© 2021 The Authors
344

Water Practice & Technology Vol 16 No 2
doi: 10.2166/wpt.2021.001

Empirical equation for the correction of fluorescence quenching of proteinaceous substance by Suwannee river natural organic matter

Kornravee Saipetch, Rajendra Khanal* and Chihiro Yoshimura

Department of Civil and Environmental Engineering, School of Environment and Society, Tokyo Institute of Technology, 2-12-1-M1-4, Ookayama, Meguro-ku, Tokyo, 152-8552, Japan

*Corresponding author. E-mail: khanal.r.aa@m.titech.ac.jp

Abstract

Fluorescence quenching of proteinaceous substances by natural organic matter is a well-known phenomenon, but there are no known methods for correcting it. The main objective of this research was to develop an empirical equation to correct the fluorescence quenching of different concentrations of bovine serum albumin (BSA – 0.15, 0.25, 0.5, 0.75, 1, 1.25 μmol/L (μM)) by Suwannee river natural organic matter (SWNOM - 0,2,4,6,8,10 mg-C/L) using the fluorescence titration method. The excitation emission matrix (EEM) data were analyzed by parallel factor analysis with inner filter effect removal. With increasing SWNOM concentration, BSA peak intensity quenching was in the range 29–85%, with a linear relationship for increment of either BSA or SWNOM concentration. A higher ratio of SWNOM to BSA resulted in greater BSA peak intensity quenching. The unquenched BSA peak (BSA (RU)) is given by the empirical equation.

$$\text{BSA (RU)} = \{2.052 \times \text{peak C (RU)} + 2.522\} \times \text{quenched peak T (RU)}^{0.624}$$

The calculated unquenched BSA peak intensities using the empirical equation agreed well with the actual unquenched peak values ($R^2 = 0.98$, mean absolute error = 0.33 RU). The equation is expected to help in rapid estimation of the quenching effect of SWNOM on BSA.

Key words: bovine serum albumin, dissolved organic matter, fluorescence titration, PARAFAC, protein-humic substance complex, excitation emission matrix

Highlights

- Empirical equation to correct the fluorescence quenching of bovine serum albumin by Suwannee river natural organic matter is discussed.
- The EEM data was analyzed by parallel factor analysis with inner filter effect removal.
- With an increase in SWNOM concentration, quenching of BSA peak intensity was in the range 29–85%.
- Higher ratio of SWNOM to BSA resulted in higher quenching of the peak intensity of BSA.

INTRODUCTION

Fluorescence quenching (quenching) is one of the commonest phenomena in fluorescence analysis, whereby any component (quencher) present in a system or related physico-chemical process reduces the intensity of fluorescence of a sample (fluorophore) (Lakowicz 1983; Eftink 1991).

Water Practice & Technology Vol 16 No 2
doi: 10.2166/wpt.2021.001

Quenching mechanisms include excited-state reactions, molecular rearrangement, energy transfer, ground-state complex formation, and collisional quenching. Collisional quenching can be further divided into static and dynamic quenching. Although there are numerous studies on quenching mechanisms (Yuan *et al.* 1998; London & Ladokhin 2002; Soares *et al.* 2007; Keppler *et al.* 2014; Joye *et al.* 2015; Wang *et al.* 2015; Heo *et al.* 2016; Ali & Al-Lohedan 2018), an empirical method for quenching correction to enable calculation of the actual concentration of proteinaceous fluorophore substances quenched by humic substances in a sample matrix is still lacking. In earlier studies, the post-chlorination fluorescence for trihalomethane formation potential was found to be quenched by humic substances, leading to reduced accuracy in predicting trihalomethane formation in water samples (Saipetch & Yoshimura 2019). In another experiment, multi-spectroscopic studies revealed static quenching between humic and protein-like substances arising from rearrangement of the secondary polypeptide carbonyl hydrogen bonding network of BSA (Saipetch *et al.* submitted).

