Environ Earth Sci (2013) 69:349–358
DOI 10.1007/s12665-013-2305-3

SPECIAL ISSUE

On-line fluorometry of multiple reactive and conservative tracers in streams

Dennis Lemke · Pierre-André Schnegg · Marc Schwientek · Karsten Osenbrück · Olaf A. Cirpka

Received: 4 July 2012 / Accepted: 2 February 2013 / Published online: 27 February 2013

Abstract Performing tracer tests using artificial tracer compounds is a common practice to characterize natural streams regarding their (reactive) transport properties. Recently, the fluorescent compound resazurin was introduced as a reactive stream tracer to quantify hyporheic exchange and metabolic activity of streams. This tracer, together with its reaction product resorufin and a conservative tracer (in our case fluorescein), provides additional information about transport properties of the stream and its hyporheic zone and can therefore overcome restrictions that are commonly affiliated with the use of conservative tracers alone. However, all previously published studies using this tracer system were based on manual sampling of the water. This usually limits the number of measurements and thus the achievable temporal resolution, and potentially endangers data quality due to inadequate handling of samples. In this paper, a modified version of the GGUN-FL30 on-line fluorometer is presented in which the optics have been modified to allow measuring the concentrations of all three tracers simultaneously at intervals of 10 s. Experiments under controlled and natural conditions showed that the performance of the on-line fluorometer regarding tracer separation efficiency and practical detection limits is comparable to a high-performance laboratory spectrofluorometer. Furthermore, suggestions are given on how to correct tracer signal fluctuations caused by temporal changes in temperature and pH that might occur during a field tracer test.

Keywords Fluorometer · Tracer test · Groundwater/surface water interaction · Resazurin

D. Lemke (✉) · M. Schwientek · K. Osenbrück
WESS, Water and Earth System Science Competence Cluster, c/o University of Tübingen, Hölderlinstr. 12, 72074 Tübingen, Germany
e-mail: dennis.lemke@uni-tuebingen.de

D. Lemke · O. A. Cirpka
Center for Applied Geosciences, University of Tübingen, Hölderlinstr. 12, 72074, Tübingen, Germany

P.-A. Schnegg
Albillia Sàrl, Rue de l'Evole 13, 2000 Neuchâtel, Switzerland

Introduction

Tracer tests are a reliable and often used method to study ground water (Einsiedl 2005; Einsiedl and Maloszewski 2005; Benischke et al. 2007; Osenbrück et al. 2013) and surface water bodies (Aumen 1990; Wagner and Harvey 1997; Grathwohl et al. 2013). In the past decades, artificial tracer tests have increasingly gained importance to characterize (reactive) transport mechanisms in natural streams (Boulton et al. 2010). In this context, typically, a tracer is introduced into the stream and the time series of the tracer concentration, denoted breakthrough curve (BTC), is detected at one or more points downstream of the injection point. Numerous well accepted mathematical models have been used to estimate the parameters describing (reactive) transport from the breakthrough curves by inverse modelling strategies (Runkel 1998; Haggerty and Reeves 2002; Liao and Cirpka 2011). As tracer material, fluorescent dyes are widely used for this purpose due to their advantageous properties regarding low background concentrations, sorption, reactivity and detection limits (Käss 2004; Leibundgut et al. 2009).

In principle, BTCs during stream-tracer tests can be obtained either by manual sampling of the stream water and subsequent analysis of the samples in a laboratory, or

by continuous on-line recording of the tracer concentrations directly in the field. On-line fluorometers have successfully been used in numerous studies to record concentration profiles of quasi-conservative fluorescent dyes such as rhodamine WT, fluorescein, naphthionate, eosin, among others (Gooseff et al. 2003, 2005, 2008; Payn et al. 2008). Continuous on-line measurements are potentially superior to manual sampling because typically a higher temporal resolution of the measurements can be achieved and possible errors due to sample storage and contamination are avoided. Additionally, the tracer concentrations are instantly available, helping to adjust and refine the sampling strategy for possible concurrent experiments, such as gas-tracer tests (Cirpka et al. 1993).

Recently, resazurin has been introduced as a reactive fluorescent tracer to investigate metabolic activity in stream ecosystems (Haggerty et al. 2008, 2009; Argerich et al. 2011). Compared to a conservative tracer, this tracer is referred to as 'smart' because it provides additional information about the flow paths along which the tracer is transported. Resazurin is weakly fluorescent and can irreversibly be reduced to the strongly fluorescent dye resorufin. Originally, resazurin was used as an oxidation-reduction indicator in cell viability assays for bacteria and mammalian cells (O'Brien et al. 2000). In stream systems, the reduction of resazurin to resorufin indicates exposure of the water to a metabolically active environment, which is believed to be concentrated in the hyporheic zone, where the large surface area of the riverbed grains facilitates the growth of biofilms and cell colonies (Fischer and Pusch 2001). Combining resazurin and a conservative compound in a stream-tracer test thus provides additional information about hyporheic exchange and metabolic activity of the hyporheic zone (e.g., Liao and Cirpka 2011).

All previously published tracer tests with resazurin were based on manual sampling of the stream water (e.g., Haggerty et al. 2009; Argerich et al. 2011) and subsequent analysis of the samples in a lab. In practice, this may imply a considerable time gap between sampling and sample analysis due to long transportation routes or availability issues of the lab instruments. It has been found that dissolved resazurin and also resorufin degrades within days, which may be in the order of the time delay between sampling during field campaigns and sample analysis. These effects are even more relevant when the samples are unfiltered and/or exposed to sunlight (Haggerty et al. 2008). Thus, the concentration data from lab analysis might not correspond to the concentrations during the tracer test, possibly leading to erroneous conclusions from the sample data.

In this paper, an on-line fluorometer is presented that is able to simultaneously measure the concentrations of resazurin, resorufin, and fluorescein with an accuracy that is comparable to high-performance lab spectrofluorometers. This on-line fluorometer is a customized version of the GGUN-FL30 on-line fluorometer (Schnegg and Flynn 2002) that has successfully been applied in numerous tracer tests and stream gauging campaigns (e.g., Goldscheider et al. 2008; Schnegg et al. 2011). The main objectives of the paper are to (1) give a brief description of the technique and the setup of the on-line fluorometer, (2) present the data processing and correction methods required for successful application, and (3) assess the performance of the fluorometer in laboratory and field studies.

