Quantitative Fluorescence Imaging of Tracer Distributions in Soil Profiles

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH
for the degree of
DOCTOR OF NATURAL SCIENCES

presented by

PHILIPP GREGOR AEBY
Dipl. Natw. ETH
born 13 December, 1968
citizen of Beinwil SO

accepted on the recommendation of

Prof. Dr. Hannes Flühler, examiner
Prof. Dr. Hubert van den Bergh, co-examiner
Prof. Dr. Hans-Gerd Löhmannsröben, co-examiner

1998
# Table of Contents

Summary 1

Zusammenfassung 3

1. Introduction 5
   1.1 Project Framework ....................................................... 5
   1.2 Project Background ................................................. 6

2. Quantitative Fluorescence Imaging of Tracer Distributions in Soil Profiles 10
   2.1 Introduction ................................................................. 10
   2.2 Experimental Methods and Equipment .................................. 13
      2.2.1 Image acquisition and processing .................................. 13
      2.2.2 Instrumentation ...................................................... 14
      2.2.3 Calibration ............................................................ 16
   2.3 Background Correction ................................................ 21
      2.3.1 Correction for surface roughness .................................. 22
      2.3.2 The penetration depth of the incident light into the soil ....... 23
      2.3.3 Correction for surface absorption .................................. 26
      2.3.4 Background correction function .................................... 31
   2.4 Field Experiment ....................................................... 35
   2.5 Conclusions ............................................................... 38

3. Fluorescent Dyes Selection for Multitracing Experiments in Soils 41
   3.1 Introduction ............................................................... 42
   3.2 Requirements for Tracer Selection ..................................... 43
   3.3 Brilliant Sulfaflavine and Sulforhodamine B .......................... 44
   3.4 Oxazine 170 ................................................................. 48
   3.5 Acute Toxicity of Oxazine 170 to Earthworms (Eisenia foetida) in a 14-Day Static Test ........................................... 50
      3.5.1 Materials and methods .............................................. 50
      3.5.2 Results and discussion ............................................. 51
   3.6 The Effects of Oxazine 170 on Microbial Respiration and Nitrification in Soil ...................................................... 52
      3.6.1 Materials and methods .............................................. 52
Summary

Solute transport in the unsaturated zone between the soil surface and the groundwater table is highly irregular and difficult to simulate or predict. The main reason for this is the fact that in naturally structured soil the solutes travel along pathways of significantly different transport characteristics and that the spatial arrangement and scale of these pathways are not known. Therefore, we need test solutes that probe the dominant properties of the travel routes. These environmental probes must, at the same time, be detectable with a high spatial resolution. Fluorescence spectroscopy is a highly sensitive and specific analytical method which is suited to measure fluorescing test solutes directly in the soil.

The aim of this study was to develop a detection device for in situ imaging of several fluorescent tracers in structured field soils. The light generated by a high-power xenon lamp is filtered according to the excitation spectra of the fluorescent dyes and used to illuminate a soil profile. The resulting fluorescence is detected by a cooled CCD camera after passing through an emission filter which closely matches the emission spectrum of the measured dye. The recorded fluorescence image is corrected for inhomogeneous lighting and soil background. This corrected fluorescence signal is proportional to the concentration of the fluorescing tracers as long as the tracer concentrations do not exceed the linear range. The correction for the inhomogeneous background is based on a theory of the optical path of the excitation and emission light in the soil which is described in detail. The applicability of this measurement technique is demonstrated using field-measured data. The combination of concentration maps of different reactive solutes after a multitracing experiment with knowledge about the soil structure based on the background images allows new approaches in solute transport modeling.

Fluorescent dye tracers employed in environmental studies, laser technology and microbiology were evaluated as test solutes. Brilliant Sulfaflavine, Sulforhodamine B, and Oxazine 170 qualified as suitable tracers based on the following criteria: high, non-overlap-
ping fluorescence in water, low toxicity, low fluorescence quenching by soil constituents, low pH dependence and photobleaching, and low interference with soil autofluorescence. For the red-emitting laser dye Oxazine 170, fluorescence, toxicity and adsorption data are presented. It shows no acute toxicity to earth worms and has no effects on soil respiration and nitrification. Oxazine 170 is highly adsorbing to inorganic soil material and organic matter, whereas Brilliant Sulfaflavine is rather mobile and Sulforhodamine B moderately adsorbing.

Fluorescence imaging is compared to concentration determinations of non-fluorescing dyes by absorption imaging. The high sensitivity and signal-to-noise ratio and the linear calibration function of the fluorescence technique make it more suitable for use in structured soils. Simple improvements are suggested to make use of absorption imaging as an efficient alternative to fluorescence imaging for homogeneous soils.
Zusammenfassung


Die Vorteile der Fluoreszenzmessung, nämlich eine hohe Sensitivität, ein hohes Signal-Rauschen-Verhältnis und eine lineare Kalibrationsbeziehung, werden im Vergleich mit Konzentrationsmessungen mittels Bildanalyse deutlich. Mit einfachen Verbesserungen erlaubt die Bildanalyse jedoch eine kosteneffiziente Messung von nichtfluoreszierenden Farbstoffen in homogenen Böden.
Chapter 1

Introduction

1.1 Project Framework

This thesis reports on the results of the project ‘Multitracing of solute transport’ of ETH Zürich. A fluorescence imaging technique was developed to measure distributions of several dye tracers in soil profiles. The report focuses on fluorescence imaging (Chapter 2) and the selection of fluorescent tracers for multitracing (Chapter 3).

Quantification of flow patterns in soils is one of the principal research lines of the soil physics group at ETH Zürich. Image analysis techniques have been used to determine concentrations of the dye Brilliant Blue in sand columns [Aeby et al., 1997] and soil profiles [Forrer, 1997]. In comparison, fluorescence imaging enables the simultaneous use of several dye tracers and simplifies the calibration procedure.

The project was initiated by Prof. Dr. Hannes Flühler (ETH Zürich, principal investigator), Prof. Dr. Kurt Roth (University Stuttgart-Hohenheim) and Prof. Dr. Hubert van den Bergh (EPF Lausanne, co-investigator). Dr. Daniel Braichotte (EPF Lausanne) designed and constructed the detection device and supervised the tracer selection. Dr. Farnaz Moser (EPF Lausanne) measured the diffuse reflectance spectra and helped me with the project proposal. Olf Kramer designed digital filters for application in tracer images. Paul Gähwiller refined the calibration procedure and Hannes Wydler maintained and improved the detection device. Björn Studer and Thomas Pfluger performed the tracer analyses.

1. Project No. 41-2713.5, ‘Characterization of mass transfer coefficients in heterogeneous soils using multiple fluorescent dye tracers’, short title ‘Multitracing of solute transport’
Dr. Ulrich Memmert and Dr. Birgit Seyfried (RCC Ltd, Itingen, BL) were in charge of the studies about the toxicity and ecotoxicity of Oxazine 170.

The fluorescence imaging technique was tested in laboratory experiments of Dr. Daniel Stadler [Stadler et al., 1998] and field experiments of Maya Bundt [Bundt et al., 1998].

1.2 Project Background

Agricultural management, soil remediation and groundwater protection require ways of quantifying transport processes in the unsaturated zone between the soil surface and the groundwater table. Flow heterogeneities in this zone have a major influence on solute residence times and the bioavailability of solutes. Therefore, averaged transport properties fail to describe the displacement of nutrients and contaminants. For instance, solute transport in unsaturated field soils does often not obey the convection-dispersion equation which relies on the assumption of a constant dispersivity.

Salt and dye tracers are commonly used to study flow patterns in porous media. To quantify solute transfer between flow paths of varying velocities, tracer concentration fields with a high spatial resolution are needed. Depending on their sorption characteristics solutes may experience flow regions of distinct particle residence times and accessibilities. Fluorescence imaging yields two-dimensional concentration distributions of several tracers with an exceptionally high spatial resolution. Using simultaneously applied tracers with differing retardation factors it should be possible to estimate the distribution of pertinent transfer coefficients.

Local flow velocities in unsaturated soils vary drastically in space and time. Mass transfer between fast and slowly moving liquid controls the macroscopic transport regime, which, in one limiting case, may appear to be a diffusion-like mixing process (hydrodynamic dispersion) or, in another limiting case, as a superposition of two or more independent or only weakly coupled flow systems having different scales of heterogeneities (e.g. macropore transport or viscous fingering).
Rapid mass exchange between regions of different flow velocities leads to a convective-dispersive transport regime. Under such conditions all solutes have a high probability to reach all possible flow regions. Rate-limited mass exchange, on the other hand, preserves the local velocity distribution of weakly connected flow paths. The mass transfer coefficients define the coupling of the flow fields in the macro- and micropore domains, and therefore quantify a dominant feature of the transport regime.

Flury et al. [1994] visualized different transport regimes in field soils under unsaturated flow conditions. Flühler et al. [1996] analyzed a variety of solute transport models that are potentially applicable to the entire spectrum of observed flow regimes. All of these models distinguish different flow domains having different flow velocities. The main conceptual difference between the models is the description of the mass transfer between regions of differing flow conditions. The coefficients describing mass transfer are, with very few exceptions, fitting parameters obtained from breakthrough curves measured at given depths, \( C_z(t) \), or from depth distributions of solute concentrations at given times \( C_z(z) \).

The correlation structure of a particular distribution \( C(x,y,z) \) is the result and not the cause of a specific transport regime. Although we ultimately want to model the underlying processes, we need first to parameterize the observed \( C(x,y,z) \)-distributions in order to compare them with the modeled distributions. Most available models only use the first and second moment of \( C_z(t) \) or \( C_z(z) \). Parameters describing the correlation structure of \( C(x,y,z) \) capture essential features of the flow field relevant for solute residence times and bioavailability.

When reactive solutes are being displaced in heterogeneous systems the resulting concentration profiles may deviate substantially from those predicted with the classical convection-dispersion equation. Due to preferential flow, i.e. continuous fast flow in the main transport direction, sorption behavior often does not significantly affect the maximum displacement distance, but rather the fraction of solute being displaced beyond a given depth. This feature has been experimentally documented by Flury et al. [1995] using two mobile tracers and three herbicides of different reactivity.

In a field soil with dominant preferential flow we should therefore distinguish between two retarding mechanisms, i) retardation in fast flow regions and ii) retardation in the course of
the penetration process into the soil matrix. Solute transfer from macropores into the surrounding matrix may be impeded by clay or bioorganic coatings deposited on the biopore walls or aggregate surfaces. Such highly sorbing barriers separate the macropore region from the soil matrix [Turner and Steele, 1988; Mallawatantri et al., 1996]. The travel paths of a particle may actually be represented by three media aligned in series with different retardation factors, the first being the fast flow region of the macro- or biopores with coatings serving as immediate boundaries, the second being the thin layer of the coatings themselves, and the third the soil matrix. Transport from the fast flow region into the soil matrix has been investigated only for very few special cases. The high retention capacity for pesticides by earthworm deposits was demonstrated by Stehouwer et al. [1994]. The highly variable accessibility of the soil matrix may determine to a large extent the retardation and residence time of solutes in the unsaturated zone.

Resident solute concentrations (expressed per unit soil solution residing in a given soil volume) are most often measured by taking soil cores and by extracting the solutes with an excess of extracting solution (e.g. water-acetone). Such procedures are time consuming and, in addition, average out concentrations of differently accessible or differently mobile zones. Other methods of measuring resident concentrations are the use of in situ solution collection or in situ electrolyte sensing with time domain reflectometry [Dalton and van Genuchten 1986]. In situ techniques as well as soil coring do not yield a high spatial density of concentration measurements due to limited access and/or experimental capacity.

By contrast, image analysis yields two-dimensional, highly resolved spatial concentration distributions in profiles [Aebly et al., 1997; Forrer, 1997]. Imaging fluorescent dyes extends the detection capacity to simultaneously applied tracers and simplifies the measurement.
References


Chapter 2

Quantitative Fluorescence Imaging of Tracer Distributions in Soil Profiles

P. Aeby, D. Braichotte, M. Bundt, P. Gähwiler, F. Moser-Boroumand, O. Kramer, H. Wydler, and H. Flühler

We describe a device for in situ imaging of several fluorescent tracers in structured field soils. With this method we conducted a multitracing infiltration experiment and determined the tracer concentrations in soil profiles. Three fluorescent dyes with excitation peaks in the blue (Brilliant Sulfaflavine), green (Sulforhodamine B), and red (Oxazine 170) part of the spectrum have been selected for their spectroscopic properties and their pH-independent fluorescence efficiency. A cooled CCD camera yields fluorescence images of a high spatial resolution. The images are corrected for variable lighting and background. The resulting two-dimensional concentration distributions of several tracers can be used to test transport models and to relate heterogeneous flow fields to observable soil characteristics.

2.1 Introduction

Solute transport studies in field soils and laboratory columns are often based on tracers like salts, radioactive isotopes or dyes. Tracer concentrations determined in soil cores or solu-
tions sampled in suction cups represent volume or flux averaged concentration measurements with a rather poor spatial resolution. Dyes visualize flow paths but in most cases yield only qualitative information. The use of fluorescence imaging to detect dye tracers combines a high spatial resolution and reliable concentration measurements over a large concentration range. Furthermore, tracers of differing transport characteristics or tracers applied at different times or locations can be detected simultaneously. The concentration data of such multitracing experiments allow the evaluation of transport models and the determination of the corresponding parameters. At the same time, the imaging technique provides ample information on soil structure. Thus, the interrelation between structure and tracer concentrations can be studied for a given soil profile.

