

Development of a “smart” tracer for the assessment of microbiological activity and sediment-water interaction in natural waters: The resazurin-resorufin system

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Received 16 November 2007; revised 25 February 2008; accepted 14 March 2008; published 23 July 2008.

[1] A “smart” tracer is a tracer that provides, directly or through measurement of its concentration or in combination with another compound, at least one “bit” more of information about the environment through which it travels than a conservative tracer. In this study we propose and present the chemical compound resazurin as a smart tracer to assess the coupling between solute transport and microbiological activity in sediment-water interfaces in freshwaters. Resazurin is a weakly fluorescent redox-sensitive dye that undergoes an irreversible reduction to strongly fluorescent resorufin under mildly reducing conditions, most commonly in the presence of living microorganisms. To investigate the suitability of resazurin as a smart tracer, we characterized the decay, sorption, reaction, and transport behavior of resazurin and resorufin in various waters and sediments using laboratory experiments. Results show that resazurin irreversibly and rapidly reacts to resorufin in colonized sediment with pseudo-first-order behavior and a rate coefficient of 1.41 h^{-1} . This reaction is 3 orders of magnitude faster than that in stream water alone, indicating the tracer is sensitive to microbiological activity and associated sediment-water interactions. The compounds are affected by significant sorption, with an approximately linear isotherm and a K_d of 6.63 mL/g for resorufin in sediment with 2.19% organic carbon. The compounds are stable over weeks in natural water, except in the presence of strong light where significant photochemical decay may occur more rapidly.

Citation: Haggerty, R., A. Argerich, and E. Martí (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.

1. Introduction

[2] Applications of tracers are one of the key techniques used in hydrological studies of surface freshwaters and groundwaters (for a review, see *Flury and Wai* [2003]). However, the information that conservative tracers provide is largely tied to water transport and arrival time. The processes available for direct study via arrival time are limited to advection, dispersion (spreading of arrival times), and transient storage or mass transfer (tailing of arrival times). In freshwater ecosystems these processes can have a large influence on biogeochemical responses. However, some hydrologic processes may be indistinguishable using arrival times of conservative tracers; for example, advection through low-permeability zones and diffusion into and out of low-permeability zones can yield the same arrival times [*Becker and Shapiro*, 2000; *Sánchez-Vila and Carrera*, 2004]; and in-stream transient storage can yield similar arrival times to hyporheic transient storage [*Gooseff et al.*,

2005]. Furthermore, biogeochemical processes have non-unique responses to arrival times due to dependence on biological community, biogeochemical heterogeneity between flow paths, and other factors [e.g., *Jones et al.*, 1995; *Kirchner*, 2003; *Nagorski et al.*, 2003]. Given this context, geo/eco-hydrology would be advanced considerably if our community could develop tracers that collect information about the environment through which they traveled, tracers that are, simply put, smarter than conservative tracers. We define a “smart tracer” as a tracer that provides, directly or through measurement of its concentration or in combination with another compound, at least one “bit” more of information about the environment through which it travels than a conservative tracer. In other words, the smart tracer provides direct information about conditions in the hydrologic system (location history, chemical conditions, biological activity, physical interactions) in addition to arrival time. In this study, we present a prototype smart tracer for the measurement of microbiological activity and associated sediment-water interactions in freshwater ecosystems.

[3] A smart tracer should have characteristics common to all good conservative tracers [*Flury and Wai*, 2003]. That is, the tracer (and, if applicable, its daughter products) should be (1) conservative, with one exception; (2) clearly discernable from the background of the system; (3) of low toxicity; and

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(4) insensitive to variations in solution or medium chemistry, also with one exception. The one exception is that the smart tracer must undergo an irreversible change in the presence of a process or condition under investigation. This irreversible change must be (1) detectable with a measurement technique or sensor; (2) rapid relative to transport rates; and (3) insensitive to likely chemical or other variations in the solution or medium. Finally, the ideal smart tracer and its measurement technique should be inexpensive, safe to handle and not harmful to the environment.

[4] We are aware of the previous use of at least one smart tracer in hydrology. *Gramling et al.* [2002] used the irreversible reaction of CuSO_4 and EDTA^{4-} to CuEDTA^{2-} and the resulting color change to measure true chemical-scale mixing and distinguish it from hydrodynamic dispersion. This smart tracer allowed direct measurement of the amount of longitudinal molecular-scale mixing that occurred in a tank experiment. Smart tracers have been used in other fields; for instance, in fluid mechanics, reacting compounds that change color have been used to measure mixing times in chemical reactors [*Melton et al.*, 2002; *Cabaret et al.*, 2007]. Though not strictly a tracer technique, smart radiotracers used in positron emission tomography preferentially lodge in tumors owing to unique metabolism found there (e.g., inefficient and higher rate of glucose use). When the radiotracer decays it emits a positron that can be used to tomographically image the tumor [*Juweid and Cheson*, 2006].

[5] The objective of this study is to identify and characterize a smart tracer to measure microbiological activity associated with stream water-sediment interaction. Because in lotic (i.e., flowing water) ecosystems, microbial activity is mostly associated with biofilms developed on sediments [*Fischer and Pusch*, 2001], this assay will be useful for increasing our understanding of the interaction between hydrology and biogeochemistry in these ecosystems, and could also extend to other aquatic ecosystems.

[6] After a search of candidates, we selected resazurin (Raz) as our potential smart tracer. This compound, also known as Alamar Blue [*O'Brien et al.*, 2000], is a redox-sensitive phenoxazine dye [*Tratnyek et al.*, 2001; *Bueno et al.*, 2002] that has been known for more than a century, for example to test the hygienic quality of milk (see discussion by *Twigg* [1945]). In the presence of mildly reducing conditions, Raz loses an oxygen ion irreversibly to become resorufin (Rru). Rru can also undergo a further, reversible reduction to hydroresorufin, but this is not favored in the presence of oxygen. The reduction of Raz to Rru is a well-documented indicator of the presence of living bacteria [*Liu*, 1983; *Peroni and Rossi*, 1986; *De Fries and Mistuhashi*, 1995; *O'Brien et al.*, 2000; *Guerin et al.*, 2001; *McNicholl et al.*, 2007], particularly of aerobic bacteria [*Karakashev et al.*, 2003]. The Rru-Raz reaction has been widely used as an indicator of bacterial growth in milk [e.g., *Ramsdell et al.*, 1935; *Moyer and Campbell*, 1963] and semen quality [e.g., *Erb and Ehlers*, 1950; *Zrimšek et al.*, 2004]. Both Raz (blue) and Rru (red) are intensely colored and fluorescent, but Rru is more fluorescent, as indicated by the quantum efficiency [*Bueno et al.*, 2002] (Figure 1). Raz and Rru are not highly toxic, and in fact are used in evaluation of toxicity to count living bacteria [*O'Brien et al.*, 2000]. *Fung and Miller* [1973] did experiments with 10,000 $\mu\text{g/L}$ Raz and found that the growth of most of the species of bacteria

considered (23 out of 30) were not inhibited by the dye. Therefore, a priori, this compound fulfilled most of our initial requirements for a smart tracer.

