AGU100 ADVANCING EARTH AND SPACE SCIENCE

Water Resources Research

RESEARCH ARTICLE
10.1029/2018WR023103

Key Points:
- Past applications of resazurin in hydrology on a range of spatial scales are summarized
- New developments and challenges of the reactive tracer system are outlined
- Details of the resazurin-resorufin "smart" tracer system for the estimation of metabolic activity are presented

Supporting Information:
- Supporting Information S1
- Movie S1
- Data Set S1
- Data Set S2
- Data Set S3
- Data Set S4
- Data Set S5

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Citation:
Knapp, J. L. A., González-Pinzón, R., & Haggerty, R. (2018). The resazurin-resorufin system: Insights from a decade of "smart" tracer development for hydrologic applications. *Water Resources Research*, *54*, 6877–6889. https://doi.org/10.1029/2018WR023103

Received 9 APR 2018
Accepted 25 JUL 2018
Accepted article online 3 AUG 2018
Published online 27 SEP 2018

The Resazurin-Resorufin System: Insights From a Decade of "Smart" Tracer Development for Hydrologic Applications

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Abstract The resazurin-resorufin tracer system has been used to quantify surface water-sediment interactions and microbial metabolic activity in stream ecosystems for one decade. This review describes the evolution of the tracer technique and summarizes how it has been used by the hydrologic and stream ecology communities. We highlight major hydrologic applications and milestones in the advancement of the reactive tracer system on scales ranging from cells to river reaches and catchments. We discuss the advantages and limitations of the resazurin-resorufin system for hydrologic applications and suggest new directions of research, including how to address existing knowledge gaps. Beyond the goal of summarizing information that is specific to the development of the resazurin-resorufin system, this review seeks to inform on the development of new "smart" tracer techniques as they, very likely, will face the same or similar challenges and opportunities encountered in the development of the resazurin-resorufin system. The supporting information furthermore contains a detailed manual for the application of the resazurin-resorufin system as hydrologic tracer and MATLAB codes for the analysis of their reactive transport.

Plain Language Summary The reactive tracer compound resazurin has been used for 10 years to investigate how surface water interacts with the sediment and to calculate rates of metabolic activity. This review paper describes the development and applications of the tracer compound resazurin in hydrology and ecology. We also discuss advantages and limitations of the tracer and show future directions of research. The development of the resazurin-resorufin system can also be used as a model for the development of other tracer techniques.

1. Introduction

The majority of the nutrient turnover, degradation of contaminants, and metabolism in river corridors takes place in metabolically active transient storage zones near the stream bed in benthic and hyporheic zones. Due to the importance of hyporheic processes for ecosystem health (Boano et al., 2014; Cardenas, 2015), numerous studies have aimed to quantify the travel times and processing of solutes through the hyporheic zone. Tracer tests with conservative compounds have been carried out to estimate travel and residence times for nearly half a century, and their signals have been analyzed using different adaptations of the transient storage model (TSM; Bencala & Walters, 1983; Runkel, 1998), which conceptualizes exchange between the main channel and transient storage zones. Conservative tracer tests, however, suffer from the disadvantage that in-stream dispersion and transient storage in dead zones of the main channel cannot be separated from storage in the subsurface, lumping parts of both contributions into the storage component of the TSM. For this reason, Haggerty et al. (2008, 2009) proposed the use of the resazurin-resorufin system, which is capable of providing additional information about surface water-sediment interactions through the differences in metabolic activity of surface and subsurface storage zones. The tracer system is therefore often referred to as "smart" tracer technique. The resazurin-resorufin system was based on the concept that an ideal tracer technique to separate surface from subsurface transient storage should mimic the behavior of the "injection" and "retrieval" of binary nano-switches. The tracer injected would begin in state 0 (zero) when injected into the surface water and would remain in that state unless it entered an environment where it was irreversibly transformed (e.g., the subsurface) and, then, register state 1 (one) thereafter. If it were possible to recover the counts of 0s and 1s resulting after a known initial number of 0s were injected upstream, the concentrations of