In water sources, the amount of each component in dissolved organic matter (DOM) varies widely (Carstea *et al.* 2016; Singh *et al.* 2017). The nature of the various DOMs affects the extent of fluorescence quenching, but no previous study has been designed to investigate the effect of DOM concentration on the level of quenching (Wang *et al.* 2015; Lin *et al.* 2017). Saipetch *et al.* (submitted) found that the protein fluorescence of bovine serum albumin (BSA) is quenched by interaction with Suwanee River natural organic matter (SWNOM), indicating the possibility that the concentration of each of BSA and SWNOM can influence the quenching uniquely. No study has yet demonstrated the correction accuracy of quenching by BSA and SWNOM using empirical methods, however.

The relationships between BSA and SWNOM concentrations on quenching were investigated in this study, leading to the proposal and verification of a method for correcting quenching.

MATERIALS AND METHODS

Fluorescence titration was carried out between varying concentrations of SWNOM (0, 2, 4, 6, 8, 10 mg-C/L), and BSA (0.15, 0.25, 0.5, 0.75, 1, 1.25 μmol/L (μM)) was performed. The concentrations of SWNOM and BSA were equivalent to NOM concentrations found in surface water samples. The excitation emission matrix (EEM) data were analyzed using parallel factor analysis with inner filter effect removal (drEEMtoolbox in MATLAB – Murphy *et al.* 2013). The effect of the BSA and/or SWNOM concentration on BSA's quenched fluorescence intensity was investigated using non-linear regression, on the basis of the hypothesis that the fluorescence intensity of BSA (unquenched BSA fluorescence, peak T location (Figure 1(a2))) in the absence of SWNOM is a function of BSA's quenched intensities in the presence of SWNOM, and the fluorescence intensity of SWNOM alone. (Saipetch & Yoshimura 2019; Saipetch *et al.* submitted).

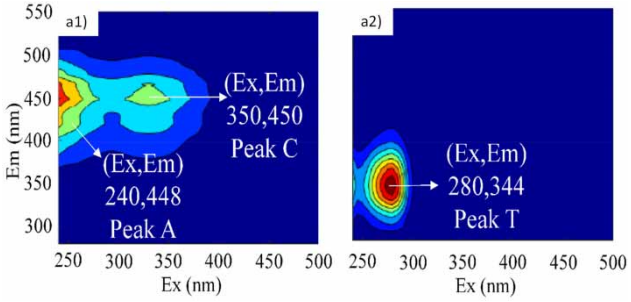

Figure 1 | PARAFAC components of fluorescence titration between – (a1) BSA and SWNOM (peaks A and C); and (a2) fluorescence intensity of BSA only (in the absence of SWNOM) (peak T).

Water Practice & Technology Vol 16 No 2
doi: 10.2166/wpt.2021.001

RESULT AND DISCUSSION

Impact of SWNOM concentration on BSA fluorescence intensity

PARAFAC components of fluorescence titration between BSA–SWNOM mixtures and BSA alone are shown in Figure 1(a1) and 1(a2). If the inner filter effect is removed by PARAFAC, three distinct peaks, A (Ex/Em 240/448), C (Ex/Em 350/450), and T (Ex/Em 280/344) appear. A clear overlap of BSA and SWNOM fluorescence intensity (Figure 2(a1)) is visible in the emission wavelength range 330–450 nm. All three peaks – A, C and T – are within this wavelength range – Figures 1 and 2(a1). An increase in BSA concentration led to a distinct increase in peak T's fluorescence