[7] To verify if resazurin could be used as a smart tracer, here we present results from laboratory experiments coupled with application of mathematical models for parameter estimation, which were used to characterize decay, sorption, and reaction of Raz and Rru.

2. Methodology

2.1. General Methods

[8] Raz, Rru, and other reagents were purchased from Panreac Química S.A. (Castellar del Vallès (Barcelona), Spain; www.panreac.com) and used as supplied. The 2007 bulk cost of Raz was slightly less than 1000 USD for 250 g in both the U.S. and Europe (experiments described in this paper required $\ll 5$ g). Initial comparison of fluorescence signals showed that Raz was contaminated by 3% Rru. *Kangasniemi* [2004] showed that the fluorescence of Rru is constant above pH 8, but that it decreases sharply below pH 6.5. *Bueno et al.* [2002] showed very similar results for the fluorescence of Raz. Consequently, water samples from the experiments were buffered to pH 8 prior to analysis of Raz and Rru. A stock solution of buffer near pH 8.0 was generated by mixing 1 molar $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$ with equal parts 1 molar NaOH, which was added to samples at a 1:10 buffer-to-sample ratio. Where noted, filtration of water samples was performed with 0.7 μm glass fiber filter (GF/F, supplied by Whatman, Kent, UK, and Albet, Sant Boi de Llobregat (Barcelona), Spain). All laboratory materials for the experiments described below were triple-washed with tap water, triple-rinsed with deionized water (DIW), and dried if needed.

[9] Fluorescence of Raz and Rru in water samples was measured on a Shimadzu RF spectrofluorometer, with excitation and emission wavelengths given in Figure 1. Samples were placed in a quartz cuvette and held within the sample chamber for less than 1 min to minimize temperature changes. Unless otherwise noted, samples were stored in the dark at room temperature prior to measurement, which was always within 24 h of sampling (and usually within 1 h). Error bars for data were calculated assuming independent contributions of error from sample preparation, sample measurement, and signal measurement (machine error). Sample preparation, sample measurement, and signal measurement were each performed a minimum of 7 times to isolate the targeted error (*Clesceri et al.* [1999], method 1020). The limit of quantitation (LOQ, approximately 3 times the limit of detection, LOD) for Rru in DIW is 0.012 $\mu\text{g/L}$ (0.05 nmol/L). Because Rru fluoresces more strongly than Raz and because the fluorescence spectra overlap, the LOD and LOQ for Raz depend on Rru concentration. In DIW the LOQ for Raz with 0 $\mu\text{g/L}$ Rru is 0.16 $\mu\text{g/L}$ (0.62 nmol/L), while the LOQ for Raz with 25 $\mu\text{g/L}$ (0.11 nmol/L) Rru rises to 0.96 $\mu\text{g/L}$ (2.2 nmol/L). The LOQ for both compounds in natural water is approximately 5 times larger. The errors reported in this manuscript account for variable Rru concentration.

[10] Change in fluorescence due to variation in temperature in the spectrofluorometer reading cell was measured by placing a sample in the spectrofluorometer and measuring

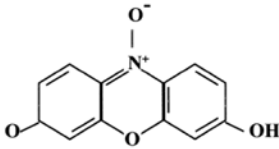
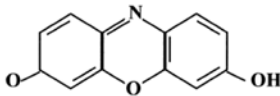
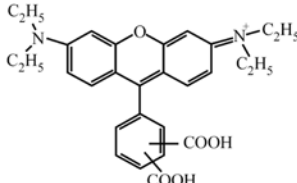
Property	Resazurin	Resorufin	Rhodamine WT
Chemical formula ¹	NaC ₁₂ H ₆ NO ₄	NaC ₁₂ H ₆ NO ₃	C ₂₉ H ₂₉ N ₂ O ₅ ClNa ₂
Structure ²			
CAS # ¹	62758-13-8	34994-50-8	37299-86-8
Molecular weight ¹	251.2 g/mol	235.2 g/mol	567.0 g/mol
Solubility @ 25°C ¹	18.4 g/L ³	20.0 g/L ³	180-220 g/L ⁴
log(<i>K_{ow}</i>) ^{3,5}	0.94	1.02	-1.3
Nominal <i>K_d</i> ⁶	n/a	6.63 mL/g ⁷	33.2 mL/g ⁸
Maximum excitation wavelength	602 nm ⁷	570 nm ⁷	555 nm ⁹
Maximum emission wavelength	616 nm ^{7,10}	585 nm ⁷	580 nm ⁹
Quantum efficiency	0.11 ¹¹	0.74 ¹¹	n/a but high
Fluorescence temperature correction constant ¹²	n/a	-0.0041 ± 0.003 /°C ⁷	-0.027 /°C ⁹

Figure 1. Properties of the resazurin-resorufin system, with rhodamine WT for comparison. Unless otherwise noted, analyses were conducted on the resazurin-resorufin system near pH 8. 1, for sodium salt; 2, Raz and Rru diagrams from *O'Brien et al.* [2000], with rhodamine WT after *Vasudevan et al.* [2001] 3, estimated for molecular compound with method of *Meylan and Howard* [1995] and *Meylan et al.* [1996] and available at <http://www.syrres.com/esc/> (accessed 19 June 2007); 4, *McVoy* [1985] reported by *Soerens and Sabatini* [1994]; 5, *K_{ow}* = octanol-water partitioning coefficient, with rhodamine WT value from *Smart* [1984]; 6, *K_d* = distribution coefficient; 7, this study; 8, *Gooseff et al.* [2005], reported for sediment containing 9% organic carbon; 9, *Smart and Laidlaw* [1977]; 10, in the case of resazurin, this emission wavelength yielded maximal difference (minimum interference) with the resorufin emission rather than being the maximum absolute value; 11, *Bueno et al.* [2002]; 12, temperature correction constant defined as by *Smart and Laidlaw* [1977].

fluorescence immediately at 23.0°C, turning off the lamp, and then measuring fluorescence again after the sample had equilibrated to the temperature inside the instrument (31.6°C) (Figure 1).

