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Interactions of dissolved humic substances with oppositely charged fluorescent dyes for tracer techniques

Akira Hafuka\textsuperscript{a,}\textsuperscript{*}, Qing Ding\textsuperscript{b}, Hiroshi Yamamura\textsuperscript{a}, Koji Yamada\textsuperscript{c}, Hisashi Satoh\textsuperscript{d}

\textsuperscript{a} Department of Integrated Science and Engineering for Sustainable Society, Faculty of Science and Engineering, Chuo University, 1-13-27 Kasuga, Bunkyo-ku, Tokyo 112-8551, Japan

\textsuperscript{b} Division of Civil and Environmental Engineering, Graduate School of Science and Engineering, Chuo University, 1-13-27 Kasuga, Bunkyo-ku, Tokyo 112-8551, Japan

\textsuperscript{c} Division of Environmental Materials Science, Graduate School of Environmental Science, Hokkaido University, North-10, West-5, Sapporo 060-0810, Japan

\textsuperscript{d} Division of Environmental Engineering, Graduate School of Engineering, Hokkaido University, North-13, West-8, Sapporo 060-8628, Japan

E-mail address:
* Corresponding author. Akira Hafuka

Tel. & fax: +81-3-3817-7283

E-mail: hafuka.14p@g.chuo-u.ac.jp
Abstract

To investigate interactions between oppositely charged fluorescent dyes and dissolved humic substances, fluorescence quenching of fluorescein and rhodamine 6G with dissolved humic substances was performed. Binding coefficients were obtained by the Stern–Volmer equation. The fluorescence of rhodamine 6G was largely quenched by the addition of humic acid and a non-linear Stern–Volmer plot was obtained. This strong quenching may be caused by the electrostatic interaction between cationic rhodamine 6G and humic acid and strengthened by the hydrophobic repulsion. In contrast, the quenching and interactive effects of dissolved humic substances for fluorescein were relatively weak.

Keywords: Tracer, Stern–Volmer plot, Fluorescein, Rhodamine 6G

1. Introduction

Fluorescent dyes have been widely employed in various technologies such as dye tracing, bio-imaging, chemical sensing, and dye lasers (Yuan et al., 2013;
Dye tracer techniques are powerful tools that enable better understanding of solute transport in the liquid phase under a variety of hydrological conditions by tracking natural tracers and/or spiked artificial tracers. In the field of water and environmental engineering, tracers have been applied to investigate water flow in surface and ground water systems, as well as in laboratory experiments (Goppert and Goldscheider, 2008; Jones and Smith, 2005; Sabaliunas et al., 2003). To be effective, tracers must be absent from (or present in very low concentrations) natural aquatic environments, have high water solubility and low sorptivity to soil and sediment, be safe, highly detectable, and chemically, physically, and biologically stable. Among a variety of tracers, fluorescent dyes composed of synthetic organic compounds possess most of these characteristics, are cost effective and easy to use, and can be easily identified on-site using a portable fluorometer. Fluorescein is a popular fluorescent dye tracer commonly used in aquatic systems (Sabaliunas et al., 2003; Harden et al., 2008). Rhodamine 6G is also sometimes used as a fluorescent dye tracer (Kwak et al., 2013); however, this compound has been shown to have toxic effects and is therefore generally limited to use in laboratory experiments (Alford et al., 2009). The difference of two dyes is that fluorescein is anionic and rhodamine 6G is cationic.
Smart and Laidlaw (1977) investigated the sensitivity, detectability, effect of water chemistry, decay rates, adsorption resistance, toxicity, and cost of eight fluorescent dye tracers, including fluorescein. Since then, ecotoxicological assessments (Behrens et al., 2001), photobleaching (Larsen and Crimaldi, 2006), and effects of solvents and pH (Saini et al., 2009; Oba and Poulson, 2012) of fluorescent dye tracers have been investigated. However, no studies have focused on the effects of dissolved humic substances (DHS) on fluorescent dyes. Although fluorescent dyes can be used for qualitative analysis, the interactions might inhibit the optical detection of fluorescent dyes due to the potential quenching caused by the presence of DHS. DHS is known to interact with organic compounds such as herbicides, pesticides, and polycyclic aromatic hydrocarbons (PAHs) in surface water (Ravacha and Rehun, 1992; Schlautman and Morgan, 1993; Hesketh et al., 1996; Spark and Swift, 2002), and to affect their fate and transport. Various methods such as simple adsorption experiments, dialysis, microcalorimetry, reverse phase separation, solubility enhancement, and fluorescence quenching have been used to investigate the interactions between DHS and these organic compounds (Hesketh et al., 1996; Spark and Swift, 2002; Raber et al., 1998; Yamamoto et al., 2003; Schlautman and Morgan, 1993). Fluorescence quenching is a
simple and useful method commonly employed to investigate interactions of DHS with PAHs because some PAHs show fluorescence and DHS acts as a quencher. The Stern–Volmer equation (also known as the fluorescence static quenching equation) can be plotted by conducting fluorescence quenching experiments (Gauthier et al., 1986). The binding coefficient can be obtained easily by determining the slope of the Stern–Volmer plot. In this study, the fluorescence quenching method was applied to analyze interactions of oppositely charged fluorescent dyes, fluorescein and rhodamine 6G, with DHS to investigate its effects on the dyes. This paper would contribute to the practice of selecting suitable fluorescent dye tracers in aquatic and hydrology studies.

