can be fixed together with k as a single variable), and $d = 1 \text{ cm}$ as the side length of the cuvette. Based on a least squares analysis an almost perfect fit was obtained with the optimized $k = 132$ (Fig. 8a). We then used those parameter values for k ($k = 132$) and I ($I = 100 \text{ photons cm}^{-2} \text{ s}^{-1} \text{ nm}^{-1}$) in all following calculations, unless stated otherwise.

For a Uranine concentration range up to $10^6 \mu\text{g L}^{-1}$, a comparison is shown (Fig. 8b) between the theoretical calibration curve and laboratory measurements for U490 using a molar extinction (absorption) coefficient of $1000 \text{ L cm}^{-1} \text{ mol}^{-1}$ (Table 3). For comparison, the theoretical calibration curve for U350 using a molar extinction coefficient of $8400 \mu\text{g L}^{-1}$ is shown. Comparing the theoretical U350 and U490 curves clearly indicates the advantage of excitation at 350 nm wavelength, since this significantly extends the linear part of the curve from 0.15 to 1.2 g L^{-1} . Discrepancies between the theoretical U490 curve

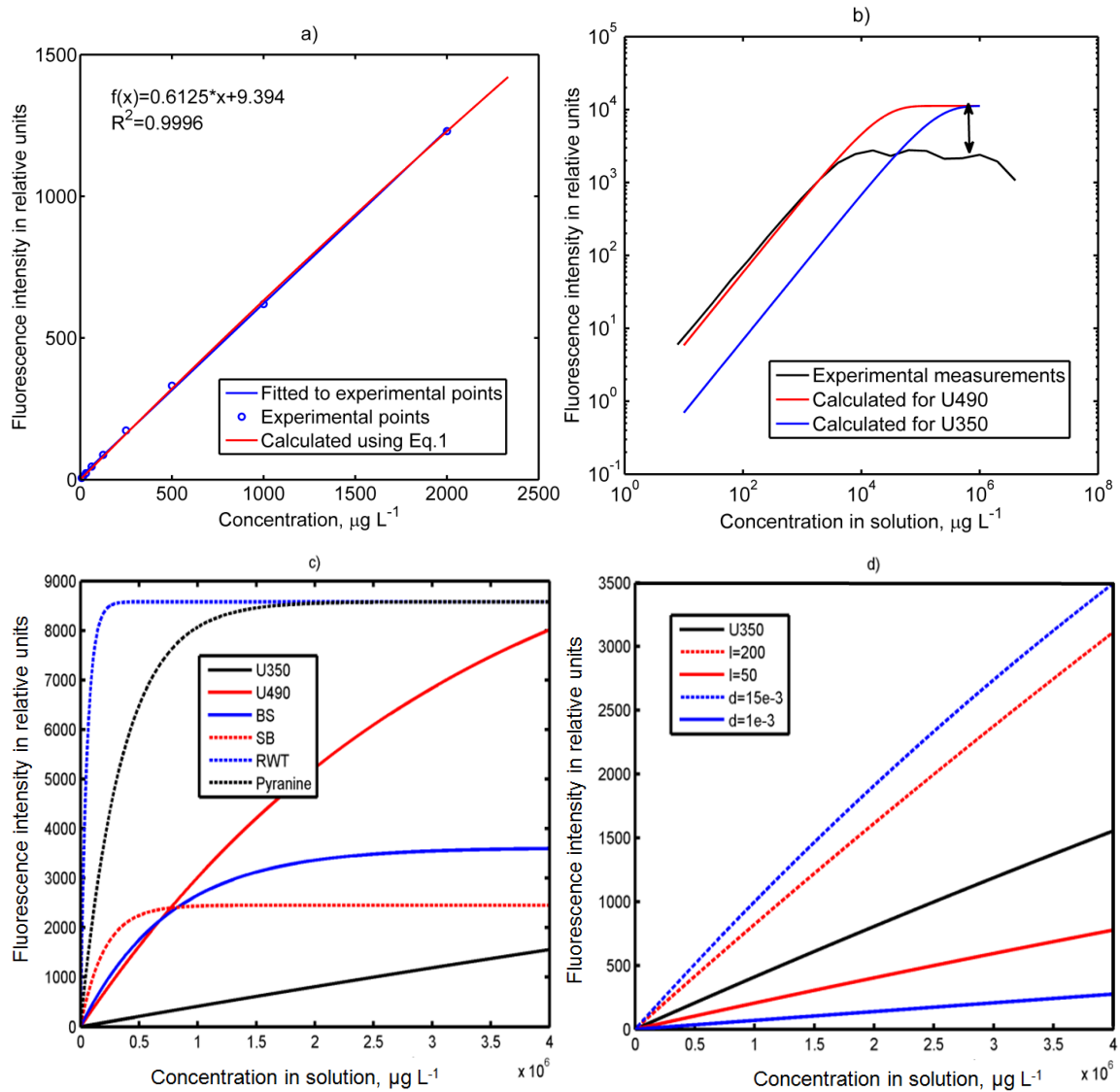


Fig. 8. Numerical study of dye fluorescence using Eq. (1): (a) Experimental and theoretical calibration curves for U490; (b) comparison of experimental (U490) and theoretical fluorescence curves (U490 and U350); (c) comparison of theoretical fluorescence curves for different fluorescent compounds; and (d) influence of incident light intensity I and fluorescent layer depth d on resulting fluorescence signal for U350.

based on Eq. (1) and measurements in the concentration range 10^4 – 10^6 $\mu\text{g L}^{-1}$ (see double arrow, Fig. 8b) are likely the result of two factors: (1) the classical theory expressed through Eq. (1) does not consider effects of collision quenching and self-filtering, both of which result in decreased fluorescence intensity attributed to solutions with high concentrations (Duwig et al., 2008); and (2) due to the angular position of the cuvette (Fig. 2b) the value used for parameter d ($d = 1$ cm) is overestimated.

Fig. 8c shows fluorescence curves calculated for different dyes: Uranine (U490 and U350), Brilliant Sulfaflavine (BS), Sulforhodamine B (SB), Rhodamine WT (RWT), and Pyranine (for each compound molar extinction coefficients m , quantum yields Q and depths of fluorescent layer d are taken from Table 3). Uranine excited at a 350 nm wavelength (U350) has the longest linear range in the concentration-fluorescence relationship, followed by U490 and Brilliant Sulfaflavine. Only these three dyes exhibit almost linear concentration-fluorescence relationships within the concentration range desired for field applications (up to 4 g L^{-1} , see Fig. 4). All other dyes (Sulforhodamine B, Rhodamine WT and Pyranine) exhibit a linear relationship only for concentrations up to 1–10 $\mu\text{g L}^{-1}$.