[11] To measure the rates of decay, sorption and reaction of the Raz-Rru chemical system and to examine if reaction rates were susceptible to water transport and biological activity we conducted three sets of experiments in the laboratory using water from two streams (“riera” in Catalan) located in the Tordera catchment (40 km NE of Barcelona, Spain) and sediment from one of them. Both streams have near-neutral pH and low total dissolved solids. The second-order Riera de Santa Fe de Montseny drains a 2.15 km² forested catchment and the third-order Riera de Gualba drains a 13.46 km² catchment of mixed forest, agriculture and urban land use. A representative sample of sediment (<2 mm size fraction) collected from the Riera de Sta. Fe was silicic and consisted of 52.8% fine-grained metasedimentary lithic fragments; 34.8% quartz grains; 6.2% feldspar grains; 3.7% granodiorite lithic fragments; and 2.38 ± 0.09% organic carbon. Inorganic sediment was manually separated by binocular microscope and weighed. Fraction of organic carbon was determined gravimetrically on 3 samples by loss on ignition at 550°C for 15 h.

[12] Prior to the experiments, the stability of a 25 μg/L solution of Rru in DIW stored in the dark at room temperature was examined. The fluorescence of this solu-

tion was measured nearly every work day for approximately 1 month. No degradation in the fluorescence was observed in this time; therefore, this solution was used as a fluorescence standard for all the subsequent experiments.

2.2. Measurement of Reaction and Decay Rates

[13] We measured the reaction, and decay of Raz and Rru in batch experiments using water from the two streams and DIW under different conditions. First, reaction and biochemical or chemical decay in natural water were measured using water from the two streams. Water from Gualba was filtered; Sta. Fe water was used unfiltered. Both were refrigerated and used within 1 week of collection. Solutions of 25 μg/L Raz and Rru were created using these waters and stored in the dark at room temperature (19°–23°C). On an approximately daily schedule for 13–22 days (depending on experiment), samples from each batch were taken, filtered in the case of Sta. Fe water, buffered, and fluorescence was measured. Second, we also measured the reaction and decay of Raz in DIW. The procedure was the same as above except that filtration was not required. No replicates were taken in these experiments.

[14] Third, we examined the effect of light (under laboratory conditions) on the decay of Raz and Rru. For this experiment we used DIW. The procedure was the same as above, except that the solutions were placed in clear glass containers and left on a counter top in the laboratory (19°–

Table 1a. Column Properties

Property	Value
Column size	15 cm × 1 cm ID
Temperature, °C	16
Porosity n , -	0.422
Fraction organic carbon, %	2.19
Raz concentration injected, $\mu\text{g/L}$	200
Dispersivity (conservative), cm	0.107
Dispersivity (sorbing), cm	0.647
Retardation factor R , Raz and Rru, -	2.07

23°C) with the lights turned on. Lights were Osram Lumilux daylight 18 W bulbs (Munich, Germany) with a broad spectrum from 400 to 600 nm and peaks at 427, 478, and 536 nm. The light intensity was $0.624 \pm 0.072 \text{ mol/m}^2/\text{d}$, monitored with a photoactive radiation (PAR) sensor (SKP215 Quantum, Skye Instruments, Powys, U.K.).

2.3. Measurement of Sorption

[15] The sorption isotherm for Rru was measured using stream sediment from Sta. Fe. Saturated sediment was collected in a plastic bag, dried at 60°C for 72 h, then sieved (Retsch AS200, Haan, Germany) to separate the fractions larger and smaller than 2 mm. The size fraction <2 mm was divided into 17 aliquots of approximately 20 g, which were weighed and placed in 250 mL Pyrex™ jars. Weighed aliquots of approximately 200 g of filtered stream water from Sta. Fe were used to prepare 0.00, 1.00, 4.64, 21.54, and 100.00 μg Rru/L solutions which were then poured into the jars. Four replicates were made of 4.64 and 100.00 $\mu\text{g/L}$, and 3 replicates were made for other concentrations. The jars were capped, kept in the dark, and manually shaken every 2 h (4 h at night) for 41 h. Fifteen mL aliquots were extracted from the jars at the end of the incubation, placed into plastic centrifuge tubes, and centrifuged 15 min at 2358 G . The supernatant was decanted, filtered, buffered and measured for aqueous Rru concentration. Sorbed Rru was calculated by difference between the initial and final concentration.

[16] The sorption isotherm for Raz was not measured because the compound reduces to Rru in the presence of sediment. However, we may expect that the sorption isotherm for Raz to be similar to Rru because the compounds have very similar octanol-water partitioning coefficients (Figure 1) and solubilities in water, and are otherwise chemically similar. Therefore, where needed in the calculations we assumed that the sorption of Raz is the same as Rru.

2.4. Characterization of Transport and Reaction

[17] Reaction and transport of Raz and Rru were quantified in a series of flow-through column experiments at 3 different flow rates. A 15-cm-long, 1-cm-ID, low-pressure glass chromatography column (chromaflex, Kontes Glass Company, New Jersey, USA) was packed underwater in the field with stream sediment from Sta. Fe on 1 March 2007. The majority of sediment (visual estimate) was <2 mm size fraction with a few pebbles. The column was capped and returned to the laboratory where it was refrigerated in the dark until use (12–21 days later). Column properties are provided in Table 1a. Column experiments were conducted in a

temperature-controlled room (16°C). A solution of Raz and NaCl in filtered Sta. Fe stream water was pumped at constant rate through the column using a high-pressure peristaltic pump (Masterflex L/S pump, Vernon Hills, Illinois, USA). Outflow was delivered to a capped flask on a balance to monitor flow rate. The tracer reservoir was shielded from the light with aluminum foil. The column was shielded from the light except for the slowest-flow experiment (completed first). However, lights were out in the room except when samples were being taken, which for the slow-flow experiment was only a few minutes at a time. Furthermore, the photodecay time for the compounds is at least many 10s of h (see results in section 3.1), so the lack of column shielding on one experiment had no effect. Electrical conductivity (EC) was monitored continuously in a chamber in front of the outflow port using an LF 340 EC meter (WTW, Weilheim, Germany) connected to a CR510 data logger (Campbell Scientific, Logan, Utah, USA). Water samples were extracted manually with a 15-mL Luer lock syringe at an in-line sample port placed in front of the EC chamber. Samples were taken at regular intervals over the duration of each experiment; sample frequency and experimental duration are given in Table 1b. Since samples were taken manually, the 2 slower flow rate experiments had 10–13 h sample gaps overnight. Samples were filtered immediately, buffered and stored (<5 h) in the dark at 16°C in 20-mL clear borosilicate vials prior to fluorescence measurement of Raz and Rru. Oxygen concentration was periodically measured in the inflow carboy and at the EC chamber (Table 1b) with a WTW (Weilheim, Germany) 340i portable oxygen meter.