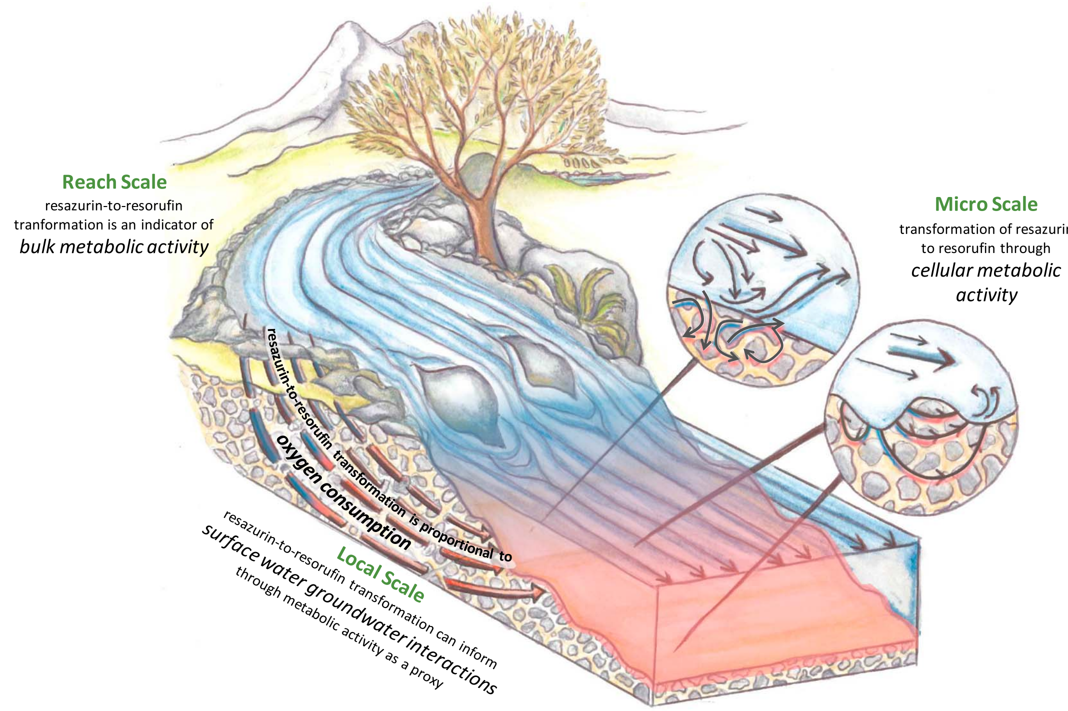

Figure 1. The resazurin-resorufin tracer system tracks metabolic processes in the river corridor from cellular to reach scales. The transformation of resazurin to resorufin is proportional to the consumption of oxygen and can thus be used as a proxy to estimate aerobic metabolism.

particles still in state 0 would represent the fraction of water that did not enter the subsurface, whereas those recording 1 would represent the fraction of water that visited the subsurface. Such an ideal smart tracer would provide information about the type of compartments through which water flows and also about the distribution of residence times associated with each compartment. Moreover, a "mass balance" would allow estimates of the amount of new, unlabeled water flowing into the stream. Although this ideal tracer would provide us with information about transport processes of interest, that is, advection, dispersion, and transient storage, it would not (yet) provide any information regarding biochemical characteristics of each compartment. For this, we would have to "engineer" the aforementioned tracer into an ideal reactive tracer—or series of reactive tracers—sensitive to biogeochemical processes of interest (Foppen et al., 2013).

In 2008, Haggerty et al. (2008) introduced the resazurin-resorufin system as a smart tracer technique because resazurin (0s in our previous analogy) undergoes an irreversible transformation to resorufin (1s in our previous analogy) in the presence of metabolic activity, which is a pathway of interest to hydrologists and stream ecologists (Figure 1). Therefore, the injection of resazurin and the recovery of resazurin and resorufin signals allow solute transport and metabolic activity to be related. Further characterizations of the resazurin-resorufin system showed that the transformation of resazurin in filtered water was negligible compared to that in sediments (Haggerty et al., 2008). Thus, as the transformation of resazurin is much higher in the subsurface than in surface storage zones or the main channel, the application of this smart tracer can also be used to identify parameters related to exchange with, and storage within, metabolically active transient storage zones of the near subsurface. This allows investigations of metabolic activity on different hydrologic scales.

Over the past 10 years, the resazurin-resorufin approach has been applied in hydrologic research to investigate the coupling of transient storage, metabolism, and nutrient cycling at multiple scales through batch experiments, laboratory columns and flumes, and field experiments in streams and wetlands. This review paper explains the details of the resazurin-resorufin tracer system, summarizes the applications of resazurin as a smart tracer in hydrologic research on a range of different spatial scales, and discusses potential future directions of smart tracer development and testing. Furthermore, we include an

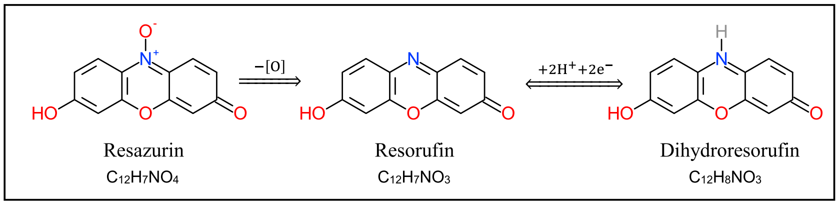

Figure 2. Structures and reactions of resazurin.

appendix with practical advice gained from 10 years of experience, helpful information and tools to guide users (practitioners and scientists) in the application and analysis of resazurin experiments in the supporting information.

2. The Resazurin-Resorufin Tracer System

Resazurin (/rə 'zazərən/) is a phenoxazine dye (commercially known as *Alamar Blue*) that can be reduced irreversibly to the daughter compound resorufin (/rɛzɔ,'ru:fɪn/; Figure 2) by aerobic and facultative anaerobic microorganisms, but not by strict anaerobes (Karakashev et al., 2003). Because the transformation of resazurin to resorufin occurs in the presence of living microorganisms (Guerin et al., 2001; O'Brien et al., 2000), it can be used as an indicator of microbial growth and cytotoxicity. Because its application is nontoxic and noninvasive, it is applied frequently in cell viability tests in the dairy industry (e.g., Erb & Ehlers, 1950; Pesch & Simmert, 1929), medicine (e.g., Carter et al., 1998; Reddy et al., 1997), and for other biochemical applications (e.g., Chen et al., 2015; Riss et al., 2016) including the assessment of metabolic activity in activated sludge (McNicholl et al., 2007). Furthermore, investigations into the reaction mechanism found that in addition to microorganisms, a number of organic chemicals (Guerin et al., 2001) and some other fluorescent dyes (O'Brien et al., 2000) may also be able to chemically reduce resazurin.

Both resazurin and resorufin are fluorescent, and their aqueous concentrations can be estimated with fluorometers via standardization (see Text S5). The quantum yields (i.e., ratios of photons emitted through fluorescence to photons absorbed) of resazurin and resorufin are 0.11 and 0.75, respectively (Bueno et al., 2002), and both compounds are not naturally present in the environment. Therefore, low concentrations in the range of micrograms per liter can be detected and are typically targeted in hydrologic applications of the tracer technique. Like any other tracer, resazurin and resorufin can be sampled manually, with automatic samplers or with mini piezometers (discrete samples), but also in situ using on-line fluorometry (semicontinuous samples). For discrete samples, a benchtop spectrofluorometer can be used to read the fluorescence intensities of the two tracers (i.e., each sample is read, in tandem, at the characteristic wavelengths for resazurin and resorufin; see Text S3). For semicontinuous signal monitoring, an on-line fluorometer (e.g., GGUN-FL30, Albillia Sàrl, Neuchâtel) option is available and has been successfully tested in column and field experiments (Lemke et al., 2013; Text S4).

