Measuring aerobic respiration in stream ecosystems using the resazurin-resorufin system

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Received 28 January 2012; revised 28 May 2012; accepted 1 June 2012; published 19 July 2012.

[1] The use of smart tracers to study hydrologic systems is becoming more widespread. Smart tracers are compounds that irreversibly react in the presence of a process or condition under investigation. Resazurin (Raz) is a smart tracer that undergoes an irreversible reduction to resorufin (Rru) in the presence of cellular metabolic activity. We quantified the relationship between the transformation of Raz and aerobic bacterial respiration in pure culture experiments using two obligate aerobes and two facultative anaerobes, and in colonized surface and shallow (<10 cm) hyporheic sediments using reach-scale experiments. We found that the transformation of Raz to Rru was nearly perfectly (min $r^2 = 0.986$), positively correlated with aerobic microbial respiration in all experiments. These results suggest that Raz can be used as a surrogate to measure respiration *in situ* and *in vivo* at different spatial scales, thus providing an alternative to investigate mechanistic controls of solute transport and stream metabolism on nutrient processing. Lastly, a comparison of respiration and mass-transfer rates in streams suggests that field-scale respiration is controlled by the slower of respiration and mass transfer, highlighting the need to understand both biogeochemistry and physics in stream ecosystems.

Citation: González-Pinzón, R., R. Haggerty, and D. D. Myrold (2012), Measuring aerobic respiration in stream ecosystems using the resazurin-resorufin system, *J. Geophys. Res.*, *117*, G00N06, doi:10.1029/2012JG001965.

1. Introduction

[2] After three decades of active research coupling hydrology and stream ecology, the connection among stream solute transport, metabolism and nutrient dynamics is still unresolved. These knowledge gaps obscure the functioning of stream ecosystems and how those ecosystems interact with other landscape processes. To date, stream metabolism has been measured with techniques that have large uncertainties and are not spatially representative. Reach-scale approaches based on oxygen mass balance require a reaeration coefficient to estimate atmosphere-stream mass transfer, and correction for groundwater oxygen flux. These quantities can be highly uncertain. Because reaeration is a major component of oxygen mass balance, uncertain reaeration coefficients negatively affect the quantification of community respiration, particularly in headwater streams [*Marzolf et al.*, 1994, 1998; *Aristegi et al.*, 2009]. Also, substantial groundwater inflows with relatively low concentrations of dissolved oxygen (DO) strongly bias estimates of community respiration [*McCutchan et al.*, 1998, 2002; *Hall and Tank*, 2005; *McCutchan and Lewis*, 2006]. Measuring accurate groundwater DO flux is technically and logistically challenging and this flux term is often neglected. Furthermore, correcting respiration rates by temperature might be misleading because metabolically active compartments typically experience significantly different diel changes [e.g., *Constantz*, 2008] than those where temperature is routinely measured during metabolism studies, i.e., above the streambed. On the other hand, direct measurement with respiration chambers is problematic because the sample volume is much smaller than the reach of interest, and because reproducing in situ hydrodynamic and heat transfer conditions is virtually impossible [*Bott et al.*, 1997; *Naegeli and Uehlinger*, 1997; *Aristegi et al.*, 2010]. Clearly, these limitations mask the true role of metabolism in nutrient processing and call for more robust techniques to improve our fundamental understanding of in-stream processes and how streams interact with other ecosystems.

[3] With outstanding redox and fluorescent properties, the resazurin-resorufin system was introduced as a "smart tracer" in hydrology [*Haggerty et al.*, 2008, 2009]. Resazurin (Raz) is a redox-sensitive phenoxazine frequently used to estimate biological activity. In appropriate reducing conditions, Raz (blue in color) irreversibly loses an oxygen ion to become resorufin (Rru) (Figure 1). Rru (pink in color) also can undergo a further reduction to colorless dihydroresorufin, but this reaction is reversible by atmospheric oxygen and is mainly favored upon total consumption of Raz [*O'Brien et al.*, 2000; *Guerin et al.*, 2001]. Raz reacts in the presence of dehydrogenases, which are enzymes involved in many of the vital metabolic processes (catabolic and anabolic) of

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0148-0227/12/2012JG001965

Figure 1. Resazurin (Raz) is irreversibly transformed to resorufin (Rru) by aerobic metabolism. The figure shows Raz being transformed by *Sinorhizobium meliloti* 1021.

living organisms [*Liu*, 1986; *Strotmann et al.*, 1993; *Zalata et al.*, 1998; *O'Brien et al.*, 2000]. *O'Brien et al.* [2000] found that Raz is reduced to Rru in a medium by cellular activity, but that a highly reduced medium without viable cells did not support the reduction.

[4] In the last 25 years, some of the applications of Raz as a bioreactive tracer have included the detection of chemical toxicity [*Liu*, 1986], sludge activity in wastewater treatment [*Strotmann et al.*, 1993; *McNicholl et al.*, 2007], cell viability in mammals [*O'Brien et al.*, 2000], abundance of contaminant-degrading microorganisms [*Guerin et al.*, 2001], differentiation of aerobic and anaerobic bacteria [*Karakashev et al.*, 2003], detection of activity of disinfectants against biofilms [*Mariscal et al.*, 2009], seed viability assays [*Min and Kang*, 2011] and, most recently, cell counting in breast cancer treatment experiments [*Ziegler et al.*, 2011]. Particularly important for applications in hydrology and biogeochemistry, these studies reported that Raz can be reduced by strict aerobes, facultative anaerobes, aerotolerant and microaerophile organisms, but not by strict anaerobes.

