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A preliminary examination of an in situ dual dye approach to measuring light fluxes in lotic systems

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Abstract

Light is a critical parameter in aquatic ecosystems, affecting primary production and in situ photochemistry. However, measuring light exposure for suspended particles or dissolved components in a dynamic water column can be challenging with existing Eulerian approaches. Here, we assess the simultaneous deployment of two dyes differing in photolability (rhodamine WT and fluorescein) as a Lagrangian measure of sunlight exposure in a lotic system. Fluorescein is sensitive to light exposure; rhodamine WT is relatively photostable. We examined dye fluorescence at various pH, salinity, and temperature conditions. We also tested dye photolability as a function of pH and wavelength range. In conjunction with this laboratory work, we performed initial field testing of the dual-dye approach in a stream on the north shore of Lake Superior, USA. Irradiation of the dyes using long-pass filters identified wavelengths ≥ 420 nm as responsible for the vast majority of the loss of fluorescein fluorescence, with rhodamine appearing relatively photostable in these short-term studies across the wavelength ranges tested. Dye response to irradiation is pH-sensitive; the dual-dye approach will require additional calibration for acidic or basic waters and should be used with caution in aquatic systems undergoing strong (several pH unit) changes in pH. Field testing showed that the fluorescein to rhodamine WT ratio decreased approximately linearly with light exposure. The dual-dye methodology shows promise as an in situ light sensor applicable to water column species in lotic systems if temperature is recorded, and the pH range is measured and relatively stable (e.g., varies by < 1 unit).

The interaction of the vertically variable UV-visible radiation field and mixing in the surface waters of aquatic systems has long been appreciated as a fundamental factor affecting photosynthesis (e.g., Falkowski 1983; Lewis et al. 1984a, 1984b; Falkowski and Wirick 1981) and nonbiological photoreactions such as photodegradation of natural and anthropogenic organic compounds in lentic environments (e.g., Miller et al. 2002; Tixier et al. 2003). However, the interaction of light and mixing remains poorly understood, especially in dynamic lotic environments, which are characterized by temporally varying advection as a function of flow, varying water clarity in high flow versus base flow conditions, and by varying canopy cover. In this study, we performed preliminary tests to examine the feasibility of a light integrator (in situ actinometer) to measure the light exposure/light history of parcels of water in a Lagrangian sense. This integrator is based on the simple idea of deploying two fluorescent dyes in a known ratio within a chosen aquatic system: one of the dyes (fluorescein) is sensitive to light exposure and one (rhodamine WT) is relatively photostable. Theoretically, by measuring the ratio of their concentration over time, one can estimate the actual light exposure of a parcel of water and its constituents. Such an in situ actinometer would complement existing Eulerian methods of light measurement techniques. A Lagrangian approach to light field characterization would be more appropriate for investigating the photochemical response of species that also "go with the flow": those found in dissolved, colloidal, and small-particle phases.

Fluorescent dyes have been commonly used as tracers in water in a variety of aquatic systems, and their applicability has been extensively evaluated for specific systems (Smart and

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Laidlaw 1977; Kasnavia et al. 1999; Klonis and Sawyer 2000; Dierberg and DeBusk 2005; Chua et al. 2007; Upstill-Goddard 2008). In order to be a good tracer, these dyes must have high fluorescence quantum yields, high solubility, negligible background concentrations, and be inert to (or predictably and reproducibly affected by) chemical and biological factors (i.e., change in pH, change in temperature, particle sorption, and microbial uptake) within the aquatic system of interest. Most tracer dyes have ionic functional groups, making them soluble in water; however, these functional groups are often pH sensitive; with the resulting change in structure causing changes in dye fluorescence and sorption characteristics (Smart and Laidlaw 1977; Kasnavia et al. 1999).

Rhodamine WT (Fig. 1) was specifically designed to be an inexpensive and effective tracer for water flow (Smart and Laidlaw 1977) and is one of the most commonly used dyes for tracer studies. While often considered conservative (Upstill-Goddard 2008), it has been shown to sorb to particles, especially organic-rich and metal-oxide coated ones (Vasudevan et al. 2001; Smart and Laidlaw 1977), and must be used with care in particle rich and organic rich systems, such as wetlands (Dierberg and DeBusk 2005). Complicating the sorption issue is that tracer grade rhodamine WT often consists of 2 isomers (Shiau et al. 1993; Vasudevan et al. 2001) that have different sorption characteristics (Vasudevan et al. 2001). Rhodamine WT is somewhat photoreactive (Abood et al. 1969), but its photolytic loss has been reported as less than five percent for an 11.5-d deployment in North Sea surface waters (Upstill-Goddard et al. 2001) placing it in a usable range as a conservative tracer in coastal marine systems. Rhodamine WT does show pH sensitivity, with its fluorescence dropping dramatically below pH 5 (Smart and Laidlaw 1977), and its partition into organic phases (and likely sorption to organic particles) increasing with decreasing pH (Kasnavia et al. 1999). The relevant pK_a for rhodamine WT in natural waters is between 4.7 and 5.1, which refers to the interchange between either the +1 charged species or the zwitterion and the –1 charged species (Shiau et al. 1993; Kasnavia et al. 1999; Vasudevan et al. 2001).

Fluorescein (Fig. 1) is another dye commonly used for aquatic tracer work. It has been shown to exhibit low particle sorption to mineral phases and moderate sorption to organic material (e.g., Smart and Laidlaw 1977), has been used with success in brackish water aquifers, and appears to be unaffected by salinity (Chua et al. 2007). Fluorescein is highly susceptible to photolytic losses (Smart and Laidlaw 1977) and is thus more commonly used in groundwater or aquifer studies rather than in surface water work (Chua et al. 2007). Fluorescein is sensitive to pH, shifting among five to six different structures, with different fluorescence characteristics as the pH changes. Martin and Lindqvist (1975) report pK_{a1} ($[H^+][H_2Fl]/[H_3Fl^+]$ where "Fl" represents a deprotonated fluorescein molecule) to be 2.2, pK_{a2} ($[H^+][HFl^-]/[H_2Fl]$) to be 4.4, and pK_{a3} ($[H^+][Fl^{2-}]/[HFl^-]$) to be 6.7, and further discuss that there are three neutral forms of fluorescein, which have varying light absorption and fluorescence properties.

Both fluorescein and rhodamine WT fluorescence have also been shown to be functions of temperature, showing decreases in fluorescence as temperature increases (Smart and Laidlaw 1977). Rhodamine WT is more sensitive to temperature changes than fluorescein (e.g., rhodamine fluorescence doubles (~100% increase) as temperature decreases from 25°C to 0°C while fluorescein fluorescence increases by approximately 10% over the same temperature range, as shown by Smart and Laidlaw 1977, and our own data).