Fluorescence spectroscopy is well established in environmental analysis. Laser-induced fluorescence is suited to detect contaminants in groundwater [Chudyk et al., 1985; Barczewski and Marschall, 1992] and soil [Kumke et al., 1995; Schade and Bublitz, 1996; Apitz et al., 1993], as well as for fluorescent tracer detection in glass-bead media [Kulp et al., 1988]. These techniques referred to above utilize fiber optics which allow in situ measurements providing local information with a good resolution in time. The objective of the present study was to measure two-dimensional concentration distributions in soil profiles. Hence, information on spatial heterogeneity is the target of the method presented here.

Fluorescence involves the emission of photons from electronically excited states following the absorption of light of shorter wavelengths. Fluorescent compounds show characteristic excitation and emission spectra. The excitation spectrum of a compound is the fluorescence signal at a given emission wavelength expressed as a function of the wavelength of the excitation light. The emission spectrum is the fluorescence signal for a given excitation wavelength expressed as a function of the wavelength of the emitted light. Fluorescent dyes can be detected and discriminated in a mixture of different dyes, provided that their spectra barely overlap.

In a solution containing a dye with a concentration \( c \), the fluorescence intensity \( I_f \) is given by

\[
I_f = \Phi_f I_0 (1 - e^{-2.303 \varepsilon_c d})
\]  
(2.1)
where $I_0$ is the intensity of the incident light and $d$ the thickness of the absorbing layer [Rendell and Mowthorpe, 1987]. The extinction coefficient $\varepsilon$ expresses the dye’s efficiency of absorption according to the Beer-Lambert law and the fluorescence efficiency $\phi_f$ is the fraction of the absorbed light which is re-emitted as fluorescence. At low concentrations, where $2.303\varepsilon cd < 0.05$, equation (2.1) can be approximated by an expression for $I_f$ which is linear in the dye concentration

$$I_f = 2.303\phi_f I_0 \varepsilon cd$$  \hspace{1cm} (2.2)

In soil $d$ is unknown and the incident light varies spatially according to the roughness of the soil surface. Because of absorption and scattering, the excitation light does not equal the incident light, and just a small portion of the emission light is collected by the detector. Nevertheless, $I_f$ is proportional to the intensity of the excitation light $E^1$ and in case of a linear detector like a CCD camera the measured fluorescence signal $F$ is proportional to $I_f$. Thus,

$$F \propto E$$  \hspace{1cm} (2.3)

A proper choice of suitable dyes minimizes difficulties which may impair fluorescence measurements in soils. These difficulties are: (i) The fluorescence may be quenched in presence of halogen ions and humic or other substances [Kumke et al., 1994]; (ii) Dissolved molecules and mineral surfaces may interact with the fluorescent dyes, altering their fluorescence; (iii) The fluorescence may decrease with time because of photodecomposition of the dye molecules when the soil is exposed to light; (iv) The fluorescence often depends on the soil pH which varies over a large range; and (v) finally, fluorescence of the soil background may interfere with the fluorescence signal.

We selected the three dyes Brilliant Sulfaflavine (Sigma Chemical Co., St. Louis, MO), Sulfurhodamine B monosodium salt (Fluka Chemie AG, Buchs, Switzerland), and Oxazine 170 perchlorate (Fluka Chemie AG, Buchs, Switzerland) for their well separated fluorescence.

1. Here and in equation (2.1) it is assumed that no ground state depopulation occurs because a xenon lamp and not a laser or flash lamp is used for illumination. In this case, the illumination intensity is sufficiently low that an excitation of a dye molecule during its fluorescence lifetime is very unlikely.
cence spectra, their solubility in water, and their favorable fluorescence properties in soil\(^2\). The fluorescence of these dyes is not quenched by soil components, does not change when illuminated for a few hours, is pH-independent in the range from 4 to 9, and the background fluorescence of the soil itself does not interfere with the dye emission. The dyes show also interesting transport behavior in soil: Brilliant Sulfaflavine is rather mobile, Sulforhodamine B is moderately adsorbing, and Oxazine 170 strongly adsorbs to organic matter and inorganic soil material.

In the following we describe a device to detect them simultaneously in soil profiles and present background corrections to cope with the changing roughness and light absorption of the soil. The applicability of this measurement technique is demonstrated using field-measured data.

### 2.2 Experimental Methods and Equipment

#### 2.2.1 Image acquisition and processing

To determine the dye concentration from the fluorescence signal, the fluorescence for a given instrumental setting should exclusively depend on the concentration. A calibration was performed to obtain the function parameters for the particular experimental conditions. For each dye, five steps were performed:

1. A fluorescence image \(F(x, y)\) and an image of the soil reflection \(R(x, y)\) were recorded. In addition, the intensity distribution of the lighting was assessed by taking an image of a grey panel covering the entire soil surface. This image is called flat field image.

2. The fluorescence and the reflection image were corrected for slight displacements against each other. This was done by evaluating the geometric relationship between the images based on spatially well defined control points in both images. Assuming simul-

---

2. See chapter 3, “Fluorescent dyes selection for multitracing experiments in soils”
taneous translation, scaling and rotation as a result of small movements of the camera head, the following image coordinate transformations describe how one of the images had to be warped to make it register over the other image:

\[ x' = a_0 + a_1 x + a_2 y \quad \text{and} \quad y' = b_0 + b_1 x + b_2 y, \]

where \( x, y \) are the original image coordinates and \( x', y' \) the transformed coordinates. The parameters \( a_0, a_1, a_2, b_0, b_1, b_2 \) were estimated by least-squares fitting [Press et al. 1992]. The grey levels, i.e. signal intensities of the transformed image, were calculated by bilinear interpolation from the original grey levels [Castleman, 1996].

3. The light intensities reflected from the homogeneous flat field panel were normalized with their median value. The fluorescence and the reflection image were divided by the normalized flat field image to correct for the inhomogeneous lighting of the light source.

4. The reflection image was employed to correct the fluorescence image for the inhomogeneous soil background. (This correction is explained in detail in section 2.3.)

5. A two-dimensional concentration distribution was derived from the fluorescence image by calibration with soil samples of known dye concentrations. This step can be omitted when only relative concentrations are required.

All image processing steps were implemented on a personal computer using IDL software (Version 5.0, Research Systems Inc., Boulder, CO). Reflection images were taken for three different tracers at the corresponding excitation wavelengths. These images are maps of reflected monochromatic light intensities, depending on surface roughness, density and absorption characteristics of soil constituents and their structural arrangement.

### 2.2.2 Instrumentation

The setup as shown in Figure 2.1 consists of an excitation and a detection module. The excitation light is generated by a xenon lamp, optically filtered according to the excitation spectra of the fluorescent dyes, and guided through a optical transmission line to the soil profile.
The resulting fluorescence is detected by a CCD (Charge-coupled device) camera and displayed on the computer screen.

Figure 2.1 Experimental setup

The xenon lamp (KiloArc, Photon Technology International, South Brunswick, NJ) has an optical output of 104 W in the range of 250-1150 nm and is water cooled by a liquid-to-air heat exchanger (MCS20H01, Lytron, Woburn, MA). The fixed elements of the optical path are the hot mirror (Tempax 113, Schott, Mainz, Germany), which removes most of the infrared light, and the lenses (01LPK061/066 and 01LAG127/066, Melles Griot, Irvine, CA). The other filters have to be exchanged for each dye and image according to Table 2.1. The dichroic mirrors pre-select the wavelengths of the light before it enters the liquid light guide (Series 380, Lumatec, Munich, Germany), whereas the transmission spectra of the excitation filters closely match the dyes' excitation spectra.
Table 2.1 Excitation and emission filters

<table>
<thead>
<tr>
<th></th>
<th>Dichroic Mirror 1</th>
<th>Dichroic Mirror 2</th>
<th>Excitation Filters</th>
<th>Emission Filters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brilliant Sulfaflavine</td>
<td>Reynard(^a) 1005</td>
<td>Reynard 1046</td>
<td>Omega(^b) 425-45 (twice)</td>
<td>Reynard 920 Schott(^c) GG 475 (twice)</td>
</tr>
<tr>
<td>Sulforhodamine B</td>
<td>Reynard 1030</td>
<td>Reynard 915</td>
<td>Omega 546-10</td>
<td>Reynard 935 Schott OG 590 (twice)</td>
</tr>
<tr>
<td>Oxazine 170</td>
<td>Reynard 1040</td>
<td>Reynard 925</td>
<td>Omega 610-20</td>
<td>Reynard 944 Schott RG 645 (twice)</td>
</tr>
</tbody>
</table>

\(^a\) Reynard Corporation, San Clemente, CA  
\(^b\) Omega Optical Inc., Brattleboro, VT  
\(^c\) Schott, Mainz, Germany

In the same way, the transmission spectra of the emission filters in front of the CCD camera closely match the dyes' emission spectra. The fluorescence images were taken by utilizing all filters given in Table 2.1, whereas for the reflection images the emission filters in front of the camera were removed.

Because of the weak fluorescence of the dyes in the opaque soil and the corresponding long exposure times, maximum sensitivity and minimum noise from readout and dark current were required. Hence, we used a slow-scan cooled CCD camera (Antares TE4 EEV CCD05-30 MPP, AstroCam Ltd, Cambridge, UK) with an image dimension of 1248 by 1152 pixels and 16-bit digitization. The CCD and the readout electronics and therefore the overall detection system are highly linear over the whole dynamic range. The camera is controlled and the images are captured with the software PixCel (Version 2.1, LSR AstroCam Ltd, Cambridge, UK) which is supplied with the camera and runs under Windows 95 (Microsoft Corporation, Redmond, WA). For field use, the whole equipment was placed on a rugged rack and powered by a gasoline-driven generator.

2.2.3 Calibration

The fluorescence signal corrected for inhomogeneous lighting and background \( F_c \) is a linear function of the dye concentration as shown in equation (2.2)

\[
F_c = kC + z
\]  

(2.4)
or an exponential function of the dye concentration as in equation (2.1)

\[ F_c = 1 - (1 - z)e^{-mC} \]  

(2.5)

Here, \( C \) represents the dye concentration in mol per kg dry soil and is a measure for the number of fluorescing molecules visible to the detector. Because the thickness of the light absorbing layer is not known, \( C \) instead of the dissolved concentration \( c \) should be used in soil. When expressed as a function of \( C \), the fluorescence \( F_c \) only moderately depends on the soil water content (Figure 2.2). The parameter \( z \) equals the background signal at zero

![Figure 2.2](image)

**Figure 2.2** The fluorescence signal \( F \) of Brilliant Sulfaflavine (BF) and Sulforhodamine B (SB) as a function of the soil water content measured with a fluorescence spectrometer. The dye concentrations are 119 (BF low), 478 (BF high), 86 (SB low), and 345 (SB high) \( \mu \)mol (kg dry soil\(^{-1} \)). The measurements for different concentrations and dyes were performed for dissimilar experimental conditions and are not directly comparable.

dye concentration. The parameters \( k \) and \( l \) are proportional to the fluorescence efficiency \( \phi_f \) in the soil, the average excitation light intensity \( E_0 \), and the sensitivity of the detector. The parameters \( m \) and \( k \) are proportional to the extinction coefficient \( \varepsilon \). As in the case of equations (2.2) and (2.1), equation (2.4) approximates equation (2.5) for small \( \varepsilon \) or \( C \). The validity of these equations is demonstrated by Figure 2.3 and Figure 2.4 which depict the fluorescence signal \( F \) as a function of \( C \) for the fluorescent dyes Brilliant Sulfaflavine and
Sulforhodamine B. The fluorescence is always expressed in relative units because it depends not only on the specimen but also on several instrumental factors such as intensity of illumination, type and number of optical filters, and the chosen sensitivity of the CCD camera. The extinction coefficient of Sulforhodamine B \( (\varepsilon = 84 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}) \) is about five times higher than the one of Brilliant Sulfaflavine \( (\varepsilon = 16 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}) \) and leads to a non-linear relationship of \( F_c \) and \( C \) at high concentrations. However, for small \( C \) the approximation of equation (2.4) is applicable for Sulforhodamine B as well.

![Graph](image)

**Figure 2.3** The fluorescence signal \( F \) as a function of the concentration \( C \) of Brilliant Sulfaflavine in soil measured with a fluorescence spectrometer. Because of the dye's low extinction coefficient \( (\varepsilon = 16 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}) \) the function is linear over the entire experimental concentration range. The dye's molecular weight is 418.41 g, excitation wavelength was 418 nm and emission wavelength was 547 nm. For emission, a cut-off filter at 515 nm was used.

Oxazine 170, having an extinction coefficient in-between the two other dyes \( (\varepsilon = 48 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}) \), has a linear relationship between dye concentration and relative fluorescence intensity except for very high concentrations.