To further evaluate the influence of other parameters in Eq. (1), we plotted fluorescence signal curves for U350 with variable incident light intensities I and depth of fluorescent layer d (Fig. 8d). In field applications, the I parameter can be varied by using more powerful UV-light sources. As shown in Fig. 8d a four-fold higher light intensity I does not change the linear relationship, but results in significantly improved visibility of staining patterns. Changes in the d parameter (from 10^{-3} to 15×10^{-3} cm) have similar effects; however, modifying this parameter under field conditions requires knowledge about numerous factors, e.g., dye chemical composition and soil optical properties. When such parameters are determined, their impact can be assessed using, for example, the Kubelka-Munk theory.

Analysis of the data in Table 3 and Fig. 8 demonstrates that for all fluorescent dyes except Uranine excited at 350 nm wavelengths, critical concentrations beyond which the linear approximation no longer holds are lower than the lowest boundary of concentrations applied in field experiments. For example, for the Garrido et al. (2000) experiments, Eq. (1) was used to calculate a critical Uranine concentration of 60 mg L^{-1} assuming $d = 15 \times 10^{-3}$ cm; the calibration relationship used by Garrido et al. (2000) displayed a slightly curved behavior for a concentration range up to 160 mg L^{-1} . Experimental difficulties occurred when dyes with a high absorption coefficient were used (e.g., Pyranine; see Table 3) or when concentrations were high (up to 40 g L^{-1} in the case of Duwig et al., 2008). The latter conditions typically result in inner filtering and self-quenching effects (Fig. 4). Aeby (1998) and Vanderborcht et al. (2002) experienced the same difficulties for high concentrations of Sulforhodamine B fluorescent dye; i.e., their calibration curve displayed an exponential shape (see Fig. 2.4 in Aeby (1998) and Fig. 3 in Vanderborcht et al. (2002)). In these same studies the calibration curves for Brilliant Sulfaflavine fluorescent dye were linear for a wide range of soils and concentrations of the staining tracer, in contradiction to our theoretical calculation of the critical concentration. A reasonable explanation for the discrepancy could be a low precision due to the noise which results in poor visibility in their work (Jan Vanderborcht, personal communication), or because the influence of adsorption of the tracer on its fluorescent properties was not considered in their analysis.