Like most hydrologic tracers, resazurin and resorufin have nonidealities that have to be considered. First, the irreversible transformation of resazurin does not uniquely yield resorufin, but also additional, unidentified byproducts that are not fluorescent and thus cannot be quantified through fluorescence spectroscopy (O'Brien et al., 2000). In a further reaction step, resorufin can be reversibly transformed to colorless dihydroresorufin (sometimes also referred to as *hydroresorufin*) under anoxic conditions. This second reaction step (see Figure 2) has been used as redox indicator and to detect oxygen "contamination" in experimental setups (e.g., Bauer et al., 2008). The reverse reaction from dihydroresorufin to resorufin takes place nearly instantaneously upon contact with oxygen, and, thus, dihydroresorufin is not likely to falsify fluorescence analyses of stream tracer tests, be them in situ with online fluorometers or ex situ through grab sampling. Second, both resazurin and resorufin are affected by sorption processes that are pH dependent (Lemke et al., 2014), with stronger sorption occurring at lower pH values. Accounting for the effect of sorption is particularly important for slug injections as the timing of the rising and notably the falling limbs of the breakthrough curves are delayed if sorption occurs. Conversely, analyses based on plateau concentrations (steady state concentration) resulting from continuous injections are not affected by sorption. Nonequilibrium (kinetic) and equilibrium sorption analyses by Haggerty et al. (2009) and Lemke et al. (2014) suggest that using linear sorption models is adequate, but emphasize differences between the sorption distribution coefficients of resazurin and resorufin. Their results highlight the importance of accounting for sorption of resazurin and resorufin in the analysis of stream-tracer tests. If this is neglected, sorption processes may erroneously be attributed to other transport parameters, which in turn may affect the interpretation of hyporheic exchange processes. Finally, like any fluorescent tracer, resazurin and resorufin are sensitive to sunlight. The rate of photodecay, however, is relatively slow (Haggerty et al., 2008, found time scales of photodecay of several tens of hours for resorufin and hundreds of hours for resazurin). While this rate is usually negligible in the stream, samples need to be stored out of the light. From the three tracer nonidealities we have highlighted here, the irreversible transformations of resazurin and resorufin to other, nonfluorescent products still need to be investigated in more detail to improve mass balance closure in the resazurin-resorufin system. Furthermore, it has not been investigated so far whether the sorption behavior of dihydroresorufin differs from the one of resorufin and resazurin, which would influence subsurface transport and total mass recovery (as pointed out by, e.g., Knapp & Cirpka, 2017).

Due to its nonconservative behavior, resazurin is typically co-injected with a conservative tracer. This provides information on conservative transport (details can be found, e.g., in Hauer & Lamberti, 2011, Chapter 8) and helps to distinguish them from reaction processes. Depending on the characteristics of the system where resazurin is injected, tracer salts like sodium bromide or sodium chloride can be used (e.g., Argerich et al., 2011; González-Pinzón et al., 2014, 2015, 2016; Haggerty et al., 2009; Knapp et al., 2017). In other studies, the fluorescent tracer fluorescein has been co-injected with resazurin (e.g., Blaen et al., 2018; Knapp & Cirpka, 2017; Lemke et al., 2013). Importantly, the use of rhodamine WT is not suggested due to its close fluorescent interaction with resazurin and resorufin. The decoupling of conservative and reactive transport is key to enabling estimates of dilution, decay, and production in slug and continuous rate injections. However, Riveros-Iregui et al. (2018) recently reported on differences in metabolism between wetland and channel transitions in a tropical, high-elevation (páramo) stream system, where the recovery of the conservative tracer NaCl failed due to high natural background values of specific conductance. Due to the lack of evaluable conservative tracer data, the differences in timing, shape of the breakthrough curves, and relative production of resorufin informed how differences in average longitudinal flow velocity and consequent residence times resulted in differences in metabolism, as seen from the production of resorufin. In this study, less than 15% of the resazurin was transformed to resorufin by aerobic respiration in the channel, compared to approximately 50% in the wetland.

Due to the dependence of the transformation of resazurin on the presence of living microorganisms (O'Brien et al., 2000), it has been hypothesized that the resazurin-resorufin system can be used in situ to quantify ecosystem respiration and to estimate reaeration and primary production from independently measured dissolved oxygen signals (González-Pinzón et al., 2014, 2016). Other common methods used to estimate metabolism in streams (e.g., diel oxygen techniques or chambers) apply an opposing approach, by using diel oxygen signals and estimates of reaeration rates to determine respiration based on nighttime recordings and primary production from daytime measurements, typically assuming constant reaeration and respiration fluxes. The advantage of the reactive tracer approach for the estimation of ecosystem respiration lies in its consideration of the whole study system, implicitly accounting for spatial heterogeneity in stream bed morphology and biogeochemistry, instead of relying on highly uncertain upscaling approaches from small-scale measurements to the reach scale.