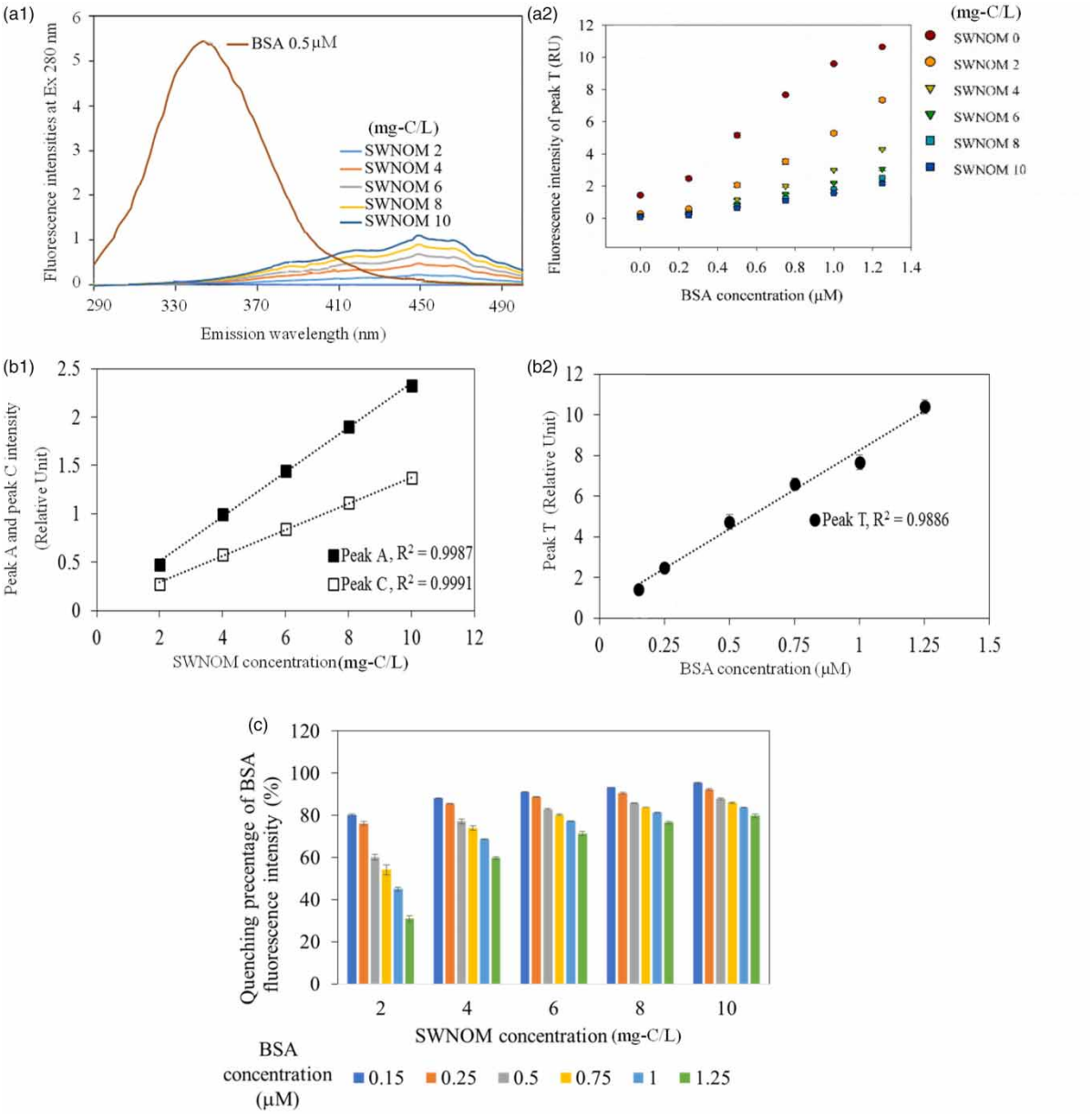

Figure 2 | (a1) Overlapping fluorescence spectrum of SWNOM and BSA, (a2) fluorescence peak intensity of BSA in the presence of varying concentrations of SWNOM; (b) relationship between fluorescence peak intensity and varying concentrations of (b1) SWNOM, and (b2) BSA. (c) Quenching percentage of BSA fluorescence intensity at different concentrations of BSA and SWNOM.