The fluorescence measurements of Figure 2.3 to Figure 2.5 were made with a Perkin-Elmer fluorescence spectrometer (LS50B, Norwalk, CT). For Brilliant Sulfaflavine the selected excitation wavelength was 418 nm. The emission was detected at 547 nm using a built-in
Figure 2.4 The fluorescence signal $F$ as a function of the concentration $C$ of Sulforhodamine B in soil measured with a fluorescence spectrometer. Because of the dye's high extinction coefficient ($\varepsilon = 84 \times 10^4$ L mol$^{-1}$ cm$^{-1}$) the function is exponential over the experimental concentration. The dye's molecular weight is 580.60 g, excitation wavelength was 565 nm and emission wavelength was 590 nm. For emission, a cut-off filter at 590 nm was used.

longpass filter with cut-off at 515 nm to get rid of the stray light scattered at the soil surface that strongly interfered with the fluorescence for small signals. For Sulforhodamine B the excitation wavelength selected was 565 nm and the emission was detected at 590 nm using a Schott filter OG 590 (Mainz, Germany) as cut-off filter. The samples were prepared by mixing 4 g of 2 mm-sieved soil$^3$ with 1.2 g of dye solution with various concentrations. For each concentration five fluorescence measurements were made with the solid surface accessory of the spectrometer. By using transparent cylinders, soil surfaces measured with the CCD camera in the field experiment were sampled and measured with the spectrometer again. The fluorescence $F_c$, measured in the field and corrected for inhomogeneous lighting and background, differed only by a constant factor from the fluorescence measured with the spectrometer (Figure 2.5). Therefore, we may safely conclude that equations (2.4) and (2.5), given the same soil and dyes, hold also for field measurements.

3. See section 2.4 for description of soil used
Figure 2.5 Comparison of fluorescence measurements of Brilliant Sulfaphilavine of the same soil samples measured in the field with the CCD imaging technique (corrected) and in the laboratory with the fluorescence spectrometer. The relative fluorescence intensities are proportional to each other.

If the background signal $z$ is known, it can be subtracted from the fluorescence in equation (2.4) so that the fluorescence signal is proportional to the dye concentration.

In short: the corrected fluorescence image is identical with the two-dimensional distribution of relative concentrations as long as the dye concentration does not exceed the linear range.

Fluorescence measurements of the soil without dyes showed that autofluorescence of soil constituents did not contribute to the background signal for the excitation and emission wavelengths used. The considerable background signal especially for Brilliant Sulfaphilavine measurements was therefore due to stray light. Just a small part of the stray light reproduced features of the soil surface, implying that the major part was multiply reflected and not correlated to the reflection image making a correction impossible. Also, the reduction of stray light by an additional emission filter would have been offset by the prolonged exposure time.

For most applications the determination of relative concentrations is sufficient. If absolute concentrations are needed, there are three ways to relate relative to absolute values:
1. *Calibration by extraction:* Small samples are scraped off from the soil surface and their dye concentrations are determined by extraction. Then, the dye concentrations are plotted against the corresponding intensities in the fluorescence image.

2. *Direct spectrometer calibration:* Undisturbed samples of the soil surface are taken with small transparent cylinders and directly measured in the fluorescence spectrometer. A calibration curve is obtained by measuring the fluorescence of cylinders which are filled with soil of known dye concentration.

3. *Direct field calibration:* Soil patches of known dye concentration are applied within the soil profile and directly measured in the field with the CCD imaging technique.

The direct calibration methods should be preferred to the calibration by extraction, because they are faster and large uncertainties may be associated with the extraction as demonstrated by Figure 2.6. There are two problems concerning the extraction:

1. The mass recoveries of the dyes used in our study depend on soil composition, sample treatment, water content and dye concentration.

2. The extraction averages the dye concentration over the whole sample volume. The volume-average concentration is weakly correlated to the concentration in the visible surface layer.

### 2.3 Background Correction

After the flat field correction, the fluorescence signal is a function of the dye concentration, of the availability of excitation light, and of the emission light absorption. Although the lighting is uniform after flat fielding, the excitation light varies due to the surface roughness and light absorption by the soil. The visibility of the resulting fluorescence is also altered by the absorption of the soil. The objective is to correct the observed fluorescence signal in a way that it only depends on the dye concentration. The reflection image \( R(x, y) \) recorded at each of the three excitation wavelengths was used to correct the corresponding fluores-
Figure 2.6 Calibration by extraction of samples from the soil profile to determine absolute concentrations of Brilliant Sulfaflavine from the corrected fluorescence signal. The extracts were obtained from sampling volumes that are large in comparison to the fluorescing surface samples. This results in a weak correlation between extract concentration and fluorescence.

cence image $F(x, y)$. Each pair of images was taken under identical lighting conditions, but the reflection image without the emission filters in front of the camera. Therefore, the reflection image measures the reflection of the excitation light at the soil surface.

In the following, we assume that $R(x, y)$ and $F(x, y)$ are already flat field corrected as described in section 2.2. We examine the impact of the surface roughness on the fluorescence signal in the case of uniform absorption. Then, we measure the penetration depth of light to derive the impact of absorption on the fluorescence signal in the case of a flat soil surface. Finally, we consider the combined effects of surface roughness and absorption and develop a correction function for these effects.

2.3.1 Correction for surface roughness

Shadows and depressions in the soil surface alter the incidence of light. If the absorption of the surface is constant over the entire image, both the excitation and the reflection light are proportional to the incident light and the following relation holds:
\[ E(x, y) = R(x, y)E_0/R_0 \]  \hspace{1cm} (2.6)

where \( E(x, y) \) is the excitation light intensity at a certain location \((x,y)\), \( R(x, y) \) the intensity of the reflected light at the same location, \( E_0 \) the average excitation light intensity in the image, and \( R_0 \) the average reflection intensity in the image. At all locations \((x,y)\), the measured fluorescence \( F(x, y) \) is then corrected for different excitations according to equation (2.3):

\[ F_g(x, y) = F(x, y)E_0/E(x, y) = F(x, y)R_0/R(x, y) \]  \hspace{1cm} (2.7)

\( F_g(x, y) \) is the fluorescence corrected for varying excitation light intensity caused by the uneven surface geometry. This formula can be applied as long as the reflection at each location exclusively depends on the geometry and not on heterogeneous absorption of the surface. In the next two sections, we study the influence of heterogeneous absorption on the excitation light.

### 2.3.2 The penetration depth of the incident light into the soil

To understand the impact of light absorption on excitation and emission of fluorescence, it is necessary to investigate the optical path of a light beam incident on the soil surface. A small portion of the light is specularly reflected at the surface, whereas most of the light is absorbed by the soil material or scattered back resulting in a diffuse reflection. In the case of specular reflection, the angle of reflection and the angle of incidence are equal and lie in the same plane. If the light is specularly reflected at several, randomly distributed faces of the soil particles and refracted at air-water-particle interfaces, the light is scattered and therefore the reflection appears as diffuse. If the incident light penetrates the soil, it may be absorbed or reflected back by particle faces within the soil. The deeper the penetration, the more dye molecules can be excited and contribute to the measured fluorescence.

We determined the penetration depth at a selected wavelength by diffuse reflectance spectroscopy using the phenomenological theory of light absorption and scattering by Kubelka and Munk [Kortüm 1969]. We assumed that the attenuation of light in the soil can be described by an absorption coefficient \( K \) [cm\(^{-1}\)] and a scattering coefficient \( S \) [cm\(^{-1}\)] which
both depend on the wavelength. The penetration of light at a given wavelength can then be described by

\[
\ln\left(\frac{I_d}{I_0}\right) = -(K + S)d
\]  

(2.8)

where \( \ln \) is the natural logarithm and \( \frac{I_d}{I_0} \) the ratio of the light intensity at depth \( d \) to the intensity of the incident light on the surface. The penetration depth \( d_p \) was set arbitrarily to the depth at which \( \frac{I_d}{I_0} \) equals 0.05 and thus \( \ln(\frac{I_d}{I_0}) = -3 \). To estimate \( d_p \), \( K \) and \( S \) were determined based on the diffuse reflection of a soil sample as described in the following.

If the sample thickness is larger than the penetration depth, the ratio of the absorption and the scattering coefficient equals a function of the diffuse reflectance \( f_0 \) of the sample. This function is called Kubelka-Munk function \( F(f_0) \) and is defined by

\[
F(f_0) = \frac{(1 - f_0)^2}{2f_0} = \frac{K}{S}
\]  

(2.9)

The diffuse reflectance \( f_0 \) represents the ratio of the diffuse reflection of the sample to that of a white reflectance standard like BaSO\(_4\), which reflects more than 98.5 % of the incident light at every wavelength of the visible spectrum. At a given wavelength \( \lambda \), one can determine the coefficients \( K \) and \( S \) by measuring the diffuse reflectance \( f_0(\lambda) \) of several samples containing different known dye concentrations and applying the Kubelka-Munk function. Once \( K \) and \( S \) are determined, equation (2.8) yields the penetration depth. The absorption coefficient \( K \) is the sum of two terms, a constant term, \( K_{\text{soil}} \), describing the background absorption due to the soil and a varying term, \( K_{\text{dye}} \), proportional to the dye concentration

\[
K = K_{\text{soil}} + K_{\text{dye}}
\]  

(2.10)

\( K_{\text{dye}} \) is related to the volumetric molar dye concentration \( c \) [mol L\(^{-1}\)] by the decimal molar extinction coefficient \( \epsilon \) [L mol\(^{-1}\) cm\(^{-1}\)]

\[
K_{\text{dye}} = 2\ln(10)\epsilon c
\]  

(2.11)
Hence,
\[
F(f_0) = \frac{K_{\text{dye}}}{S} + \frac{K_{\text{soil}}}{S}
\]  
(2.12)
is a straight line with slope \(1/S\) and intercept \(K_{\text{soil}}/S\).

The diffuse reflectance of soil samples was measured with a Varian Cary 1E spectrophotometer (Varian, Palo Alto, CA) equipped with a diffuse reflection accessory similar to the Praying Mantis of Harrick Scientific (Ossining, NY). The sample was held horizontally so that no reflection-distorting silica lamella was necessary to keep the soil in the sample holder. By adjusting the accessory’s mirrors, the specular reflection from the sample was rejected. The samples were taken at 30 - 40 cm depth from a soil\(^4\) classified as a typic Eutrochrept (coarse-loamy, mesic). Its particle size distribution is 58% sand, 31% silt, 11% clay with a bulk density of 1.54 g cm\(^{-3}\) and an organic matter content of 0.64%. Samples of 5.7 g of soil were mixed with 2.3 g of a Sulforhodamine B solution with different concentrations and 0.7 g of the mixture was put onto the sample holder. The sample surface was flattened by applying a constant pressure on top of it.

The diffuse reflectance \(f_0\) was determined at the wavelength of maximum absorption of Sulforhodamine B at 564 nm. The corresponding molar extinction coefficient \(\varepsilon\) in water is \(84 \times 10^3\) L mol\(^{-1}\) cm\(^{-1}\). Figure 2.7 shows \(F(f_0)\) as a function of the absorption coefficient \(K_{\text{dye}}\), which in turn is a function of the concentration of the dye Sulforhodamine B. The linear regression through the mean values of three measurements at five dye concentrations gave the coefficient of soil absorption \(K_{\text{soil}} = 145\) cm\(^{-1}\) and the scattering coefficient \(S = 62\) cm\(^{-1}\). From this, the mean penetration depth of light of the wavelength 564 nm into the soil was computed with equation (2.8) to be only 145 \(\mu\)m. Taking into account the rough soil surface and the particle size of sand grains and soil aggregates, it seems that just a very small fraction of the incident light passes through the outmost particle layer.

---

\(^4\) Andelfingen test site, see Forrer [1997] and Kasteel [1997]
2.3.3 Correction for surface absorption

Because of the very small penetration depth of the light, we assume that most excited molecules are at or close to the surface and directly exposed to the incident light. This agrees with the results of Apitz et al. [1992] who showed that only the molecules on the clay and sand particles or in the interstices between the first particle layer contribute to measurable fluorescent signals. Hence, the excitation light \( E(x, y) \) at a certain location \((x,y)\) consists of two components, namely the incident light coming directly from the light source and the indirect light which was reflected by the soil. If we assume further that only a very small fraction of the incoming light is reflected in the thin water film which contains the dye molecules and that just a very small fraction is absorbed by the dye molecules themselves, we get the following relation:

\[
E(x, y) = E_{\text{direct}} + E_{\text{reflected}}(x, y) = E_{\text{direct}} + f_{EX}(x, y)E_{\text{direct}}
\]

(2.13)
where $E_{direct}$ is the incident light from the light source which is supposed to be constant after flat fielding. $E_{reflected}(x, y)$ is the part of the incident light which is reflected by the soil surface and $f_{EX}(x, y)$ is the reflectance of the soil surface at the excitation wavelength at a certain location $(x, y)$, i.e. the fraction of light which is reflected and not absorbed.

The absorption corrected excitation light $E_a$ should be constant over the whole image. It is calculated by using equation (2.13) and the average reflectance $f_{EX,0}$:

$$E_a = E_{direct} + f_{EX,0}E_{direct}$$

(2.14)

The average diffuse reflectance $f_{EX,0}$ can be determined at the excitation wavelength of a given dye by diffuse reflectance spectroscopy (see preceding section and Figure 2.8).

![Graph](image)

**Figure 2.8** Average diffuse reflectance $f_{EX,0} = (R_{soil}/R_{reference})$ of the soil in the visible and near infrared. $R_{soil}$ is the diffuse reflection at the soil surface, which is standardized by the reflection of the white reflectance standard $R_{reference}$ ($BaSO_4$).