Given that the resazurin-resorufin system can measure respiration at cell to reach scales, in situ, it can be used to investigate biogeochemical processes that require the sampling of relevant or realistic transport systems (i.e., contrary to conditions encountered in chamber systems where hydrodynamic and thermal regimes are highly altered), at or across more representative spatial scales (González-Pinzón et al., 2012, 2014). For example, the resazurin-resorufin system can be applied (1) in situations where the determination of metabolic activity from diel oxygen signals is not ideal, such as when oxygen is constantly at or near saturation conditions due to cold temperatures and high turbulence, that is, when rapid reaeration occurs; (2) when instantaneous rates are of interest instead of diel averages obtained from measurements of dissolved oxygen; (3) when spatial changes in respiration near the streambed or across meanders are needed and sampling induces reaeration, thus altering the samples; (4) in spatially or temporally intermittent streams without surface flow when only hyporheic samples can be taken; (5) when flow paths between groundwater and surface water need to be understood from both transport and metabolism perspectives; (6) in wetlands and stream systems with point or diffuse sources; among others. In these and similar systems, applying resazurin can help to estimate metabolic activity.

3. Review of Hydrologic Applications of Resazurin Across Spatial Scales

This section summarizes hydrologic studies that made use of the resazurin-resorufin system on scales ranging from individual cells to stream reaches. We start with microscale studies serving as proof of concept and then focus on studies conducted in the field applying the technique to investigate ecosystem respiration.

3.1. Micro and Local Scales: From 10^{-6}- to 10^{1}-m Scales

Before Haggerty et al. (2008) introduced the use of resazurin to hydrologic applications, McNicholl et al. (2007) showed that measurements of metabolic activity in activated waste water treatment sludge using resazurin correlated well with respiration obtained from direct measurements of dissolved oxygen. Based on these findings and preliminary tests of Haggerty et al. (2008, 2009), González-Pinzón et al. (2012) used pure cultures of different strains of obligate and facultative anaerobic microorganisms commonly found in sediments to establish a quantitative relationship between the transformation of resazurin and dissolved oxygen consumption as measure of cellular aerobic respiration. The study found positive, linear relationships between the resazurin-to-resorufin transformation and respiration rates, which suggests that the transformation of resazurin can be used as a proxy to quantify aerobic respiration in flow systems at different spatial scales. Although the transformation of resazurin was always positively correlated with microbial respiration, the molar processing ratio of the target to the proxy tracer (i.e., moles of dissolved oxygen processed per moles of proxy-tracer resazurin processed) was neither equal to one (ideal case where resazurin transformation could be directly related to oxygen consumption) nor constant for all pure cultures. Therefore, follow-up modeling work by González-Pinzón et al. (2012) focused on establishing a relationship between the processing rates of the two solutes when they are transported in a flow system and undergo advection, dispersion, transient storage, and reaction. This work derived algebraic equations related to the TSM demonstrating that the relationship between the two processing rates is primarily a function of the molar processing ratio of dissolved oxygen consumption to that of resazurin transformation. Importantly, the study also found that the uncertainty in the estimation of the TSM parameters is less significant than the uncertainty in the molar processing ratio (cf. equation 13 in González-Pinzón & Haggerty, 2013). These proof-of-concept studies showed that resazurin can be used to quantify aerobic respiration in flow systems and estimate the consumption of dissolved oxygen (see Figure 1). The development of this novel technique allows measurements in situ and in vivo, as water flows through surface and hyporheic flow paths, with sampling resolution at multiple spatial scales, that is, from cells to reaches.

Based on the knowledge that the transformation of resazurin is proportional to respiration, Stanaway et al. (2012) used resazurin to investigate the effects of persistent anthropogenic metal deposition (As, Cd, Cu, Pb, and Zn, among others) on hyporheic microbial communities exposed to differing magnitudes of chronic metal stress. For this, hyporheic sediments from sites with high, medium, and low contamination along the Clark Fork River in Montana, USA, and nearby reference sites with similar elevations and hydrogeological properties were used to quantify differences in sediment respiration in controlled column experiments. The obtained transformation rate constants of resazurin suggest that metals inhibited respiration by 13–30% and that the observed inhibition was directly related to the level of in situ metal stress. Thus, there is a legacy of impaired hyporheic aerobic microbial community functioning due to heavy metal contamination, even after 100 years of adaption opportunity. In this study, resazurin brought a novel ecological monitoring tool for geochemically complex environments as this was the first application of a hydrologic smart tracer as a functional indicator of ecological integrity within anthropogenically influenced flowing water systems.

Since microbial respiration is much greater than that from macroinvertebrates in natural systems, the transformation of resazurin can be used to understand how macropore formation by animal mobility (bioirrigation) in streambed or lakebed sediments affects the dynamics of oxic and anoxic zones and, thus, microbial respiration. Baranov et al. (2016) quantified the effects of bioirrigation of Chironomidae larvae (a type of nonbiting midge) on aerobic respiration in sediments from a eutrophic lake in Germany using resazurin. For this, lakebed sediments were collected, homogenized, and sieved for complete defaunation. Then, following standardized bioassays, the authors quantified resazurin-to-resorufin transformation rates in

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column experiments as a function of Chironomidae density. Resazurin transformation rates differed between bioirrigated and nonbioirrigated sediments by up to a factor of 3. This difference was attributed to the enhanced transport conditions brought by the larvae movement, which increased microbial respiration rates, likely due to a displacement of the oxic-anoxic boundary through biologically driven reaeration. The increased transformation of resazurin was correlated to increased microbial respiration because control experiments using water columns with and without larvae suggested negligible transformations of resazurin. In this study, the novelty brought by the resazurin-resorufin system was the capability of quantifying the impacts of bioirrigation on bacterial respiration as other classic measurements based on oxygen consumption failed to separate the respiration impacts of Chironomidae and sediment microbial communities. Differentiation of these pathways previously relied on separate measurements of animal respiration that are prone to artifacts caused by animal stress, and result in overestimations of respiration rates. Thus, the use of resazurin was advantageous because its transformation offered a direct relationship with microbial respiration, isolating animal respiration and chemical oxygen demands that would have affected oxygen mass balances.