2.5. Data Analysis

[18] In the experiments, Raz and Rru underwent the following processes: reaction of Raz to Rru, decay of both Raz and Rru to other unquantified byproducts; sorption of both Raz and Rru if sediment was present; and in the columns, both advection and dispersion. To estimate the different rates of these processes, we assumed that decay and reaction followed a first-order law and that sorption was at equilibrium. The system of equations for these processes is as follows:

$$\frac{\partial C_{Raz}}{\partial t} = \frac{\alpha_L v}{R} \frac{\partial^2 C_{Raz}}{\partial x^2} - \frac{v}{R} \frac{\partial C_{Raz}}{\partial x} - k_1 C_{Raz} - k_{12} C_{Raz} \quad (1a)$$

$$\frac{\partial C_{Rru}}{\partial t} = \frac{\alpha_L v}{R} \frac{\partial^2 C_{Rru}}{\partial x^2} - \frac{v}{R} \frac{\partial C_{Rru}}{\partial x} - k_2 C_{Rru} + k_{12} \frac{M_{Rru}}{M_{Raz}} C_{Raz}, \quad (1b)$$

where C_{Raz} [ML^{-3}] is the concentration of Raz; C_{Rru} [ML^{-3}] is the concentration of Rru; t [T] is time; x [L] is distance

Table 1b. Experimental Conditions

Condition	Low Flow	Medium Flow	High Flow
EC above background, $\mu\text{S/cm}$	115.3	139.5	131.8
Flow rate, mL/h	3.39	15.5	34.5
O ₂ concentration in/out, mg/L	8.4/5.1	9.1/7.2	9.4/8.7
Velocity v , cm/h	13.6	32.9	80.4
Travel time ($\tau = L/v$), h	1.10	0.455	0.187
Sample frequency (approx.), min	130	35	15
Total experiment time, h	46.9	24.9	6.65

from the upstream boundary; α_L [L] is the longitudinal dispersivity; v [LT^{-1}] is the mean pore water velocity; R [-] is the retardation coefficient due to sorption; k_{12} [T^{-1}] is the reaction rate coefficient for Raz to Rru; k_1 [T^{-1}] is the decay rate coefficient for Raz; k_2 [T^{-1}] is the decay rate coefficient for Rru; M_{Rru} [mol M^{-1}] is the molecular weight of Rru; and M_{Raz} [mol M^{-1}] is the molecular weight of Raz. We assume here that molecular diffusion is negligible compared to dispersion and that the dispersion coefficient $D_L = \alpha_L v$. The retardation coefficient is equivalent to $K_d \rho_b / n$, where K_d is the distribution coefficient; ρ_b is the bulk density; and n is the porosity.

[19] For the batch experiments, $v = 0$ m/s, boundary conditions are unnecessary and the initial conditions are as follows:

$$C_{Raz}(t = 0) = (1 - F_{Rru})C_{Raz,0} \quad (2a)$$

$$C_{Rru}(t = 0) = F_{Rru}C_{Raz,0}, \quad (2b)$$

where $C_{Raz,0}$ [ML^{-3}] is the initial concentration of Raz; and F_{Rru} [-] is the mass fraction of Rru contamination in Raz, which we measured as 0.03.

[20] The solution of (1)–(2) for $v = 0$ is as follows [e.g., Levine, 1988, p. 522]:

$$C_{Raz} = C_{Raz,0}(1 - F_{Rru}) \exp[-k_{1Tot}t] \quad (3a)$$

$$C_{Rru} = C_{Raz,0}(1 - F_{Rru}) \frac{M_{Rru}}{M_{Raz}} \frac{k_{12}}{k_2 - k_{1Tot}} \{ \exp[-k_{1Tot}t] - \exp(-k_2t) \} + C_{Raz,0} F_{Rru} \exp(-k_2t), \quad (3b)$$

where k_{1Tot} is the combined reaction and decay rate coefficient for Raz,

$$k_{1Tot} = k_1 + k_{12}. \quad (3c)$$

[21] For the column experiments we developed a semi-infinite model, with the following boundary and initial conditions.

$$C_{Raz}(x, t = 0) = C_{Rru}(x, t = 0) = 0, x \geq 0, \quad (4a)$$

$$C_{Raz}(x = 0, t) = C_{Raz,0}, t > 0, \quad (4b)$$

$$C_{Rru}(x = 0, t) = F_{Rru}C_{Raz,0}, t > 0, \quad (4c)$$

$$C_{Raz}(x \rightarrow \infty, t) = C_{Rru}(x \rightarrow \infty, t) = 0, t > 0. \quad (4d)$$

[22] The solution to (1)–(2) and (4a)–(4d) can be developed from the basic solution to the advection-dispersion equation without reaction using the method of Sun *et al.* [1999]. The solution is as follows:

$$C_{Raz}(x, t) = \frac{C_{Raz,0}(1 - F_{Rru})}{2} \left\{ \exp \left[\frac{Pe}{2} (1 - B_{Raz}) \right] \cdot \operatorname{erfc} \left[\frac{1 - A_{Raz}t^{1/2}}{2(t/R\tau Pe)^{1/2}} \right] + \exp \left[\frac{Pe}{2} (1 + B_{Raz}) \right] \cdot \operatorname{erfc} \left[\frac{1 + A_{Raz}t^{1/2}}{2(t/R\tau Pe)^{1/2}} \right] \right\} \quad (5a)$$

$$C_{Rru}(x, t) = \frac{C_{Raz,0}}{2} \left[\frac{(1 - F_{Rru})y_{12}k_{1Tot}}{k_{1Tot} - k_2} + F_{Rru} \right] \cdot \left\{ \exp \left[\frac{Pe}{2} (1 - B_{Rru}) \right] \operatorname{erfc} \left[\frac{1 - A_{Rru}t^{1/2}}{2(t/R\tau Pe)^{1/2}} \right] + \exp \left[\frac{Pe}{2} (1 + B_{Rru}) \right] \operatorname{erfc} \left[\frac{1 + A_{Raz}t^{1/2}}{2(t/R\tau Pe)^{1/2}} \right] \right\} - \frac{C_{Raz}(x, t)y_{12}k_{1Tot}}{k_{1Tot} - k_2}, \quad (5b)$$