Note that theoretical critical concentrations based on Eq. (3) are approximate; experimental evidence shown here illustrates that useful calibrations can sometimes be obtained when concentrations exceed the critical value c_{crit} . For instance, a calibration relationship for concentrations beyond the critical value of Eq. (3) for Brilliant Sulfaflavine ($c_{\text{crit}} = 0.04$ g L^{-1}) will still be quite accurate, while errors for Sulforhodamine B ($c_{\text{crit}} = 0.001$ g L^{-1}) can be considerable (Fig. 8c). Indeed, reasonable estimations can still be achieved for higher concentrations for SB (up to 0.25 g L^{-1}) and for BS (1 g L^{-1}), however the accuracy can be significantly compromised, especially if signal-to-noise ratios are poor for the given experimental condition. The SB fluorescence signal reaches an asymptotic value beyond a concentration of 0.5 g L^{-1} , making any quantification impossible, while for BS, the curve is almost flat and hence useless for concentrations higher than 2 g L^{-1} (Fig. 8c).

Among the most frequently used fluorescent tracers for subsurface hydrology, Uranine has the lowest molar absorption coefficient, m (Table 3). A low m value allows more precise quantification for a wider range of concentrations in natural soil profiles. A low molar absorption coefficient should also positively affect the depth, d , of the fluorescent layer, since dye substances with low m values will be less affected by soil profile texture, increasing the accuracy. Thus, having a low molar absorption coefficient is the second important criterion we elucidate for a successful dye tracer (in addition to the first criterion of high quantum yield established earlier). As mentioned before, temperature changes can drastically alter fluorescence intensity of dye solutions, which yields another important tracer criterion: fluorescence should be temperature independent. Low photodecomposition rates also have field implications because concentrations should remain stable during soil profile treatments and photographing. The last criterion concerns the dependency of fluorescence on pH; this relationship should be stable to minimize measurement uncertainties and simplify calibrations. An optimal dye satisfying all these properties probably does not exist and tracers should be carefully selected with criteria developed here and analysis of soil properties and fluorescence relationships based on Eq. (1) (see Appendix).

CONCLUSIONS

The fluorescent dye Uranine has been used successfully as a hydrological tracer for more than a century, but its usefulness as a vadose zone tracer has not been discussed much. We summarized the physical and chemical properties of Uranine that are relevant to its use as a vadose zone tracer. By undertaking laboratory experiments we demonstrated that Uranine has negligible fluorescence dependency on temperature, and negligible influence of photodecomposition on fluorescence. On the other hand, Uranine fluorescence was shown to depend strongly on pH, but the observed dependency was different from previously reported values in studies where only one excitation wavelength was used. The observed pH dependency was the lowest at the excitation wavelength of 350 nm. Exciting fluorescence on wavelengths other than its excitation maximum was extremely useful in determining beneficial Uranine properties for soil tracing tests. For example, lower wavelengths can be used to obtain a smaller molar absorption coefficient or adjust other fluorescent properties (such as fluorescence intensity) of the dye or reflection properties of the soil profile.

A calibration curve developed for Uranine was found to be linear for concentrations < 2 mg L^{-1} and our results agree with published values (Käss, 1998). Uranine has characteristics that

makes it a promising staining tracer for vadose zone hydrological applications: (1) good water solubility; (2) visibility under UV light that is easily discernible in any soil and has no background concentrations; (3) low toxicity; (4) inexpensive compared to other fluorescent dyes (factor of 10); and (5) sorption characteristics for forest soils comparable to brilliant blue (BB FCF). A disadvantage of Uranine is its sensitivity to pH which can significantly reduce detection limits for acidic soils. One advantage of a fluorescent dye like Uranine over common dyes like BB FCF is that its calibration curve can be obtained with high accuracy using only one grey-scale intensity instead of three levels (RGB).