Whereas both dyes have often been used as water tracers, the potential of fluorescein and rhodamine WT as light sensors is just beginning to be explored. The first attempt to apply these in a quantitative sense has been to develop inexpensive Eulerian sensors. Bechtold and coworkers (2012) placed multiple vials or plastic bags containing either fluorescein or rhodamine in fixed locations on a stream bed and the loss of fluorescence over time was related to PAR sensor data to determine fluorescence decay rates as a function of light exposure. These dyes are also beginning to be applied as light sensors in pulsed injection approaches (Austin et al. 2010; Welsh 2012; Cullin et al. 2013), but these attempts have not yet addressed critical dye parameters, such as the wavelength range of light affecting fluorescence response and pH effects on dye fluorescence, and they have been applied in more qualitative rather than quantitative approaches.

The goal of this study is to further the coupling of fluorescein and rhodamine WT to make a Lagrangian light integrator. We performed preliminary lab testing to evaluate the dyes' sensitivity to salinity, pH, temperature, and the presence of natural stream dissolved material. We also constrained the integrator's wavelength range of response and pH effects on dye photodegradation. We then conducted field testing in Amity Creek (Duluth, Minn., USA), located on the north shore of Lake Superior, across a range of water flows that included both base flow and high flow conditions. Finally, we performed calibrations of dye response relative to PAR irradiation. This study, therefore, represents a proof of concept application of the Lagrangian dual dye approach.

Materials and procedures

Reagents and samples

For all lab and field experiments, the following chemicals were used: fluorescein ("Uranine K Liquid," manufactured by Keystone, item ID 801-073-42, lot number A208F209, where the fluorescein is present as the dipotassium salt) and Keyacid Rhodamine WT Liquid ("rhodamine WT," manufactured by Keystone, item ID 703-010-27, lot number A207K221). These liquids were viscous, making precise delivery of volumes challenging; thus they were diluted into working solutions of dye in tap water or deionized water, and these solutions were then used for the experiments. For each experiment or deployment, the same working solution was used for initial, exposed, and control samples (as described below). We present concentra-

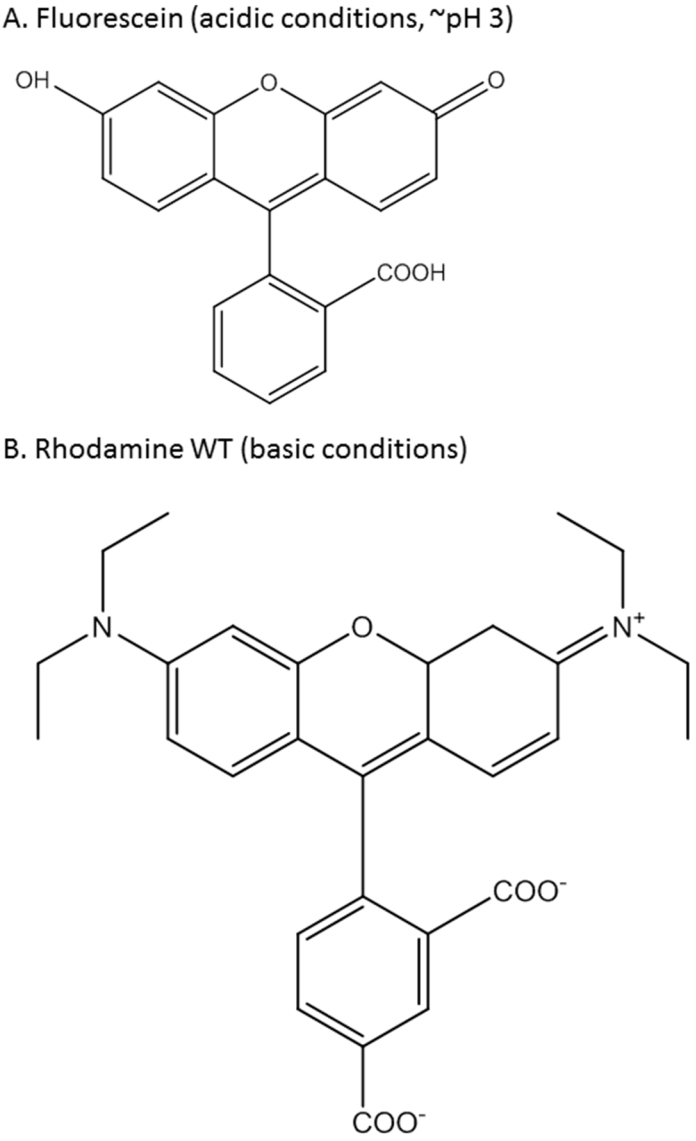

Fig. 1. The molecular structures of the dyes used in this study.

tions as both volume dye liquid per volume total solution (as is usual in the tracer dye community) and as moles per liter. Molarity (M) was determined from UV-visible analysis of selected dye solutions (absorbances at 490 and 550 nm for fluorescein and rhodamine WT, respectively) and published extinction coefficients (65,000 $M^{-1}cm^{-1}$ at pH 7 and 490 nm for fluorescein, Mota et al. 1991; and 87,000 $M^{-1}\,cm^{-1}$ for rhodamine WT at pH 5.6 and 550 nm, Tai and Rathbun 1988). Shiau et al. (1993) report that the different isomers of rhodamine WT, while varying in light absorption characteristics in the UV range, show very similar absorption at higher wavelengths; therefore we feel confident applying a published extinction coefficient at 550 nm even if the proportional isomer composition may be different in our rhodamine WT solution. Solutions and their UV-visible absorbances were used to calculate the relationship between dye liquid concentration and dye molarity, which was then used to calculate all other concentrations.

For lab tests of natural stream water photoresponse (i.e., the photobleaching of colored dissolved organic matter or CDOM), an Amity Creek sample was taken during high flow on 30 September 2010. The sample was filtered through a Whatman GF/F filter and then a Whatman Polycap aqueous solution 0.2 μm-filter capsule (Type 18056) within 24 hours of sampling. It was then stored in the dark at 4°C for several months until use. It was filtered again (0.2 μm) less than 24 hours before the irradiation experiments (described below). It is acknowledged that natural samples will change in both dissolved organic carbon (DOC) and UV-visible absorbance characteristics upon storage, but these changes are within (and often much less) than variations seen within the same stream under different flow characteristics (Macdonald and Minor 2013). While not performed on this sample, we have tested storage effects on samples from 5 local streams stored 52 to 119 days (Macdonald and Minor 2013); the average DOC loss upon storage was 15% (n = 12, range 0% to 30%); the ratio of absorbance at 250 nm to 365 nm (e2/e3) went up in 2 of 13 samples (average increase 25%) and down in 11 of 13 samples (average decrease 5%, range 0% to 13%). We felt that such variations were acceptable in a proof of concept test of the wavelength response of CDOM photobleaching relative to dye photobleaching.