[5] Raz is a "smart" tracer because it provides information about the environment through which it travels that is specific to the process of interest [*Haggerty et al.*, 2008]. Raz is a promising tool to investigate physically different environments with respect to transient storage and stream metabolism (Figure 2). Using column experiments and batch reactors, the transformation rate of Raz was found to be ~1400-fold faster in hyporheic sediments than in the water column [*Haggerty et al.*, 2008], which agrees with the larger biomass abundance observed in sediments or surfaces, compared to microbes suspended in the water column [*Findlay*, 2010]. A metabolically active transient storage (MATS) model [*Haggerty et al.*, 2009; *Argerich et al.*, 2011] based on the Raz-Rru system was developed to organize transient storage from a metabolically based perspective, rather than from a physically based perspective, as it was conceptualized before [*Bencala and Walters*, 1983; *Harvey et al.*, 1996; *Gooseff et al.*, 2005]. In a reach-scale study comparing distinctive types of transient storage, Raz was sensitive to differences in channel configuration and morphology [*Argerich et al.*, 2011].

[6] Although Raz has been widely used to assess metabolic activity, to our knowledge no study has addressed how the transformation of Raz is related to cellular respiration. In this paper, we want to answer two questions. First, is the Raz-Rru transformation related quantitatively to aerobic respiration?

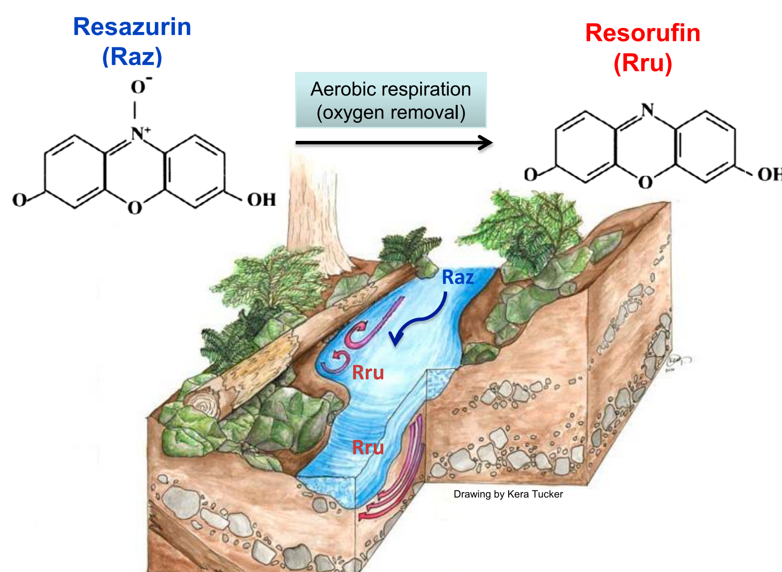


Figure 2. The resazurin-resorufin (Raz-Rru) system can be used to estimate aerobic respiration in stream ecosystems. Raz is transformed to Rru in surface and hyporheic metabolically active zones.

Table 1. Experimental Configuration in Pure Culture Experiments

Experiment Number	Species of Bacteria	Raz Initial Concentrations (μg L^{-1})	Average DO Initial and Final Concentrations (mg L^{-1})
1	*B. subtilis*	0 (control), 50, 100, 150	8.2–1.5
2	*A. chlorophenolicus*	0 (control), 55, 110, 160	8.3–1.7
3	*P. putida*	0 (control), 100, 200, 300	8.5–4.8
4	*S. meliloti*	0 (control), 125, 235, 345	8.6–6.2

Second, what does reach-scale Raz-Rru transformation tell us about reach-scale respiration?

2. Materials and Methods

2.1. Pure Culture Experiments

2.1.1. Bacteria Species

[7] Four species of bacteria were used. Two species are obligate aerobes: *Arthrobacter chlorophenolicus* A6, ATCC (700700) (*A. chlorophenolicus*), and *Sinorhizobium meliloti* 1021, ATCC (51124) (*S. meliloti*); the others are facultative anaerobes: *Bacillus subtilis* 168, ATCC (23857) (*B. subtilis*), and *Pseudomonas putida* KT2440, ATCC (47054) (*P. putida*). *A. chlorophenolicus* is a Gram-positive soil-dwelling bacterium with the ability to degrade chlorophenol [*Sahoo et al.*, 2011a, 2011b]. *S. meliloti* is a Gram-negative soil bacterium capable of fixing nitrogen in root nodules [*Marketon et al.*, 2002]. *B. subtilis* is a Gram-positive soil and water-dwelling bacterium, commercially important for its highly concentrated production of the enzymes amylases and proteases [*Itaya and Tanaka*, 1991]. *P. putida* is a Gram-negative, soil-dwelling bacterium, with the ability to degrade organic solvents [*Hill and Robinson*, 1975; *Jiménez et al.*, 2002].

[8] Two to four days prior to each experiment, Luria Broth (LB) bacterial culture medium was prepared and sterilized. The medium was inoculated and incubated in the dark for two days at 24°C room temperature for *A. chlorophenolicus, S. meliloti* and *B. subtilis*, and 37°C for *P. putida.* From here on, we will refer to the use of each species of bacteria as one experiment.