Because the ratio of the local reflectance at a given location in the image to the average reflectance, $f_{EX}(x, y)/f_{EX,0}$, is equal to the ratio of the local reflection to the average reflection, $R(x, y)/R_0$, we can substitute $f_{EX}(x, y)$ in equation (2.13) and obtain
\[ E_{\text{direct}} = \frac{E(x, y)}{1 + f_{EX}(x, y)} = \frac{E(x, y)}{1 + f_{EX,0}R(x, y)/R_0} \] (2.15)

Combining equations (2.14) and (2.15) we can correct the excitation light for the different absorption at every location in the image:

\[ E_a = E(x, y)\frac{1 + f_{EX,0}}{1 + f_{EX,0}R(x, y)/R_0} \] (2.16)

The measured fluorescence \( F(x, y) \) can then be corrected for different excitations at every location according to equation (2.3):

\[ F_{a,EX}(x, y) = F(x, y)E_a/E(x, y) = F(x, y)\frac{1 + f_{EX,0}}{1 + f_{EX,0}R(x, y)/R_0} \] (2.17)

However, not only the excitation light is absorbed but also the resulting emission. As for the excitation light, we can think of the measured emission light \( F(x, y) \) to consist of two components:

\[ F(x, y) = F_{\text{direct}}(x, y) + F_{\text{reflected}}(x, y) \]

\[ = F_{\text{direct}}(x, y) + f_{EM}(x, y)F_{\text{direct}}(x, y) \] (2.18)

\( F_{\text{direct}}(x, y) \) is that part of the fluorescence which is emitted in direction of the camera, whereas \( F_{\text{reflected}}(x, y) \) is reflected by the soil surface before being directed towards the camera. It depends on the reflectance of the surface at the emission wavelength, \( f_{EM}(x, y) \).

The average reflectance at the emission wavelength, \( f_{EM,0} \), can be obtained from Figure 2.8. Contrary to the reflectance at the excitation wavelength, the reflectance at the emission wavelength is not proportional to the reflection \( R \) at the excitation wavelength. However, the reflection images at different wavelengths are correlated to a large extent. The coefficients of correlation for reflection images illuminated with light of different mean wavelengths are given in Table 2.2. A reason for this strong correlation is the big pixel dimension in comparison to the individual soil constituents. Minerals which possess sharp absorption bands are embedded in a large number of other soil particles. Hence, surface geometry, par-
Table 2.2 Coefficients of correlation for reflection images illuminated with light of different mean wavelengths

<table>
<thead>
<tr>
<th>nm</th>
<th>425</th>
<th>490</th>
<th>545</th>
</tr>
</thead>
<tbody>
<tr>
<td>490</td>
<td>0.93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>545</td>
<td>0.88</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>610</td>
<td>0.87</td>
<td>0.92</td>
<td>0.93</td>
</tr>
</tbody>
</table>

ticle size distribution, water and organic matter content usually mask the absorption characteristics of individual constituents.

This is not true for certain iron oxides which show strong absorption in the shorter wavelength range. Therefore, soils have often brownish or reddish color and an increasing reflectance for wavelengths longer than 500 nm as shown in Figure 2.8. But if these compounds are homogeneously distributed over the soil profile, they influence just the average reflectance, whereas local reflection variations are due to the non-chemical factors mentioned above.

We made use of the strong correlations between the reflection images at different wavelengths and estimated the local reflection at the emission wavelength $R_{EM}(x, y)$ from the corresponding value at the excitation wavelength by two equations. The first equation is based on the assumption that the deviation from the average reflection is the same for both wavelengths:

$$R_{EM}(x, y) - R_{EM,0} = R(x, y) - R_0$$ (2.19)

The second equation implies that the ratio of the local reflection to the average reflection is the same at both wavelengths:

$$R_{EM}(x, y)/R_{EM,0} = R(x, y)/R_0$$ (2.20)

To test the two assumptions, we determined mean values and standard deviations of the absolute pixel-wise differences between the measured and estimated reflection images. We found that equation (2.20) leads to smaller differences and is therefore considered to be the better estimate. As a consequence, the local reflectance at the emission wavelength, $f_{EM}$,
was estimated from the average reflectance at the emission wavelength, \( f_{EM,0} \), and the excitation light reflection image by the following formula:

\[
f_{EM}(x, y) = f_{EM,0} \times \frac{R(x, y)}{R_0}
\]  

The fluorescence corrected for the absorption of the emission, \( F_{a,EM}(x, y) \), is calculated by replacing \( f_{EM} \) in equation (2.18) with the average reflectance \( f_{EM,0} \). To account also for the absorption of the excitation light, \( F_{direct}(x, y) \) is corrected according to equation (2.17):

\[
F_{direct, a,EX}(x, y) = F_{direct}(x, y) \frac{1 + f_{EX,0}}{1 + f_{EX,0} \frac{R(x, y)}{R_0}}
\]  

Therefore, the absorption corrected fluorescence \( F_{a,EXM}(x, y) \) is

\[
F_{a,EXM}(x, y) = F_{direct}(x, y) \frac{1 + f_{EX,0}}{1 + f_{EX,0} \frac{R(x, y)}{R_0}} (1 + f_{EM,0})
\]  

Finally, equation (2.18) is solved for \( F_{direct}(x, y) \) and inserted into equation (2.23) and we obtain the absorption corrected fluorescence \( F_{a,EXM}(x, y) \) as a function of the measured fluorescence \( F(x, y) \) and the measured reflection \( R(x, y) \) at all locations in the image:

\[
F_{a,EXM}(x, y) = F(x, y) \left( \frac{1 + f_{EM,0}}{1 + f_{EM,0} \frac{R(x, y)}{R_0}} \right) \left( \frac{1 + f_{EX,0}}{1 + f_{EX,0} \frac{R(x, y)}{R_0}} \right)
\]  

If the absorption by the dye molecules cannot be neglected as in equation (2.13), it can be lumped into the local reflectance \( f_{EX} \). Because a dye does not absorb at its emission wavelength, the relationship of the reflections at excitation and emission wavelengths may be different from that given in equation (2.20). If for some reason, e.g. for a less absorbing background, the penetration depth of light becomes larger and a considerable amount of dye molecules residing inside the soil are excited as well, the increase in reflection may be under-corrected by equation (2.24). This is because an increase in reflectance is normally related to a decrease of absorption according to equation (2.9). This further increases the
contribution of fluorescence from those dye molecules inside the soil not taken into account by equation (2.24). This equation describes therefore the limiting case of minimal impact of change of reflection on change of fluorescence.

2.3.4 Background correction function

As has been shown in the preceding sections, the fluorescence image has to be corrected for the differing background of the soil profile. We obtain the background corrected fluorescence \( F_c(x, y) \) by multiplying the measured fluorescence \( F(x, y) \) with the local value of a correction function \( c(R(x, y)/R_0) \), which depends on the relative reflection \( R(x, y)/R_0 \)

\[
F_c(x, y) = F(x, y) c(R(x, y)/R_0)
\]  

(2.25)

The correction function \( c_g \) for a varying surface roughness and uniform light absorption, given by equation (2.7), and the correction function \( c_a \) for varying light absorption and a flat surface, given by equation (2.24), are two limiting cases. In the first case, the detected fluorescence varies proportional to the detected reflection, whereas in the second case a change in the detected reflection implies merely a small change in the detected fluorescence. The upper graph in Figure 2.9 depicts the reciprocal values of \( c_g \) and \( c_a \) as a function of \( R(x, y)/R_0 \) in the range 1 to 12, i.e. the local reflection exceeds the average reflection. The correction function \( c_a \) is shown for different average reflectances \( f_{EX,0} \) and \( f_{EM,0} \) corresponding to the main excitation and emission wavelengths of the fluorescent dyes Brilliant Sulfaflavine, Sulforhodamine B and Oxazine 170 (Table 2.3, compare Figure 2.8). The lower graph in Figure 2.9 represents the same correction functions as a function of \( R_0/R(x, y) \) in the range 1 to 12, i.e. the local reflection is smaller than the average reflection.

| Table 2.3 Average reflectances \( f_{EX,0} \) and \( f_{EM,0} \) corresponding to the main excitation and emission wavelengths \( \lambda_{EX} \) and \( \lambda_{EM} \) of Brilliant Sulfaflavine, Sulforhodamine B, and Oxazine 170 |
|---------------------------------|-----------------|-----------------|-----------------|
| \( f_{EX,0} (\lambda_{EX}) \)   | 0.07 (418 nm)   | 0.13 (565 nm)   | 0.18 (618 nm)   |
| \( f_{EM,0} (\lambda_{EM}) \)   | 0.09 (514 nm)   | 0.15 (580 nm)   | 0.19 (650 nm)   |
Figure 2.9 Background correction functions \( c_g \), \( c_a \), and \( c_m \) in the range \( 1 < R(x, y)/R_0 < 12 \) and \( 1 < R_0/R(x, y) < 12 \). The correction functions \( c_g \) and \( c_a \) are shown for the dyes Brilliant Sulfaflavine (BF), Sulforhodamine B (SB), and Oxazine 170 (OX). The correction function \( c_m \) is shown for Sulforhodamine B only. Note that the vertical axis of the upper graph refers to the inverse of the correction functions, whereas in the lower graph the vertical axis refers to the correction functions.

In Figure 2.9 we see that the maximum value of \( 1/c_a \) is limited by the maximum value of \( R(x, y)/R_0 = 1/f_{EM} \) because the reflectance cannot exceed 1. However, in real images \( R(x, y)/R_0 \) can be larger than this theoretical maximum value, because the effects of absorption and scattering of the soil material interfere with the effects of surface roughness. If the local reflection is smaller than the average reflection, \( c_a \) is, according to equation (2.24), confined in a even narrower range by the limit \( (1+f_{EM,0})(1+f_{EX,0}) \) for \( R(x, y)/(R_0) \to 0 \). Accordingly, there is a large difference between \( c_a \) and \( c_g \) for local reflections which deviate substantially from the average reflection. This difference would
still exist in case we had a larger penetration depth of light or other reasons for an increased impact of changing light absorption on fluorescence.

Unfortunately, surface roughness and absorption vary both, but independently over the soil profile. As a consequence, a change in reflection cannot be assigned to a change in roughness or absorption only and an intermediate correction function $c_m$ between $c_a$ and $c_g$ has to be applied in equation (2.25). For field soils, we employed the mean value of $c_a$ and $c_g$ as intermediate correction function:

$$c_m = \frac{1}{2} \left( \frac{1 + f_{EM,0} R(x, y) / R_0}{1 + f_{EM,0}} \right) \left( \frac{1 + f_{EX,0} R(x, y) / R_0}{1 + f_{EX,0}} \right) + 1/2 \left( \frac{R_0}{R(x, y)} \right) \quad (2.26)$$

The function $c_m$ is shown in Figure 2.9 for the dye Sulforhodamine B.

The differences between $c_m$ and $c_a$ as well as between $c_m$ and $c_g$ for a very high or very low reflection may introduce large correction errors. At locations of very high reflectance of the soil material, the fluorescence signal is over-corrected, because $c_a$ instead of $c_m$ should be used for such spots. Similarly, at very dark spots in local depressions of the soil surface, the fluorescence is under-corrected, because $c_g$ instead of $c_m$ should be used for these locations. If the areas of extreme, i.e. very high or very low reflection are small, as in case of strongly reflecting soil components, specular reflection or little holes, they can be smoothed out by averaging over the neighboring image elements (pixels).

Smoothing is applied before background correction and therefore the reflection as well as the fluorescence signal have to be smoothed in the same way. If the fluorescence signal were smoothed and the reflection signal left as such, the subsequent background correction would account for reflection variations without the corresponding fluorescence variations. On the other hand, if the reflection image were smoothed and the fluorescence signal left as it is, the subsequent background correction would not account for background induced fluorescence changes still being present in the fluorescence image.

Smoothing leads to a loss in spatial resolution and should therefore be employed in critical regions only. Critical regions are detected by a high local variance of reflection which exceeds the variance of a reference region without spots of very high or very low reflection.
Both, detection of critical regions and their smoothing is performed by a locally adapting digital filter:

\[
S_s(x, y) = \left(1 - \frac{v_n}{v_w(x, y)}\right)S_{w0}(x, y) + \left(\frac{v_n}{v_w(x, y)}\right)S(x, y)
\]  

(2.27)

where \(S_s\) is the filter output at a certain location \((x, y)\), i.e. the smoothed signal, \(S(x, y)\) the original signal at the same location, \(S_{w0}\) the local mean of the signal in a region around that particular location \((x, y)\), \(v_w(x, y)\) the local variance of reflection in this region, and \(v_n\) the standard reflection variance, i.e. the reflection variance in a reference region. The region to calculate the local mean and variance is a quadratic area of \(7 \times 7\) pixels surrounding the central location \((x, y)\). Such a region is called filter window. To calculate the filter output in the whole image, the filter window moves over the whole image and applies the filtering equation (2.27) to the central locations. This filter is applied to both signal images, the reflection and the fluorescence image. However, standard and local variances are determined from the reflection image only. The filter output in the fluorescence image depends therefore on the corresponding values in the reflection image.

The selection of the reference region in the reflection image to calculate the standard reflection variance is crucial, first, to avoid unnecessary filtering and related losses of resolution, and second, to detect the critical regions where the background correction function would lead to very large uncertainties without smoothing. The reference region should therefore contain the whole range of reflection values except the very high or very low values due to strongly reflecting soil components, specular reflection or little holes. If the variance of reflection values in the reference region, the standard reflection variance \(v_n\), is larger than the reflection variance \(v_w(x, y)\) in a \(7 \times 7\) pixels neighborhood of a location \((x, y)\), the value at this location is left unchanged in the reflection and the fluorescence image. By contrast, if \(v_w(x, y)\) is larger than \(v_n\), the value at \((x, y)\) is replaced by the mean of the surrounding pixels in the reflection and the fluorescence image. Because the filter output depends on the local statistics of the reflection image, it belongs to the class of adaptive filters based on local statistics [Pitas and Venetsanopoulos, 1990]. The filtering equation (2.27) is notably inspired by the design of the Local Minimum Mean Square Error.
(LMMSE) filter [Kleihorst, 1994; Pitas and Venetsanopoulos, 1990]. However, the underlying assumptions are completely different.