where k_{1Tot} and F_{Rru} are the same as in (2–3), and

$$A_{Raz} = \sqrt{\frac{Pe}{R\tau} + 4k_{1Tot}}, \quad (5c)$$

$$A_{Rru} = \sqrt{\frac{Pe}{R\tau} + 4k_2}, \quad (5d)$$

$$B_{Raz} = \sqrt{1 + \frac{4k_{1Tot}R\tau}{Pe}}, \quad (5e)$$

$$B_{Rru} = \sqrt{1 + \frac{4k_2R\tau}{Pe}}, \quad (5f)$$

$$y_{12} = \frac{k_{12}}{k_{1Tot}} \frac{M_{Rru}}{M_{Raz}}, \quad (5g)$$

$$Pe = x/\alpha_L = vx/D_L, \quad (5h)$$

$$\tau = x/v. \quad (5i)$$

[23] It is also worth noting the asymptotic values of (5a) and (5b) for an injection of constant concentration,

$$C_{Raz}(x, t \rightarrow \infty) = C_{Raz,0}(1 - F_{Rru}) \cdot \exp \left[\frac{1}{2} Pe \left(1 - \sqrt{1 + \frac{4k_{1Tot}R\tau}{Pe}} \right) \right] \quad (6a)$$

$$C_{Rru}(x, t \rightarrow \infty) = C_{Raz,0} \left[\frac{(1 - F_{Rru})y_{12}k_{1Tot}}{k_{1Tot} - k_2} + F_{Rru} \right] \cdot \exp \left[\frac{Pe}{2} \left(1 - \sqrt{1 + \frac{4k_2R\tau}{Pe}} \right) \right] - \frac{y_{12}k_{1Tot}}{k_{1Tot} - k_2} C_{Raz}(x, t \rightarrow \infty) \quad (6b)$$

[24] Parameters (i.e., decay, sorption, and reaction) were estimated by fitting the appropriate model and minimizing the sum of squared error. In the case of the column experiments, velocities, travel times and a dispersivity for a conservative tracer were estimated from the breakthrough curves (BTCs) of EC. These values were then used in the Raz and Rru models. All Raz and Rru models were used simultaneously to estimate k_1 , k_{12} , k_2 , and a new value of dispersivity. A different dispersivity was fit to the EC data than to the Raz/Rru data because sorption increases dispersion and because rate limitations were not included in the model, which also increases dispersion [Kuřera, 1965; Goltz and Roberts, 1987; Sánchez-Vila and Carrera, 2004]. It has been shown that sorbing and nonsorbing tracers should have different dispersivities, even if non-equilibrium effects are properly modeled, owing to differ-

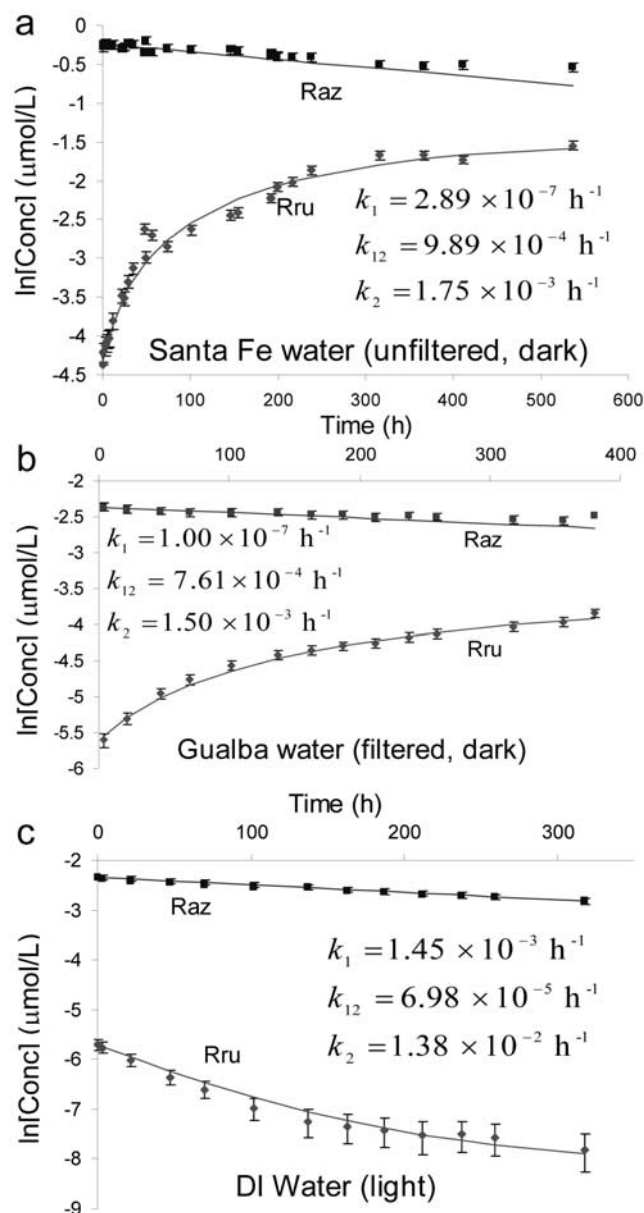


Figure 2. Reaction of resazurin (Raz) to resorufin (Rru) versus time. (a) Raz in water from Riera de Sta. Fe de Montseny, unfiltered and stored in the dark. (b) Raz in water from Riera de Gualba, filtered and stored in the dark. (c) Raz in deionized water stored under laboratory lights. Data are shown with error bars indicating 95% confidence; some error bars are not visible because they are smaller than the data symbol. Corresponding models from equations (3a) and (3b) are shown as solid lines.

ences in the boundary conditions at the sediment grain–water interface [Smith, 1983; Shapiro and Brenner, 1988; Grosser et al., 1991; Buckley and Loyalka, 1994].

3. Results

3.1. Raz and Rru Parameter Estimates From Batch Experiments

[25] Reaction of Raz to Rru and the decay of both Raz and Rru were all well-mo s first-order processes (3a)

and (3b). A representative set of concentration histories of both Raz and Rru from batch reactions are shown in Figure 2. The reaction and decay processes can all fairly be called pseudo-first-order. The model for Rru generally fit within the data error bars, with the exception of a few data points for water from Sta. Fe, which were our “noisiest” data set, probably due to mistakes in sample preparation. Data for water from Gualba were not noisy. The model for Raz fit within the error bars, except for data from close to the end of the experiment for both Sta. Fe and Gualba water. This late-time discrepancy appears to be systematic in natural water, but is probably negligible for most lab and field applications because it is only apparent after many days.