Materials and methods

Description of the on-line fluorometer

The GGUN-FL30 on-line fluorometer was designed to replace the mechanical sampler in tracer tests using one to three simultaneously injected fluorescent tracers. Water temperature and turbidity are also measured. At the highest sampling rate (2 s when measuring a single tracer), this instrument has successfully been used for stream gauging (Schnegg et al. 2011). The original probe contains LEDs and optical filters conveniently selected for conventional dye tracers, such as fluorescein, eosin, and any brand of rhodamine and colourless tracers such as amino G acid, Tinopal or Na-naphthionate. Only a few optical parts have been replaced to fit the spectral characteristics of the reactive tracers, resazurin and resorufin. The GGUN-FL30 fluorometer contains an optical cell (Pyrex tube) placed along the vertical axis of a waterproof stainless steel casing (Fig. 1). The fluorometer is deployed in the stream and automatically analyzes and records the signals of the three tracers in the water that flows through the optical cell. The optical components used for the measurement of dye concentrations are installed along the orthogonal axes in two different planes (Fig. 1). Each measuring section consists of (1) an excitation branch, comprising a quasi-monochromatic light source (LED), a filter and a condenser lens, and (2) a detection branch, perpendicular to the excitation beam, with a lens, a filter and a photodetector. The light sources and the filters are selected according to the absorption-emission spectra of the dyes. This geometry allows the installation of up to four measuring axes. One of them is used to measure the water turbidity using a light source that is beyond the excitation spectra of all tracers used, and therefore only measures the light that is scattered by the suspended particles in the water. The other axes are used to measure the fluorescent dye concentrations. LEDs with spectral maxima at 470, 525 and 570 nm are used to excite fluorescein, resorufin, and resazurin, respectively. The excitation wavelengths of

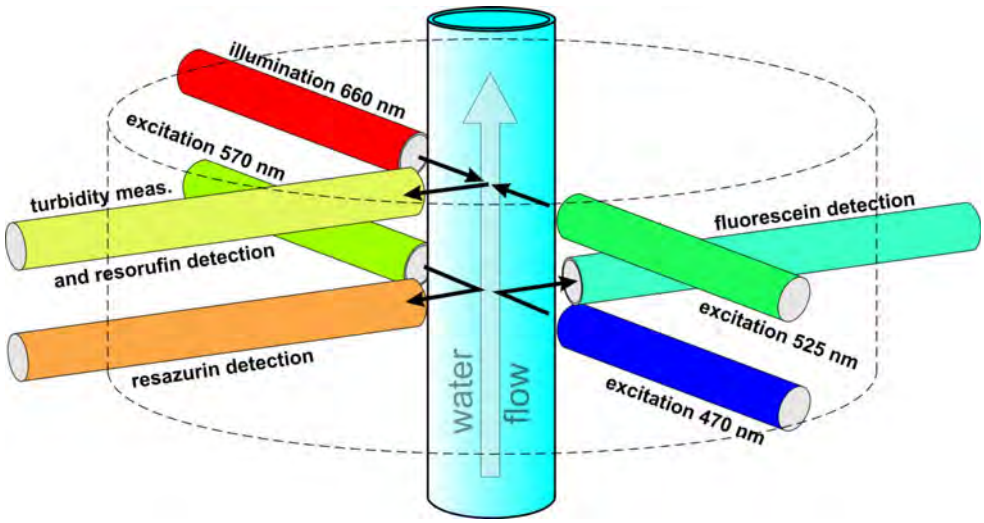

Fig. 1 Optical setup of the GGUN-FL30 on-line fluorometer

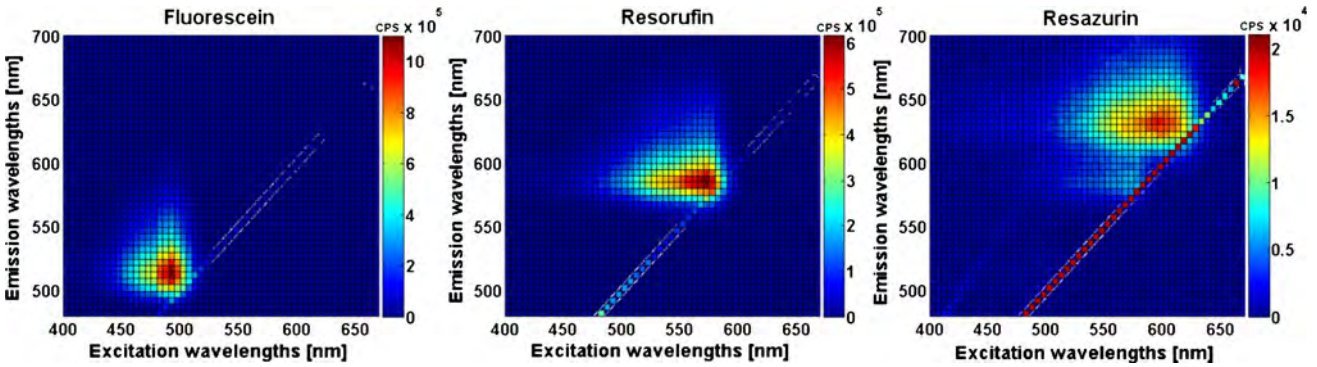

Fig. 2 Fluorescence excitation-emission spectra of all tracers used. Measurements were done using a lab fluorometer (Horiba FluoroMax-4 Spectrofluorometer). The *colors* represent the emission light intensity in counts per second (cps) increasing from *blue* to *red*. Note that the *peak intensity lines* seen at the spectra for resorufin and resazurin (here the maximum intensities were capped to bring out the resazurin signals) are due to Rayleigh scattering in the sample and are unconnected to the tracer

resorufin and resazurin have been intentionally shifted to shorter wavelengths [compared to the excitation maxima of 570 and 602 nm, respectively, found by Haggerty (2008), see Fig. 2] to assure better spectral separation from the fluorescence signals.

When using the supplied software, 100 ppb solutions of every tracer are used for calibration by default. In principle, any other concentration can be used as long as it is the same for all three tracers. These solutions are consecutively filled into the on-line fluorometer and the signal at every detector is recorded for each tracer. The on-line fluorometer records the fluorescence signals from the tracers as well as the dark signals (signal detected at the detectors when no excitation lamp is active) as electrical voltages (mV). The water temperature is measured with a probe fastened to the Pyrex tube. An external logger connected to the probe by a cable acts as a power source and data storage device. The supplied software converts the raw data into intelligible text files and also performs the tracer signal separation and the conversion of signal intensities into tracer concentrations. It also has a built-in display that shows all values of the parameters mentioned above in real-time. The logger can be connected to a computer to allow for convenient graphics-based monitoring of the tracer concentrations. On-line monitoring by a computer, however, is only possible when the computer is connected to the logger prior to the start of the experiment.