2. Materials and methods

2.1 Fluorescent dyes

The chemical structures of fluorescein, rhodamine 6G, and rhodamine B are shown in Fig. 1. All fluorescent dyes are xanthene derivatives with many valuable characteristics, including sharp and intense absorption and fluorescence bands, high fluorescence quantum yields, high molar absorption coefficients, good photo-chemical stability,
relatively long emission wavelength, and good water solubility (Chen et al., 2012). Fluorescein sodium salt (MW: 376.27), rhodamine 6G (MW: 479.01), and rhodamine B (MW: 479.01) were purchased from Sigma-Aldrich (St. Louis, United States) and used without further purification.

2.2 Sample preparation and fluorescence quenching experiments

Fluorescence quenching experiments were conducted in batch experiments. Stock solutions of the fluorescent dyes (330 μM) were prepared by dissolving the dyes separately in Milli-Q water (18.25 MΩcm). IHSS Suwannee River Humic Acid Standard II (2S101H) and Fulvic Acid Standard II (2S101F) were used as humic substances standards. The humic substances were dissolved in Milli-Q water, filtered through a 0.45-μm-pore-size membrane (A045A047A; Toyo Roshi Kaisya, Ltd., Tokyo, Japan) and stored separately as DHS stock solutions. Dissolved organic carbon (DOC) concentrations of the stock solutions were determined using a total organic carbon analyzer (TOC-L CSH; Shimadzu Corporation, Kyoto, Japan). Test solutions were prepared by adding 30 μL of the dye stock solution and an appropriate volume of the DHS stock solution into 200 μL of sodium phosphate buffer (10 mM, pH 6.9) in a
10-mL volumetric flask. The mixture was then diluted in Milli-Q water and mixed gently. Final concentrations of the dye and phosphate were 1 μM (0.38 mg/L for fluorescein and 0.48 mg/L for rhodamine 6G and rhodamine B) and 0.2 mM (19 mg-PO₄³⁻/L), respectively. The linearity of fluorescence intensities in these dye concentrations was confirmed (see figure S1 in supplementary data). The test solutions were incubated at room temperature under dark conditions for 1 h. The pH of the solutions was maintained after the incubation. The fluorescence and absorption spectra were measured using a fluorescence spectrophotometer (F-2700; Hitachi High-Technologies Corporation, Tokyo, Japan) and UV-Vis spectrophotometer (UV-1800; Shimadzu Corporation, Kyoto, Japan), respectively. Both the excitation and fluorescence slit widths were 5.0 nm. All experiments were performed in triplicate.

For the inner filter effect, measured fluorescence spectra were corrected. When DHS absorbed the excitation and emission light, the measured fluorescence spectra were corrected by equation 1 (Lakowicz, 2006):

$$F_{corr} = F_{obs} \frac{A_{ex} + A_{em}}{2}, \quad (1)$$

where $F_{corr}$ and $F_{obs}$ are the corrected and observed fluorescence intensity, respectively, and $A_{ex}$ and $A_{em}$ are the absorbance of the test solution at excitation and emission.
wavelengths, respectively.