2.1.2. Bioreactors Setup

[9] The day of each experiment, absorbance of the culture medium was measured on a Beckman Coulter (DU® 530) spectrophotometer at a wavelength of 660 nm (hereafter Abs_{660}). Abs_{660} showed no interferences with Raz or Rru signals, allowing measurement of growth for all four species. An initial optical density $Abs_{660} = 0.050$ was used as a target to determine the volume of inoculated medium to be added. Sterile media and a known concentration of Raz were also added to sterilized 500-mL Erlenmeyer flasks. All flasks were stocked with an initial liquid phase (i.e., LB medium, bacterial inoculum in LB medium, and DI-based Raz solution) of about half their total capacity. Flasks were wrapped in aluminum foil to prevent any photo-decay of Raz and Rru, as well as to provide a dark environment for the bacteria. Once the LB medium and Raz solution were added and mixed, flasks were sealed with septum stoppers. Within 20 min of the Raz addition, inoculated LB was injected through the septa to complete the total liquid. To maintain atmospheric pressure during the inoculated LB injection, an exit needle was inserted temporarily across the septum and was removed immediately afterwards. The flasks were then set on a rotary shaker table (Orbit Shaker, Lab-line) and agitated at 100 rpm. We refer to each flask filled with LB medium, Raz and bacterial inoculum as a bioreactor. Each experiment with a given species of bacteria had four replicates with identical volumes of LB medium and bacterial inoculum, but with different Raz concentrations (Table 1).

2.1.3. Experimental Sampling and Readings

[10] The experiments were run at room temperature (21°C to 23°C) for about six hours. The sampling in each of the four bioreactors consisted of taking eight to ten rounds of 1 mL gas samples to estimate CO_2 concentrations, and 8 mL of liquid samples. The liquid samples were taken for making measurements in three aliquots. The aliquots were 1 mL for cell growth measurement, 2 mL for Raz, Rru and dissolved oxygen (DO) concentrations, and 5 mL for temperature and *pH.*

[11] Gas samples were taken from the headspace of the bioreactor with a Hamilton Gastight® 500-μL syringe and stored in 2-mL septum vials (National Scientific), previously purged with 99.9% Argon. CO_2 samples were read within 24 h on a Hach-Carle (Series 100 AGC) gas chromatograph. Liquid samples were taken with BD™ Luer-Lock disposable syringes. Abs_{660} was read within 2 min of sampling from VWR® two-sided polystyrene cuvettes containing 1 mL of the unfiltered sample. The rest of the liquid samples were filtered with 0.2-μm polycarbonate Whatman® filter membranes to remove cells. DO was measured with a YSI ProODO probe and *pH* and temperature with a VWR® sympHony® *pH* Meter. The Raz and Rru samples were standardized to *pH* 8.5 by pouring 2 mL of the filtered sample into VWR® four-sided cuvettes and buffered (1:10 buffer-to-sample ratio) with ~1 M sodium phosphate [*Haggerty et al.*, 2008]. After each sample was buffered and vortexed (Fisher Scientific Vortex Genie 2), the fluorescence signals were measured with a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) within 10 min of sampling. The excitation/emission wavelengths for Raz and Rru were set to 602/632 nm and 571/584 nm, respectively. Initial conditions for all monitored parameters were measured before bacteria were added and before sealing the flasks with rubber stoppers.

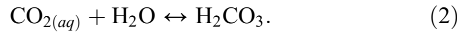
2.1.4. Estimation of CO_2 Production and DO Consumption

[12] We measured DO consumption from the amount of CO_2 generated. Before describing this method, we explain why we chose it. We first attempted to measure DO continuously inside the bioreactors using dip-type oxygen microelectrodes (MI-730 Microelectrodes Inc.); however, measurements with all bacteria species generated irregular readings that were affected by the agitation of the bioreactors and by biofouling of the probe membranes. We next tried to measure DO from unfiltered and filtered liquid samples extracted from the bioreactors, but these methods suffered from inconsistencies due to continued cellular respiration (unfiltered samples) and reoxygenation (filtered samples). To overcome the difficulties of directly measuring DO consumption, we used the carbonate system as a proxy [e.g.,

Morel and Hering, 1993; *Goudar et al.*, 2011]. The assumption in this approach is that 1 mol CO_2 generated in the system resulted from cellular respiration of 1 mol O_2, i.e., we assumed a respiratory quotient of 1.0. Otherwise, equilibrium between the gas and liquid phases was guaranteed by the reaction rates and the mixing effects of the shaker table.

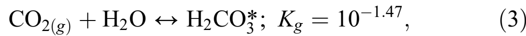
[13] The carbonate system, i.e., gaseous carbon dioxide $CO_{2(g)}$, carbonic acid H_2CO_3, bicarbonate HCO_3^- and carbonate CO_3^{2-}, was balanced using the following reactions:

$$CO_{2(g)} \leftrightarrow CO_{2(aq)} \quad (1)$$



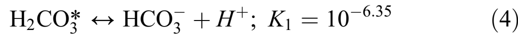
$$CO_{2(aq)} + H_2O \leftrightarrow H_2CO_3. \quad (2)$$

For aqueous solutions, it is common to express (1) and (2) as:

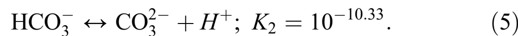


$$CO_{2(g)} + H_2O \leftrightarrow H_2CO_3^*;\ K_g = 10^{-1.47}, \quad (3)$$

where $H_2CO_3^* = CO_{2(aq)} + H_2CO_3$ and K_g is an equilibrium constant for standard conditions. The dissociation of carbonic acid and bicarbonate are shown in (4) and (5), where K_1 and K_2 are also equilibrium constants for standard conditions.

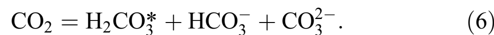


$$H_2CO_3^* \leftrightarrow HCO_3^- + H^+;\ K_1 = 10^{-6.35} \quad (4)$$



$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+;\ K_2 = 10^{-10.33}. \quad (5)$$

The total concentration of CO_2 in the system is:



$$CO_2 = H_2CO_3^* + HCO_3^- + CO_3^{2-}. \quad (6)$$

In these experiments we measured $CO_{2(g)}$ directly from the gas samples and estimated CO_2 using the previous equations and the *pH* of the liquid samples. To estimate the change of DO due to respiration, we assumed that DO consumption was inversely proportional to CO_2 production:

$$\frac{-dO_2}{dt} = \frac{dCO_2}{dt}. \quad (7)$$

The time-varying concentration of DO was estimated from (7), using the measured initial concentration of DO. For all experiments, the medium was saturated with DO before the addition of bacteria and DO concentration was a function of the temperature of the medium. To validate the use of the carbonate system to measure DO change, the final concentration of DO in each of the four bioreactors was measured directly in the liquid-phase with the YSI ProODO, no later than 1 min from the time that the bioreactors were opened.