Fluorescence measurements

Fluorescein and rhodamine fluorescence was monitored using Sea Point immersible fluorometers and an Onset thermister (temperature probe), with voltages recorded with an Onset 4-channel datalogger. The Sea Point rhodamine WT fluorometer had an excitation wavelength of 540 ± 15 nm and emission wavelength of 610 ± 15 nm. The Sea Point fluorescein fluorometer had an excitation wavelength of 475 ± 10 nm and an emission wavelength of 530 ± 20 nm. Raw fluorescence voltages were corrected for background fluorescence and converted to dye concentration using temperature data and a lab-determined temperature calibration. The changes in these fluorescence-based concentrations as a function of sample treatment (e.g., irradiation) are presented as the ratio of fluorescein (F) to rhodamine WT (R) in treated sample normalized to the ratio of fluorescein to rhodamine WT in the initial untreated sample (hence $(F/R)/(F/R)_o$). This ratio is introduced because it acts to isolate light-mediated effects from those of water motion. Within in situ dye deployments, both dyes will diffuse and advect similarly. Therefore, in the absence of light, the ratio will remain the same during a downstream deployment; a change in the ratio indicates light exposure.

Ancillary measurements

UV-visible absorbance measurements of dye samples and stream water samples were performed using a Genesys 6 spectrophotometer (Thermo Fisher Scientific) and 1-cm quartz

cuvettes. MilliQ water was used as the blank. A Eutech Instrument Waterproof pHTestr 20 calibrated with WTW Technical Buffers (pH of 4.01 and 7.00) was used to determine pH. Rapid lower-resolution pH measurements (~ ±0.5 pH units) were taken using Whatman indicator papers.

Laboratory irradiations

Lab irradiations were performed in either matching 500-mL quartz round-bottom flasks (with foil-wrapped 500-mL borosilicate flasks as dark controls) or in matching wide-mouth 20-mL vials wrapped in foil, with irradiation coming from overhead into the openings, which were either left uncovered, covered with foil, or covered with filters allowing specific wavelengths of light to pass. The irradiations were performed in a QSun Solar Simulator, producing 0.56 W/m^2 per nm at 340 nm, and samples were cooled by partial immersion in a water bath set at approx. 25°C. The solar simulator provides approximately twice the spring to summer irradiance seen in northern Minnesota. For example, noon direct normal irradiance at 332 nm on 21 June 2012 in Grand Rapids, Minn., USA, 47°10.8' N, 93°31.8' W, was 0.33 W/m^2 per nm, (http://uvb.nrel.colostate.edu/UVB/da_LangleyIrradiance.jsf) and comparison of dye response in the solar simulator versus natural mid-day spring sunlight in Duluth, Minn., USA (10 Apr 2009) showed that the rate of fluorescein photodegradation in the solar simulator was approximately twice the rate in natural sunlight.

Stream deployments

For stream deployments, 500 mL dye solution (22 parts per thousand (ppt) Keyacid Rhodamine WT liquid, i.e., 2×10^{-2} M rhodamine WT, and 78 ppt Uranine K Liquid, i.e., 6×10^{-2} M fluorescein; in tap water) was added to Amity Creek surface water. The same fluorometers used in lab tests were deployed, along with a temperature probe, 41 m downstream of the initial dye deployment, to read initial dye concentrations in the stream. Voltage measurements were sent to a data logger every 2 seconds. After the initial dye mixture passed, the fluorometers were taken to sites farther downstream, where they were placed in the stream until the dyes passed. The total reach for all deployments (n = 6) was 1.70 km.

During four of the Amity Creek dye deployments, two controls were also monitored. These controls consisted of 6 parts per million (ppm) Keyacid Rhodamine WT liquid (4×10^{-6} M) and 20 ppm Uranine K Liquid (1×10^{-5} M fluorescein) in creek water (4 L aliquots) placed in matching open-topped polypropylene containers (33 cm × 13.5cm × 12 cm). One container was placed in the stream in a place exposed to ambient sun, thus acting as a light control (LC). The other container was placed in the stream in the shade and was wrapped with a black plastic bag. This container acted as a dark control (DC). The control containers were sampled at the beginning, partway through and at the end of the deployment. These samples were brought back to the lab where they were analyzed using the field fluorometers and temperature probe. Data were then temperature and background corrected, as well as converted to dye concentration based upon calibration curves.

Statistical analysis (t-tests and linear correlations) was performed using SPSS and Excel. Unless otherwise stated, significance was determined with a probability value (p value) of $p < 0.05$.

Calibration of fluorescein response relative to PAR exposure

To test dye photobleaching in response to natural sunlight exposure, we irradiated duplicate aliquots of a known concentration of fluorescein dye (0.077 ppm or 1.9×10^{-7} M) in phosphate buffer (pH 7.5, 0.1M) and duplicate aliquots of dye mixture (0.08ppm (2×10^{-7} M) fluorescein and 0.09 ppm (6×10^{-8} M) rhodamine) in the same phosphate buffer. The irradiations were performed using matching 500 mL borosilicate round bottom flasks placed in a flow-through water-cooled chamber (18 to 23°C) filled with Lake Superior surface water and exposed to natural sunlight at 47°10'N, 91°15'W on 19 Aug 2013, starting at 12:24 PM Central Daylight Savings time and continuing to 4:23 PM. Fluorescein fluorescence was measured using a Turner 10AU Fluorometer. The change in fluorescein fluorescence response was measured as a function of natural sunlight photon dose measured with a surface PAR sensor placed less than 100 feet from the water-cooled chamber (QSR 2200 Biospherical, calibrated January 2013).