Water Practice & Technology Vol 16 No 2
doi: 10.2166/wpt.2021.001

intensity (Figure 2(a2)). The quenching effect of increases in SWNOM concentration can also be seen in the BSA peak intensity (Figure 2(a2)). There was a linear relationship between SWNOM concentration and the fluorescence intensity of peaks A ($R^2 = 0.9987$) and C ($R^2 = 0.9991$) (Figure 2(b1)), and peak T's fluorescence intensity ($R^2 = 0.9886$) with BSA concentration (Figure 2(b2)).

The fluorescence intensities of BSA at peak T, in the absence of SWNOM, and of SWNOM at peak C were used to represent the concentrations of BSA and SWNOM due to their linear relationship (Figure 2(b1) and 2(b2)). As the SWNOM concentration increased, the quenching of BSA's peak T was between 29 and 85% (Figure 2(c)), and the relationship was linear for every concentration increment of either BSA or SWNOM. Hence, the hypothesis that BSA's fluorescence intensity in the absence of SWNOM is a function of the intensities of quenched fluorescence of BSA in the presence of SWNOM and the fluorescence intensity of SWNOM alone can be said to be true.

Empirical equation for the quantitative impact of SWNOM concentration on BSA fluorescence intensity

Higher ratios of quencher (SWNOM) to proteinaceous fluorophore (BSA) resulted in greater quenching of the intensity of BSA's peak T. The effects of BSA and SWNOM concentration on the quenched and unquenched intensities of BSA's peak T are shown in Figure 3. There were linear relationships between BSA peak intensity and the concentrations of either BSA or a BSA-SWNOM mixture (Figure 3(b)). At the same initial concentration of BSA, an increase in SWNOM concentration lowered the fluorescence intensity of peak T linearly (Figure 3). At fixed SWNOM concentration, however, an increase in BSA concentration reduced the quenching of BSA's peak T. The relationship between the quenched intensity of peak T, and the concentration of either BSA or the BSA-SWNOM mixture, can be explained by the power function Equation (1) ($R^2 = 0.71$).

$$T\ (RU) = \{-0.4367 \times SWNOM(mg - C/L)\} + 5.4806) \times BSA\ concentration\ (\mu M)^{1.5437} \quad (1)$$

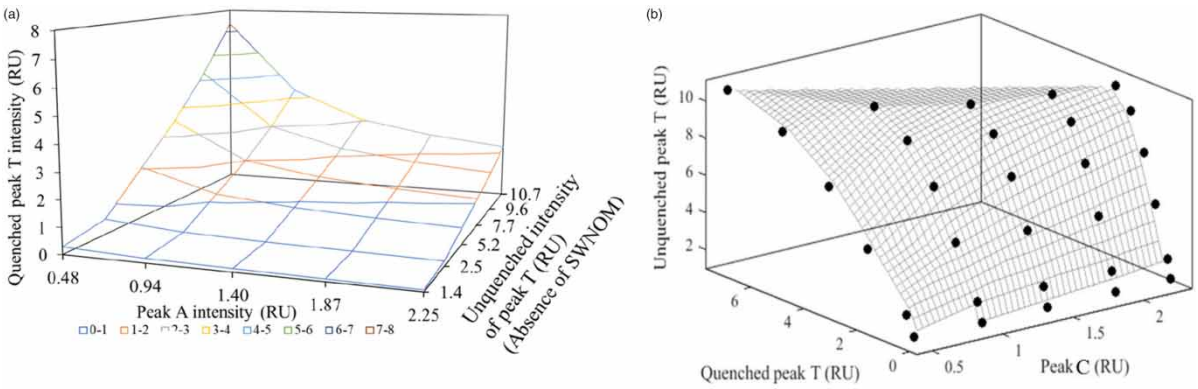

Figure 3 | (a) Effect of BSA and SWNOM concentration on peak T's quenched intensity. BSA concentration presented in terms of peak T's unquenched intensity, T (RU); SWNOM concentration in terms of peak C (RU). (b) Relationship between BSA's unquenched peak T and its quenched peak T and peak C (the grid pane is for visualization of the data pattern).