[26] A summary of reaction and decay coefficients obtained from all experiments is given in Figure 3. Raz and Rru have very little decay or reaction in either DIW or water from the two streams. In DIW, all rate coefficients are well below 10^{-4} h^{-1} , indicating that laboratory standards are stable over periods of several weeks if stored in the dark. In the light, Raz and Rru both undergo photochemical decay, within many tens of hours for Rru and hundreds of hours for Raz in our experiments. Biochemical/chemical decay and reaction in the two stream waters was similar. Biochemical/chemical decay of Raz was negligible, while biochemical/chemical decay of Rru was $1.50\text{--}1.75 \times 10^{-3} \text{ h}^{-1}$. In stream water, reaction of Raz to Rru was slower than biochemical/chemical decay, and was slightly below 10^{-3} h^{-1} . Water filtration with a $0.7 \mu\text{m}$ glass fiber filter made no difference in the results.

[27] The sorption isotherm (Figure 4) was fit very well by a Freundlich isotherm with $1/n = 0.89 \pm 0.04$ and a Freundlich coefficient (K_f) of $5.15 \pm 0.63 \text{ mL/g}$. Since the isotherm is nearly linear, we also fit a linear isotherm model to the data, yielding a distribution coefficient of $6.63 \pm 0.40 \text{ mL/g}$.

3.2. Raz and Rru Parameter Estimates From Column Experiments

[28] Results from column experiments with stream sediment and water (Figure 5) show that reaction of Raz to Rru is more than 3 orders of magnitude faster than reaction measured in batch experiments with water alone, with $k_{12} = 1.41 \text{ h}^{-1}$ (compare all rate coefficients in Figure 3). A large fraction of Raz was converted to Rru even with water residence times in column as short as 0.19 h ($\sim 11 \text{ min}$). Nearly all of the Raz was removed from solution in the column with a 1.1 h water residence time, and much of this was converted to Rru.

[29] Travel times cause plateau concentrations to change nonlinearly. Figure 6 shows that maximum Rru concentration was achieved in the column experiment with intermediate travel time (i.e., 0.46 h for water). Shorter travel time do not allow sufficient time for Raz to Rru reaction. Longer time (i.e., slower flow) allows more conversion of Raz to Rru, but also more time for biochemical/chemical decay and sorption of both Raz and Rru. On the other hand, the loss of oxygen across the column is approximately proportional to the travel time (Table 1b), consistent with microbial respiration.

[30] Nonequilibrium sorption, though not modeled, affects column BTCs. The fastest pumping rate ($Q = 34.5 \text{ mL/h}$, $\tau = 0.19 \text{ h}$) has an early breakthrough relative to the

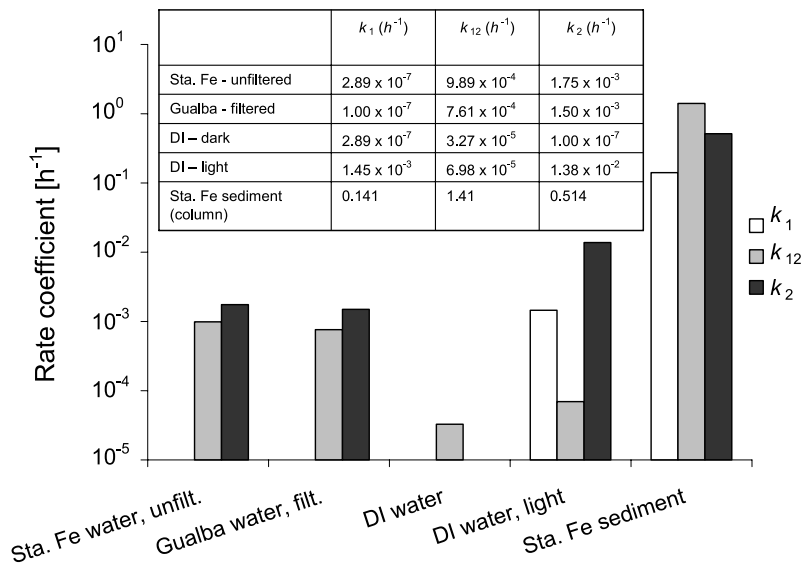


Figure 3. Summary of reaction rates (k_{12}), decay rates for Raz (k_1), and decay rates for Rru (k_2) under various conditions. All samples were stored in the dark except “DI water, light.” All values were obtained from a batch reactor except “Sta. Fe sediment,” which were obtained from a series of column experiments (see text).

equilibrium sorption model while the slow pumping rate ($Q = 3.39$ mL/h, $\tau = 1.1$ h) has a late breakthrough relative to the equilibrium sorption model. Furthermore, even the conservative tracer BTC displays rate-limited mass transfer for all pumping rates, indicating that the mobile aqueous and immobile sorbed phases are certainly out of equilibrium. We expect that the addition of a first-order or other rate-limited mass transfer mechanism [e.g., Haggerty and Gorelick, 1995; Haggerty et al., 2000] to the model would allow a much better fit to the early time arrivals, with the expense of a more complicated model and the loss of a fully analytical solution. However, since our primary interest is the data from plateau concentrations, which are not affected by rate limitations in mass transfer, we chose not to add this complexity to the model.

4. Discussion

4.1. Resazurin as a Smart Tracer

[31] Results from the experiments cautiously support the feasibility of using Raz as a smart tracer, and show that it can provide at least one “bit” more of information than current hydrological tracers in the assessment of the coupling between solute transport and biogeochemical processes in freshwater ecosystems, with particular promise for water-sediment interactions.

[32] Our results indicate that Raz and Rru are relatively stable and nonreactive in water (without sediment) for days to weeks, a period long enough to complete laboratory or field experiments, provided that samples are stored out of the light (Figure 3). With the exception of long residence times (hundreds of hours) or water with high microbial abundances (e.g., untreated sewage), it appears that significant biochemical/chemical decay and Raz-to-Rru reaction in natural water is not likely. However, sample exposure to intense light (e.g., bright sunlight during field experiments) can result in significant biochemical decay. Therefore,

caution should be taken with sample storage, especially in the field.

[33] In contrast, Raz reacts to Rru quickly, in less than an hour, when water interacts with sediment (i.e., in the flow-through sediment columns). The fact that the reaction in sediment is much faster than without sediment makes Raz highly attractive as a smart tracer for water-sediment interaction.