Assessment

Lab experiments

Fluorescence characteristics of the two dyes

The first questions addressed were whether the fluorescence of the two dyes was separable using appropriate excitation and emission wavelengths and whether there was quenching of fluorescence when the two dyes were mixed together. These questions were addressed by measuring known phosphate-buffered (pH 6.7) solutions (n = 3) of fluorescein using the excitation and emission characteristics of rhodamine WT and vice versa, and then by measuring mixtures of the two dyes (in triplicate) targeting either the fluorescein or rhodamine fluorescence (Table 1). There was no measurable fluorescence of fluorescein (when compared with the buffer blank) using the rhodamine fluorometer and very little rhodamine fluorescence when the rhodamine solution was measured with the fluorescein fluorometer. t tests were used to compare the results from the single dye replicates versus the dye mixture replicates (using the appropriate fluorometers for each dye); these showed that the data means for each dye were statistically different for the 2 dye mixture versus the single dye solutions at $\alpha = 0.05$ but not at $\alpha = 0.01$. However, although the difference is significant at $\alpha = 0.05$, it is small, leading to < 4% increases in rhodamine WT measurements and < 7% increases for fluorescein measurements when the 2-dye mixtures are measured relative to the single mixtures. The single-dye versus dye mixture test was then repeated by adding the dyes to sterile-filtered stream water from Oregon Creek (Duluth, Minn., USA, pH = 8.6). With stream water solutions, the fluorescence voltage responses for each dye and the

Table 1. Relative fluorescence of standard dye solutions (prepared in triplicate in phosphate buffer or sterile-filtered water from Oregon Creek, Duluth, Minn., USA). A test of peak-overlap and quenching issues.

Sample	Fluorescence response (V) from the rhodamine fluorometer corrected to 0° using Eq. 1	Fluorescence response (V) from the fluorescein fluorometer corrected to 0° using Eq. 1
Rhodamine WT solution (2×10^{-7} M rhodamine WT in phosphate buffer, pH 6.74)	4.53 ± 0.03	0.0102 ± 0.0006
Fluorescein solution (6×10^{-8} M in phosphate buffer, pH 6.74)	0.022 ± 0.001	2.308 ± 0.09
Mixed dye solution (Rhodamine WT (at 2×10^{-7}M and fluorescein at 6×10^{-8} M in phosphate buffer, pH 6.74)	4.71 ± 0.09	2.47 ± 0.04
Phosphate buffer, pH 6.74	0.020 ± 0.001	0.0051 ± 0.0006
Rhodamine WT solution (2×10^{-7} M rhodamine WT in filtered stream water, pH = 8.6)	4.50 ± 0.06	0.043 ± 0.001
Fluorescein solution (6×10^{-8} M in filtered stream water, pH = 8.6)	0.040 ± 0.000	2.79 ± 0.03
Mixed dye solution (Rhodamine WT (at 2×10^{-7}M and fluorescein at 6×10^{-8} M in filtered stream water, pH = 8.6)	4.55 ± 0.11	2.98 ± 0.08
Filtered stream water, pH = 8.6	0.034 ± 0.000	0.038 ± 0.000

"cross-talk" results were very similar to those seen in the buffer solutions (Table 1). The results for fluorescein again showed increases of < 7% for the dye mixture versus the single dye sample; however, the same concentrations of rhodamine alone (n = 3) and rhodamine in the dye mixture (n = 3) showed no difference in fluorescence response (4.50 ± 0.05 V versus 4.5 ± 0.1 V).

The effect of temperature on dye fluorescence was examined between 2°C to 40°C. Fluorescein and rhodamine stock solutions were diluted separately to make 0.1 ppm (9×10^{-8} M fluorescein) and 10 ppm (7×10^{-6} M rhodamine WT) solutions, consecutively. Each working solution (1.0 L) was placed separately into a beaker and cooled to approximately 2°C. A stir bar was added to each beaker, which was then placed on a hot plate/stirrer. Temperature and fluorescence probes were also placed into each beaker, and data were recorded every 2 seconds until the solutions reached 40°C. Both rhodamine WT and fluorescein showed an exponential relationship between fluorescence and temperature (Eq. 1) as reported by Smart and Laidlaw (1977). Our temperature exponent (n) for fluorescein was 0.0036°C^{-1}, which agreed with Smart and Laidlaw's (1977) value using the equation

$$F_s = Fe^{n(t_s - t)} \quad (1)$$

where F_s is the fluorescence at a standard temperature (t_s), and F is fluorescence at sample temperature (t). Our rhodamine WT temperature exponent was 0.026°C^{-1}, comparable with Smart and Laidlaw's (1977) value of 0.027°C^{-1} using the same equation. Note that Smart and Laidlaw (1977) used a t_s of 0°C, while data in this study (unless otherwise stated) was converted to fluorescence at a standard temperature of 25°C.

Additional lab tests showed that the fluorescence of fluorescein increased by over a factor of 2 with pH over the pH range of 4.89–9.04, while the fluorescence of rhodamine WT was unaffected over this range, which is consistent with Smart and Laidlaw's (1977) findings that rhodamine WT fluorescence is affected significantly only below pH 5.0. Thus, for field work, it is recommended to normalize fluorescent response to initial in situ dye fluorescence (as done in this study) and to measure the in situ pH as well.

Dye response upon irradiation; the effect of wavelength

The wavelength range of light to which the dye pair is sensitive was examined using the QSun solar simulator. To determine if the dye pair would act as an appropriate light sensor for use in studies of the photobleaching of natural dissolved organic matter, we also assessed the wavelength range and time of response for photobleaching of DOM (as assessed by changes in UV-visible absorbance) from our experimental stream system (Amity Creek). Sterile-filtered stream water from Amity Creek (sampled 30 September 2010, in conjunction with one of the high flow field deployments described below) was irradiated using the same irradiation period and wavelength ranges as used for studying the dye solutions. To examine the wavelength response of the dyes' fluorescence ratio, phosphate-buffered (0.24 M, pH 7, made in deionized water) dye solutions (9.7 ppm Uranine K Liquid [7×10^{-6} M fluorescein] and 2.7 ppm Rhodamine WT Liquid [2×10^{-6} M rhodamine WT]) were prepared. Aliquots of the dye solution were placed into wide-mouthed (2.1 cm diameter) clear-glass 20-mL vials (5.5 cm in length). The vials were wrapped in aluminum foil, leaving the top exposed to light. Exposures were performed in duplicate using four long-pass filters (345, 360, 400, and 420 nm) placed over the vial openings. Two sample vials were left open to act as full-light controls. An additional set of vials (n = 2, "initial") was placed in a refrigerator, while

a third set (n = 2, "dark control") was wrapped completely in foil and placed in the solar simulator. The sample and dark control vials were partially submerged in a water bath at approx. 25°C in the QSun Solar Simulator for an exposure time of 50 min. After exposure, fluorescence was measured on both the exposed and control samples.