2.1.5. Estimation of Raz Transformation (ΔRaz, ΔRru) and Respiration (ΔDO)

[14] We calculated the transformation of Raz as ΔRaz and ΔRru, and respiration activity (ΔDO) as normalized transformations with respect to their initial concentration. These values allowed us to compare the transformation of Raz by different microorganisms, independently of their metabolic patterns and the initial concentration of Raz:

$$\Delta Raz_t = \frac{Raz_0 - Raz_t}{Raz_0} \quad (8)$$

[0 at $t = 0$ and 1 when all Raz is transformed to Rru]

$$\Delta Rru_t = \frac{Rru_t}{Raz_0} \quad (9)$$

[0 at $t = 0$ and 1 when all Raz is transformed to Rru]

$$\Delta DO_t = \frac{DO_0 - DO_t}{DO_0} \quad (10)$$

[0 at $t = 0$ and 1 when all DO is consumed],

where subscript t represents a time-dependent variable and subscript 0 represents the initial value of each state-variable.

2.2. Reach-Scale Experiments

[15] To study the rates of Raz transformation mediated by diverse microorganism communities in stream ecosystems, we used benthic and shallow hyporheic sediments (<10 cm depth) collected in two watersheds located in the H. J. Andrews Experimental Forest. Watershed 1 (WS1) and Watershed 3 (WS3) are second-order tributaries of Lookout Creek, located in the western Cascade Mountains (Oregon, USA). A detailed description of the study sites has been presented by *Kasahara and Wondzell* [2003] and *Argerich et al.* [2011]. The two watersheds are less than 2 km apart and the stream reaches investigated were 81 m and 160 m long. Sediments smaller than pebbles were collected by hand from each watershed and placed in three rectangular fiberglass mesh bags of 25 cm × 45 cm × 7 cm. These sediments were incubated in situ for 10 d (WS1) and 7 d (WS3) (Figure 3). At each watershed, respiration chamber experiments were run for about 8 h, i.e., incubated sediments were placed in recirculating chambers closed to the atmosphere where Raz was injected and sampled through time. Oxygen consumption was measured instantaneously inside the chambers with YSI ProODO and HACH HQ40D probes, whereas Raz samples were collected, filtered, refrigerated at 4°C and then read within 72 h of sampling. Filtering and reading procedures were performed as described for pure culture experiments. ΔRru and ΔDO were estimated as previously described.

[16] Although the experiments with respiration chambers are not accurate to estimate *in situ* respiration rates, these experiments provided an ideal set-up to eliminate reaeration effects from the oxygen mass balance. This allowed us to directly correlate the transformation of Raz and oxygen consumption by field microbial communities.

3. Results and Discussion

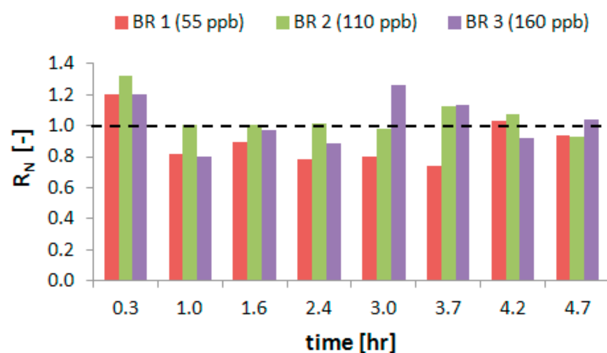
3.1. CO_2 Production and Cell Growth in Pure Culture Experiments

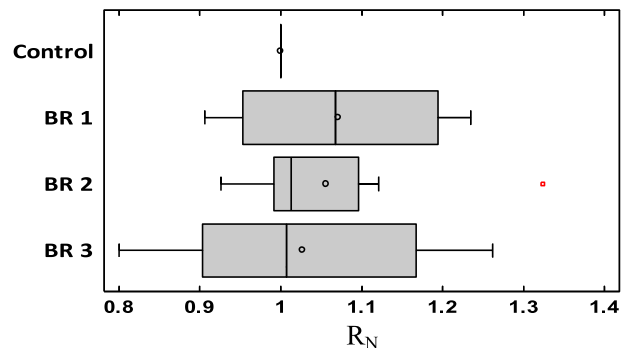
[17] We estimated cellular CO_2 production ratios through time in the bioreactors, i.e., R_t [mol L^{-1} abs^{-1}], to detect changes in metabolic activity due to the addition of the organic compound Raz. We used absorbance as a proxy to estimate the relative number of cells in each bioreactor [*Nerbrink et al.*, 1999; *Dalgaard and Koutsoumanis*, 2001].

$$R_t = CO_2(t)/Abs_{660}(t). \quad (11)$$

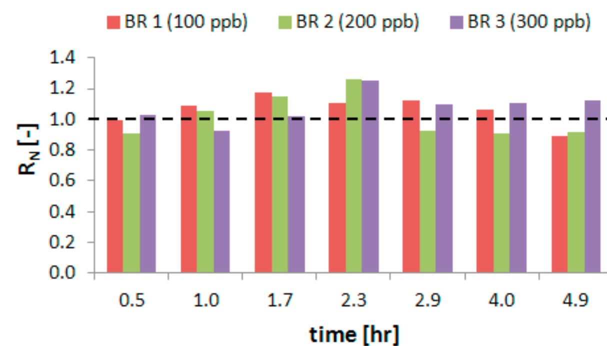
Figure 3. Respiration chamber experiments conducted at the H. J. Andrews Experimental Forest to test the correlation between ΔRru and ΔDO: (left) fiberglass incubation meshes; (right) recirculating chamber with incubated sediments.