[34] The sorption of Raz and Rru may be of concern when considering this system as a hydrologic tracer. The breakthrough of Raz and Rru in the intermediate flow rate

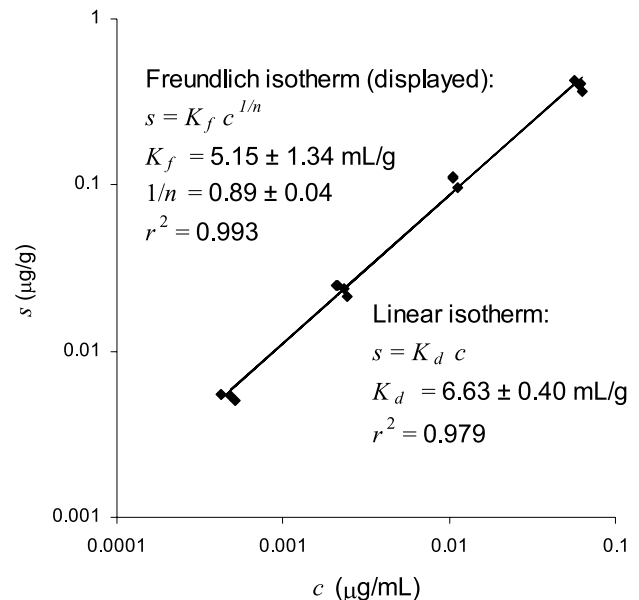


Figure 4. Sorption isotherm for Rru where s is sorbed concentration (MM^{-1}) and c is aqueous concentration (ML^{-3}).

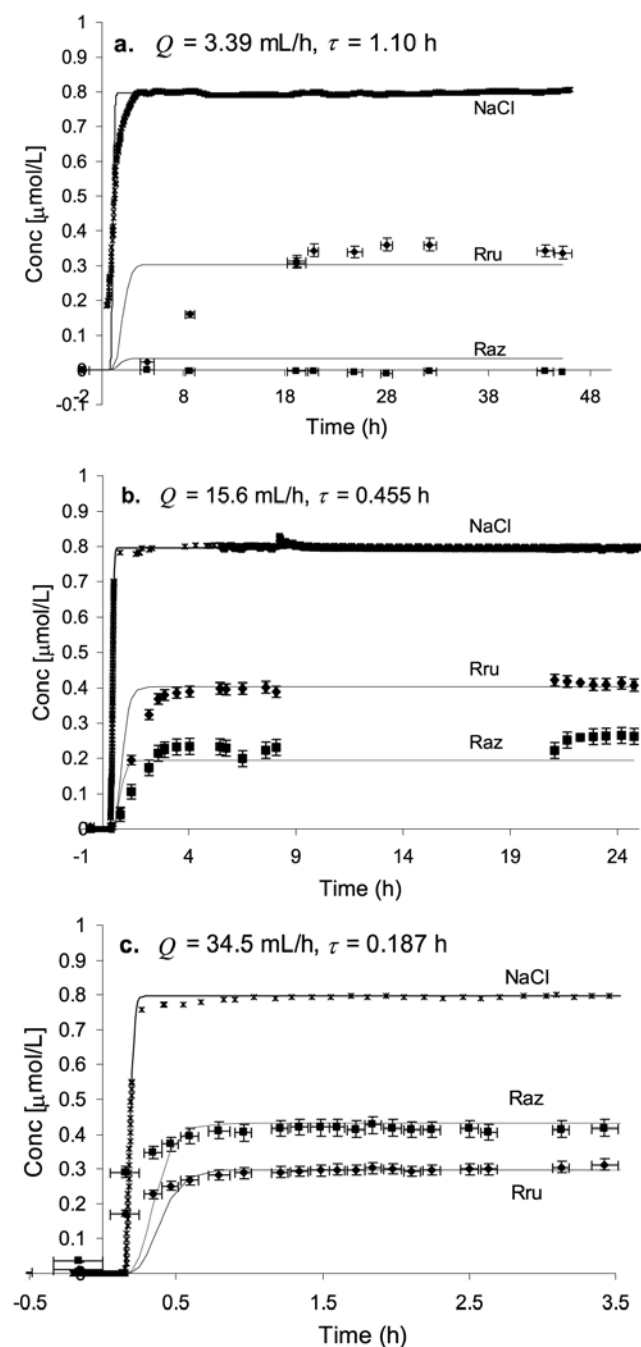


Figure 5. Breakthrough curves for Raz, Rru and NaCl (conservative tracer). Concentrations for NaCl have been rescaled so that plateau concentrations are the same as the injected concentration of Raz. Models are those given in equations (5a) and (5b). Error bars in time show time needed to extract sample.

column was retarded relative to that of a purely conservative tracer (chloride) from travel times of approximately 30 min to travel times of approximately 90 min; the K_d value of 6.63 mL/g (Figure 1) for Rru could generate retardation factors at equilibrium as high as 60 in unconsolidated sediment. This K_d is probably maximal for our sediment because we obtained the sorbed concentration by difference from the aqueous. Since we know that Rru decays in addition to sorbs, the actual concentration is probably

overestimated. In any case, other compounds currently used as hydrological tracers, such as rhodamine WT, can have equally strong sorption (Figure 1). For example, *Gooseff et al.* [2005] measured a K_d of 33.2 mL/g for rhodamine WT in sediment with 9% organic carbon (Figure 1), and *Vasudevan et al.* [2001] reported a host of similar or larger values. We expect that sorption of Raz and Rru will be weak when the fraction of organic carbon in the sediment is relatively small. However, measurements of sorption characteristics of Raz and Rru using a range of sediment composition and organic carbon content will help evaluate the level of sorption for this tracer. This is particularly critical if Raz-Rru is used in groundwater studies.

[35] Raz is a likely smart tracer for microbial activity in freshwater ecosystems. Previous studies have shown that the reduction of Raz to Rru takes place in the presence of living bacteria [*Guerin et al.*, 2001; *Karakashev et al.*, 2003; *Liu*, 1983; *McNicholl et al.*, 2007; *Moyer and Campbell*, 1963; *O'Brien et al.*, 2000; *Peroni and Rossi*, 1986; *Twigg*, 1945; *Zrimšek et al.*, 2004]. This suggests that the Raz to Rru reaction in our columns is probably driven by the redox conditions generated by microbial activity associated with the sediment. This is consistent with metabolic activity in the columns indicated by our oxygen consumption measurements.