The dye test described above was performed using solutions visibly colored by the dyes themselves. The experiment was repeated with a dilute dye solution containing 1 ppm Uranine K Liquid (9×10^{-7} M fluorescein) and 0.3 ppm Keyacid Rhodamine WT Liquid (2×10^{-7} M rhodamine WT). Using the same long-pass filters as before, aliquots of this dye solution were irradiated for three hours in the QSun Solar Simulator, and initial, control, and exposed samples were analyzed. For both dye irradiation experiments, the dark control and initial samples gave the same fluorescence response, indicating no degradation of dye fluorescence.

To compare the wavelength response and photodegradation rates of the dual-dye indicator with those for the photobleaching of stream DOM, the irradiation experiment was replicated replacing the dye solutions with 20-mL aliquots of sterile-filtered Amity Creek water. The photobleaching of DOM in the stream water aliquots was assessed by UV-vis spectrophotometry and the resultant rates of photobleaching and wavelength ranges responsible were compared with the changes in dye fluorescence determined in the previous experiment.

Both CDOM and the dyes were measurably affected over the same timescale of exposure in the QSun Solar Simulator, but differed in wavelength response (Fig. 2 versus Fig. 3). CDOM responded mainly to the UVA range of light (345-400 nm range), consistent with results reported in Granéli et al. (1998) for lake samples from Sweden and Brazil, and Larson et al. (2007) for water from forested streams in the Lake Superior region. In contrast, the dye solutions, both concentrated and dilute, were degraded at wavelengths at and/or above 420 nm (our longest wavelength range long-pass filter). The fluorescence loss was mainly due to fluorescein degradation (Fig. 3). The dyes are thus applicable to assessing the photosynthetically active radiation (PAR) rather than the UV portion responsible for the majority of CDOM photobleaching.

Dye response upon irradiation; pH effects

The effect of pH on dye photodegradation was examined by irradiating solutions of the dye buffered at pH levels ranging from 5 to 9 (Table 2). Ninety minute irradiations were performed in the QSun solar simulator (water bath temperature 23°C to 27°C) using 500-mL quartz round bottom flasks (diameter 10.9 cm) containing 300-mL of buffered dye solutions (4×10^{-2} ppm of Uranine K Liquid [3×10^{-8} M fluorescein], and 1×10^{-2} ppm of Rhodamine WT Liquid [8×10^{-9} M Rhodamine WT]). Dark controls consisted of 300-mL aliquots of each buffered dye solution in 500-mL borosilicate round bottom flasks covered with aluminum foil and placed in the solar simulator. All irradiated and control samples were measured using Sea Point fluorescein and rhodamine fluorometers. Temperature and pH were also recorded at the time of the fluorescence measurements.

Fluorescein was photo-labile across the entire pH range, but showed maximum photo-lability at pH 7.2 (Fig. 4). Rhodamine WT was photo-stable from pH 4.9 to pH 8.1, but was photolabile from pH 8.3 to 9.0. The apparent photo-lability of rhodamine at pH values of 8 to 9 may be due to the carbonate buffer used. Carbonate radicals may have been formed from interaction with photochemically produced OH•, thus leading to indirect photolysis of the rhodamine WT (e.g., Schwarzenbach et al. 2003), rather than a direct photochemical reaction

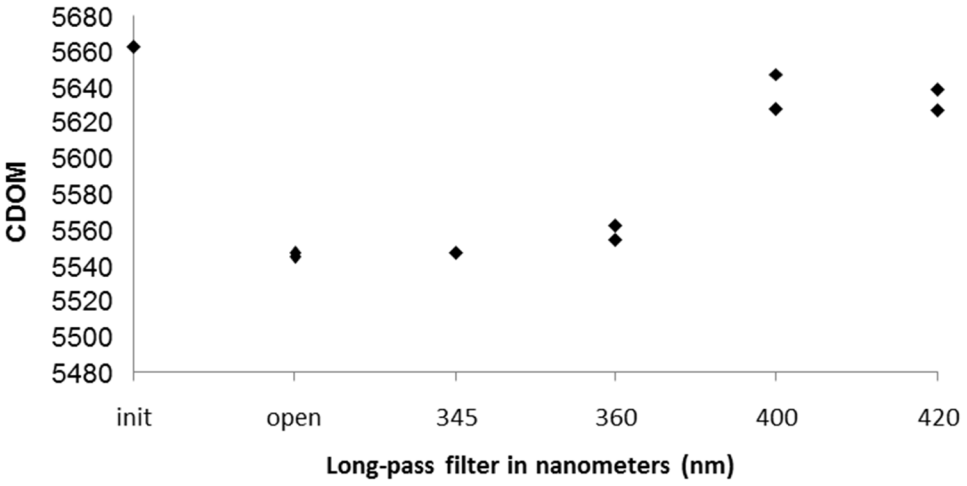

Fig. 2. Solar simulator irradiations (performed on 11 Jan 2011) of < 0.2 μm filtered Amity Creek water (sampled on 30 September 2010) for 50 min using long-pass filters (345, 360, 400, and 420 nm). The amount of colored dissolved organic matter (CDOM) present was measured using absorbance coefficients (at 1-nm increments) summed over 250-400 nm. For comparison unirradiated sample ("init") and sample irradiated with the full wavelength range of the Q sun solar simulator ("open") are also included.