Mathematical principle for separating the tracer signals

Fluorescent compounds are able to absorb light of a specific spectrum (excitation light) that temporarily transforms the molecules to an electronically excited state by shifting electrons to higher energy levels. The system immediately tends to jump back to the initial stable state, releasing energy partially as light typically at longer wavelengths compared to the absorbed light due to the Stokes shift (emission spectrum). The intensity of the emitted light is a

measure for the concentration of the fluorescent tracer (Leibundgut et al. 2009).

Figure 2 shows the resulting fluorescence emission intensity from different combinations of excitation and emission wavelengths. The intensity of the light source was constant for all three tracers and the emission light intensities were normalized by the tracer concentrations used, so that the colour scales provide information about the relative fluorescence intensities of the three tracers.

Due to the overlapping excitation and emission spectra of the tracers (cross-sensitivity, significant for resazurin and resorufin, but only marginal for fluorescein), it is inevitable that the signal received by the photo-detectors is a mixture of the emission lights when more than one tracer is present. The separation of the three tracers is achieved within the fluorometer software by solving a set of three linear equations (since three tracers are simultaneously analyzed by the instrument). Each equation gives the intensity of signal U_i (mV) of photo-detector P_i produced by each tracer under excitation by lamp L_{ij}, with $i,j = 1, 2, 3$. For small tracer concentrations such as found in many hydrological applications (<1 ppm), tracer signals are additive and the system is almost linear. As an example we take the case of water containing three different tracers with unknown concentrations C_1, C_2 and C_3 (ppb). Previous calibration of the fluorometer using solutions of single tracers yielded the fixed coefficients k_j^i (mV/ppb) of the three different sets i of lamps, filters and photo-detectors for a fixed concentration of each tracer j. The observed voltages depend on the concentrations in a linear way:

$$\underbrace{\begin{bmatrix} k_1^1 & k_2^1 & k_3^1 \\ k_1^2 & k_2^2 & k_3^2 \\ k_1^3 & k_2^3 & k_3^3 \end{bmatrix}}_{\mathbf{K}} \begin{bmatrix} C_1 \\ C_2 \\ C_3 \end{bmatrix} = \begin{bmatrix} U_1 \\ U_2 \\ U_3 \end{bmatrix} \quad (1)$$

which needs to be inverted in order to determine the concentrations from measured voltages. The coefficients k_j^i of the matrix $\mathbf{K}$ can be determined from three calibration experiments with a single, non-zero concentration each.

Table 1 shows an example of the coefficient matrix $\mathbf{K}$ as acquired upon calibration. The quality of tracer separation can be quantified following the sensitivity analysis described in Wagner and Harvey (1997) using $\mathbf{K}$ and the standard deviations of the tracer signals (in mV):

$$\mathrm{Cov}(\mathbf{C}) = \mathbf{K}^{-1}\mathrm{Cov}(\mathbf{U})\left(\mathbf{K}^{-1}\right)^T \quad (2)$$

in which Cov($\mathbf{U}$) and Cov($\mathbf{C}$) are the covariance matrices expressing uncertainty in the measured voltages and the resulting concentrations, respectively. If we assume that the errors in the voltages are uncorrelated and identical for each channel, the corresponding covariance is a diagonal matrix, $\mathrm{Cov}(\mathbf{U}) = \mathbf{I}\,\sigma_U^2$, with the uncertainty in voltage σ_U. Then, the diagonal of Cov($\mathbf{C}$) contains the estimation variances, or squared estimation standard deviations of the resulting tracer concentrations. In the device, voltages can be measured to an accuracy of 0.05 mV, resulting in standard deviations of the tracer concentrations as low as 0.0004 ppb for fluorescein, 0.0017 ppb for resorufin and 0.0277 ppb for resazurin, indicating good separation. The remaining uncertainty of the concentrations measurements is highly correlated: The correlations coefficient of the uncertainty in resazurin and resorufin is −0.93, that of fluorescein and resazurin −0.36, and that of fluorescein and resorufin 0.07. That is, if the concentration of resazurin is smaller than 0.05 ppb, the measurement is highly uncertain and strongly correlated with that of resorufin. For values of more than 1 ppb resazurin, the relative uncertainty of the measurement is smaller than 1/36, and the correlation of that uncertainty with the uncertainty of another measurement is irrelevant.

Correction of tracer signals for temperature fluctuations

The intensity of the fluorescence signal strongly depends on temperature (Käss 2004; Leibundgut et al. 2009). Differences in temperatures between the conditions during calibration and those during the tracer test itself can lead to significant over- or underestimation of the tracer concentrations, so that a correction of the acquired raw data for temperature is advisable. The fluorescence intensity $U^i_{j\,\mathrm{meas}}$ (mV) of detector i caused by tracer j at the measurement temperature T (K) is related to the intensity $U^i_{j\,\mathrm{c}}$ (mV) at the calibration temperature T_c (K) by (modified after Leibundgut 2009):

$$U^i_{j\,\mathrm{meas}} = U^i_{j\,\mathrm{c}} \times \exp\left(h^i_j(t - t_\mathrm{c})\right) \quad (3)$$

where h^i_j (K^{-1}) is a correction factor that is specific for the device, the fluorescence detector i and the tracer j, because it includes temperature effects of the quantum yields of the tracers as well as the excitation light intensity. An easy procedure to estimate these coefficients is to fill the fluorometer with a tracer solution and store the device in a cooling chamber for a sufficient time to let it cool down to approx. 5 °C. Subsequently the device will be removed from the cooling chamber and the fluorescence intensities are measured until the probe has reached ambient room temperature (Fig. 3).