Correction of fluorescence quenching by SWNOM

In order to develop the correction method, the term α was defined to represent the ratio between BSA's unquenched and quenched peak Ts, and assumed to be a function of quenched peak T intensity and peak A intensity (Equation (2)). The plots derived using Equation (2) are shown in Figure 4(a). which shows clearly that α is a power function of quenched peak T. The values of the constants in

Water Practice & Technology Vol 16 No 2
doi: 10.2166/wpt.2021.001

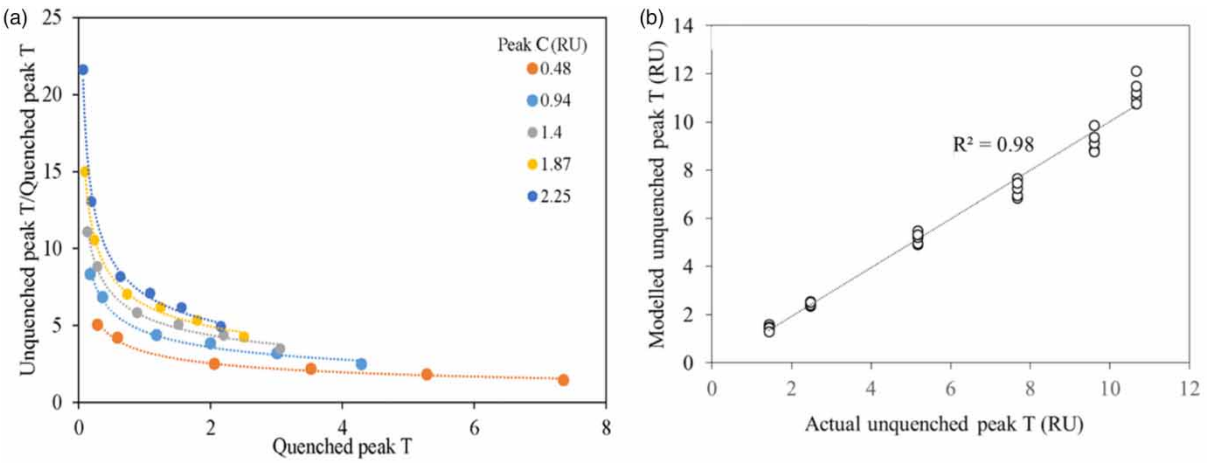

Figure 4 | (a) Relationship between unquenched peak T/quenched peak T vs. quenched peak at different peak C levels, (b) modelled vs. actual unquenched peak T fluorescence intensity.

Equation (2) were derived by selecting the empirical values of the slope (a) and constant (b) from Figure 4(a) – Table 1. It is to be noted that peak C is the direct representative of SWNOM concentration, as the intensity of SWNOM's peak C is not quenched by BSA addition.

$$\propto = \frac{\text{Unquenched peak T}}{\text{Quenched peak T}} = \text{f(quenched peak T, peakA)} = \text{a} \times \text{quenched peak T}^{\text{b}} \quad (2)$$

where a and b are constants.

Table 1 | Values of empirical constants for correcting fluorescence quenching by SWNOM

Peak C (relative unit)	a	B
0.48	3.3006	−0.375
0.94	4.6083	−0.362
1.4	5.5677	−0.350
1.87	6.3514	−0.364
2.25	7.0224	−0.402

Values of both the empirical constants 'a' and 'b' changed with an increase in peak C's relative intensity (Table 1). Coefficient 'a' was a linear function of peak C, whereas 'b' was a third-order of a polynomial function. Application of a polynomial function of peak C for 'b' was considered unnecessary as it produced no improvement in R^2 between the modelled and actual fluorescence intensities of unquenched peak T compared to the use of an average value for 'a'. The calculated intensities of unquenched peak T also agreed well with its actual intensities ($R^2 = 0.98$ and mean absolute error 0.33 RU (Figure 4(b))).