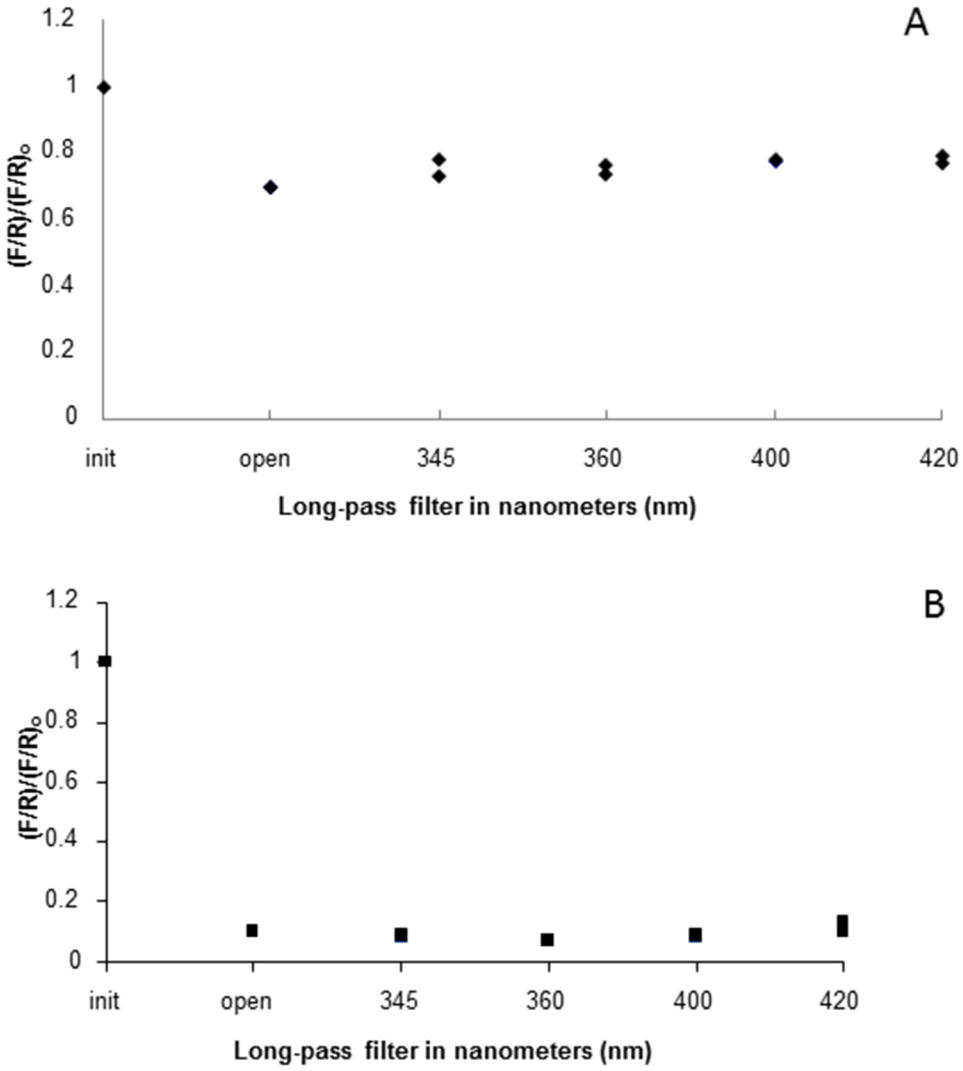

Fig. 3. The ratio of buffer-solution (pH 7) dye concentrations normalized to their initial ratio $[(F/R)/(F/R)_0]$ after A. 50 min of irradiation and B. 3 h of irradiation. Long-pass filters (345, 360, 400, 420 nm) were used to determine the wavelength ranges responsible for maximum dye response. For comparison, unirradiated dye solution ("init") and dye solution irradiated with the full wavelength range of the Q sun solar simulator ("open") are also included.

Table 2. Buffer solutions used in testing pH response of dye photodegradation. Dye concentrations for all solutions were 4.1×10^{-2} ppm Uranine K Liquid (3×10^{-8} M fluorescein) and 1.12×10^{-2} ppm Rhodamine WT Liquid (8×10^{-9} M rhodamine WT).

pH	Reagents	Moles/L	Number irradiated, number control
4.9	A.C.S. Fisher Scientific sodium acetate	0.50	2, 2
	17.4 M Fisher Scientific acetic acid, HPLC grade	0.35	
6.2	Arcos Organics ammonium phosphate	0.44	1, 1
	17.4 M Fisher Scientific acetic acid, HPLC grade	0.35	
7.2	Fisher Scientific HPLC grade ammonium acetate	0.10	1, 1
8.1	Fisher Scientific A.C.S. certified sodium bicarbonate	0.10	2, 2
	Fisher Scientific A.C.S. certified 6 M HCl	Small additions	
8.3	Fisher Scientific A.C.S. certified sodium bicarbonate	0.10	1, 1
	Fisher Scientific A.C.S. certified 6 M HCl	Small additions	
9.0	Fisher Scientific A.C.S. certified sodium bicarbonate	0.10	1, 1
	Fisher Scientific certified A.C.S. sodium hydroxide beads	Small additions	

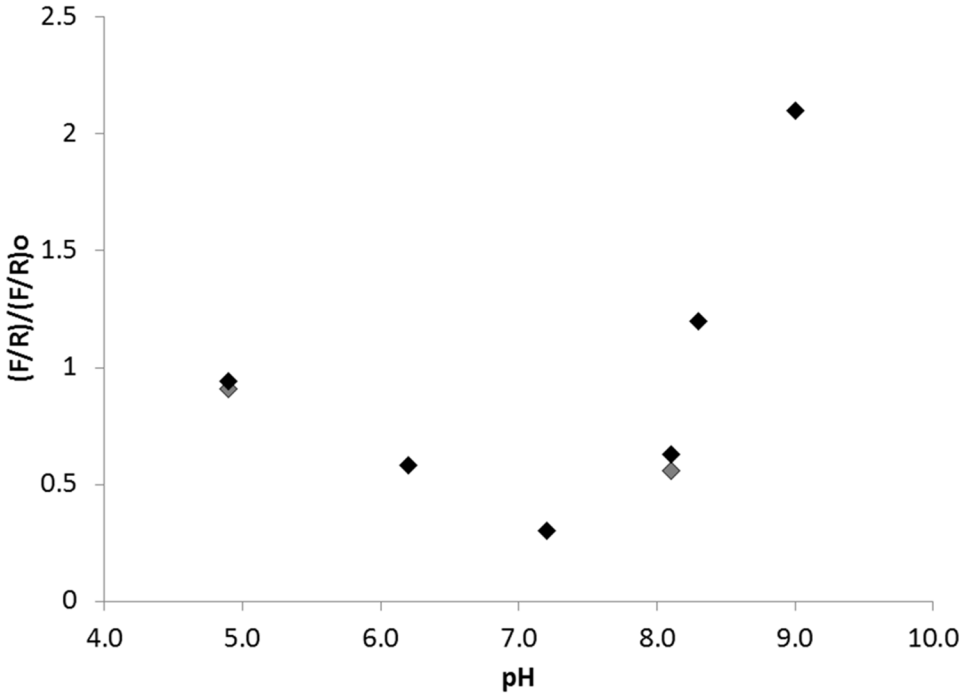

Fig. 4. Corrected dye ratio ([F/R]/[F/R]0) versus pH value after irradiation for 90 min in a solar simulator. Replicate samples were treated at pH 4.9 and 8.1 and are shown in gray.

of rhodamine WT. Further studies are needed to evaluate the high pH-response of rhodamine WT, and its relevance to natural water systems, especially those buffered by the carbonate system.

Based upon the above results, the practical pH range for the dual-dye approach considering rhodamine WT as a photoinert tracer is approximately 6 to 8 (Fig. 4), which is suitable for many natural waters, although seawater is on the edge of this range (Upstill-Goddard et al. 2001). The dual dye approach can be extended outside this range; however, to do so, both tracers may need to be modeled as photoreactive species differing in response rate (and possibly in wavelength range of response) from those reported here for the pH range 6-8. Our field testing site (Amity Creek, pH range 6.99-7.54 for the summer of 2012, LakeSuperiorStreams [2009]) is within the working range of the dual dyes using the simpler modeling approach (i.e., assuming rhodamine WT to be a photoinert tracer).