The fluorescence intensities of the tracers react to changes in temperature in a different way. The fluorescence intensity of fluorescein decreases with increasing temperature, which is in agreement with previous findings (Leibundgut et al. 2009). The resazurin signal shows the same trend, but with a much more distinct slope. In contrast, there is a positive correlation between fluorescence

Table 1 Example of coefficients k_j^i (mv/ppb) acquired in calibration of the on-line fluorometer using standard solutions with 69.24, 103.49 and 91.03 ppb for fluorescein, resorufin and resazurin, respectively

Excitation/ emission wavelength	Tracer 1: Fluorescein (mV/ppb)	Tracer 2: Resorufin (mV/ppb)	Tracer 3: Resazurin (mV/ppb)
Channel 1: 470 nm/512 nm	27.939	7.269	0.700
Channel 2: 525 nm/585 nm	0.348	5.566	0.411
Channel 3: 570 nm/616 nm	0.003	1.260	0.273

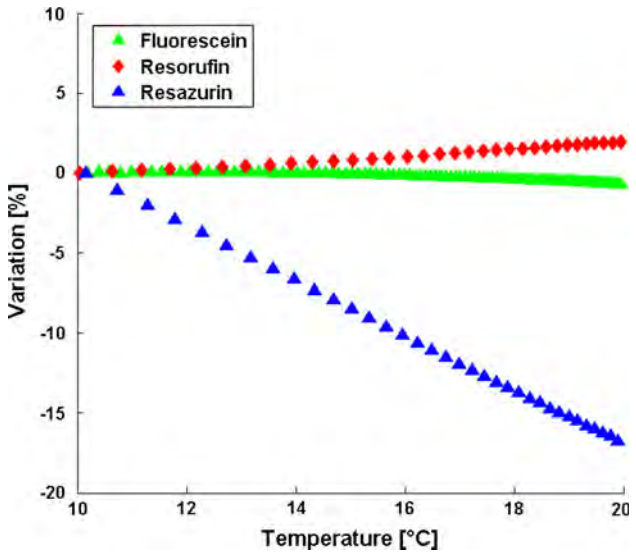

Fig. 3 Dependency of the fluorescence intensity on temperature. Displayed are the data from the respective channels of the tracers

intensity and temperature for resorufin. This behaviour was also found for other dyes such as eosine (Leibundgut et al. 2009), but is in disagreement with the findings of Haggerty (2008). However, as stated above, the effect of temperature on the fluorescence intensity does not only depend on the compound but also to the light source. Repetitive measurements of this relationship yielded the same outcome. The coefficients h_j^i shown in Table 2 were determined by fitting Eq. 3 to the acquired data shown in Fig. 3.

Table 2 Temperature correction coefficients h_j^i (/K) associated with the experiment shown in Fig. 3

	Tracer 1: Fluorescein	Tracer 2: Resorufin	Tracer 3: Resazurin
Channel 1:	−0.0004/K	0.0011/K	−0.0029/K
Channel 2:	0.0195/K	0.0017/K	−0.0039/K
Channel 3:	0.0000/K	−0.0039/K	−0.0183/K

Correction of tracer signals for pH fluctuations

The net charge of dye molecules depends on the pH of the solution. Since the pK_a values (i.e., the negative decadic logarithm of the acid dissociation constant) of resazurin and resorufin are 6.71 (Erban and Hubert 2010) and 5.7 (Kangasniemi 2004), respectively, both compounds appear as neutral molecules or anions under most natural conditions. Fluorescein can occur either in the protonic, neutral, monoanionic or dianionic form (Zanker and Peter 1958). While the pK_a values for the transitions reported in the literature are inconclusive (Kasnavia et al. 1999; Klonis and Sawyer 1996; Zanker and Peter 1958), we use a single pK_a of 6.4 for fluorescein, as suggested by Thanh (2005), in the following.

Figure 4 shows the fluorescence intensities (left y axis, normalized for maximum intensities) of the three tracers as a function of pH. If the pK_a values for all three tracers are known, the fraction of the anionic form of the tracers can easily be calculated as:

$$F_{base}^{j}(pH) = \left(1 + 10^{pK_a^j - pH}\right)^{-1} \tag{4}$$

in which $F_{base}^{j}(pH)$ is the fraction of the anionic form of tracer j and pK_a^j is the negative decadic logarithm of the acid dissociation constant of that tracer. The values are shown on the right y axis of Fig. 4. There is a strong correlation between the fluorescence intensities and the fractions of the anionic forms of the tracers with respect to changes in pH value, indicating that it is predominately the anionic species that causes the fluorescence signal. This is in agreement with the findings of Kangasniemi (2004) who came to the same conclusion regarding resorufin. However, our data from lab experiments (Fig. 4) cannot be described with the pK_a values stated above. Instead, we fitted apparent pK_a values of 6.00, 6.75 and 5.38 for fluorescein, resorufin and resazurin, respectively, accounting for all effects (temperature, ionic strength, etc.) on the pK_a values under our laboratory conditions.

Now the fluorescence intensity $U_{j\,\mathrm{meas}}^{i}$ of detector i according to the presence of tracer j that was measured at the field pH is related to the fluorescence intensity $U_{j\,\mathrm{c}}^{i}$ under calibration conditions by:

$$U_{j\,\mathrm{meas}}^{i} = U_{j\,\mathrm{c}}^{i} \times \frac{F_{\mathrm{base}}^{j}(pH_{\mathrm{field}})}{F_{\mathrm{base}}^{j}(\mathrm{pH_c})} \tag{5}$$

which results from a correction of the anion fraction for the same total concentration of the tracer. pH_c is the pH value under calibration conditions, whereas pH_{field} is the pH observed in the field.

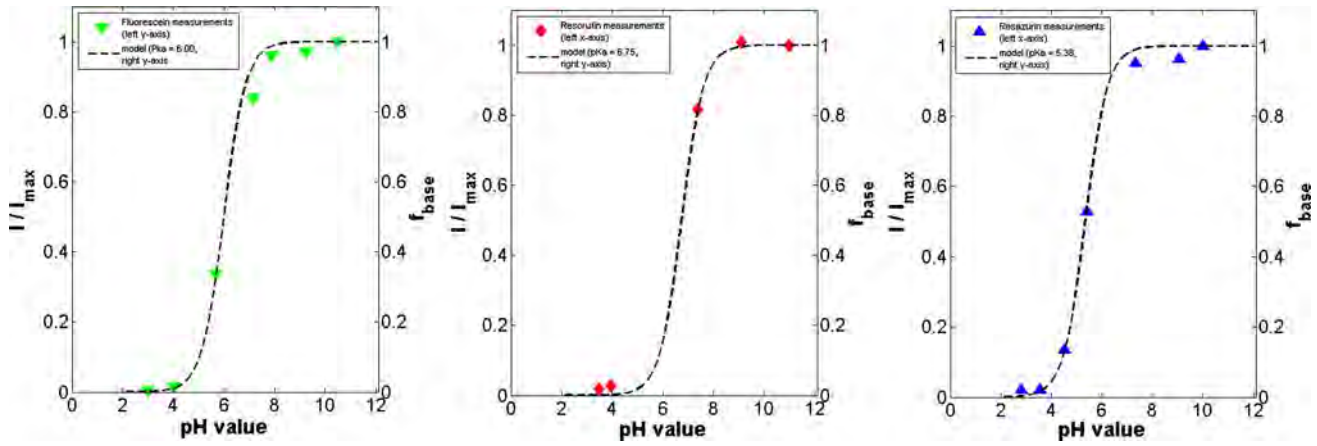

Fig. 4 Effect of pH on the fluorescence intensity and the fraction of the anionic species of the dyes in solution

Applying the corrections to the data

Ideally, the calibration of the fluorometers should be done using the medium that is to be tested, that is, the water of the aquatic system where the tracer test will be conducted. Furthermore, the temperature and the pH value of the water should be stable over the duration of the whole experiment. In practice these two parameters may change over the duration of the tracer test, and logistic reasons may require calibrating the fluorometers in the lab using standard solutions based on tap water, so that differences in temperature and pH may occur between the calibration and the application in the field.