The application of resazurin can also be helpful to quantify metabolic processes in different compartments of the river corridor. A field, local-scale application of resazurin was presented by Knapp et al. (2017). The study described the injection of the tracer into an urban stream in Virginia (USA), where tracer breakthrough curves were recorded at several depths in multiple hyporheic zone locations around the stream's thalweg, near side pools, and upstream and downstream of fallen logs using USGS MINIPOINT samplers (Harvey et al., 2013; Harvey & Fuller, 1998). The vertical transformation of resazurin was used as an indicator of metabolism, and high-reactivity zones were identified from depth profiles of tracer concentrations. The results from this subsurface analysis indicated that the potential for tracer transformation varied with depth in the hyporheic zone, and highlighted the importance of the benthic biolayer as a metabolically active region. The novelty brought to hyporheic research by the use of resazurin in this study was the ability to identify layers of higher and lower reactivity in the subsurface from the reaction rates of resazurin, and the results indicated the importance of the benthic biolayer in controlling substrate supply and subsequent microbial metabolism. While the data helped in localizing layers with increased turnover, they did not allow a quantitative resolution of the relationship between biomass abundance and/or function, and hydrological substrate supply. Therefore, that is a research opportunity where resazurin can be used in the future.

Resazurin has also been applied on a slightly larger scale, in mesocosm and flume experiments. These settings are closer to nature than column and batch tests, but the conditions can still be better controlled than in a field experiment. Haggerty et al. (2014) investigated how flow heterogeneity caused by impermeable bed forms affected ecosystem respiration in two streamside flumes. Biofilms were allowed to grow in two flumes with either flat or dune-shaped beds covered with a single layer of previously cleaned gravel. The flumes were fed with stream water at a constant discharge, and ecosystem respiration was estimated at different stages of biofilm growth through resazurin injections. The study found higher ecosystem respiration in the flume with dune-shaped bedforms than in the flume with a flat gravel layer. Furthermore, biofilm growth in both flumes resulted in an increase in ecosystem respiration that was attributed to both the increased number of respiring cells and an observed increased in transient storage. Resazurin does not undergo reaeration, for which reason the reactive tracer allowed a more accurate determination of ecosystem respiration than would have been possible from oxygen measurements.

In another study using artificial mesocosms, Kurz et al. (2017) investigated the joint effect of water level changes and submerged vegetation on transient storage and metabolic activity in a set of flumes controlled for different water level conditions. Resazurin was injected continuously into the flumes, and the ratios of resazurin and resorufin concentrations were used to assess ecosystem respiration. In a second step, acetate was co-injected as labile carbon source together with resazurin to stimulate metabolic activity. Additionally, through the whole course of the experiment, oxygen concentrations were recorded at the outflows of the flumes. The study found higher amounts of submerged vegetation and resazurin transformation at increased water column depth, indicating that metabolically active transient storage was a combination of vegetated surface waters and water-sediment interactions. Comparisons of resazurin processing rates before and during the acetate injections showed a slight increase in metabolic activity in some of the flumes due to the addition of acetate. Furthermore, the study found that the instantaneous rate of respiration obtained from the reactive tracer injection correlated with water depth, whereas the diel rates of ecosystem respiration determined from oxygen measurements did not scale with water levels. This study thus highlights the demand for a substantial comparison of the two techniques for estimating ecosystem respiration.

3.2. Reach Scale: From 10^1- to 10^3-m Scale

For the most part, the applications of the reactive tracer resazurin have focused on the characterization of reach sections in different geological and morphological settings. For these stream-tracer tests, resazurin is injected into the stream channel together with a conservative tracer, and the concentrations of the three tracers are recorded over time at one or more measurement stations downstream of the injection site. Extensions of the TSM accounting for the turnover of resazurin in metabolically active zones are commonly used for the analysis of mass exchange, residence time distributions and reactivity (e.g., Haggerty et al., 2009; Knapp & Cirpka, 2017; Lemke, Liao, et al., 2013).

The first field studies applying resazurin at the reach scale aimed to show that resazurin can indeed be used as proxy for metabolism in larger systems. One of the first applications took place in a headwater stream in the Cascade Range in Oregon, USA. Argerich et al. (2011) injected resazurin together with the conservative tracer NaCl at a constant rate. The study investigated the turnover of resazurin to quantify the fractions of metabolically active and inactive storage in a reach with bedrock and alluvium beds, finding faster resazurin turnover in the alluvial section, which also featured a larger metabolically active transient storage area. Furthermore, they determined ecosystem respiration based on recorded diel concentrations of dissolved oxygen and found it to be proportional to the recorded resazurin transformation, indicating that the reactive tracer system can be applied to assess reach-scale respiration.

To investigate the applicability of the reactive tracer in bigger streams, resazurin was injected and recorded in a low gradient stream in Germany (Lemke, Liao, et al., 2013) injected and recorded resazurin in a low gradient stream in Germany. In the same study the authors presented the development and application of a field on-line fluorometer (GGUN-Fl30; described in further detail in Lemke, Schnegg, et al., 2013). In this field study, resazurin, resorufin, and the conservative tracer fluorescein were injected jointly for the first time. The use of an on-line field fluorometer allowed the collection of high-frequency data, for which reason errors typically caused by manual sampling (e.g., mix up of the samples, sample contamination, and altering of the tracer concentrations in the samples due to long transport/storage times) can be avoided. The authors analyzed the reactive and conservative tracers jointly using a modified transient storage approach, and compared the outcomes to those of transient storage modeling of the conservative tracer alone. The joint fit yielded higher hyporheic exchange rates and lower dispersion than model results from the conservative tracer alone, indicating that the application of the reactive tracer indeed helps to separate metabolically active in-stream from metabolically inactive subsurface transient storage.