a) Aerobe. *A. chlorophenolicus* A6





b) Facultative anaerobe. *P. putida KT2440*



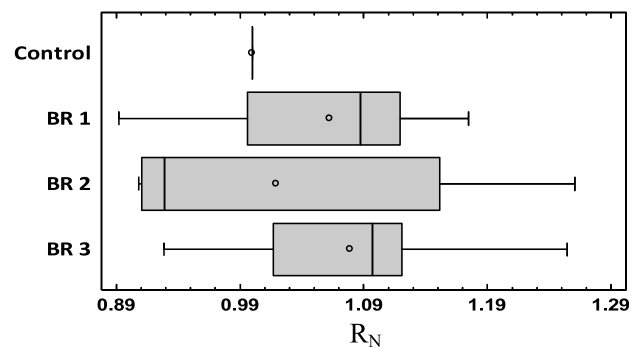


Figure 4. At the 95% confidence level, respiration is not a function of Raz at concentrations <300 μg L^{-1}. The figure shows normalized cellular CO_2 production rates (R_N) relative to the control, for two species of bacteria and three different concentrations of Raz (cf. Table 1). (a) *A. chlorophenolicus* A6; (b) *P. putida KT2440*; (left) temporal variations with respect to the control bioreactor (dashed-line); (right) box-and-whisker plots of the same data.

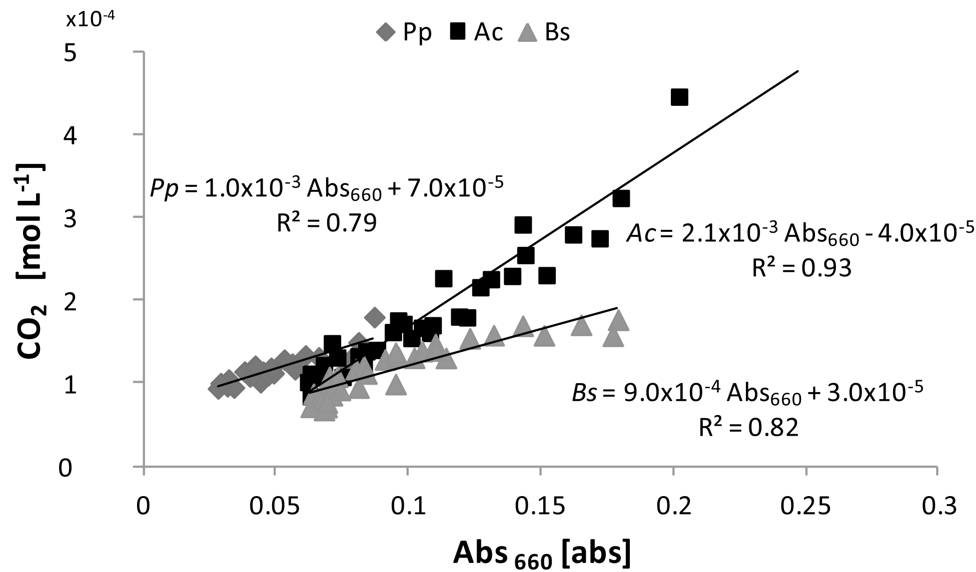


Figure 5. Correlation between CO_2 production and cell growth for *P. putida* (*Pp*), *A. chlorophenolicus* (*Ac*) and *B. subtilis* (*Bs*). Data from the four bioreactors are included for each species.

These ratios were calculated for every sample and then normalized (R_N) with respect to those from the control bioreactor (R_{tc}) for each experiment.

$$R_N = R_t/R_{tc}. \qquad (12)$$

Although this indirect estimator was not standardized for every species, i.e., we did not correlate Abs_{660} with absolute cell concentrations, a comparison of the normalized cellular CO_2 production rates (R_N) is adequate to evaluate changes in metabolic activity by adding Raz to the different species. We