Field experiments

Initial field deployments of the dual dye integrator were performed in Amity Creek, a local Lake Superior tributary (Fig. 5). Although the stream is located within the city limits of Duluth, Minn., USA, its watershed is primarily undeveloped (71% forested, 19% grassland, 4% developed, and 3% wetland, LakeSuperiorStreams [2009]). The dye deployment reach (1.70 km long) was upstream of the confluence of Amity Creek and Lester River, whose combined flow, with a watershed area of 134.8 square kilometers, then drains into Lake Superior. The dye deployments were performed across a range of flow conditions, from 0.071 to 0.364 m^3/s. The change in the fluorescence ratio of the dyes along the reach was compared to solar radiation and PAR exposure (Table 3), which was estimated using data from a nearby meteorological buoy. Whereas PAR was not directly measured in either year, a PAR sensor (LI-COR LI190, spectral response 400-700 nm) had been deployed in 2007 adjacent to a shortwave radiation sensor (Kipp and Zonen CM3, spectral response 305-2800 nm) and a strong linear relationship ($R^2 = 0.99$) between the two was found. This allowed PAR to be estimated in 2010 and 2011 from shortwave radiation data.

For each dye deployment, a 500-mL solution (2×10^{-2} M rhodamine WT, 6×10^{-2} M fluorescein) was added to the stream. The SeaPoint immersible fluorometers and Onset thermister were submerged 41 m downstream of the initial dye deployment, in order to read initial dye concentrations in the stream, with voltages reported to a data logger every two seconds. Downstream measurements were then taken midway through the reach and at the end (after 1.70 km, Fig. 5).

During four of the dual dye deployments, two controls consisting of both dyes plus creek water (4×10^{-6} M rhodamine and 1×10^{-5} M fluorescein dipotassium salt in matching polypropylene containers, 33 cm × 13.5cm × 12 cm) were also monitored to compare with the more complex in-stream condition. These controls were not subject to variable particle loadings downstream, had controlled depths (without riffle/pool dynamics), and eliminated advection as a variable.

Fig. 5. Dye deployment area along Seven Bridges Road (Duluth, MN) as seen in Google Earth showing dye input (Dye In) and the location of fluorescence measurements, which were performed at 46.8608°N, 92.0132°W (first pass), 46.85816°N, 92.0131°W (Bridge 4), and 46.8538°N, 92.0113°W (Bridge 2). Solar irradiance data were taken from a Lake Superior buoy deployed at 46.864°N, 91.929°W.

Table 3. The deployment date, duration, solar radiant exposure, PAR exposure, and weather conditions. Lake Superior solar radiation data were taken from a buoy deployed at 46.864°N 91.929°W and was averaged over the deployment time and multiplied by the time length of the deployment (h). PAR was converted from the solar radiation data as stated in the text.

Date	Start time	Total deployment time (min)	Solar radiant exposure (W/m^2) × h	PAR exposure, μEinsteins/m^2	Conditions
30 Sep 2010	13:45	92.9	1205.3	6.5×10^6	Sunny with intermittent clouds
17 Jun 2011	10:00	180.8	932.5	5.8×10^6	Cloudy
24 Jun 2011	10:00	42	821.1	3.5×10^6	Sunny day after large rain event
6 Jul 2011	10:40	223.6	2789.2	2.3×10^7	Sunny
13 Jul 2011	9:30	282.5	3685.4	2.8×10^7	Sunny
29 Jul 2011	13:40	268.6	3593.2	2.2×10^7	Sunny

One container, the light control (LC) was left in the stream (for cooling) and exposed to ambient sunlight. The other container, the dark control (DC) was placed in the stream in the shade, covered, and wrapped with a black plastic bag. Each control was sampled two to three times throughout the deployment time, using 500 mL plastic bottles. The first sample was taken at the beginning of the deployment, a second was taken about half-way through the deployment (when possible), and the last sample was taken at the end of the deployment. These samples were brought back to the lab where they

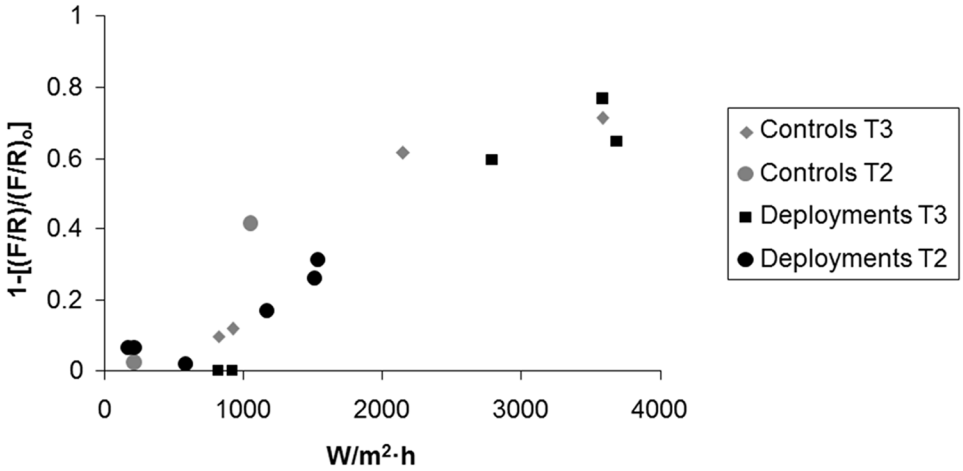

Fig. 6. The loss of normalized fluorescence response (F/R), i.e., fluorescence "photobleaching" versus solar radiation in W/m² × h for the light control samples taken at the mid-point sample time (T2, indicated by a light circle symbol) and end-point sample time (T3, indicated by a diamond symbol) and the deployment measurements taken at the mid-point sample location (T2, indicated by a dark circle symbol) and end-point sample location (T3, indicated by a dark square symbol.) The R^2 values for linear fits to the controls and deployments were 0.861 and 0.921, respectively.

were analyzed using the field fluorometers and temperature probe. Data were then temperature and background corrected, and converted to dye concentration.

For statistical analyses, the dye concentration ratios were normalized by the initial ratio of the dyes at the beginning of the deployment. Three time points from each dye pass were taken, one at half the peak height on the rising limb, one at peak height, and one at half of the peak height on the falling limb of the fluorescence response. These were averaged and then plotted versus time and solar insolation in (W/m^2)*h and compared with the light control samples. In Amity Creek, the dyes showed a strong photochemical response across the tested reach that was positively correlated with solar radiation; the light control samples showed a very similar correlation to that seen in situ (Fig. 6).