### 2.4 Field Experiment

We conducted a field experiment to demonstrate fluorescence imaging of tracer distributions in a soil profile. The experiment was carried out in a forest stand at Unterehrendingen, AG, Switzerland, on 24/25 June 1998. The soil is a Typic Haplumbrept [Soil Survey Staff, 1994]. Selected physical and chemical properties are given in Table 2.4.

#### Table 2.4 Soil properties at the experimental site

<table>
<thead>
<tr>
<th>depth [cm]</th>
<th>bulk density [g cm$^{-3}$]</th>
<th>Clay</th>
<th>Silt</th>
<th>Sand</th>
<th>pH$^b$</th>
<th>C$_{org}$ [g kg$^{-1}$]</th>
<th>CEC$_{eff}$ [mmol$_c$ kg$^{-1}$]</th>
<th>base saturation [%]$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-9</td>
<td>1.22</td>
<td>21</td>
<td>34</td>
<td>45</td>
<td>3.6</td>
<td>19.9</td>
<td>72.52</td>
<td>27</td>
</tr>
<tr>
<td>9-20</td>
<td>1.22</td>
<td>21</td>
<td>33</td>
<td>46</td>
<td>3.6</td>
<td>11.6</td>
<td>65.69</td>
<td>25</td>
</tr>
<tr>
<td>20-50</td>
<td>1.40</td>
<td>22</td>
<td>32</td>
<td>46</td>
<td>3.8</td>
<td>6.6</td>
<td>73.49</td>
<td>49</td>
</tr>
<tr>
<td>50-100</td>
<td>1.51</td>
<td>28</td>
<td>37</td>
<td>35</td>
<td>4.0</td>
<td>3.1</td>
<td>114.60</td>
<td>79</td>
</tr>
</tbody>
</table>

a. Expressed on dry soil weight basis.

b. 0.01 M CaCl$_2$.

During 6 hours, a plot of size 0.5 by 1.5 m was irrigated with 40 L of a solution of the fluorescent dyes Brilliant Sulfaflavine (2.39 mmol L$^{-1}$), Sulforhodamine B (1.72 mmol L$^{-1}$), and Oxazine 170 (0.035 mmol L$^{-1}$) with a portable sprinkling device [Flury et. al., 1994]. The gravimetric water contents before and after irrigation are given in Table 2.5. One day after the irrigation we prepared a vertical soil profile of size 1 by 1 m in the middle of the irrigated plot. The images were taken during the night and a black blanket covered the soil pit to prevent any light to infiltrate into the pit from the outside.

The corrected fluorescence images and therefore the relative concentration distributions for Brilliant Sulfaflavine and Sulforhodamine B are shown in Figure 2.10. For high concentra-
Table 2.5 Gravimetric water content before and after irrigation

<table>
<thead>
<tr>
<th>depth [cm]</th>
<th>before irrigation [kg kg⁻¹]</th>
<th>after irrigation [kg kg⁻¹]</th>
<th>difference [kg kg⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-9</td>
<td>0.314</td>
<td>0.366</td>
<td>0.052</td>
</tr>
<tr>
<td>9-20</td>
<td>0.237</td>
<td>0.294</td>
<td>0.057</td>
</tr>
<tr>
<td>20-50</td>
<td>0.217</td>
<td>0.259</td>
<td>0.042</td>
</tr>
<tr>
<td>50-100</td>
<td>0.269</td>
<td>0.256</td>
<td>-0.013</td>
</tr>
</tbody>
</table>

tions of Sulforhodamine B outside the linear range of the fluorescence-concentration function, the amount of Sulforhodamine B in Figure 2.10 is underestimated. Oxazine 170 was strongly adsorbed by the organic materials and did not infiltrate into the mineral soil in the 24 hours between irrigation and measurement. One pixel corresponded to 1 mm² in the soil profile, i.e. one million tracer concentration measurements were obtained for a soil profile of 1 m². The exposure time for Brilliant Sulfaflavine was 400 s and for Sulforhodamine B 110 s. We needed approximately 2 hours to take all images for the detection of three dyes. The image processing to obtain relative concentrations took another 5 hours.

The detection limit was about 0.05 mmol kg⁻¹ for Brilliant Sulfaflavine and about 0.02 mmol kg⁻¹ for Sulforhodamine B. The detection limit was not determined by the sensitivity of the fluorescence signal but by the high level of stray light. The dyes were still detectable for concentrations 10 times less than the detection limit, but the signal-to-noise ratio became too low for these concentrations. The lower detection limit of Sulforhodamine B is due to the much higher extinction coefficient which reduces exposure time and therefore the level of stray light.

5. The amount of applied Sulforhodamine B was too high in the presented experiment. To obtain a linear fluorescence-concentration function, the concentration of the irrigated Sulforhodamine B solution should not exceed 1.0 mmol L⁻¹. Sulforhodamine B has a high fluorescence and can be easily detected in soil even if applied with much lower concentrations.
Figure 2.10 Corrected fluorescence images of the dyes Brilliant Sulfaflavine and Sulforhodamine B corresponding to their relative concentrations in a soil profile of size 1 by 1 m after a multitracing infiltration experiment in a forest.
2.5 Conclusions

Fluorescence imaging provides a means of determining two-dimensional concentration distributions of the dyes Brilliant Sulfaflavine, Sulforhodamine B, and Oxazine 170 in soil profiles. By the selection of suitable optical filters, a powerful xenon lamp, and a sensitive CCD camera, a high signal-to-noise ratio is achieved. Correction procedures based on images showing light distribution and background reflection allow to cope with the highly variable lighting and background. Consequently, the fluorescence signal is a well defined function of the dye concentration. For small dye concentrations, the fluorescence signal is linear in the concentration and relative concentrations are obtained directly from lighting and background corrected fluorescence images. Absolute concentrations are derived by calibration, e.g. by comparing the fluorescence imaging signals with measurements on a fluorescence spectrometer for samples taken from the soil profile.

The fluorescence images have a resolution of 1248 by 1152 pixels which corresponds to more than one million concentration measurements per soil profile. The measurements for the three dyes and the subsequent image processing are performed within one day. The detection limit is in the range of 0.02 to 0.05 mmol dye tracer per kg dry soil.

The correction procedure of the fluorescence signal is based on various reflection images showing the soil background at different wavelengths. These images can also be used to obtain information about the soil structure. The combination of information about structure and distributions of several tracers might provide a powerful tool to understand solute transport in soils.
References


Chapter 3

Fluorescent Dyes Selection for Multitracing Experiments in Soils

P. Aeby, D. Braichotte, U. Memmert\textsuperscript{1}, B. Seyfried\textsuperscript{1}, B. Studer, and H. Flühler

We evaluated fluorescent dye tracers which can be simultaneously applied and detected to visualize and quantify flow paths in the soil. In addition to the dye tracers already known in hydrology and soil science, we tested laser dyes and fluorescent compounds employed in microbiology and related fields. The dyes to be used for multitracing in the field must have high, non-overlapping fluorescence in water and must not be toxic nor light sensitive. Furthermore, their fluorescence should not be quenched by soil constituents, interfere with the autofluorescence of the soil, or depend on the pH. Based on these criteria, Brilliant Sulfaflavine, Sulforhodamine B, and Oxazine 170 qualify as suitable tracers. For the red-emitting laser dye Oxazine 170, fluorescence, ecotoxicity and adsorption data are presented. It shows no acute toxicity to earthworms and has no effects on the respiration and nitrification rates of soil micro-organisms. Oxazine 170 is highly adsorbing to inorganic soil material and organic matter, whereas Brilliant Sulfaflavine is rather mobile and Sulforhodamine B moderately adsorbing. The three tracers are recommended for use in multitracing experiments to probe the properties of travel routes of different reactive solutes.

\textsuperscript{1} RCC Ltd, Itingen, BL, Switzerland
3.1 Introduction

Transport processes in soils are complex and not satisfactorily understood, partly because of the inhomogeneity of the soil matrix, the intricate arrangement of three phases, and the soil's continual change in time. Many approaches successfully applied to transport problems in chemical and mechanical engineering and related fields like hydrology are only useful in special cases. Models which seem to describe transport experiments reasonably well, may fail when predictions are made based on the assumptions and model parameters of these particular experiments. Tracers are needed to evaluate transport models in the field and laboratory and to determine model parameters. They are also needed to investigate the processes involved in transport of water and solutes and to visualize flow patterns of differently reactive compounds.

Dye tracers have the obvious advantage of staining flow paths. Their concentrations can be measured in solutions extracted from soil cores or sampled with suction cups. Among the dye tracers, the fluorescent dyes have the additional advantage that they can be determined in situ with fluorescence imaging or fiber-optic spectroscopy. Imaging methods have a considerably higher spatial resolution than e.g. bulk extraction of salt tracers.

Fluorescence spectroscopy is a highly sensitive and specific analytical method which is suited to measure fluorescing tracers in solution or directly in the soil. The fluorescent dyes have specific wavelengths of excitation and emission which makes it possible to distinguish several dyes at the same time and in the presence of other absorbing compounds. For this reason, fluorescent tracers have been employed in hydrology since the last century [Knop, 1878]. In 1965, Reynolds [1966] tested various fluorescent dyes for percolation experiments through soils. Up to now, many fluorescent tracers have been utilized to study water infiltration and solute movement in soils. Among them are Fluorescein and Pyranine [Omoti and Wild, 1979], Rhodamines, Lissamine FF, and Amino G Acid [Smettem and Trudgill, 1983; Trudgill, 1987], optical brighteners [Smart, 1976], and Naphtionate [Behrens et al., 1986; Wernli, 1986]. An overview about the properties of the most common flu-

---

2. See chapter 2, “Quantitative Fluorescence Imaging of Tracer Distributions in Soil Profiles”
orescent tracers is given by Smart and Laidlaw [1977]. However, recent research has shown
that transport experiments carried out with Rhodamine B and Rhodamine WT are difficult
to interpret, because these dyes form structural isomers with identical fluorescence but dif-
ferent adsorption characteristics [Shiau et al., 1993; Lopez Arbeloa and Rohatgi-Mukher-
jee, 1986].

In the following, we evaluate fluorescent tracers which can be simultaneously applied and
detected to visualize and quantify flow paths in the soil. In addition to the tracers already
known in hydrology and soil science, we tested laser dyes and fluorescent compounds
employed in microbiology and related fields. In the case of lacking ecotoxicological infor-
mation, we tested their potential to harm organisms in the natural environment.

3.2 Requirements for Tracer Selection

The dyes to be used for multitracing in the field must have high, non-overlapping fluores-
cence in water and must not be toxic. A high fluorescence results from high efficiencies to
absorb and re-emit photons. The absorption efficiency is expressed by the extinction coef-
ficient $\varepsilon$ and the re-emission efficiency by the fluorescence efficiency or fluorescence quan-
tum yield $\phi_f$. These properties change depending on the solvent. Thus, the fluorescence of
many strongly fluorescing dyes is too weak in water and cannot be measured in soil or soil
solution. Moreover, many dyes are poorly or not soluble in water.

Spatial and temporal changes in fluorescence impair the determination of the dye concen-
trations. The following factors modify the fluorescence and interfere with the concentration
analysis: (i) Fluorescence quenching by soil constituents like humic substances or the dye
molecules themselves; (ii) Background fluorescence of the soil or soil solution at wave-
lengths of excitation and emission; (iii) Light sensitivity; and (iv) finally, the pH depen-
dence of fluorescence. The pH determines the charge of a dye molecule and consequently
its adsorption and fluorescence characteristics. The fluorescence is at best constant over the
pH range of the soil. Fluorescence depends also on temperature. However, since temperature is almost constant in the upper parts of a soil, its effects can be neglected.

### 3.3 Brilliant Sulfaflavine and Sulforhodamine B

Based on the criteria given in the preceding section, Brilliant Sulfaflavine (Sigma Chemical Co., St. Lois, MO) and Sulforhodamine B monosodium salt (Fluka Chemie AG, Buchs, Switzerland) qualify as suitable tracers (Figure 3.1). Brilliant Sulfaflavine has almost identical properties as Brilliant Sulfoflavine (Lissamine FF) which contains only one methyl group, para to the nitrogen. Sulforhodamine B (Pontacyl Brilliant Pink B, Amidorhodamine B) belongs to the xanthene dyes which comprise other widely used rhodamines like Rhodamine B and Rhodamine WT. Selected properties of Brilliant Sulfaflavine and Sulforhodamine B are given in Table 3.1.