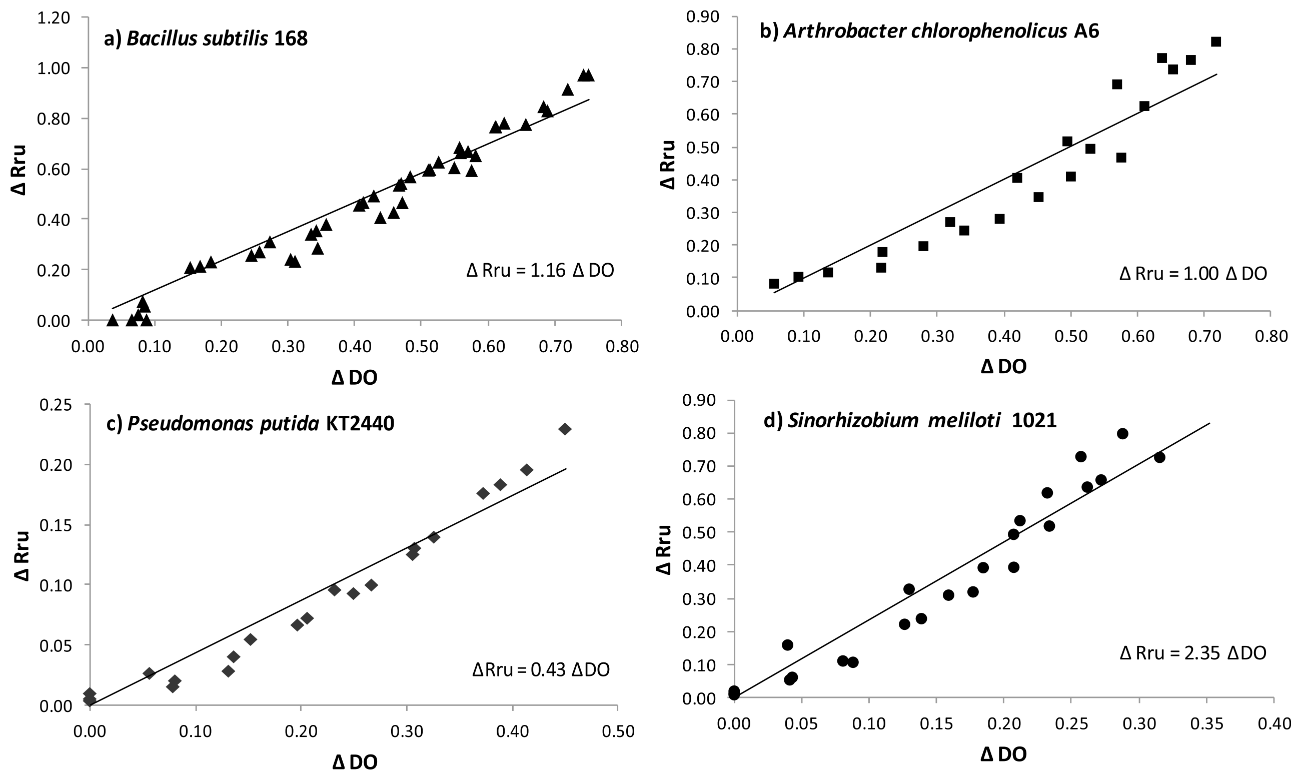


Figure 6. Raz transformation is linearly proportional to aerobic respiration in pure cultures. The figures show correlations between Raz transformation (as ΔRru) and cellular respiration (ΔDO) in pure culture experiments. Linear trend-lines, valid over the entire range of DO, are presented to facilitate the discussion. Power law trend-lines with exponents between 1 and 1.3 also fit the data, and such behavior was previously discussed by *Haggerty et al.* [2009, cf. equations (5)–(7)].

Table 2. Statistical Results From ΔRru Versus ΔDO Linear Regressions in Pure Culture and Field Experiments[a]

Species or Site	Correlation Coefficient	K_{Raz}^{DO} = Slope of Trend Line	Standard Error
B. subtilis	0.994	1.162	0.020
A. chlorophenolicus	0.986	1.004	0.038
P. putida	0.989	0.435	0.015
S. meliloti	0.988	2.348	0.076
WS1	0.997	1.717	0.038
WS3	0.990	1.428	0.058
WS1 and WS3	0.989	1.552	0.046

[a]All regressions have a p-value <0.001.

did not find significant changes, or patterns, in (R_N) as a function of the initial concentration of Raz at the 95% confidence interval (Figure 4), i.e., p-values from ANOVA F tests >0.05 in all cases (STATGRAPHICS® Centurion XVI). This suggests that all bioreactors had similar metabolic activity; i.e., Raz neither stimulated nor inhibited respiratory activity.

[18] Correlations between CO_2 concentration and cell growth are presented in Figure 5. The linear trends observed are due to the exponential CO_2 production (or oxygen consumption) and exponential cell growth observed during the experiments. This behavior is due to the short times elapsed between the incubation of the microorganisms and the performance of the experiments (2 d), and confirms that the microorganisms were in the exponential phase of growth [*Zwietering et al.*, 1990]. The differences in the slopes most likely reflect the efficiency with which the different bacterial species utilized the substrate found in the medium used and would likely vary if the medium constituents were changed.

3.2. Raz Transformation and DO Consumption

[19] To relate quantitatively the transformation of Raz to cellular respiration, we plotted the results of ΔRru versus ΔDO obtained in the pure culture experiments (Figure 6). Each plot shows results from all bioreactors used for a given species, i.e., three different initial concentrations of Raz for each. Table 2 shows the statistical significances of the regressions presented in Figure 6.

[20] Figure 6 shows strong quantitative relationships between the transformation of Raz and cellular respiration for each of the species studied. However, the relationships were organism-dependent. These differences in the ability of bacteria to transform one mole of Raz, per mole of oxygen consumed, could be due to variations in the electron transport chains of the different bacterial species or, more likely, differences in Raz and DO uptake diffusivities for each bacterium. These results suggest that a quantitative relationship between Raz and DO must be found via calibration, i.e., a standard curve will have to be developed for Raz to be used as a bioassay for aerobic respiration. However, a Raz-based bioassay is not restricted to the use of pure cultures, because the transformation of Raz for multiple species can be characterized by a linear combination of the type of signals shown in Figure 6. We tested this hypothesis with field experiments.

[21] Figure 7 and Table 2 present the results obtained from field chamber incubation experiments in WS1 and WS3 at the H.J. Andrews Experimental Forest, where Raz was transformed by a mixture of microbial communities present in the natural biofilms developed on the sediments. The field results are qualitatively similar to the lab results and are quantitatively bracketed by the lab results. Further, the field results are similar to each other. Although little can be concluded based on only two sets of field data, it does suggest strong similarities between the microbial communities present in the two streams, or at least that the rates of transformation of Raz were very similar. However, no information about microbial communities present at the sites was available from this or previous studies to resolve this duality. Regardless of the microbial communities present, we found a strong quantitative relationship between the transformation of Raz and microbial respiration.