Results from these first reach-scale applications illustrated the possibility of using resazurin to investigate the functional relationship between metabolism and geomorphic units, bed materials, and type of transient storage. González-Pinzón et al. (2014) used the resazurin-resorufin system to estimate metabolism at different spatial scales from the habitat to the reach scale in two headwater streams in Oregon, USA. The study found higher respiration rate coefficients in subreaches with extensive hyporheic flow and flow through large woody debris, and higher reaeration in subreaches with intensive respiration activity and higher flow velocities. The metabolic hot spots detected by the resazurin-resorufin system in both watersheds were related to hydrodynamic conditions known to increase biological processing, but that are difficult to quantify in headwater streams without the use of tracer techniques. For example, the reactive tracer technique allowed quantifying metabolism associated with pure hyporheic flow. Here monitoring changes in dissolved oxygen would have been impractical due to artificial reaeration through manual or pump-driven sampling or difficulty in installing oxygen sensors in undisturbed hyporheic flow paths, that is, disconnected from the atmosphere. Similarly, the reactive tracer injection tracked metabolic activity in natural, fractal geometries that provide surface area for biofilm growth such as fallen trees and algae growing on irregular rocks. Finally, these experiments with resazurin allowed, for the first time, the generation of longitudinal oxygen profiles separating respiration, reaeration, and primary production in streams. More recently, Blaen et al. (2018) investigated the dependence of ecosystem respiration on woody debris in a lowland stream in the UK. In this study resazurin and fluorescein were co-injected in reaches with different amounts of large woody debris. They concluded that differences in resazurin transformation observed between different reaches could not be explained by differences in hydraulic properties of the individual study sections, indicating that metabolic processes are not primarily controlled by transport, as is sometimes assumed. Instead, the rate of resazurin transformation was purely related to differences in large woody debris accumulation, with higher transformation rates observed in reaches with larger amounts of woody debris.

In another reach-scale experiment with consecutive daytime and nighttime injections of resazurin, González-Pinzón et al. (2016) showed that daytime and nighttime respiration in two reaches did not differ in spite of temperature differences observed in the streams' thalwegs, where temperature is routinely measured in ecohydrology studies. The majority of stream respiration usually takes place within hyporheic zones, where temperature fluctuations are damped. These results therefore suggest that community respiration in headwater streams may not need to be corrected for temperature between daytime and nighttime. Therefore, assuming similar respiration rates during daytime and nighttime is reasonable, even though instantaneous changes in respiration are expected to occur from a pure biological perspective. This highlights the potential for using resazurin to examine seasonal differences in ecosystem metabolism directly, without relying on temperature corrections of reaeration and respiration.

Resazurin has also been used to compare local- to reach-scale reactivity. In a study in a third-order stream in Pennsylvania, USA, González-Pinzón et al. (2015) found that the average molecule of resazurin that entered the hyporheic zone and traveled along ~9-cm-deep flow paths was completely transformed to resorufin. While the transformation of resazurin was significant at the local scale, reaching 100% transformation potential within the shallow hyporheic zone, only ~20% of the injected resazurin was transformed along the ~450-m study reach. This result highlighted the importance of linking local- and reach-scale observations in analyses of biogeochemical processing. More specifically, resazurin proved to be a tool to address the common question: When/where do groundwater-surface water interactions matter for biogeochemical cycling and when/where are they less important? In a similar fashion, Knapp et al. (2017) combined sampling at two in-stream stations with subsurface monitoring of resazurin at three stations located near the stream banks and thalweg, each one with USGS MINIPOINT samplers taking samples at five different depths, following a continuous injection in a third-order stream in Virginia, USA. From these, transformation rates of the tracer were estimated at the local and reach scale. Using a relatively extensive sampling resolution, this study showed that tracer recordings in the hyporheic zone lead to estimates of higher hyporheic reaction rates than if the tracer is recorded in the stream channel, validating the findings presented in González-Pinzón et al. (2015). Through a side-by-side comparison based on temporal moments of resazurin data, Knapp et al. (2017) demonstrated that, unlike previously thought, the use of different measurement techniques applied at different scales is not the main reason why results between local and reach-scale reactivity are irreconcilable. Instead, the results from this study demonstrate that the observations at different scales provide snapshots of disparate parts of one large system that cannot be reconciled. The water sampled by in-stream stations has not seen any of the deep subsurface, and, thus, in-stream monitoring cannot provide a good quantification of the depth of reaction and much less the gradient of reactivity in the subsurface; on the other hand, point-scale subsurface profiles tell us little about what parts of the subsurface are relevant for reach-scale processes. Therefore, only by combining different sampling approaches we can gain information about hydrological and biochemical variability and their cumulative effects at multiple scales.

Expanding the application of resazurin from isolated reach-scale studies to stream networks can help shed further light onto the evolution of stream water quality and the localization and timing of metabolically active zones. One such example is a study by Gootman et al. (personal communication, July 5, 2018) that investigated hyporheic zone processing controls using results from instantaneous co-injections of resazurin and bromide along the Jemez River, a major tributary of the Rio Grande, located in northwestern New Mexico (USA). In this study, reach-scale tracer observations at representative sites across the stream network were combined with tracer recordings in the subsurface from multiple locations and hyporheic depths within different reaches spanning from first- to fifth-order streams, during summer baseflow and spring snowmelt. The study assessed the hyporheic zone net functional behavior across the stream network, revealing that hyporheic zone processing controls varied with depth and stream order. The findings further indicated that seasonal stream flow differences influence hyporheic processing throughout the majority of the studied stream reaches. The application of resazurin enabled the investigation of hyporheic zone processing controls from two spatial scales of interest, and a longitudinal comparison by season, which resulted in a clearer understanding of hyporheic zone net functional behavior. Although these findings demonstrated that different stream orders have unique processing controls, additional research opportunities still exist to improve assessments and predictions of hyporheic zone contributions across the river corridor. This study highlights the potential for the application of reach-scale knowledge for larger-scale predictions. This could prove helpful in the context of management decisions, for example, for evaluating stream restoration efforts with respect to their impact on ecological functioning.