The standard software of the GGUN-FL30 on-line fluorometer does not correct the raw data for temperature or pH fluctuations. This needs to be done by post-processing. Neglecting the temperature dependence of the pK_a, the corrections to be applied to the coefficients k_j^i occurring in Eq. 1 with respect to temperature and pH, respectively, are multiplicative. This is so, since the pH correction applies to the concentration of the fluorescent-active species of each tracer, whereas the temperature correction concerns the linear voltage yield for a given concentration of the active species.

The coefficients k_j^i are defined as the ratio of the signal intensity of detector i in response to the concentration of tracer j. In the following, the subscript c indicates conditions at calibration for k_j^i, temperature, and pH. For any $T \neq T_c$ and $\mathrm{pH} \neq \mathrm{pH_c}$, each element k_j^i of the coefficient matrix $\mathbf{K}$ in Eq. 1 needs to be corrected by:

$$k^i_{j_{t\neq t_c},\mathrm{pH}\neq\mathrm{pH_c}} = k^i_{j_c} \times \exp\left(h^i_j(t-t_c)\right) \times \frac{1+10^{\mathrm{pK}^j_a-\mathrm{pH_c}}}{1+10^{\mathrm{pK}^j_a-\mathrm{pH_{field}}}} \tag{6}$$

Using the corrected coefficients of Eq. 6, the concentrations of fluorescein, resorufin and resazurin are obtained by solving Eq. 1 for the vector of concentrations.

Results and discussion

Comparison of field and lab fluorometer

The performance of the on-line fluorometer was checked regarding sensitivity, detection limits and accuracy compared to a lab fluorometer under controlled lab conditions. For the tests described below, tap water with an adjusted pH of 8.4 was used to prepare the samples and standard solutions. All solutions were constantly shielded from light and given enough time to equilibrate with the room temperature of about 20 °C.

First, a set of solutions with concentrations ranging from 0.1 ppb to 100 ppb of each individual tracer was prepared and the measurements using the on-line fluorometer were compared with those of the lab fluorometer (Horiba FluoroMax-4 Spectrofluorometer). The results are shown in Fig. 5, where the signal intensity is plotted against the known tracer concentration. For fluorescein and resorufin, a clear linear relationship ($R^2 = 0.999$) between tracer concentration and signal intensity is obtained over the chosen concentration range of 4 orders of magnitude with both measurement devices. For resazurin, this relationship is similarly good for concentrations of 1 ppb and higher, and again there is no substantial difference in accuracy between the lab fluorometer and the on-line fluorometer.

In a real tracer test, all three tracers (fluorescein, resorufin and resazurin) are measured simultaneously. Hence, in order to test the accuracy of the tracer signal separation, a set of samples with different combinations of concentrations of the three tracers (see Table 3 for concentrations) was prepared, and their concentrations were subsequently measured with both the on-line fluorometer and the lab fluorometer. The results shown in Fig. 6 indicate a good agreement between the known and the measured concentrations with no significant difference in accuracy between both measurement devices.

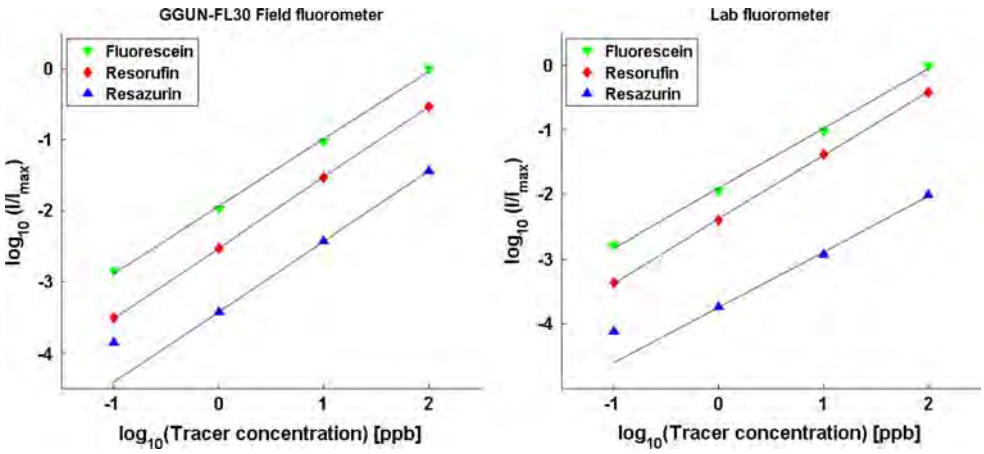

Fig. 5 Comparison between tracer concentrations and the corresponding signal intensities. Note that all intensities (y axis, denoted as I) are normalized for the maximum signal of fluorescein and the numbers on the y axis and x axis are the decadic logarithm of the intensities and the tracer concentrations, respectively. *Black lines* indicate linear fits for all points of the tracers, fluorescein and resorufin, and for concentrations of ≥1 ppb of resazurin

For concentrations below 1 ppb, the relative deviation between the true and measured values increased significantly. This holds for all tracers and both measurement devices. We conclude from this, that concentrations of >1 ppb can be measured fairly accurately when all three tracers are applied.

Application to a tracer test in a small stream

The previous results show that the GGUN-FL30 fluorometer can provide reliable data when used as a lab fluorometer. However, the main intention for developing such a fluorometer was to apply it in field tracer tests. In contrast to the controlled conditions in the lab, there are numerous factors in streams or groundwater that can affect the measurements. While we have outlined approaches to correct for temperature and pH variations above, also the turbidity can affect the measurements because particles can scatter the excitation light and therefore artificially increase the light detected.