3.3. Implications of Hydrologic Processes on Field-Scale Use of Raz for Measuring Respiration

[22] We now turn our attention to the how stream hydrology modifies respiration at the field scale and on the implications for use of Raz to measure respiration. In contrast to pure culture and chamber experiments where respiration is controlled by biologically mediated reactions, in stream ecosystems respiration is also controlled by hydrological processes [for related conclusions for both nutrients and respiration see *Harvey and Wagner*, 2000; *Gooseff et al.*, 2005; *Runkel*, 2007; *Argerich et al.*, 2011]. Assuming first-order

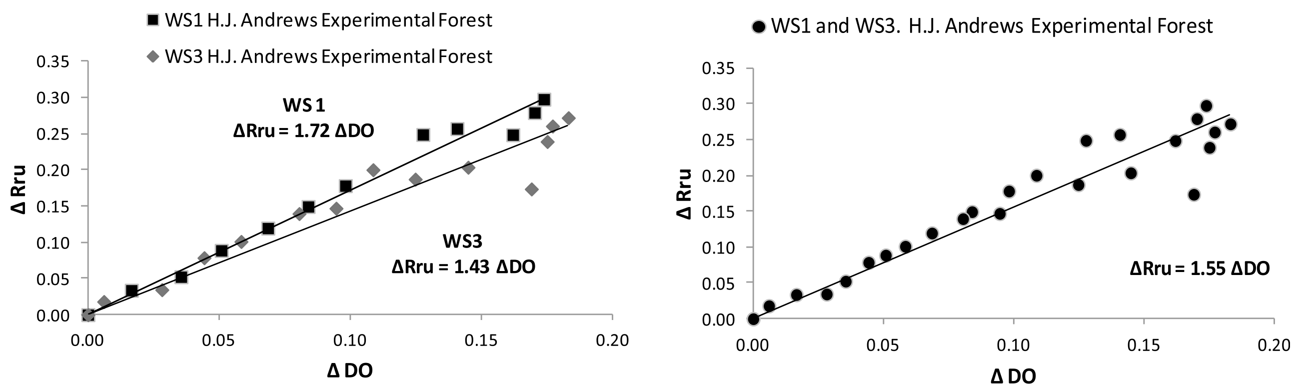


Figure 7. Raz transformation is linearly proportional to aerobic respiration in stream sediments. The figure shows a reach-scale experimental correlation between Raz transformation (as ΔRru) and cellular respiration (ΔDO): (left) regressions for experiments in two streams; (right) regression including both streams.

Table 3. Quantitative Analysis of Rate Control on Effective Respiration Rates in Stream Ecosystems[a]

Biological Rate Control[b] $\lambda_{eff}^{DO} \rightarrow \lambda_b^{DO}$	Transport Rate Control $\lambda_{eff}^{DO} \rightarrow \alpha_2$	φ = Fraction of Respiration Controlled by Smaller Rate $\varphi = \lambda_{eff}^{DO}/\min(\lambda_b^{DO}, \alpha_2)$
$\lambda_b^{DO}/\alpha_2 = 1/49$	$\lambda_b^{DO}/\alpha_2 = 49$	0.98
$\lambda_b^{DO}/\alpha_2 = 1/9$	$\lambda_b^{DO}/\alpha_2 = 9$	0.90
$\lambda_b^{DO}/\alpha_2 = 1/4$	$\lambda_b^{DO}/\alpha_2 = 4$	0.80
$\lambda_b^{DO}/\alpha_2 = 1$	$\lambda_b^{DO}/\alpha_2 = 1$	0.50

[a]This analysis can be extended to other bioreactive compounds by substituting DO.
[b]$\lambda_{eff}^{DO} \rightarrow K_{DO}^{Raz}\lambda_b^{Raz}$.

reaction rates, quantitative approaches to estimate volume-weighted effective respiration rates yield expressions of the form [*Runkel*, 2007; *Botter et al.*, 2010; *Argerich et al.*, 2011]:

$$\lambda_\theta^{DO} = \beta f \frac{\lambda_b^{DO}\alpha_2}{\lambda_b^{DO} + \alpha_2} = \beta f \lambda_{eff}^{DO}, \quad (13)$$

where λ_θ^{DO} $[T^{-1}]$ is the volume-weighted effective respiration rate in metabolically active zones or hot spots; β $[-]$ is the volumetric ratio of transient storage and in-channel transport zones (or A_s/A as described by *Runkel* [2007] and *Botter et al.* [2010]); f $[-]$ is the fraction of the transient storage zone that is metabolically active (or "hot"; e.g., [*McClain et al.*, 2003]); λ_b^{DO} $[T^{-1}]$ is the biological respiration rate in metabolically active zones; α_2 $[T^{-1}]$ is the mass transfer rate between in-channel transport and transient storage zones (or $\alpha A/A_s$ as described by *Runkel* [2007] and *Botter et al.* [2010]); and λ_{eff}^{DO} $[T^{-1}]$ is the effective respiration rate. Equation (13) could be applied to any dissolved bioreactive compound that is taken-up through metabolic activity, but we restrict our discussion to DO.