In addition to conventional critical properties based on rigorous analysis of Uranine's properties we have developed the following new criteria for selection of a successful fluorescent staining tracer for soil hydrological applications:

- high quantum yield,
- low molar absorption coefficient,
- fluorescence independent of temperature in the relevant temperature range for field or laboratory applications,
- negligible photodecomposition during field and laboratory applications,
- fluorescence that exhibits high intensities for all pH values.

We have demonstrated that Uranine conforms to all these criteria and is therefore a potentially useful tracer for vadose zone applications. Additional studies to verify its potential under field conditions are under way.

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Appendix

This section contains the code that was used to plot Fig. 8c. It is written for Octave and Matlab environments. Each segment of the code is explained in the comments. The last section "Potential staining dye" is an easy-to-plug snippet for any potential fluorescent staining dye to be tested.

```
clear all
k=132; %This parameter can be tuned using an existing calibration curve
I=100; %Take 100 for simplicity, as  $kxI = \text{const}$ 
```

```
%Uranine350
c_min=0; %Starting concentration
c_max=0.0027/1000000*4000000; %Up to what concentration do we want to plot the relationship
%for Uranine 1 g = 0.0027 mol of dye; normalized to be in  $\mu\text{g}$ ;
%concentrations up to  $4 \times 10^6 \mu\text{g}$  (=4 g)
c=c_min:(c_max-c_min)/100000:c_max;
Q=0.85; %Uranine's quantum yield
m=1000; %Uranine's molar absorption (estimated for 350 nm excitation wavelength)
d=6e-3; %Fluorescent depth layer
F=k*Q*I*(1-exp(-2.303.*c*m*d)); %Equation 1 in this paper
cg=c./(0.0027/1000000); %Convert from mol back to  $\mu\text{g}$ 
plot(cg,F, '-', 'Color','black'); %Plot the curve
```

```
%Uranine490 (all parameters' meanings are similar to that of Uranine350 example)
```

```
Q=0.85;
m=8400;
d=6e-3;
F=k*Q*I*(1-exp(-2.303.*c*m*d));
hold on;
cg=c./(0.0027/1000000);
plot(cg,F, '-', 'Color','red');
```

```
%Brilliant Sulfaflavine
```

```
c_min=0;
c_max=0.0024/1000000*4000000;
c=c_min:(c_max-c_min)/100000:c_max;
Q=0.274;
m=16e3;
d=15e-3;
F=k*Q*I*(1-exp(-2.303.*c*m*d));
hold on;
cg=c./(0.0024/1000000);
plot(cg,F, '-', 'Color','blue');
```

```
%Sulphorhodamine B
```

```
c_min=0;
c_max=0.0017/1000000*4000000;
c=c_min:(c_max-c_min)/100000:c_max;
Q=0.186;
m=84e3;
d=15e-3;
F=k*Q*I*(1-exp(-2.303.*c*m*d));
hold on;
cg=c./(0.0017/1000000);
plot(cg,F, '-', 'Color','red');
```

```
%Rhodamine WT
```

```
c_min=0;
c_max=0.0016/1000000*4000000;
c=c_min:(c_max-c_min)/100000:c_max;
Q=0.65;
m=50e3;
d=0.1;
F=k*Q*I*(1-exp(-2.303.*c*m*d));
hold on;
cg=c./(0.0016/1000000);
plot(cg,F, '-', 'Color','blue');
```

```
%Pyranine
```

```
c_min=0;
c_max=0.0019/1000000*4000000;
c=c_min:(c_max-c_min)/100000:c_max;
Q=0.65;
m=43e3;
d=15e-3;
F=k*Q*I*(1-exp(-2.303.*c*m*d));
hold on;
cg=c./(0.0019/1000000);
plot(cg,F, '-', 'Color','black');
```

```
%Potential staining dye
```

```
%c_min=0;
%c_max=[1 g = ? mol]/1000000*4000000;
%c=c_min:(c_max-c_min)/100000:c_max;
%Q=[dye quantum yield];
%m=[molar absorption];
%d=[depth of the fluorescent layer];
%F=k*Q*I*(1-exp(-2.303.*c*m*d));
%hold on;
%cg=c./([1 g = ? mol]/1000000);
%plot(cg,F, '-', 'Color','red');
```