We conducted a tracer test in the 3rd-order stream Wiesaz within the Neckar catchment in southern Germany injecting 3.36 g of fluorescein and 31.50 g of resazurin as a pulse. We placed a GGUN-FL30 fluorometer in the river approximately 1,700 m downstream of the injection site and manually collected water samples at the same location for subsequent analysis with the lab fluorometer. The samples were analyzed within 3 h after finishing the experiment to make sure that degradation of the tracers in the collected samples was negligible. Prior to analysis, all samples were equilibrated to room temperature to exclude temperature effects on fluorescence intensity. The concentrations of the tracers in the samples were calculated using Eq. 1. The recordings from the on-line fluorometer were corrected for temperature changes (the water temperature during the experiment ranged from 13.7 to 16.3 °C) using Eq. 3. Because the stream pH was >8, corrections for pH changes were not necessary (see next section).

Table 3 Concentrations of the tracer mixtures used for validation of tracer separation

	Fluorescein (ppb)	Resorufin (ppb)	Resazurin (ppb)
Mix 1	1	0.1	10
Mix 2	10	1	100
Mix 3	100	10	0
Mix 4	0	100	0.1
Mix 5	0.1	0	1

The analysis of the data yielded a mean stream velocity of 0.26 m/s and a discharge of 0.212 m^3/s; 11.7 % of the injected resazurin was removed from the stream where almost all of it (9.9 %) was transformed to resorufin.

Figure 7 shows the BTCs of the three tracers determined by the on-line fluorometer compared to the results based on analyzing of the samples with the lab fluorometer. Without the application of the temperature correction to the tracer signals (see Eq. 3), the peak concentration of resazurin is about 2.5 ppb higher and the peak concentration of resorufin is about 0.1 ppb lower compared to the corrected breakthrough curve. The curve for fluorescein, however, remains virtually unaffected. For all three tracers, the BTCs obtained by the two methods agreed very well when concentrations exceeded 1 ppb, which is in agreement with our findings from the lab experiments described above. However, the accuracy of the measurements is expected to improve significantly when measuring less than three tracers in a stream simultaneously.

Practical advices and conclusions

It has been shown that the GGUN-FL30 on-line fluorometer is able to measure the concentrations of the tracers fluorescein, resorufin and resazurin with an accuracy that is comparable to high-performance laboratory spectrofluorometers. It therefore combines the advantages of both

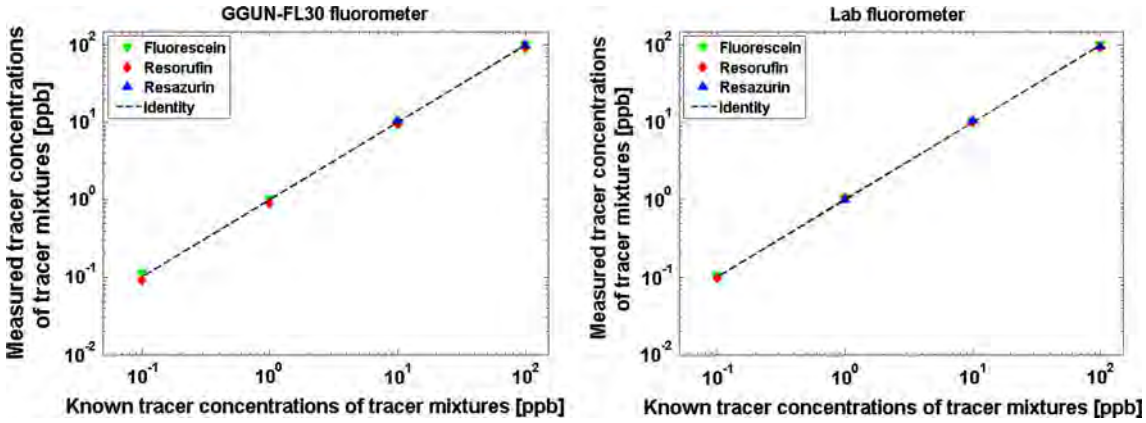

Fig. 6 Comparison between measured and known concentrations in solutions with mixed tracers

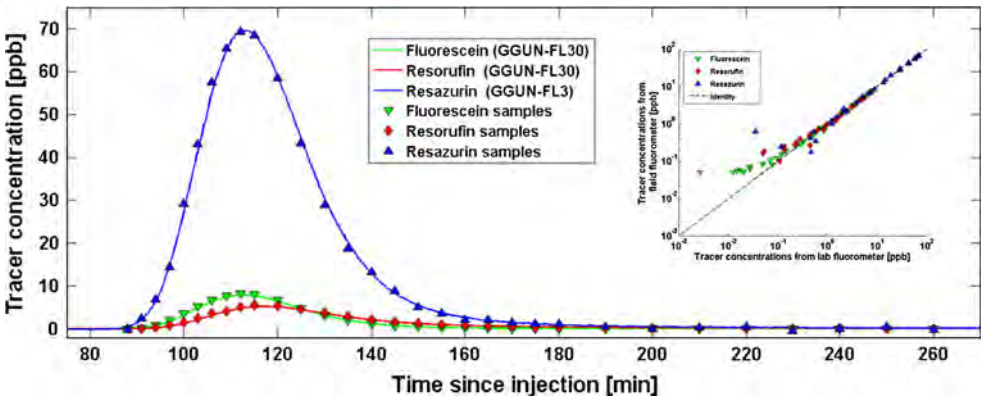

Fig. 7 Comparison of tracer breakthrough curves acquired by the on-line fluorometer and the lab fluorometer, including the direct comparison of the measurements from both devices in a log scale

measurement devices. However, depending on the conditions of the tracer test and the calibration strategy used, several steps of post-processing of the raw data acquired in the field might be necessary. Figure 8 shows a schematic overview of the work flow for the application of the on-line fluorometer and sums up the different individual operations described in the previous sections.

The maximum temporal resolution of the on-line fluorometer is 10 s when all three tracers and the turbidity are measured simultaneously. This exceeds by far the resolution that can be achieved by manual sampling. The advantage of using the on-line fluorometer is thus a better resolved, high-quality data set of multiple tracer concentrations that is available for subsequent data analysis, potentially decreasing the uncertainty of the parameters derived by the analysis, such as velocity or dispersion coefficients (Wagner and Harvey 1997). A highly resolved and potentially longer extended data set of conservative and reactive tracers may also contribute to refining the conceptual model of stream-tracer transport, particularly with respect to the description of processes related to the hyporheic zone (e.g., Liao and Cirpka 2011).