The apparent fluorescence quantum yields were obtained by comparing the area under the observed fluorescence spectrum of the test solution with that of the solution of fluorescein in 0.1 M NaOH or rhodamine 6G in ethanol, which have a reported fluorescence quantum yield ($\Phi_R$) of 0.91 and 0.95, respectively (Valeur and Berberan-Santos, 2012). The fluorescence quantum yield ($\Phi_S$) for each test solution was calculated using equation 2 (Lakowicz, 2006):

$$\Phi_S = \Phi_R \times \frac{S_S}{S_R} \times \frac{A_R}{A_S} \times \left(\frac{\eta_S}{\eta_R}\right)^2,$$

where $\Phi$ is the quantum yield, $S$ is the integrated area of the corresponding fluorescence spectrum, $A$ is the absorbance at the excitation wavelength, $\eta$ is the refractive index of the solvent used, and $S$ and $R$ refer to the sample and the reference fluorescent dyes, respectively.

2.3 Binding analysis

We analyzed the binding interaction of fluorescent dyes and DHS using equation 3 and 7. The Stern–Volmer equation is expressed as (Gauthier et al., 1986):
\[
\frac{F_0}{F} = 1 + K_{doc}[DOC], \quad (3)
\]

where \(F_0\) and \(F\) are the fluorescence intensity of the fluorescent dye in the absence (\(F_0\)) and presence (\(F\)) of DHS, respectively, and \(K_{doc}\) is the binding coefficient. Plotting \(F_0/F\) against the DHS concentration ([DOC]) revealed a linear relationship between these values with an intercept of 1 and a slope that represents the value of \(K_{doc}\). Pan et al. (2007) demonstrated the limitations of the Stern-Volmer plot for obtaining the binding coefficients between phenanthrene and DOM because the binding coefficients can vary with the concentrations of free phenanthrene as well as different DOM concentrations. Other studies reported non-linear binding interactions between PAHs and DOM and their binding coefficients depends on free solute concentration (Laor and Rebhun, 2002; Borisover et al., 2006). Laor et al. (2002) suggested the Freundlich-type equation to explain the non-linear binding. Considering these limitations, we evaluated the binding coefficients of two dyes with humic and fulvic acid under same experimental condition in which the total dye concentrations were 1 \(\mu\)M and the range of DHS concentrations were 0 – 5 mg-C/L. These DOC concentrations were used considering natural river water in Japan. If non-linear binding is expected, equation 4 is applied:

\[
C_S = K_F C_{free}^n, \quad (4)
\]

where \(C_S\) is the DHS-bound fluorescent dye concentration (w/w), \(C_{free}\) is the free dye
concentration (w/v), and $K_F$ is the Freundlich coefficient. Equation 4 can be expressed as:

$$C_S = \frac{C_{\text{bound}}}{[DOC]} = \frac{C_{\text{total}} - C_{\text{free}}}{[DOC]} = K_F C_{\text{free}}^n, \quad (5)$$

where $C_{\text{bound}}$ and $C_{\text{total}}$ are the bound and total dye concentrations (w/v), respectively.

Equation 5 can be rearranged as:

$$\frac{C_{\text{total}}}{C_{\text{free}}} = 1 + K_F [DOC] C_{\text{free}}^{n-1}. \quad (6)$$

The fluorescent intensity of a dye is proportional to its free concentration. Therefore, equation 6 can be rearranged as follows:

$$\frac{F_0}{F} = 1 + K_F' [DOC] F^{n-1}. \quad (7).$$

We calculated the values of $K_F'$ and $n$ and obtained a non-linear model by Origin Pro 9.1J software. $K_F'$ was modified to $K_F$ by a factor of $f^{n-1}$. The $f$ value is the specific fluorescence activity of a fluorescent dye, which was given by fluorescence intensity divided by its concentration.

3. Results and discussion

Figure 2 shows the absorption spectra of fluorescein and rhodamine 6G in the presence of humic acid or fulvic acid. Fluorescein and rhodamine 6G exhibited strong absorption bands with large molar absorption coefficients around 489 nm ($\varepsilon = 5.3 \times 10^4 \text{ L mol}^{-1}$...
cm$^{-1}$) and 526 nm ($\varepsilon = 8.5 \times 10^4$ L mol$^{-1}$ cm$^{-1}$), respectively. In the wavelength region below 450 nm, absorbance gradually increased with increasing DOC concentrations due to chromogenic dissolved organic matter (CDOM; Zhang et al., 2009). Interestingly, the absorption peak of rhodamine 6G at 526 nm shifted significantly to 536 nm upon addition of humic acid (Fig. 2-(c)). This peak shift indicates that the mechanism of binding interaction between rhodamine 6G and humic acid is different from that of fluorescein-DHS complexes and rhodamine 6G-fulvic acid complex.