[23] Based on (13), we can infer an effective respiration rate (λ_{eff}^{DO}) in streams from the Raz-Rru system. To do so, we introduce a constant of proportionality to relate the biotic transformation rate of Raz (λ_b^{Raz}) with oxygen consumption (λ_b^{DO}). This constant, K_{DO}^{Raz}, is the inverse of the slope of the correlation between ΔRaz (or ΔRru) and ΔDO (c.f., Figures 6 and 7 and Table 2), i.e., $K_{DO}^{Raz} = (K_{Raz}^{DO})^{-1}$:

$$\lambda_\theta^{DO} = \beta f \lambda_{eff}^{DO} = \beta f \frac{\left(K_{DO}^{Raz}\lambda_b^{Raz}\right)\alpha_2}{\left(K_{DO}^{Raz}\lambda_b^{Raz}\right) + \alpha_2}. \quad (14)$$

According to (14), the effective respiration rate (λ_{eff}^{DO}) will be reaction-limited when reaction rates (λ_b^{DO}) are small compared to mass-transfer rates (α_2), i.e., for relatively large values of α_2 (14) becomes $\lambda_{eff}^{DO} \rightarrow \lambda_b^{DO}$ (or $\lambda_{eff}^{DO} \rightarrow K_{DO}^{Raz}\lambda_b^{Raz}$). Conversely, the system will shift to a transport-limited condition when reaction rates are relatively large, i.e., $\lambda_{eff}^{DO} \rightarrow \alpha_2$ [*Argerich et al.*, 2011]. Using φ as the relative fraction of effective uptake controlled by either λ_b^{DO}or α_2, Table 3 presents a quantitative analysis of biological and transport rate-control for effective respiration rates; note that λ_b^{DO}/α_2 is used as a descriptor of rate-control in this analysis.

[24] Table 3 shows that when biological and mass transfer rates differ by one order of magnitude, the smallest rate controls the effective rate, i.e., for $\varphi > 0.9$, $\lambda_{eff}^{DO} \approx \min(\lambda_b^{DO}, \alpha_2)$. The maximum efficiency of effective respiration for a particular reach (or stream) is achieved when biological and mass transfer rates are balanced (i.e., $\lambda_b^{DO}/\alpha_2 = 1$). This suggests that instances of rate-control ($\varphi > 0.9$) may be most prevalent after significant disturbances [e.g., *Sabater and Tockner*, 2010]. At other times, the metabolic activity of microbial communities (represented by λ_b^{DO}) is likely synchronized with the supply of nutrients and dissolved oxygen (regulated by α_2) to achieve a dynamic equilibrium [*Peterson et al.*, 2001; *Orr et al.*, 2009].

[25] The use of smart tracers such as Raz and, in the near future, others such as synthetic DNA tracers [e.g., *Foppen et al.*, 2011], will help us to illuminate how effective respiration rates (or effective uptake rates in the case of nutrients) are affected at different temporal and spatial scales by stream management programs. Such effective uptake rates are direct indicators of stream functioning.

[26] Estimating effective respiration rates with a tracer technique offers important advantages, compared to traditional methods. Raz allows us to separate and quantify with improved certainty the relative effects of biology and hydrologic exchange in stream respiration at different spatial and temporal scales. Tracer injections of Raz and a conservative tracer avoid the difficulties of measuring lateral inflows of dissolved oxygen by tributaries and groundwater seepage, which is particularly required to calculate oxygen mass balances in gaining streams [*McCutchan et al.*, 2002; *Hall and Tank*, 2005; *Reichert et al.*, 2009]. Since the transformation of Raz is directly proportional to oxygen consumption, regardless of the rate of metabolic activity, no assumption has to be made about the importance of diurnal temperature fluctuations controlling respiration rates, i.e., respiration can be actually measured at day and nighttimes, which might be particularly important in streams with significant temperature differences from day to night.

4. Conclusions

[27] We have introduced an alternative approach to estimate aerobic respiration in stream ecosystems using the smart tracer resazurin. This approach seeks to overcome important limitations of current methods in stream ecology, particularly, the sampling disturbance of in situ biological and hydrodynamic conditions, and the overlooking of well-understood biophysical controls. The use of Raz as a bioreactive tracer to estimate metabolic activity enables us to integrate our knowledge of solute transport and stream metabolism, thus advancing our understanding of stream ecosystem functioning. Although this new approach needs further validation, it appears to have several advantages: (1) Raz is not naturally present in streams, the atmosphere or groundwater; (2) Raz is a compound that allows *in vivo* and *in situ* assays without altering microbial communities and hydrodynamic conditions; (3) Raz can be used at very low concentrations (~200 ppb at plateau concentrations), thus being a cost-effective technique, and 4) Raz follows the same flow paths as conservative tracers, allowing the partitioning between metabolically active and inactive transient storage.

[28] Further research is needed to understand the nature of the different rates of Raz and oxygen uptake observed at the cellular scale, as well as the significance of such differences under natural conditions, i.e., in biofilms. Experimental research in different stream compartments and biomes is warranted to advance the use of Raz as a tracer to quantify

stream respiration. Interestingly (and perhaps ironically), if Raz can be used to measure respiration in stream ecosystems, it could be used as a technique to estimate reaeration rates in reaches with negligible inputs of groundwater, i.e., a combination of upstream-downstream oxygen signals and Raz-based respiration measurements could be used to isolate the reaeration flux term in oxygen mass balances.

[29] **Acknowledgments.** This work was funded by NSF grant EAR 08–38338. Funding for work in WS1 and WS3 was provided by the HJ Andrews Experimental Forest research program, funded by the National Science Foundation's Long-Term Ecological Research Program (DEB 08–23380), U.S. Forest Service Pacific Northwest Research Station, and Oregon State University. We thank Sarah Acker and Jon Sanfilippo for lab and field assistance. We also thank Brian Wood and Stanley Gregory for allowing us to use lab equipment. Finally, we thank the anonymous reviewers who provided insightful comments on the manuscript. Bacterial strains were the gift of Janet Jansson (Lawrence Berkeley National Laboratory, *A. chlorophenolicus* A6), Peter Zuber (Oregon Graduate Institute, *B. subtilis* 168), and Sharon Long (Stanford University, *S. meliloti* 1021).

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