3.3. Transport Modeling of the Resazurin-Resorufin System

During the development of the resazurin-resorufin system, particular attention has been paid to the development of adequate models and tools for the estimation of parameters representing exchange between the surface and hyporheic zones, for example, exchange rates, hyporheic travel times, and reactivity of the hyporheic zone. Among others, finding an adequate parameterization of hyporheic travel time distributions and the partitioning between metabolically active and inactive zones have opened opportunities to classify stream systems according to physical and biochemical functioning. Most of the modeling efforts up-to-date have focused on the reach scale and use recorded time series of tracer concentrations as inputs to find the transport and reaction parameters resulting in the best match between measured and simulated breakthrough curves. These modeling efforts are challenged by the many compound-specific properties of resazurin and resorufin, which increase the dimensionality of the parameter space and thus the number of parameters that need to be estimated in order to fit a given model to the recorded concentration data. For example, Argerich et al. (2011) estimated the transport parameters using only information from the conservative tracer and kept them fixed in a second step where reaction and sorption parameters were estimated from resazurin and resorufin signals. This approach assumes that the conservative and reactive transport follow the same advection, dispersion, and exchange processes, and that transport and reaction parameters are not correlated. However, Lemke, Liao, et al. (2013) showed that the estimates of transport parameters based on a joint fitting of conservative and reactive tracer may differ from those obtained by solely fitting conservative data. In a different approach, Liao and Cirpka (2011) and Liao et al. (2013) combined the estimation of the transport parameters with compound-related parameters, as well as a nonparametric travel time distribution, but found the results to be highly dependent on the initial guess. Lemke, Liao, et al. (2013) circumvented this problem by using a Markov chain Monte Carlo approach to estimate the parameters and their uncertainties. However, this was only possible for a simplified model with an exponential travel time distribution due to the high computational demand of the search algorithm. For this reason Knapp and Cirpka (2017) developed a local-in-global approach coupling the local estimation of the nonparametric, continuous transfer function to a global, Markov chain Monte Carlo estimation of all other parameters. This approach provides parameter uncertainties and is faster than estimating all parameters and the travel time distribution through a Markov chain Monte Carlo approach, but still requires large computational time.

A common issue regarding TSMs lies in the high correlation of the estimated parameters. This is rarely discussed in tracer test modeling, but correlations play an important role when interpreting obtained results. It has been argued that an analysis based directly on the observed breakthrough curves (e.g., through the analysis of plateau concentrations or temporal moments) instead of model-derived parameters should be preferred since it does not rely on the identification of poorly constrained, correlated, and calibration-dependent parameters (e.g., see discussion by González-Pinzón et al., 2013). In the sections S6–S8 we present a summary of the quantitative methods available to estimate transport and reaction parameters using temporal moment analyses and transient storage modeling (Haggerty, 2013). Furthermore, we also include MATLAB scripts where these mathematical quantities and models are numerically computed.

4. New Developments and a Way Forward

Resazurin has been used extensively as redox indicator in biochemistry and to test for the presence of living cells. Over the past decade, the reactive tracer has also been applied in hydrology on a range of scales from individual cells to multiple reaches across catchments (see Figure 1). The resazurin tracer system, as smart tracer, provides opportunities to improve our understanding of ecosystem processes, because it offers key advantages to hydrologists and stream ecologists, e.g., (1) neither resazurin nor resorufin are naturally present so they guarantee precise end-member information; (2) the concentrations of these compounds can be quantified via fluorescence analysis at higher resolutions than those offered by other typical analysis techniques, e.g., ion chromatography; (3) the tracer concentrations can be quantified in real time using online fluorometry or in the laboratory much faster than other tracers. For example, ion chromatography typically takes ~20 min per sample whereas both resazurin and resorufin are read in <30 s; and (4) injecting, sampling, and analyzing the samples is less expensive than, e.g., using stable isotopes or ion chromatography.

To date, resazurin has proven useful in quantifying hydroecological differences between contrasting ecosystem conditions (e.g., transformation rates in river column water are orders of magnitude smaller than in active sediments) at multiple sites in freshwater systems across the United States, Europe, and in Colombia (South America). However, insights into and better knowledge of the reaction pathways and their connections to metabolic activity are still needed to use the transformation of resazurin as an absolute measure of metabolic activity, instead of relying on relative comparisons between systems. Previous work by González-Pinzón et al. (2012) has shown that the structure of the microbial community is essential in this regard, but little is known as to whether other controls exist and how important they are.

Looking forward, when more information becomes available to warrant statistically meaningful comparisons, the transformation rates of the resazurin-resorufin tracer system measured in different ecosystems could be organized by geomorphology, climate, geographical location (latitude, longitude, and altitude), and stream power conditions, among others, to search for patterns of and controls on transport and microbial metabolism. For example, differences in transformation rates between ecosystems could be used to confirm or update our current understanding of when and where microbial metabolism is reaction or transport limited, or none. Key in this development will likely be the quantification of resazurin transformation in hyporheic zones, either through the injection of the tracer into the main flow compartment (e.g., stream or lake surface) with recording of tracer concentrations in the streambed, or through push-pull experiments directly in the subsurface. Another innovation to explore systematically in years to come is the variability of respiration responses under dynamic flow conditions. Constant rate injections of the reactive tracer could help to identify changes in metabolism based on changing "plateau" concentration ratios of resazurin and resorufin relative to a conservative tracer, offering insights into instantaneous rates, rather than diel averages provided by other in situ methods. Furthermore, experiments using resazurin under changing flow conditions could help us to quantify the "fertilization" effect caused by lateral runoff mobilizing sediment, ash (after forest fires), and nutrients. Similarly, resazurin could be used to quantify microbial contamination, like *Escherichia coli* loading, in urban drainage systems after "first flush" and subsequent drainage cycles.