The fluorescence spectra of fluorescein and rhodamine 6G in the presence of humic acid or fulvic acid are shown in Fig. 3. The inner filter effect correction factors were around 1.1 for all test solutions (data not shown). This value indicated that the inner filter effects of DHS were negligible because the excitation and emission bands of DHS did not overlap with those of fluorescent dyes (Henderson et al., 2009), and we prepared the test solutions of the dyes with the absorbance under 0.1. As shown in Fig. 3, fluorescein and rhodamine 6G produced sharp fluorescence bands with high fluorescence quantum yields around 512 nm ($\Phi = 0.82$) and 551 nm ($\Phi = 0.86$), respectively. Fluorescence quenching was observed with increasing DHS concentrations, with the fluorescence of rhodamine 6G strongly decreasing upon addition of humic acid (Fig. 3-(c)). Apparent
fluorescence quantum yields of fluorescein and rhodamine 6G in the presence of humic acid or fulvic acid (5 mg-C/L) are listed in Table 1. Fluorescence quantum yields also decreased in response to the addition of DHS. Among them, the quantum yield of rhodamine 6G decreased significantly from 0.86 to 0.10 in response to the addition of humic acid. These results also indicate a strong fluorescence quenching effect of humic acid on rhodamine 6G.

The Stern–Volmer plots of the fluorescent dyes are shown in Fig. 4. Linear relationships were observed for fluorescein with humic and fulvic acids and rhodamine 6G with fulvic acid. The $K_{\text{doc}}$ values are summarized in Table 2. The $K_{\text{doc}}$ value of rhodamine 6G with fulvic acid ($1.34 \times 10^{-1}$ L/mg-C) is slightly larger than that of fluorescein with humic and fulvic acids ($7.57 \times 10^{-2}$ L/mg-C and $1.00 \times 10^{-1}$ L/mg-C, respectively). Conversely, a non-linear curved plot towards the y-axis was obtained for rhodamine 6G with humic acid. Different concentration of rhodamine 6G also resulted in the non-linearity (see figure S2 in supplementary data). Some studies have attributed the non-linear binding to different causes. For example, combination of static and dynamic quenching could result in the non-linear plot (Pan et al., 2007). Another study also attributed the non-linear Stern-Volmer plot to the inherent heterogeneity of DOM (Laor...
and Rebhun, 2002). We tried to evaluate the Stern-Volmer constant ($K_{SV}$) and the stability constant ($K_S$) by using the equation considering static and dynamic quenching. However, we could not get those values from the equation (see figure S3 in supplementary data). Therefore, we concluded that static quenching was the primary mechanism and we applied the Freundlich-type equation to explain the non-linear binding. Previous researchers also often excluded dynamic quenching. The Freundlich coefficient ($K_F$) of rhodamine 6G with humic acid was $7.84 \times 10^{-1}$ L/mg-C and the $n$ value was 0.67. This strong quenching of rhodamine 6G with humic acid may indicate the electrostatic interaction between cationic rhodamine 6G and anionic groups of humic acid. Furthermore, this interaction could be strengthened by the hydrophobic repulsion of less polar moieties from aqueous bulk. As compared with fulvic acid, the increased interaction between rhodamine 6G and humic acid is in well agreement with the significance of less hydrated hydrophobic moieties in its components.

Rhodamine B was also used for fluorescence quenching experiments with DHS. Figure 5 shows the Stern–Volmer plot of rhodamine B with DHS. The $K_{doc}$ values for humic and fulvic acids were $4.11 \times 10^{-2}$ L/mg-C and $1.12 \times 10^{-2}$ L/mg-C, respectively. Interestingly, the non-linear binding was observed for rhodamine 6G but not for
rhodamine B. As compared with rhodamine 6G, rhodamine B has carboxylic group. The carboxylic group was supposed to be ionized under the experimental pH condition and the presence of ionized carboxylic group resulted in a greater aqueous solubility of the dye as well as in an enhanced electrostatic rejection from DHS. In addition, the fact that rhodamine B does not donate H-bond from its diethyl amino groups may also reduce the binding affinity to DHS compared with rhodamine 6G.