<table>
<thead>
<tr>
<th>Property</th>
<th>Sulfaflavine</th>
<th>Sulforhodamine</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAS No</td>
<td>[2391-30-2]</td>
<td>[3520-42-1]</td>
<td></td>
</tr>
<tr>
<td>sum formula</td>
<td>C_{20}H_{15}N_{2}NaO_{5}S</td>
<td>C_{27}H_{29}N_{2}NaO_{7}S_{2}</td>
<td></td>
</tr>
<tr>
<td>molecular weight</td>
<td>418.41 g mol(^{-1})</td>
<td>580.66 g mol(^{-1})</td>
<td></td>
</tr>
<tr>
<td>solubility (in water)</td>
<td>20 g L(^{-1})</td>
<td>20 g L(^{-1})</td>
<td>Green, 1990</td>
</tr>
<tr>
<td>extinction coefficient (\epsilon) (in water)</td>
<td>(16 \times 10^3) L mol(^{-1}) cm(^{-1})</td>
<td>(84 \times 10^3) L mol(^{-1}) cm(^{-1})</td>
<td>Haugland, 1996</td>
</tr>
<tr>
<td>fluorescence efficiency (\phi_f) (in water)</td>
<td>0.274</td>
<td>0.186</td>
<td>Bedrick et al., 1979</td>
</tr>
</tbody>
</table>

The excitation and emission spectra of Sulfaflavine and Sulforhodamine B are well separated as shown in Figure 3.2. Their fluorescence is nearly independent of the pH (Figure 3.3) and not or only little quenched by salts (Figure 3.4) and humic acids (Figure 3.5). The photodecomposition of Brilliant Sulfaflavine and Sulforhodamine B is negligible if
Figure 3.1 Chemical structure of Brilliant Sulfaflavine (a) and Sulforhodamine B (b) exposed for a few hours to daylight [Laidlaw and Smart, 1982; Behrens and Teichmann, 1982; Bedrick et al., 1979] and these tracers are not toxic [Field et al., 1995].

Figure 3.2 Excitation (Ex) and emission (Em) spectra of Brilliant Sulfaflavine (BF) and Sulforhodamine B (SB) in water at pH 7
**Figure 3.3** Effect of pH on the fluorescence of the tracer dyes

**Figure 3.4** Effect of NaCl on fluorescence of tracer dyes in water at pH 7. The dye concentration is 12.8 μmol L⁻¹ for Brilliant Sulfaflavine, 0.94 μmol L⁻¹ for Sulforhodamine B, and 3.6 μmol L⁻¹ for Oxazine 170.

In most soils, Brilliant Sulfaflavine is rather mobile and Sulforhodamine B is moderately adsorbing [Smettem and Trudgill, 1983; Finkner and Gilley, 1986; Laidlaw and Smart, 1982; Smart and Laidlaw, 1977; Trudgill, 1987; Aldous and Smart, 1988].
Figure 3.5 Effect of Aldrich humic acid (Techgrade, Sigma-Aldrich, Gillingham, Dorset, UK) on fluorescence of tracer dyes in water at pH 7. The dye concentration is 5.3 mg L\(^{-1}\) for Brilliant Sulfaflavine, 0.546 mg L\(^{-1}\) for Sulforhodamine B, and 1.56 mg L\(^{-1}\) for Oxazine 170. The decrease in fluorescence for humic acid concentrations of more than 20 mg L\(^{-1}\) is due to excitation light absorption of the dark humic acid suspension.

The excitation of Brilliant Sulfaflavine at a wavelength around 418 nm is likely to excite fluorescing soil constituents giving rise to a high background fluorescence. However, the dye’s stokes shift, i.e. the difference between the peaks of excitation and emission, is much larger than the stokes shifts of the soil constituents and the background fluorescence can be cut off by an emission filter.

Other tracers with excitation and emission spectra between 400 and 600 nm are more sensitive to irradiation and pH (Fluorescein, Pyranine [Behrens, 1986]), have a weak fluorescence in water (Lucifer Yellow CH, Sulforhodamine 101) or form structural isomers (Rhodamine B and Rhodamine WT [Shiau et al., 1993; Lopez Arbeloa and Rohatgi-Mukherjee, 1986]). Also, in case of the rhodamines the fluorescence spectra of Sulforhodamine B are best separated from Brilliant Sulfaflavine.
3.4 Oxazine 170

As shown in the preceding section, Brilliant Sulfaflavine and Sulforhodamine B are suitable tracers in soil. If a third tracer is applied at the same time, its fluorescence has to be in the UV/blue or red part of the spectrum.

Optical brighteners and the tracers Naphtionate and Amino G Acid are excited between 300 and 400 nm and can be detected simultaneously with Brilliant Sulfaflavine. However, these compounds are prone to photobleaching [Smart and Laidlaw, 1977; Behrens and Teichmann, 1982; Wernli, 1986]. Furthermore, excitation in the UV results in high background fluorescence in soil and soil solution.

No tracers with emission wavelengths longer than those of the rhodamines have been used in environmental sciences so far. Thus, we tested red-emitting dyes as utilized in dye lasers and biological sciences. Most of these dyes were too expensive for field use and others did not or only weakly fluoresce in water (Basic Blue 3, Copper Phthalocyanine Tetrasulfonic Acid, 1,1'-Diethyl-4,4'-Carbocyanine, Evans Blue, Fast Green FCF, Light Green SF Yellowish, Lissamine Green B, Nile Blue A, Pyridine 1, Rhodamine 800, Styrl 7), were toxic (Diethyloxadicarbocyanine, Diethylthiadicarbocyanine), decomposed rapidly (3,3’-Diethyl-oxatricarbocyanine), or did not dissolve in water (1,1’,3,3,3’,3’-Hexamethylindodicarbocyanine).

Three tracers fluoresced strongly: Oxazine 1, Oxazine 170, and Hexamethylindodicarbocyanine. Of these, Oxazine 170 perchlorate (Fluka Chemie AG, Buchs, Switzerland), also called Oxazine 720, had the highest fluorescence and best matched the tracer requirements discussed above. Selected properties are given in Table 3.2 and the chemical structure is depicted in Figure 3.6.

Figure 3.7 shows the absorption and fluorescence spectra of Oxazine 170. For high concentrations, the absorption spectrum does not coincide with the excitation spectrum because Oxazine 170 forms dimers via hydrogen bonds which have two absorption peaks. For alkaline conditions (pH > 9), deprotonation removes the positive charge on nitrogen and the flu-
Table 3.2  Selected properties of Oxazine 170 perchlorate

<table>
<thead>
<tr>
<th>Property</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAS No</td>
<td>[62669-60-7]</td>
</tr>
<tr>
<td>chemical name</td>
<td>5,9-bis(ethylamino)-10-methyl-benzo(a)phenoxazin-7-ium perchlorate</td>
</tr>
<tr>
<td>sum formula</td>
<td>C_{21}H_{22}ClN_{3}O_{5}</td>
</tr>
<tr>
<td>molecular weight</td>
<td>431.88 g mol^{-1}</td>
</tr>
<tr>
<td>solubility (in water)</td>
<td>approximately 15 mg L^{-1}</td>
</tr>
<tr>
<td>extinction coefficient ε (in water)</td>
<td>48 \times 10^3 L mol^{-1} cm^{-1}</td>
</tr>
<tr>
<td>fluorescence efficiency ϕ_f (in water)</td>
<td>0.24 ± 0.05 (cationic form, pH 2-9)</td>
</tr>
<tr>
<td>pK_a (cationic -neutral form)</td>
<td>10.0 ± 0.2</td>
</tr>
</tbody>
</table>

Figure 3.6 Chemical structure of Oxazine 170 perchlorate

Fluorescence decreases (Figure 3.3). The various forms of Oxazine 170 in solution and their spectra were investigated by Gvishi and Reisfeld [1989].

The fluorescence of Oxazine 170 is independent of the salt concentration (Figure 3.4) but is quenched by humic acids (Figure 3.5). Apart from that, Oxazine 170 is among the most stable dyes under intense light [Antonov and Hohla, 1983; Drexhage, 1977].

Little is known about toxicity and ecotoxicity of Oxazine 170 except for a study of its mutagenicity [Wuebbles and Felton, 1985]. Therefore, its acute toxicity to earthworms and its impact on microbial respiration and nitrification in soil was evaluated and is reported in the following sections.
Figure 3.7 Absorption, excitation and emission spectra of Oxazine 170 in water at pH 7. The dye concentration is 3.6 μmol L⁻¹.

3.5 Acute Toxicity of Oxazine 170 to Earthworms (Eisenia foetida) in a 14-Day Static Test

3.5.1 Materials and methods

The acute toxicity of Oxazine 170 to Eisenia foetida was tested according to the OECD [1984] guidelines for testing chemicals.

According to the testing guideline, we used an artificial soil substrate with 10% ground peat, 20% kaolin and 70% sand. The pH was adjusted to about 6.5. The earthworms were incubated in this soil in three parallel treatments: 21 and 210 mg Oxazine 170 per kg dry soil and a control containing no test substance. The test substance was applied by mixing Oxazine 170 stock solutions with 555 g dry soil in a 1 L glass beaker. The moisture content of the soil mixture was adjusted to about 0.35 kg kg⁻¹ with deionized water and losses of water during incubation were compensated after seven days by adding deionized water. The
test was run over 14 days, at a temperature of 21-24°C and under permanent illumination with a light intensity of 400-800 Lux.

Adult earthworms with a clitellum and a body wet weight of 300-400 mg per worm were utilized. Per treatment, 40 test animals were randomly distributed to four test beakers, i.e. 10 per beaker. At the start of the test, the worms were placed onto the surface of the soil. After 7 and 14 days of exposure, the content of each test beaker was emptied. The living worms were counted and any abnormal behavior of the test animals was recorded. After the 7-day assessment, worms and soil were filled back into the test beakers.

The average body wet weight of the test animals was determined at the start and at the end of the test by weighing all living worms of each test beaker and dividing the weight by the number of worms. Before weighing, the worms were rinsed with water and quickly dried with filter paper.

The pH of the untreated and treated soils was measured at the end of the test in a soil extract of 0.1 M KCl and ranged from pH 5.8 to 6.0. The water content of the soil was 0.32-0.33 kg kg\(^{-1}\) after 14 days.

### 3.5.2 Results and discussion

After 7 and 14 days of exposure, all test animals had survived in the control and in both treatments. Moreover, no abnormal behavior or symptoms of toxicity were recorded.

The mean body wet weight of the worms in the control beakers was 325 mg at the beginning of the test, and 289 mg at the end. Hence, the mean body wet weight of the worms in the control decreased slightly by 11% due to starvation during the exposure period of 14 days. The reduction of the mean wet weight of the worms in the treated soil was in the same range (9% to 8%).

Consequently, Oxazine 170 proved to be nontoxic to the earthworm *Eisenia fetida* up to the concentration of 210 mg per kg dry soil. Based on this, the 14-day LC0 (concentration without any lethality) and NOEC (no observed effect concentration) is at least 210 mg kg\(^{-1}\) or higher. The 14-days LOEC (lowest observed effect concentration), LC50 and LC100
(concentrations with 50 and 100% lethality) were not determined, but are clearly higher than 210 mg kg\(^{-1}\).

3.6 The Effects of Oxazine 170 on Microbial Respiration and Nitrification in Soil

3.6.1 Materials and methods

3.6.1.1 Microflora study with Oxazine 170

The effects of Oxazine 170 on soil micro-organisms were tested according to the European Commission Directive 96/12/EC (March 1996) based on the methods given in SETAC - Europe [1995].

An agricultural soil (9.5% clay, 27.4% silt, 63.2% sand; 1.2% organic matter; pH 5.5-7.0) was sieved to 2 mm and mixed with alfalfa (*Medicago sativa*) meal (0.5 g per 100 g dry soil) as a nitrogen source. Samples equivalent to 100 g dry soil were mixed with Oxazine 170 solution to contain 21 (low dose treatment) and 210 (high dose treatment) mg Oxazine 170 per kg dry soil and adjusted to about 40% of its maximum gravimetric water content. Controls for the low and the high dose treatment were prepared in the same way but contained no dye. The test was performed under standard laboratory conditions in an air-conditioned room at 18-22 °C in the dark. Erlenmeyer flasks (250 ml) were used as test vessels. Samples were analyzed within 6 hours and after 14 and 28 days. All tests were made in duplicates.

**Short-term respiration:** Soil aliquots were amended with 115 mg glucose per 100 g dry soil. Substrate induced short-term respiration [Gerber et al., 1991; Malkomes, 1986; Anderson & Domsch, 1978] was monitored for approximately 16-24 hours by measuring the CO\(_2\) development on-line with an IR-gas analyzer (UNOR 6N, Maihak, Hamburg, Germany). The respiration results were compared to the scheme given by Malkomes [1986].

**Soil nitrification:** Soil aliquots equivalent to 40 g dry soil were extracted twice with 55 mL 2 \(M\) KCl solution by shaking 15 min at room temperature. Ammonium, nitrate and nitrite
were measured colorimetrically with a flow-injection analyzer (Tecator FIAstar 5010 flow injection analyzer equipped with a Tecator 5032 controller and a Tecator 5017 sampler, Foss Tecator, Hoganas, Sweden).

### 3.6.1.2 Degradation of Oxazine 170

At day 0, 7, 14 and 28 subsamples (10 –20 g) of the microflora samples treated with 210 mg kg\(^{-1}\) Oxazine 170 were taken in duplicate and immediately deep-frozen. The Oxazine 170 concentrations in the samples were measured with a fluorescence spectrometer (LS50B, Perkin-Elmer, Norwalk, CT).

### 3.6.2 Results and discussion

#### 3.6.2.1 Microbial biomass

For a glucose amendment of 115 mg per 100 g dry soil, the maximum initial CO\(_2\) production rate from 100 g dry soil equivalents was 0.611 mL h\(^{-1}\). Applying the formula given by Anderson and Domsch [1978], the microbial biomass was calculated as 25 mg microbial carbon per 100 g dry soil.