At lower intensities of unquenched peak T fluorescence (1.4–5.7 RU), the variation in modelled unquenched peak T was lower than that at unquenched intensities of 7.7–10.7 RU. The fluorescence intensities in the lower range are comparable to the protein fluorescence intensity of clean water (Tama River, Japan), while intensities higher than that can be considered to arise from increases in organic quencher concentrations from wastewater.

Use of Equation (3) is expected to improve the quality of estimates of the unquenched intensity of peak T in surface waters. The proposed empirical correction equation (Equation (3)) is also expected

Water Practice & Technology Vol 16 No 2
doi: 10.2166/wpt.2021.001

to be applicable to various types of protein-related compound other than BSA, as BSA is the standard used to represent substances like algae cell proteins (Hong *et al.* 2009; Wei *et al.* 2011), cyanobacterium (*Microcystis aeruginosa*) (Pivokonsky *et al.* 2015) and membrane foulants (Guan *et al.* 2018).

$$\text{T (RU)} = \{2.052 \times \text{peak C (RU)} + 2.522\} \times \text{quenched peak T (RU)}^{0.624} \quad (3)$$

CONCLUSIONS

The impact of natural organic matter (SWNOM) concentrations on the fluorescence quenching of a proteinaceous substance (BSA) was studied using fluorescence titration. Increasing the concentration of SWNOM increased its quenching effect on BSA fluorescence intensity, and the magnitude of the quenching was proportional to the SWNOM/BSA ratio in solution. At fixed BSA concentrations, a linear increase in SWNOM concentration lowered the intensity of BSA's peak T fluorescence, while, at fixed SWNOM concentration, an increase in BSA concentration reduced the fluorescence quenching level in line with a power function. When used to correct for fluorescence quenching, Equation (3) fitted well with the actual unquenched and modelled intensities of peak T (Figure 4).

ACKNOWLEDGEMENTS

This study was supported financially by the Ministry of the Environment of Japan [S-13-2-3] and JSPS KAKENHI [grant number 18H01566].

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

REFERENCES

Ali, M. S. & Al-Lohedan, H. A. 2018 Spectroscopic and computational evaluation on the binding of safranal with human serum albumin: role of inner filter effect in fluorescence spectral correction. *Spectrochim. Acta, Part A* **203**, 434–442.

Carstea, E. M., Bridgeman, J., Baker, A. & Reynolds, D. M. 2016 Fluorescence spectroscopy for wastewater monitoring: a review. *Water Res.* **95**, 205–219. doi:10.1016/j.watres.2016.03.021.

Eftink, M. R. 1991 Fluorescence quenching reactions. In: *Biophysical and Biochemical Aspects of Fluorescence Spectroscopy* (Dewey, T. G., ed.). Springer, Boston, MA, USA. https://doi.org/10.1007/978-1-4757-9513-4_1

Guan, Y. F., Qian, C., Chen, W., Huang, B. C., Wang, Y. J. & Yu, H. Q. 2018 Interaction between humic acid and protein in membrane fouling process: a spectroscopic insight. *Water Res.* **145**, 146–152. doi:10.1016/j.watres.2018.08.019.

Heo, J., Yoon, Y., Kim, D. H., Lee, H., Lee, D. & Her, N. 2016 A new fluorescence index with a fluorescence excitation-emission matrix for dissolved organic matter (DOM) characterization. *Desalin. Water Treat.* **57**(43), 20270–20282.

Hong, H. C., Wong, M. H. & Liang, Y. 2009 Amino acids as precursors of trihalomethane and haloacetic acid formation during chlorination. *Arch. Environ. Contam. Toxicol.* **56**, 638–645. doi:10.1007/s00244-008-9216-4.