4. Conclusions

In this study, the binding interactions between oppositely charged fluorescent dyes (fluorescein and rhodamine 6G) and DHS (dissolved humic and fulvic acid) were investigated by the fluorescence quenching method. The fluorescent intensities of dyes decreased in response to the addition of DHS, and the binding coefficients were obtained from the Stern–Volmer plots. Among them, the fluorescence of rhodamine 6G was strongly quenched by humic acid and the non-linear Stern–Volmer plot was obtained. This strong quenching may be caused by the electrostatic interaction between cationic rhodamine 6G and anionic groups of humic acid. Then, the interaction could be strengthened by the hydrophobic repulsion of less polar moieties from aqueous bulk. As
compared with rhodamine 6G, the quenching effects and binding coefficients of fluorescein with dissolved humic substances were relatively weak. These results would contribute to the practice of selecting suitable fluorescent dye tracers in aquatic and hydrology studies.

Acknowledgments

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Figure and table captions

Fig. 1 Chemical structures of fluorescein, rhodamine 6G, and rhodamine B.

Fig. 2 Absorption spectra of fluorescein (a, b) and rhodamine 6G (c, d) with increasing DOC concentrations of humic acid or fulvic acid. The spectra were taken at DOC concentrations of 0, 0.5, 1, 2, and 5 mg-C/L. The concentration of the fluorescent dye was 1.0 μM (0.38 mg/L for fluorescein and 0.48 mg/L for rhodamine 6G).

Fig. 3 Fluorescence spectra of fluorescein (a, b) and rhodamine 6G (c, d) with increasing DOC concentrations of humic acid or fulvic acid. The spectra were taken at DOC concentrations of 0, 0.5, 1, 2, and 5 mg-C/L. The concentration of the fluorescent dye was 1.0 μM (0.38 mg/L for fluorescein and 0.48 mg/L for rhodamine 6G). The excitation wavelengths of fluorescein and rhodamine 6G were 480 nm and 520 nm, respectively.

Fig. 4 Stern-Volmer plots for fluorescein (a) and rhodamine 6G (b) with increasing DOC concentrations of humic acid or fulvic acid. Fluorescence intensities of fluorescein
and rhodamine 6G were plotted at 512 nm and 551 nm, respectively. Solid lines represent linear relationships. A non-linear relationship is shown as a dashed line.

Fig. 5 Stern–Volmer plots for rhodamine B with increasing DOC concentrations of humic acid or fulvic acid. Fluorescence intensities of rhodamine B were plotted at 574 nm.

Table 1 Apparent fluorescent quantum yields of fluorescein and rhodamine 6G in the presence of DHS (5 mg-C/L).

Table 2 $K_{\text{doc}}$, Intercept, $K_F$, n, and $r^2$ value obtained in this study.
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<table>
<thead>
<tr>
<th>Fluorescent dye</th>
<th>DHS</th>
<th>Φ</th>
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<tbody>
<tr>
<td>Fluorescein</td>
<td>–</td>
<td>0.82 ± 0.01</td>
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<td>Humic acid</td>
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<td>Fulvic acid</td>
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<tr>
<td>Rhodamine 6G</td>
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</tr>
<tr>
<td>Humic acid</td>
<td>0.10 ± 0.00</td>
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<tr>
<td>Fulvic acid</td>
<td>0.58 ± 0.02</td>
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</table>
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<table>
<thead>
<tr>
<th>Fluorescent dye</th>
<th>DHS</th>
<th>$K_{doc}$ (L/mg-C) (= Slope)</th>
<th>Intercept</th>
<th>$K_F$ (L/mg-C)</th>
<th>$n$</th>
<th>$r^2$</th>
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<tbody>
<tr>
<td>Fluorescein</td>
<td>Humic acid</td>
<td>$7.57 \times 10^{-2}$ ($\pm 1.60 \times 10^{-3}$)</td>
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<td>$-$</td>
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<tr>
<td>Rhodamine 6G</td>
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<td>$-$</td>
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<td>$0.67$ ($\pm 0.090$)</td>
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<tr>
<td></td>
<td>Fulvic acid</td>
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<td>$-$</td>
<td>$-$</td>
<td>$0.998$</td>
</tr>
</tbody>
</table>
Fluorescein

Rhodamine 6G

Rhodamine B

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