#### 3.6.2.2 Short-term respiration rates

Respiration can be regarded as a measure of the general turn-over of organic matter in soil. Respiration levels were determined by monitoring glucose-induced evolution of CO\(_2\) resulting from microbial activity during short-term experiments. The initial respiration rate, an indicator of the actual microbial biomass of the samples, was used to investigate possible side effects of the chemical on micro-organisms by comparing data from treated soil with those of untreated soil. In the present study, the influence of Oxazine 170 on glucose-induced soil respiration was monitored in short-term experiments over an incubation period of 28 days. The rates of glucose-induced soil respiration at the start of the test and after 14 and 28 days are given in Table 3.3.

No significant influence of Oxazine 170 on the respiration rates of soil micro-organisms was observed for the two treatments. For the high dose treatment, the differences between untreated controls and treated samples were more pronounced than for the low dose treatment. After 28 days of incubation, the respiration rates were slightly increased in treated
soil by 0.8% and 7.9% for the low and the high dose treatment, respectively. These influences are negligible according the Malkomes scheme [Malkomes, 1986].

**Table 3.3** Influence of Oxazine 170 on glucose-induced short-term respiration rates in soil. Respiration is expressed in amount of carbon dioxide evolved during one hour of the initial respiration phase.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation Time [d]</th>
<th>0</th>
<th>14</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[ml CO₂ h⁻¹]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (low dose)</td>
<td></td>
<td>0.876</td>
<td>0.863</td>
<td>0.760</td>
</tr>
<tr>
<td>21 mg (kg dry soil)⁻¹</td>
<td></td>
<td>0.869</td>
<td>0.879</td>
<td>0.766</td>
</tr>
<tr>
<td>% Deviation</td>
<td></td>
<td>-0.8</td>
<td>1.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Control (high dose)</td>
<td></td>
<td>1.020</td>
<td>0.938</td>
<td>0.743</td>
</tr>
<tr>
<td>210 mg (kg dry soil)⁻¹</td>
<td></td>
<td>0.948</td>
<td>1.008</td>
<td>0.802</td>
</tr>
<tr>
<td>% Deviation</td>
<td></td>
<td>-7.1</td>
<td>7.5</td>
<td>7.9</td>
</tr>
</tbody>
</table>

3.6.2.3 Nitrogen transformation measurement

The microbial conversion of organic nitrogen to nitrate is a multi-step process. In a primary reaction, soil organic matter is mineralized to ammonia (ammonification). A wide range of possible sinks of ammonia are reported [Paul and Clark, 1989], e.g. immobilization by soil micro-organisms by incorporation and formation of amino acids, ion exchange or adsorption with soil constituents. Furthermore, ammonia is converted via nitrite to nitrate, a process which is designated as nitrification. This is the second step in organic matter conversion.

The following scheme shows the main pathway of the conversion of organic matter [e.g. Sprent, 1987]:

\[
\text{organic matter} \xrightarrow{\text{MINERALIZATION}} \text{NH₄}^+ \xrightarrow{\text{NITRIFICATION}} \text{NH₂OH} \xrightarrow{[\text{HNO}]} \text{NO}_2^- \xrightarrow{\text{NO}_3^-}
\]
Chemicals may affect the various micro-organism species/populations, which mediate the conversion. The occurrence of ammonium and nitrate as well as the extent of nitrate formation with respect to the control are assumed to show possible side effects on mineralization/nitrification.

Oxazine 170 had no influence on the ammonification and nitrification processes in soil at the concentrations tested. For the low dose treatment, the concentrations of the different nitrogen species are presented in Table 3.4. Ammonium-nitrogen (NH$_4^+$-N) concentrations decreased from maximum 0.11 mg per 100 g dry soil to 0.01-0.02 mg at the end of the tests (day 28). Nitrite-nitrogen (NO$_2^-$-N) concentrations were 0.02 mg or lower throughout the whole test period. The slightly decreased NO$_3^-$-N levels at day 14 indicate a net-assimilation of nitrogen by soil micro-organisms.

Table 3.4 Influence of Oxazine 170 on the content of ammonium, nitrite, nitrate, and total nitrogen in a soil treated with 21 mg (kg dry soil)$^{-1}$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Parameter</th>
<th>Incubation Time [d]</th>
<th>0 [mg N (100 g dry soil)$^{-1}$]</th>
<th>14</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (low dose)</td>
<td>ammonium</td>
<td>NH$_4^+$</td>
<td>0.11</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>21 mg (kg dry soil)$^{-1}$</td>
<td></td>
<td></td>
<td>0.11</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>% Deviation</td>
<td></td>
<td></td>
<td>0.0</td>
<td>-40.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Control (low dose)</td>
<td>nitrite</td>
<td>NO$_2^-$</td>
<td>0.02</td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>21 mg (kg dry soil)$^{-1}$</td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>% Deviation</td>
<td></td>
<td></td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Control (low dose)</td>
<td>nitrate</td>
<td>NO$_3^-$</td>
<td>5.27</td>
<td>3.98</td>
<td>5.00</td>
</tr>
<tr>
<td>21 mg (kg dry soil)$^{-1}$</td>
<td></td>
<td></td>
<td>4.07</td>
<td>2.27</td>
<td>4.68</td>
</tr>
<tr>
<td>% Deviation</td>
<td></td>
<td></td>
<td>-22.8</td>
<td>-43.0</td>
<td>-6.4</td>
</tr>
<tr>
<td>Control (low dose)</td>
<td>Total N</td>
<td></td>
<td>5.40</td>
<td>4.04</td>
<td>5.02</td>
</tr>
<tr>
<td>21 mg (kg dry soil)$^{-1}$</td>
<td></td>
<td></td>
<td>4.20</td>
<td>2.31</td>
<td>4.70</td>
</tr>
<tr>
<td>% Deviation</td>
<td></td>
<td></td>
<td>-22.2</td>
<td>-42.8</td>
<td>-6.4</td>
</tr>
</tbody>
</table>

For the high dose treatment, the concentrations of the different nitrogen species are provided in Table 3.5. The results obtained were comparable to those obtained for the low dose
treatment. After 28 days of incubation, the low dose treatment with Oxazine 170 caused a slight but negligible decrease in NO$_3^-$-N levels of 6.4%, while the high dose treatment caused a slight but also negligible increase of 5.7%.

Table 3.5 Influence of Oxazine 170 on the content of ammonium, nitrite, nitrate, and total nitrogen in a soil treated with 210 mg (kg dry soil)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Parameter</th>
<th>Incubation Time [d]</th>
<th>0</th>
<th>14</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>[mg N (100 g dry soil)$^{-1}$]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (high dose)</td>
<td>ammonium</td>
<td></td>
<td>0.06</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>210 mg (kg dry soil)$^{-1}$</td>
<td>NH$_4^+$</td>
<td></td>
<td>0.08</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>% Deviation</td>
<td></td>
<td></td>
<td>33.3</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Control (high dose)</td>
<td>nitrite</td>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>210 mg (kg dry soil)$^{-1}$</td>
<td>NO$_2$</td>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>% Deviation</td>
<td></td>
<td></td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Control (high dose)</td>
<td>nitrate</td>
<td></td>
<td>4.87</td>
<td>5.71</td>
<td>12.07</td>
</tr>
<tr>
<td>210 mg (kg dry soil)$^{-1}$</td>
<td>NO$_3$</td>
<td></td>
<td>4.65</td>
<td>5.65</td>
<td>12.76</td>
</tr>
<tr>
<td>% Deviation</td>
<td></td>
<td></td>
<td>-4.9</td>
<td>-1.1</td>
<td>5.7</td>
</tr>
<tr>
<td>Control (high dose)</td>
<td>Total N</td>
<td></td>
<td>4.93</td>
<td>5.73</td>
<td>12.09</td>
</tr>
<tr>
<td>210 mg (kg dry soil)$^{-1}$</td>
<td></td>
<td></td>
<td>4.71</td>
<td>5.67</td>
<td>12.78</td>
</tr>
<tr>
<td>% Deviation</td>
<td></td>
<td></td>
<td>-4.5</td>
<td>-1.0</td>
<td>5.7</td>
</tr>
</tbody>
</table>

The total inorganic nitrogen was calculated from the ammonium-, nitrite- and nitrate-nitrogen levels. Since the NH$_4^+$-N and NO$_2^-$-N levels were negligible, the results obtained for Total-N were comparable to those obtained for NO$_3^-$-N, i.e. -6.4% and 5.7% deviation from the controls after 28 days for the low and the high dose treatment, respectively.

Oxazine 170 showed negligible effects on the soil respiration and on the ammonification/nitrification processes in soil at the concentrations tested. Therefore, no adverse effects of Oxazine 170 on organic matter turnover, and hence on soil micro-organisms and fertility, are to be expected from its use at a concentration in the soil of up to 210 mg per kg dry soil.
3.6.2.4 Degradation study
In all treated samples we measured the same Oxazine 170 concentration. Consequently, no degradation occurred during the 28-day microflora study.

3.7 Adsorption of Oxazine 170 to Soil

Oxazine 170 strongly adsorbs to inorganic soil material and organic matter. To characterize its adsorption behavior, we performed batch experiments and determined various hydrocarbon-water partition coefficient.

3.7.1 Materials and methods
The adsorption measurements were performed under consideration of the OECD [1981] guidelines for testing chemicals. The soil material stems from a forest soil in Unterehrendingen, AG, Switzerland, classified as a Typic Haplumbrept [Soil Survey Staff, 1994]. Selected physical properties are given in Table 3.6.

Table 3.6 Properties of the soil used for the adsorption/desorption study

<table>
<thead>
<tr>
<th>depth [cm]</th>
<th>bulk density [g cm⁻³]</th>
<th>Clay [%]</th>
<th>Silt [%]</th>
<th>Sand [%]</th>
<th>pHᵇ</th>
<th>Cₓ [g kg⁻¹]</th>
<th>CECeff [mmol c kg⁻¹]</th>
<th>base saturation [%]³</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-9</td>
<td>1.22</td>
<td>21</td>
<td>34</td>
<td>45</td>
<td>3.6</td>
<td>19.9</td>
<td>72.52</td>
<td>27</td>
</tr>
<tr>
<td>9-20</td>
<td>1.22</td>
<td>21</td>
<td>33</td>
<td>46</td>
<td>3.6</td>
<td>11.6</td>
<td>65.69</td>
<td>25</td>
</tr>
<tr>
<td>20-50</td>
<td>1.40</td>
<td>22</td>
<td>32</td>
<td>46</td>
<td>3.8</td>
<td>6.6</td>
<td>73.49</td>
<td>49</td>
</tr>
<tr>
<td>50-100</td>
<td>1.51</td>
<td>28</td>
<td>37</td>
<td>35</td>
<td>4.0</td>
<td>3.1</td>
<td>114.60</td>
<td>79</td>
</tr>
</tbody>
</table>

a. Expressed on dry soil weight basis.
b. 0.01 M CaCl₂.

For 24 h, 5 g dry soil was equilibrated with 5 ml 0.01 M CaCl₂. Then, 20 mL of 6.25 mg L⁻¹ Oxazine 170 solution was added to obtain a total solute concentration of 5 mg L⁻¹. Several samples of 1 mL were taken from the supernatant solution at different times while the
soil-dye suspension was shaken for 24 h. The samples were filtered with a 0.45 μm filter and the Oxazine 170 concentration in the samples was measured with a fluorescence spectrometer (LS50B, Perkin-Elmer, Norwalk, CT). However, the dye concentration was below the detection limit of 10 μg L⁻¹ for topsoil (0-9 cm depth) and subsoil (20-50 cm depth). This experiment was repeated with a total solute concentration of 22.5 mg L⁻¹. However, no dye could be detected in the supernatant solution either. The experiments were made in duplicates.

Because all Oxazine 170 molecules were adsorbed to the soil in the presence of water, we performed a desorption batch experiment with a water-acetone extraction solvent. First, 5 g dry soil was mixed with 1.4 ml Oxazine 170 solutions with concentrations of 0, 0.025, 0.25, 0.5, 1.25, 2.5, 12.5, and 25 mg L⁻¹. Samples of 1 g were taken from the dye-soil mixture and shaken with 10 mL water-acetone (4 to 1 volume ratio) solvent for 12 h. The suspension was filtered and the dye concentration determined fluorometrically. Based on mass balance, the adsorbed concentrations were calculated from three replicates of the total concentrations and of the concentrations in the liquid phase.

We employed the hydrocarbons octanol (n-Octyl alcohol), hexane, and isooctane (2,2,4-Trimethylpentane) as measures for the affinity of Oxazine 170 to soil organic matter. Buffered at pH 7, 25 mL Oxazine 170 solutions with the concentrations 0.7 and 1.2 mg L⁻¹ were mixed with 25 mL octanol, hexane, isooctane, respectively, and shaken for 4 h. Then the concentrations of Oxazine 170 were measured in the aqueous phases and the hydrocarbon-water partition coefficients were calculated. Three replicates were made for all measurements.