We foresee that among the many applications of the resazurin-resorufin tracer system in hydrology, the most strategic advances will come through collaborative research efforts linking this smart tracer technique with other techniques capable of resolving or visualizing complementary processes. For example, we are currently using resazurin to quantify differences in stream metabolism in four stream compartments (main channel, lateral cavities, and shallow and deep hyporheic zones) of three contrasting streams in New Mexico, Colorado, and Iowa (NSF awards 1642399, 1642368, 1642402, and 1642403). These streams vary not only morphologically (from meandering to highly confined and from open to closed canopy) but also biochemically (pristine to agricultural). The novelty of our ongoing tracer studies is the linkage of resazurin with nutrient experiments exploring the role of stoichiometry on nutrient and carbon uptake, which we expect will help us to better understand patterns that have not been resolved by nutrient-specific tracer experiments. This collaborative research also couples the tracer experiments with electrical resistivity analyses that will inform the spatial and temporal extent of hyporheic zones, offering independent physical characterizations of the complex transport and uptake processes we are investigating.

While we continue to develop the resazurin-resorufin tracer system, we need to continue to pay attention to key unknowns. For example, the studies performed until now have consistently found that the total amount of resazurin and resorufin recovered is very often smaller than the mass of resazurin injected after accounting for dilution. Typically, this difference is larger than the uncertainty in the concentration measurements, indicating that other unmeasured reactions occur. It is unclear whether this gap in the mass balance can be explained by the additional reaction products that are produced during the transformation of resazurin to resorufin, or the decay of resorufin, or both. For example, Twigg (1945) showed in titration experiments that during the transformation of resazurin to resorufin, a small amount of dihydroresorufin was also produced. To close the mass balance of resazurin and resorufin, microbiological efforts are required to identify the different reaction byproducts. In a more conceptual context, we (arguably) may need to start reaching a consensus on what is the best way to report the use of the tracer system to make intersite comparisons more meaningful and consistent. To date, hydrologic studies using resazurin have reported resazurin loss, resorufin production, or resazurin-to-resorufin transformation in their results. At times, resorufin is preferred as its more intense fluorescence makes it easier to quantify. Since resorufin is both produced and degraded, however, its production is difficult to quantify. On the other hand, while resazurin is less fluorescent, it tends to sorb less and often yields more complete breakthrough curves in a given sampling period.

Further consistency may also be necessary with respect to the mathematical analysis of reactive stream tracer tests. A problem of particular relevance, but not unique to this tracer system, is the large problem dimensionality caused by the compound-specific properties, and the methods used to estimate reaction parameters. The questions of nonuniqueness and equifinality need to be acknowledged and parameter uncertainties quantified to avoid an overinterpretation of highly uncertain results. Due to the large number of compound related parameters, the use of parsimonious models should be of particular relevance in the representation of reactive tracer behavior. Simply put, the line between a realistic representation of the processes that the tracer system undergoes and the emergence of a model too complex and overparameterized to yield meaningful results is thin.

Interestingly, since the development of the resazurin-resorufin tracer system has been led by research groups from different countries and specializations, it has been challenged by and benefited from the complexity of human ingenuity, similarly to other developments in hydrological monitoring and modeling developments, or languages and systems of units. Perhaps most importantly, intellectual diversity has brought resilience to the development of the smart tracer technique, which may have otherwise become obsolete when challenged by knowledge frontiers not mastered by a single research group.

In spite of a number of questions that remain to be answered, using resazurin as a reactive tracer in streams and sediments has yielded promising results. The introduction of the reactive tracer system has increased our possibilities for investigating hyporheic exchange and metabolic activity in river corridors and thus can help us to better understand the processes governing nutrient turnover and contaminant degradation. However, this new approach is not meant to replace well-established methods for the estimation of metabolic activity, such as the determination of respiration from one- or two-station measurements of dissolved oxygen. Instead, the use of resazurin should be regarded as an additional method with its own advantages and limitations.

Resazurin is, arguably, the first type of reactive tracer in a line of smart tracers yet to come. We have known for more than 50 years that automatic computations can be performed with chemicals, at least in principle (Bennett, 1973, 1982; Feynman, 1960), and there is now a rich knowledge based on computations that can be completed with DNA molecules e.g., Benenson et al. 2001, or use scholar.google.com and search "DNA computing. Detection of chemicals and other lab operations are also possible at the molecular scale. At some point in the future, it appears likely that we will be able to run complex detection and computation algorithms on molecular-scale devices. Whether these novel tracers are synthetically engineered DNA tracers (Foppen et al., 2011) or some other, yet unknown molecular device, such truly smart tracers will likely transform hydrology and environmental science. These new approaches will have to be tested rigorously for their applicability and feasibility in a similar way as resazurin has been tested in the past decade. Thus, the development of the resazurin-resorufin tracer system will likely offer a case study for the development of future, smarter hydrologic tracers.

Acknowledgments
The development of the resazurin-resorufin system was initially funded by NSF grant EAR08-38338. Recent developments have been made available through NSF grant EAR 1642399. We are grateful to Lisa Hall for the drawing of Figure 1, to Hanna Schulz for comments on individual parts of the manuscript, and to González-Pinzón's students for testing the MATLAB codes. We thank three anonymous reviewers for thoughtful and constructive remarks. The supporting information contains detailed instructions on the planning, execution, and evaluation of resazurin tracer tests, and a number of MATLAB codes for the quantitative analysis of stream tracer tests with resazurin.

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