Joye, I. J., Davidov-Pardo, G., Ludescher, R. D. & McClements, D. J. 2015 Fluorescence quenching study of resveratrol binding to zein and gliadin: towards a more rational approach to resveratrol encapsulation using water-insoluble proteins. *Food Chem.* **185**, 261–267.

Keppler, J. K., Stuhldreier, M. C., Temps, F. & Schwarz, K. 2014 Influence of mathematical models and correction factors on binding results of polyphenols and retinol with β-lactoglobulin measured with fluorescence quenching. *Food Biophys.* **9**(2), 158–168.

Lakowicz, J. R. 1983 Quenching of fluorescence. In: *Principles of Fluorescence Spectroscopy* (Lakowicz, J. R., ed.). Springer, Boston, MA, USA, pp. 257–301. https://doi.org/10.1007/978-0-387-46312-4_8.

Water Practice & Technology Vol 16 No 2
doi: 10.2166/wpt.2021.001

Lin, T., Hou, B., Wang, J., Xu, Y. & Chen, W. 2017 Characterization of interaction between amino acids and fulvic-like organic matter by fluorescence spectroscopy combining thermodynamic calculation. *Environ. Sci. Pollut. Res.* **24**, 7226–7235. doi:10.1007/s11356-017-8444-4.

London, E. & Ladokhin, A. S. 2002 Measuring the depth of amino acid residues in membrane-inserted peptides by fluorescence quenching. *Curr. Top. Membr.* **52**, 89–115.

Murphy, K. R., Stedmon, C. A., Graeber, D. & Bro, R. 2013 Fluorescence spectroscopy and multi- way techniques. PARAFAC. *Anal. Methods* **5**, 6557. doi:10.1039/c3ay41160e.

Pivokonsky, M., Naceradska, J., Brabenec, T., Novotna, K., Baresova, M. & Janda, V. 2015 The impact of interactions between algal organic matter and humic substances on coagulation. *Water Res.* **84**, 278–285. doi:10.1016/j.watres.2015.07.047.

Saipetch, K. & Yoshimura, C. 2019 Importance of correcting for fluorescence quenching in fluorescence-based prediction of trihalomethane formation potential. *Water Supply* **19**(6), 1677–1685.

Saipetch, K., Khanal, R., Yamazaki, M., Qing-Long, F., Yoshimura, C. & XinKai, J. submitted Fluorescence quenching mechanism of amino acids and protein with natural organic matter by multi-spectroscopic method. *Water Supply* (submitted – November 2020).

Singh, S., Dash, P., Silwal, S., Feng, G., Adeli, A. & Moorhead, R. J. 2017 Influence of land use and land cover on the spatial variability of dissolved organic matter in multiple aquatic environments. *Environ. Sci. Pollut. Res.* **24**, 14124–14141. doi:10.1007/s11356-017-8917-5.

Soares, S., Mateus, N. & de Freitas, V. 2007 Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary α-amylase (HSA) by fluorescence quenching. *J. Agric. Food. Chem.* **55**(16), 6726–6735.

Wang, Z., Cao, J. & Meng, F. 2015 Interactions between protein-like and humic-like components in dissolved organic matter revealed by fluorescence quenching. *Water Res.* **68**, 404–413. doi:10.1016/j.watres.2014.10.024.

Wei, Y. Y., Liu, Y., Dai, R. H., Liu, X., Wu, J. J., Shi, Z., Ren, J. & Zhang, Y. 2011 Trihalomethanes and haloacetic acid species from the chlorination of algal organic matter and bromide. *Water Sci. Technol.* **63**, 1111–1120. doi:10.2166/wst.2011.348.

Yuan, T., Weljie, A. M. & Vogel, H. J. 1998 Tryptophan fluorescence quenching by methionine and selenomethionine residues of calmodulin: orientation of peptide and protein binding. *Biochemistry* **37**(9), 3187–3195.

First received 17 November 2020; accepted in revised form 19 December 2020. Available online 2 January 2021