As an additional test of dye response, the dye concentration ratios of the initial and last measurements for each in-stream deployment were used in a fluorescein photodegradation rate equation (Bechtold et al. 2012):

$$Dye_L = Dye_{init} e^{-k(light)} \quad (2)$$

where Dye_L is the concentration (based upon fluorescence measurement) of the light-exposed dye, Dye_{init} is the concentration (again based upon fluorescence measurement) of the initial dye solution, k is the experimentally determined rate constant, and "light" is the PAR dosage. We used two different k values, the value published in Bechtold et al. (2012) (k = 0.014 mol photons^{-1} m^{-2}) and the average k generated by our on-deck irradiation experiments (k = 0.20 mol photons^{-1} m^{-2}). The resulting calculated PAR exposures were compared with those from the buoy data (Table 4 and Fig. 7). Calculated PAR exposures for both k values were well correlated with buoy data (R^2 = 0.96, n = 6, p < 0.05). In-stream dye degradation using the k value of Bechtold et al. (2012) yielded a roughly 3-fold higher exposure rate than given by the buoy data, while using the k value from our calibration yielded values approximately one fifth of those seen at the buoy. As measured whole-water Naperian absorption coefficients (in m^{-1}) for 400-nm light in Amity Creek range from 3.45 to 26.63 (n = 9, sampling from Sep through Nov 2007, Minor unpubl. data), absorption and back-scattering by stream components are likely to lead to an in-stream exposure for the dyes that is less that surface PAR. Shading effects from canopy cover cannot be completely discounted; however, "full-sunlight" controls of stream water and the in situ deployments show similar losses of fluorescein fluorescence (Fig.6); thus, in-water shading appears more likely. Because of such absorption and backscatter potential in Amity Creek water, the k value determined in our calibration, as compared to that of Bechtold et al. (2012) seems more representative.

Comments and recommendations

In this proof-of-concept study, a dual-dye system consisting of known ratios of fluorescein to rhodamine WT has been shown to be applicable to studies of light history in natural waters and to indicate light exposures of wavelengths ≥ 420 nm. It thus shows promise as a Lagrangian PAR sensor. To make this system an accurate PAR actinometer will require careful calibration of the dual dyes by comparison with existing actinometers and by determining wavelength-based fluorescence bleaching quantum yields. To apply the dual-dye integrator within a larger range of natural water systems, more work needs to be done calibrating the dual-dye approach at basic pH levels (pH > 8). In addition, and most

Table 4. The deployment date, average stream discharge, buoy PAR exposure, PAR exposure measured from dye degradation and the ratio of calculated PAR exposure versus buoy PAR exposure. Discharge data are from LakeSuperiorStreams (2009).

Date	Average discharge (m^3/s)	PAR exposure from buoy data (mol photons/m^2)	PAR exposure calculated from dye response using k from Bechtold et al. (2012) (mol photons/m^2)	PAR exposure calculated from dye response using k from this study (mol photons/m^2)	Ratio of calculated PAR exposure (k from this study) to buoy PAR exposure
30 Sep 2010	0.26	6.5	No exposure	No exposure	0*
17 Jun 2011	0.071	5.8	15	1.0	0.17
24 Jun 2011	0.36	3.5	1.4	0.1	0.03*
6 Jul 2011	0.15	23	73	5.1	0.22
13 Jul 2011	0.076	28	82	5.7	0.20
29 Jul 2011	0.096	22	72	5.0	0.22

*high flow days

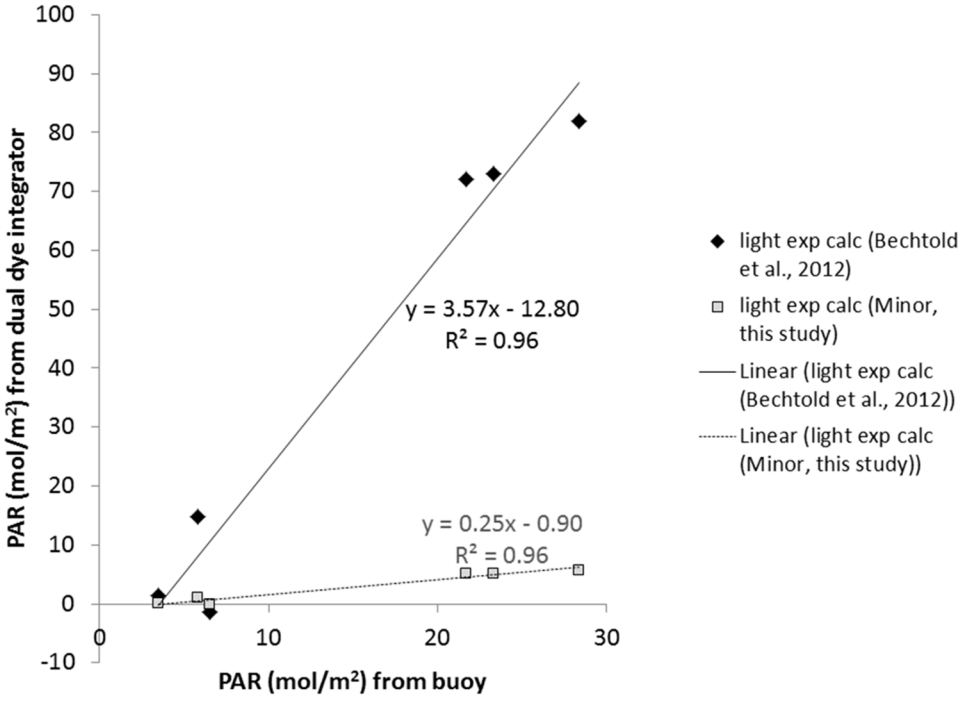

Fig. 7. The relationship between PAR (in mol photons/m^2) from buoy measurements and from in-stream use of the dual-dye light integrator. The in-stream numbers were calculated using Eq. 2 and k from Bechtold et al. 2012 (solid symbols and line) and k from this study (open symbols and dotted line).

relevant for longer term deployments (days to weeks), further work should be done to estimate potential confounding effects from particle absorption and/or preferential diagenesis of one of the dyes during field deployments. Finally, the dye deployments described here were small-scale studies across approximately 2 km of stream reach. The applicability of this approach at larger scales will require careful attention to dye dispersion and the use of ship-based or AUV-based fluorescence measurements.

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