3.7.2 Results and discussion

The batch experiments showed that Oxazine 170 strongly adsorbs to the top- and subsoil. The concentration in water was below the detection limit of 10 μg L⁻¹ even for high Oxazine 170 concentrations. On the other hand, Oxazine 170 could be extracted with a water-acetone solvent (Figure 3.8) which can be attributed to a cosolvency effect [Soerens and Sabatini, 1994].
Oxazine 170 is lipophilic having an average octanol-water partition coefficient of 38, an average hexane-water partition coefficient of 3.5, and an average isoctane-water partition coefficient of 2.0. This agrees with its low solubility in water of approximately 15 mg L\(^{-1}\). Therefore, the strong adsorption to soil material is probably mainly because of hydrophobicity and the interaction of the positively charged Oxazine 170 molecule with the negative charges of the organic matter and clay minerals.

### 3.8 Multitracing using Brilliant Sulfaflavine, Sulforhodamine B, and Oxazine 170

The red-fluorescing dye Oxazine 170 was selected to complement Brilliant Sulfaflavine and Sulforhodamine B in multitracing studies for its photochemical stability and the low background fluorescence in soil at wavelengths longer than 500 nm. Simultaneous fluorescence measurements of all three dyes in solution showed that the emission of Brilliant Sulfaflavine (200 \(\mu\)mol L\(^{-1}\)) was 10% lower in the presence of Sulforhodamine B (15 \(\mu\)mol L\(^{-1}\)). This was probably because a part of the emission light was absorbed by the Sul-
forrhodamine B molecules. In the same way, the emission of Sulforhodamine B (15 \( \mu \text{mol L}^{-1} \)) was 15\% lower in the presence of Oxazine 170 (35 \( \mu \text{mol L}^{-1} \)). In this case, excitation and emission light was presumably absorbed by Oxazine 170 molecules. In soil, the absorption of the dyes is negligible if measured against the background absorption. Furthermore, emitted photons travel very short distances in the thin surface layer of the soil or are absorbed by the background and never reach the detector. In comparison, the optical path in the cuvet is very long. Hence, the small overlap of the spectra does not pose a problem for quantitative analysis in soil.

Multitracing allows to probe the properties of travel routes with different reactive solutes at the same time for the same initial and boundary conditions. In soil, Brilliant Sulfaflavine is rather mobile, Sulforhodamine B is moderately adsorbing and Oxazine 170 is strongly adsorbing. If a second rather mobile tracer is required, the non-fluorescing dye Brilliant Blue [Flury and Flühler, 1994, 1995] instead of Oxazine 170 can be applied together with Brilliant Sulfaflavine and Sulforhodamine B. It has a similar absorption spectrum as Oxazine 170 and is well suited to stain flow paths [Flury et al., 1994]. The application of Brilliant Blue is also recommended, if quantitative analysis of Oxazine 170 is difficult because of high content of dissolved organic matter in the soil solution and consequent fluorescence quenching.

In general, however, the three tracer dyes Brilliant Sulfaflavine, Sulforhodamine B, and Oxazine 170 are recommended for quantitative multitracing experiments in soil, because of their strong, non-overlapping and stable fluorescence and low toxicity. Last but not least, their use is not expensive, especially when one considers the little quantities needed.
References


Green, F.J. 1990. The Sigma-Aldrich Handbook of Stains, Dyes and Indicators. Aldrich Chemical Company, Milwaukee, WI.


Malkomes, H.P. 1986. Effect of the quality of glucose on the reaction of short term respiration in soil compared with plant protection products using a herbicide as an example. (In German.) Nachrichtenblatt Deutsch. Pflanzenschutz. 38: 113-120.


Chapter 4

Quantitative Absorption Imaging of Tracer Distributions in Soil Profiles

4.1 State of the Art

Over the last four years, three imaging techniques have been developed at the soil physics group of ETH Zürich to measure dye tracer concentrations in soil profiles. Two of these techniques quantify the concentration of the non-fluorescing tracer Brilliant Blue by image analysis. They are based on the fact that brightness and color of a medium change with the concentration dependent light absorption of Brilliant Blue at its surface. The media used were sand [Aeby et al., 1997] and soil [Forrer, 1997; Forrer et al., 1998].

The third method, fluorescence imaging, is presented in the two preceding chapters. The fluorescence technique is sensitive and designed to maximize the signal-to-noise ratio, which results in a well-defined calibration function, namely a linear relationship between dye concentration and measured fluorescence. The signal is maximized by a high-power excitation, a highly sensitive camera, and strongly fluorescing tracers. The noise is reduced by optical filters, minimal noise of detection, and corrections for inhomogeneous lighting and background.

In contrast to fluorescence imaging, the relationship between the Brilliant Blue concentration and the absorption images is much more complex. The reflection of light at the soil or sand surface was recorded for three (color film) or four (color and near IR film) different ranges of the spectrum (image channels) according to the light sensitive layers of the film.
The reflection intensities are called grey levels of the image channels. The statistics of the grey levels of sand or soil regions with known dye concentrations (training areas) yield spectral signatures. Every spectral signature characterizes regions of known Brilliant Blue concentration for given experimental conditions and can be used to identify other regions of similar Brilliant Blue concentrations. However, a spectral signature does not only depend on the dye concentration but also on

1. background surface roughness
2. background absorption (color and water content)
3. illumination
4. measurement (response) function of the detection device

In the case of sand [Aeby et al., 1997], it was technically feasible to produce fairly flat and smooth surfaces. The background absorption varied because dark grains were present among the bright quartz grains. A median filter reduced this background absorption noise effectively. Only the illumination could not be corrected properly due to the signal dependent sensitivity of the film. For calibration, samples of known dye concentration were prepared by mixing sand with dye and used as training areas.

In the case of soil [Forrer et al., 1998], the spectral signatures of small sample areas were determined and compared to the dye concentrations extracted from these samples. The extraction produces additional errors due to varying mass recovery, slightly shifted sampling locations in the image and, most important, differences between the dye concentration in the sampling volume and the very surface.

Indirect diffuse light resulted in quite homogeneous illumination. But even for these conditions, a lighting correction was necessary. For this, a grey frame surrounding the soil profile was used. The light distribution on the grey frame was extrapolated to the area of the soil profile. Here, as in the case of the sand, lighting corrections can be improved by using a linear detection device like a CCD camera. The light intensities reflected from a grey
panel covering the entire front of the soil profile are normalized with their median value and used to correct the inhomogeneous lighting of other images by division.

Even if the illumination were absolutely homogenous, the light reflection from the soil surface is not only a function of the dye concentration but to a large extent determined by surface roughness and background absorption. The soil color is highly variable and the soil surface rugged. A large number of spectral signatures were required to obtain a representative spectral signature for every combination of soil color, roughness and dye concentration. By extracting 155 surface samples, a representative calibration set was available and the subsequent assignment of dye concentrations to grey levels was successful. This method does not work for regions in the soil profile whose soil color, roughness and dye concentration is not represented in the calibration set. An obvious disadvantage of this method is therefore the time consuming extraction of a large number of soil samples. Additional difficulties occurred because the variable film material and processing produced different spectral signatures for the same soil color, roughness and dye concentration. This problem can also be avoided by the use of a digital camera.

The structure of the calibration function was not well defined since grey levels of different channels are strongly correlated. Optical filters in front of the camera prevent an overlap of the wavelengths recorded by the different channels. However, as the grey-brownish color of the soil indicates, grey levels correlate with $R^2 > 0.85$ even for non-overlapping parts of the spectrum (see Table 2.2).

For a linear concentration-intensity relationship as in the case of fluorescence imaging, the parameters of the calibration determined by training areas can be applied to individual image elements. By contrast, point measurements are not possible for calibration functions derived from absorption imaging. This is also true because the high variability of the grey levels for the same concentration leads to wrong concentration assignments (inverse prediction problem [Neter et al., 1990]). The high variability is a consequence of the high background noise generated by the changing soil background, roughness and illumination.

An alternative to the calibration function is classification. Each point in the image is assigned to a concentration class which is characterized by its spectral signature. Here,
point measurements are possible but the increased spatial resolution is at the expense of accuracy in the determination of the concentration.

4.2 Suggestions for Improvement

Few years ago, digital cameras were expensive, technically delicate and had a low spatial resolution. Nowadays, digital imaging is a prerequisite for quantitative image analysis. The measurement function of a digital camera is well-defined and linear for the whole dynamic range. This allows an accurate illumination correction by flat-fielding and the use of optical filters to tailor image channels for a specific dye and background. By contrast, the sensitivity curves of photographic material depend on many factors, overlap strongly, and limited exposure time impedes employment of bandpass filters.

The separability of spectral signatures is increased if the image channel corresponds to the maximum absorption of the dye (signal channel). This improves the signal-to-noise ratio and consequently reduces the measurement error. In addition, a further image channel could be used where the dye is not visible (background channel). In absorption imaging, though, the background of the signal channel is different to the one characterized by the background channel, because the signal and the background are measured at different wavelengths. Since sand does not have spots with specific absorption bands and is therefore rather homogeneous, the signal background can be estimated from the background channel and subtracted form the signal channel which leads to a further noise reduction.

In sand, training areas are easily prepared for a range of dye and water contents. After flat fielding, median filtering, and background subtraction spectral signatures are mainly a function of the dye concentration. The logarithm of the dye concentration might even be proportional to the intensity of the signal channel according to the law of Beer-Lambert and a linear calibration function could be applied.
In soil, background subtraction is more difficult and noise filtering cannot be applied in structured soil. Hence, a large number of extraction samples is needed and fluorescence imaging might be the better choice.
References


Chapter 5

Concluding Remarks

5.1 Soil Structure and Solute Transport

The multitracing detection device described in chapter 2 is not only suited to detect fluorescent tracers, but also non-fluorescing materials by absorption imaging. One application is the concentration determination of non-fluorescent tracers (chapter 4). Another essential feature is its capabilities to characterize the soil background, i.e. soil material, water content, and soil structure. The equipment is designed in a way that any combination of illumination and detection wavelengths between 250 and 1250 nm can be selected with the appropriate optical filters. In fluorescence spectroscopy, the wavelengths of illumination (excitation wavelengths) are shorter than the wavelength of detection (emission wavelengths). In absorption imaging, reflection patterns of the incident light are recorded, so that illumination and detection wavelengths should overlap. A proper choice of the illumination wavelengths reduces the noise by minimizing the stray light passing through the detection filters in front of the camera. As in remote sensing, interesting features of the soil background are recognized by their spectral signature, i.e. their specific reflection spectrum [Richards, 1993]. Information about soil structure is obtained by pattern recognition, where not only spectral signatures of individual image elements are analyzed, but also their spatial arrangement [Gonzalez and Woods, 1992]. The full potential of the multitracing detection device is exploited when structure and tracer concentrations in soil are determined at the same time.

In a first step, soil regions of different hydraulic conductivities are distinguished based on their brightness at several parts of the visible spectrum. This information can be used as
input for a solute transport model. Finally, the model output can be compared to the concentrations after a tracer experiment.

5.2 Validation

The developed device yields point measurements of tracer concentrations at soil surfaces. Other techniques, e.g. extraction of small soil cores, average the concentrations over much larger measurement volumes and can therefore not be used to check local measurements. Therefore, concentration maps were validated indirectly by comparing CCD imaging and fluorescence spectrometer measurements and by preparing samples of known dye content. In soils with spatially changing or nonlinear adsorption isotherms, the varying relationship between adsorbed and dissolved dye concentration may result in different fluorescence signals for the same dye content. For that reason and considering the complex interactions between the dye molecules and soil constituents, concentration maps should always be used with caution, especially when the multitracing detection device is employed in a new soil.

5.3 Other Tracers

Depending on the specific research questions, we may require other dye tracers than Brilliant Sulfaflavine, Sulforhodamine B, Oxazine 170, and Brilliant Blue. For alkaline and moderately acid soils, Amino G Acid and Naphtionate should be tested. Their fluorescence spectra coincide with the excitation spectrum of Brilliant Sulfaflavine, but this is not expected to be a problem in soil. If they turn out to be suitable, four tracers could be used simultaneously in soil.

I hope that in near future specially tailored tracers will be available for applications in environmental physics.
References


Dank

Herzlich bedanken möchte ich mich bei Hannes Flühler für die ausgezeichnete Betreuung und das optimale Arbeitsumfeld und bei Daniel Braichotte, Hannes Wydler, Maya Bundt, Paul Gähwiller, Björn Studer, Olf Kramer, Daniel Stadler, Thomas Pfluger und Farnaz Moser für die tolle Zusammenarbeit.

Für wertvolle Hilfe bedanke ich mich vielmals bei Thomas Gimmi, Jörg Leuenberger, Nadia Ursino, Andreas Papritz, Jan Vanderborght und Hanspi Läser.

Der Firma RCC, Birgit Seyfried und Ulrich Memmert sei gedankt für die Laborstudien und Hans-Gerd Löhmansröben und Hubert van den Bergh für die Übernahme der Korreferate und die konstruktive Kritik.

Für das gute Umfeld am Institut und die vielseitige Unterstützung gilt Jeannette Hollinger, Hans Feyen und allen anderen ITOe-Gschpänli ganz lieben Dank.
Curriculum Vitae

1968
Born 13 December in Dornach SO

1975-1987
Elementary School and Junior College
in Münchenstein BL

1987-1988
Self-Studies, Federal Matura Type C

1988-1991
Undergraduate Studies in Social and Natural Sciences,
University of Basel, cand. phil.

1991-1994
Graduate Studies in Earth Sciences,
ETH Zürich, Dipl. Natw. ETH

1994-1995
Visiting Researcher, Centro Internacional de
Agricultura Tropical, Cali, Colombia

1995-1998
Research assistant and Ph.D. student, Soil Physics,
Institute of Terrestrial Ecology, ETH Zürich