[36] Our proposal to use Raz as a smart tracer for water-sediment interaction is supported by our findings of fast reaction in water with sediment and very slow reaction in water alone. Other research suggests that this may be expected in field studies as well. *Fischer and Pusch* [2001] found that bacterial production was 17–35 times higher in the upper 2 cm of sediment than in the overlying water column in a study of sixth-order River Spree in Germany. If this pattern holds across many streams, then Raz should be viable as a field tracer of water-sediment interaction.

4.2. Potential Applications and Future Needs

[37] Although Raz has been used in previous biological studies [e.g., *Ramsdell et al.*, 1935; *Moyer and Campbell*, 1963; *Erb and Ehlers*, 1950; *Zrimšek et al.*, 2004; *O'Brien et*

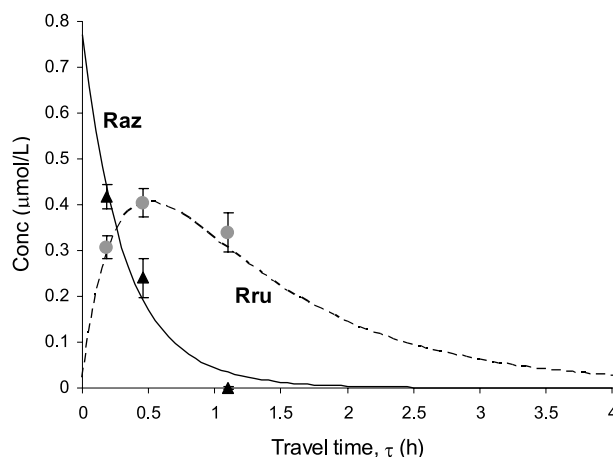


Figure 6. Concentration at plateau (averaged over plateau) versus travel time for conservative tracer ($\tau = L/v$) through column as modeled by equations (6a) and (6b). The model and data shown in Figure 5, averaged over the plateau, are the same as shown here.

al., 2000], to our knowledge this is the first time it has been assessed for use in hydrology. There is a great potential for application and further development of Raz and Rru as a smart “hydrobiological” tracer in both laboratory and field studies. We foresee contributions using this tracer to help bridge the gap between hydrological and biogeochemical studies in stream ecosystems. In a literature review, *Pusch et al.* [1998] indicated that respiration in the hyporheic zone could account for 40 to 90% of total stream ecosystem respiration. This microbial activity is likely to influence nutrient retention at the ecosystem scale if surface-subsurface hydrological exchange is extensive. The use of Raz combined with a conservative tracer could help identify not only the magnitude of water exchange, but also could provide qualitative information on redox conditions (indicative of microbial activity) in the exchange compartments. Raz may be used in hydrologic studies to help differentiate solute transport and transient storage in parts of the system with less microbial activity from those parts with more activity, especially those associated with heterotrophic processes. Interpretation of Raz-Rru dynamics with respect to a conservative tracer (e.g., chloride) could be an indicator of sediment-water interaction in streams because of the generally greater microbial population associated with sediment than with flowing water. Last, the Raz-Rru system may prove useful to the assessment of hyporheic processes.

[38] Raz may also be useful as a hydrological tracer for investigation of purely in-channel transport. For example, in-channel dispersion could be isolated with Raz. Because Raz disappears rapidly in the subsurface, the BTC of Raz would represent transport in the channel only.

[39] One advantage of its use in field studies is the fact that Rru is highly fluorescent (quantum efficiency of 0.74) in moderately basic conditions; and thus, it is possible to be measured even at very low concentrations ($\ll 1 \mu\text{g/L}$) on most spectrofluorometers. This characteristic reduces the cost of the experiments and increases the sensitivity of the results.

[40] To make the Raz-Rru system truly effective as a smart tracer, further laboratory work is necessary to help refine the properties and to quantitatively correlate the reactions with processes of interest. For instance, we need to quantify the relationship between Raz-to-Rru reaction rates, microbial activity, and sediment surface area. This is likely to be different from one location to another, so we will need to develop a standard and simple methodology for making the correlations. The relative contribution of aerobic and anaerobic respiration to reaction rates should also be quantified. It will be important to understand which organisms are mediating the Raz-Rru reaction and through which mechanisms. As already mentioned, further work on the sorption will be necessary, particularly if it is used in groundwater. Finally, while we know that Raz and Rru are not highly toxic, further work should be done to establish details of toxicity.

[41] We view the development of the Raz-Rru tracer system as a step forward in hydrology, because it provides more “bits” of information than a simple conservative tracer. Although in the present study we present Raz as a smart tracer, we propose that other compounds, nanoparticles, or even “nanobots” [Wood, 2005] could become smart tracers. Other materials may also work well as smart tracers of microbial activity or sediment-water interaction on the basis of their properties (e.g., nonsorbing, less expensive, etc.).

Most importantly, while our example may prove useful for characterization of the coupling between solute transport and biological activity or the amount of sediment-water interaction, other smart tracers may be able to be developed to assay other conditions of interest such as temperature, pH, or presence of a nonaqueous phase.

5. Conclusions

[42] This study shows that Raz is a feasible smart tracer for the assessment of sediment-water interactions and associated biological activity in freshwater ecosystems. Raz reduces rapidly, as a pseudo-first-order process, with a rate coefficient of 1.41 h^{-1} in flow-through experiments with colonized sediment. The reaction in sediment is much faster than in water without sediment and is also a much faster than decay. Both compounds can be measured at low concentrations ($<1 \mu\text{g/L}$) with a standard spectrofluorometer without background interference in natural waters. The compounds are stable over times long enough to complete lab or field work, but care should be taken to avoid samples exposure to intense light. Sorption of both Raz and Rru could be an issue of concern in some settings. Further work is necessary to quantitatively relate the Raz-Rru reaction to sediment-water interaction and microbial activity.

[43] **Acknowledgments.** We thank Jim Butler, Jesús Carrera, Xavi Sánchez-Vila, Nancy Grimm, Michael Gooseff, the members of the limnology group (CEAB-UB), and reviewers for insights and discussions on how to develop a smart tracer and ideas to improve the paper, and Lynn Melton and Kim Kangasniemi for tips on the Raz-Rru system. We thank the Centre d'Estudis Avançats de Blanes (CSIC) for hosting R.H.'s sabbatical where this work was primarily completed. This work was supported by sabbatical funding from the Ministerio de Educación y Ciencia of Spain and Oregon State University, by funding from the NICON (MEC, Spain, ref: CGL2005-7362) and EUROLIMPACS (EC 6th Framework Program, ref: GOCE-CT-2003-505540) projects, and by materials and conference support from the National Science Foundation (EAR 04-09534).

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