We advise performing the calibration of the on-line fluorometer directly in the field prior to the actual tracer test using the water of the river where the tracer test is performed. We have presented how raw data can be corrected when temperature and pH differ between calibration and measurement conditions. However, the procedure potentially introduces a bias in the corrected concentration values due to the uncertainty in the coefficients for the corrections and/or the uncertainty in the measured temperature and pH values. These uncertainties directly scale with the differences between the calibration solutions and the river water, so that it is desired to keep these differences small. In our lab experiments, we found that the fluorescence intensity for all three tracers is stable when the pH value exceeds 8, which is consistent with previous findings (Haggerty et al. 2008). This implies that a correction for pH is not necessary if the calibration standards and the river water (over the whole duration of the tracer test) have a pH value larger than 8. These stream conditions would be typical for a river with carbonate-rich sediments.

All three detectors have a certain upper detection limit that might be reached when concentrations of highly fluorescent dyes such as fluorescein are high. The separation of the tracer signals requires that none of the detectors are fully saturated. This limit might already be reached

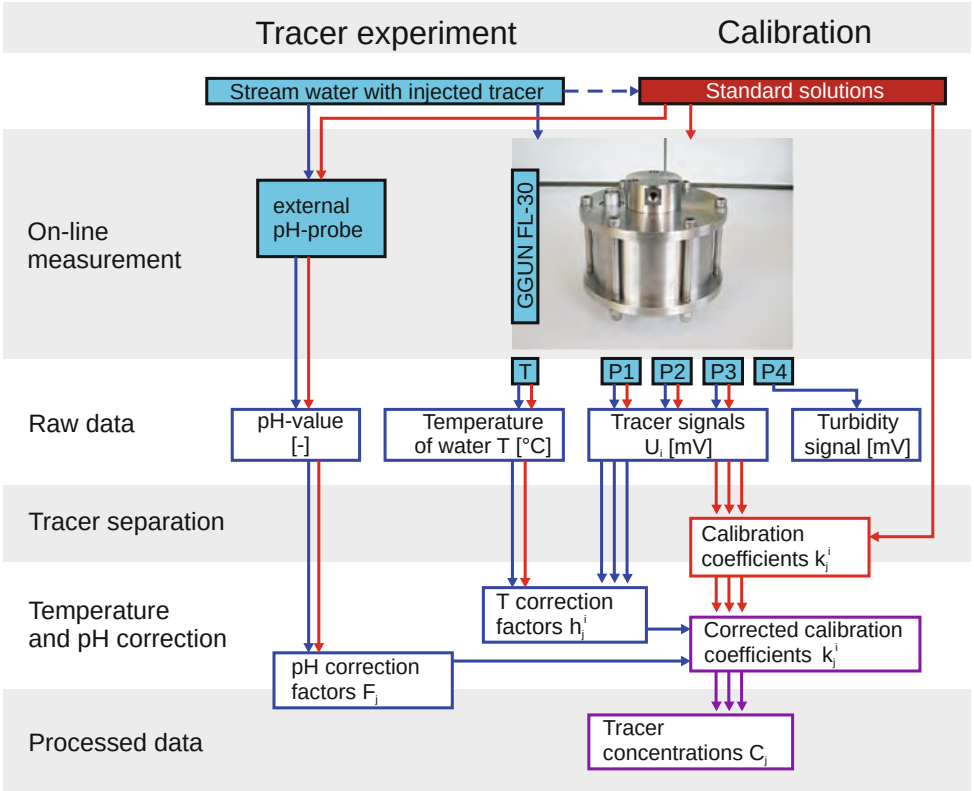

Fig. 8 Schematic work flow and data post-processing using the on-line fluorometer. P1 to P4 refer to the four optical sets of LED, filter and detector

when using a 100 ppb calibration solution of fluorescein as set by default in the GGUN-FL30 software. Hence, in such a case calibration should be done with smaller concentrations. Also, the tracer test should be designed such that the expected concentration range of fluorescein is well between 1 ppb and the concentration used for the calibration. Because their quantum yield is much lower compared to fluorescein, the upper detection limits for resazurin and resorufin are approximately 5 and 0.5 ppm, respectively.

The output of the on-line fluorometer may show sporadic outliers when applied to stream-tracer tests. We have not observed such outliers under controlled lab conditions. They may be caused by air bubbles or small particles entering the fluorometer. However, the tracer signals immediately return to the previous values, indicating that these outliers have no effect on subsequent measurements. Thus, they can easily be identified and removed either manually or by applying a statistical filter.

The design of the fluorometer requires a minimal water depth of 20 cm to ensure that the device is completely submerged. This could pose a problem especially when the tracer tests are to be carried out in streams with very low discharge. Alternatively, one could use pumps to maintain water flow through the fluorometer. A similar approach has successfully been used to monitor the three tracers in riparian piezometers, where water was pumped out of the piezometer, led through the fluorometer, and released back into the piezometer (data not shown here).

The turbidity of the river and bubbles in the water strongly determine the quality of the measured tracer signal and the tracer separation. Thus, when performing tracer tests in high-turbidity streams, extended manual sampling with subsequent analysis of filtered samples may be necessary to check the validity of the breakthrough curves obtained by the on-line fluorometer. Alternatively, one may pump out water and filter it before leading it through the on-line fluorometers.

Altogether, the use of an on-line multi-tracer fluorometer, customized for the conservative tracer fluorescein and the reactive resazurin/resorufin system, extends our capabilities of probing stream systems with respect to solute transport, hyporheic exchange, and metabolic activity.

Acknowledgments We are grateful to Roy Haggerty at Oregon State University for his advice on the resazurin/resorufin tracer system. This work was supported by a grant from the Ministry of Science, Research and Arts of Baden-Württemberg (AZ Zu 33-721.3-2), the Helmholtz Centre for Environmental Research, Leipzig (UFZ), and the National Science Foundation grant EAR 08-38338 to Roy Haggerty at Oregon State University.

References

Argerich A, Haggerty R, Marti E, Sabater F, Zarnetske J (2011) Quantification of metabolically active transient storage (MATS) in two reaches with contrasting transient storage and ecosystem respiration. J Geophys Res-Biogeo 116:G03034. doi:10.1029/2010jg001379

Aumen NG (1990) Concepts and methods for assessing solute dynamics in stream ecosystems. J N Am Benthol Soc 9:95–119

Benischke R, Goldscheider N, Smart C (2007) Tracer Techniques. In: Robins NS (ed) Methods in Karst Hydrology. Taylor & Francis/Balkema, Wallingford

Boulton AJ, Datry T, Kasahara T, Mutz M, Stanford JA (2010) Ecology and management of the hyporheic zone: stream-groundwater interactions of running waters and their floodplains. J N Am Benthol Soc 29:26–40. doi:10.1899/08-017.1

Cirpka O, Reichert P, Wanner O, Muller SR, Schwarzenbach RP (1993) Gas-Exchange at River Cascades—field experiments and model-calculations. Environ Sci Technol 27:2086–2097

Einsiedl F (2005) Flow system dynamics and water storage of a fissured-porous karst aquifer characterized by artificial and environmental tracers. J Hydrol 312:312–321. doi:10.1016/j.jhydrol.2005.03.031

Einsiedl F, Maloszewski P (2005) Tracer tests in fractured rocks with a new fluorescent dye—pyrene-1, 3, 6, 8-tetra sulphonic acid (PTS). Hydrolog Sci J 50:543–554

Erban T, Hubert J (2010) Determination of pH in regions of the midguts of acaridid mites. J Insect Sci 10:42

Fischer H, Pusch M (2001) Comparison of bacterial production in sediments, epiphyton and the pelagic zone of a lowland river. Freshwater Biol 46:1335–1348. doi:10.1046/j.1365-2427.2001.00753.x

Goldscheider N, Meiman J, Pronk M, Smart C (2008) Tracer tests in karst hydrogeology and speleology. Int J Speleol 37:27–40

Gooseff MN, Wondzell SM, Haggerty R, Anderson J (2003) Comparing transient storage modeling and residence time distribution (RTD) analysis in geomorphically varied reaches in the Lookout Creek basin, Oregon, USA. Adv Water Resour 26:925–937. doi:10.1016/S0309-1708(03)00105-2

Gooseff MN, LaNier J, Haggerty R, Kokkeler K (2005) Determining in-channel (dead zone) transient storage by comparing solute transport in a bedrock channel-alluvial channel sequence, Oregon. Water Resour Res 41:W06014. doi:10.1029/2004wr003513

Gooseff MN, Payn RA, Zarnetske JP, Bowden WB, McNamara JP, Bradford JH (2008) Comparison of in-channel mobile-immobile zone exchange during instantaneous and constant rate stream tracer additions: implications for design and interpretation of non-conservative tracer experiments. J Hydrol 357:112–124. doi:10.1016/j.jhydrol.2008.05.006

Grathwohl P, Rügner H, Wöhling T, Osenbrück K, Schwientek M, Gayler S, Wollschläger U, Selle B, Pause M, Delfs J-O, Grzeschik M, Weller U, Ivanov M, Cirpka OA, Maier U, Kuch B, Nowak W, Wulfmeyer V, Warrach-Sagi K, Streck T, Attinger S, Bilke L, Dietrich P, Fleckenstein JH, Kalbacher T, Kolditz O, Rink K, Samaniego L, Vogel H-J, Werban U, Teutsch G (2013): Catchments as reactors—A comprehensive approach for water fluxes and solute turn-over. Environ. Earth Sci., 69(2), doi:10.1007/s12665-013-2281-7

Haggerty R, Reeves P (2002) STAMMT-L version 1.0 user's manual. Sandia National Laboratories, p 76

Haggerty R, Argerich A, Marti E (2008) Development of a ''smart'' tracer for the assessment of microbiological activity and sediment-water interaction in natural waters: the resazurin-resorufin system. Water Resour Res 44:W00D01. doi:10.1029/2007wr006670

Haggerty R, Marti E, Argerich A, von Schiller D, Grimm NB (2009) Resazurin as a "smart'' tracer for quantifying metabolically active transient storage in stream ecosystems. J Geophys Res-Biogeo 114:G03014. doi:10.1029/2008jg000942

Kangasniemi KH (2004) Fluorescent diagnostics for imaging dissolved oxygen. University of Texas at Dallas, Richardson

Kasnavia T, Vu D, Sabatini DA (1999) Fluorescent dye and media properties affecting sorption and tracer selection. Ground Water 37:376–381. doi:10.1111/j.1745-6584.1999.tb01114.x

Käss W (2004) Geohydrologische Markierungstechnik. Lehrbuch der Hydrogeologie, 2 edn. p 557

Klonis N, Sawyer H (1996) Spectral Properties of the Prototropic Forms of Fluorescein in Aqueous Solution. J Fluoresc 6(3):147–157

Leibundgut C, Maloszewski P, Külls C (2009) Tracers in Hydrology. Wiley, New York

Liao ZJ, Cirpka OA (2011) Shape-free inference of hyporheic traveltime distributions from synthetic conservative and "smart'' tracer tests in streams. Water Resour Res 47:W07510. doi:10.1029/2010wr009927

O'Brien J, Wilson I, Orton T, Pognan F (2000) Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 267:5421–5426. doi:10.1046/j.1432-1327.2000.01606.x

Osenbrück K, Wöhling T, Lemke D, Rohrbach N, Schwientek M, Leven C, Castillo Alvarez C, Taubald H, Cirpka OA (2013) Assessing hyporheic exchange and associated travel times by hydraulic, chemical, and isotopic monitoring at the Steinlach Test Site, Germany. Environ Earth Sci. doi:10.1007/s12665-012-2155 (in press)

Payn RA, Gooseff MN, Benson DA, Cirpka OA, Zarnetske JP, Bowden WB, McNamara JP, Bradford JH (2008) Comparison of instantaneous and constant-rate stream tracer experiments through non-parametric analysis of residence time distributions. Water Resour Res 44:W06404. doi:10.1029/2007wr006274

Runkel L (1998) One-dimensional Transport with Inflow and Storage (OTIS): a Solute Transport Model for Streams and Rivers. Water Res Investigat 98:80

Schnegg P-A, Flynn R (2002) Online field fluorometers for hydrogeological tracer tests. Isotope und Tracer in der Wasserforschung 19:8

Schnegg PA, Perret C, Hauet A, Parrel D, Saysset G, Vignon P (2011) Stream gauging by dilution of fluorescent tracers and state of the art of the EDF hydroclimatological observation network. In: 9th Conference on Limestone Hydrogeology, Besançon

Thanh NTK (2005) Gold nanoparticles in bioanalytical assays and sensors. Ser Chem Sens Biosens 3:16. doi:10.1007/5346_008

Wagner BJ, Harvey JW (1997) Experimental design for estimating parameters of rate-limited mass transfer: analysis of stream tracer studies. Water Resour Res 33:1731–1741

Zanker V, Peter W (1958) Die prototropen Formen des Fluoresceins. Chemische Berichte 91(3):572–580