



Article Binding of Calcium and Magnesium Ions to Terrestrial Chromophoric Dissolved Organic Matter (CDOM): A Combination of Steady-State and Time-Resolved Fluorescence Study

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Abstract: Revealing the binding properties of calcium ion (Ca²⁺) and magnesium ion (Mg²⁺) to terrestrial chromophoric dissolved organic matter (CDOM) facilities understanding the effect of natural water components on the photophysics of dissolved organic matter. Steady-state and time-resolved fluorescence spectrometry, and dynamic light scattering were applied to investigate the fluorescence quenching process of CDOM by Ca²⁺ and Mg²⁺. Due to a remarkable decrease of the steady-state fluorescence intensity and a slight decrease of fluorescence lifetime, the fluorescence quenching of CDOM by cations mainly occurred through a static process. The fluorescence quenching was profound under longer excitation and emission wavelengths. The binding constant (*K*, L/mol) for Ca²⁺ to CDOM ranged from 4.29 to 5.09 (lg*K*), which was approximately one order of magnitude higher than that of Mg²⁺ to CDOM (3.86 to 4.56). Nevertheless, the efficiency of CDOM fluorescence quenching by Ca²⁺, Mg²⁺ was much lower than that by Cu²⁺. Fluorescence decay became faster in the presence of a high concentration of Ca²⁺ (>20 mg/L) and Mg²⁺ (>50 mg/L). In the presence of these two metal ions, particularly for Ca²⁺, the lifetime of CDOM mainly occurred through the interaction of Ca²⁺ /Mg²⁺ with relatively long-lived fluorophores.

Keywords: CDOM; calcium; magnesium; fluorescence quenching; lifetime distribution

1. Introduction

Natural organic matter (NOM) is ubiquitous in surface water. NOM plays a key role in biological and chemical processes because of the competition for solar irradiance and the reactive species generated upon irradiation [1–5]. Metal ions tend to bind to NOM, mainly through the phenolic and carboxylic functional groups [6]. The binding of metal ions to NOM not only altered the bioavailability of metal ions, but also affected the photophysical and photochemical properties of NOM [5,7,8].

Copper ion (Cu^{2+}) [9–11] and mercury ion (Hg^{2+}) [12,13] were the most frequently investigated B-type metal ions. Previous studies demonstrated that binding constants between Cu^{2+} and NOM, and Hg^{2+} and NOM, could be up to 10^6 L/mol [14,15], leading to a decreased fluorescence intensity of NOM. The binding constants were dependent on NOM source (structure) and experimental conditions such as solution pH and salinity [9,16,17]. The competition of co-existing cations for binding sites in NOM was used to explain the inhibition effect of salinity on the binding of metal ions to NOM [9,18–20]. For example, the presence of up to 11×10^{-6} mol/L Ca²⁺ broke the Hg–NOM complex and promoted the fluorescence intensity, whereas Ca²⁺ exhibited no apparent effect on NOM fluorescence intensity [19]. On the contrary, Gao et al. reported that Ca²⁺ could bind to Suwannee River humic acid (SRHA) accompanied by the replacement of protons in the phenolic or carboxylic functional group [21]. However, the binding properties of alkaline earth metals



Citation: Liu, J.; Zhou, R.; Zhang, X. Binding of Calcium and Magnesium Ions to Terrestrial Chromophoric Dissolved Organic Matter (CDOM): A Combination of Steady-State and Time-Resolved Fluorescence Study. *Water* **2021**, *13*, 2182. https:// doi.org/10.3390/w13162182

Academic Editor: Micòl Mastrocicco

Received: 30 June 2021 Accepted: 5 August 2021 Published: 9 August 2021

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Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). such as Ca^{2+} and Mg^{2+} to NOM have not been fully documented, although they are the most common hard metal ions in natural water.

Notably, the binding properties of metal ions to NOM were determined by the Excitation–Emission Matrix (EEM) and followed by parallel factor analysis (PARAFAC). Static quenching through the formation of the metal–NOM complex was proposed to explain the fluorescence quenching. However, the knowledge about the effects of metal ions on NOM's fluorescence lifetime was still quite limited [22]. In a previous study [23], we found that fluorophores with a relatively short lifetime (<1 ns) dominated CDOM (the major component of NOM)-excited states, while these short-lived excited states could not be well documented by steady-state fluorescence because of their minor contribution. Nevertheless, certain fluorophores had a relatively long lifetime (approximately 10 ns), and collision quenching would not be excluded in the presence of millimolar level metal ions. Therefore, the investigation of the effects of metal ions on CDOM or other NOM.

In this study, interactions between Ca^{2+}/Mg^{2+} and terrestrial source CDOM were systematically investigated by steady-state and time-resolved fluorescence spectrometry. The first objective was to quantify fluorescence-quenching extents and determine the binding constant. The second objective was to reveal the mechanism for fluorescence quenching based on the effects of Ca^{2+} and Mg^{2+} on the quenching of steady-state fluorescence intensity and fluorescence lifetime and the change of CDOM dispersity in H₂O.

2. Materials and Methods

2.1. Chemicals

Analytical grade $CaCl_2$ and $MgSO_4$ were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and used as received. CDOM reference materials, Elliott Soil HA Standard IV (ESHA, Lot 5S102H) and Pahokee Peat Humic Acid Standard (PPHA, Lot 1S103H) were purchased from the International Humic Substance Society. CDOM was added to pH 10.0 water and stirred for two hours. Then, the pH of CDOM solutions was adjusted to 7.0 and filtered by a 0.22 μ m nylon membrane (Shanghai Xingya Purification material Factory, Shanghai, China). All the CDOM stock solutions were stored in the dark at 4 °C and used within one month. The concentrations of CDOM stock solutions were analyzed on an Elementa total organic carbon analyzer (Vario TOC; Elementar, Langenselbold, Germany).

2.2. Experimental Procedure

Working solutions of CDOM (10 mg C/L), Ca^{2+} (0–30 mg/L), and Mg²⁺ (0–70 mg/L) were prepared by dilution. The resulting solutions were shaken at 25 ± 0.2 °C for at least 12 h to reach binding equilibrium. The pH values of the working solutions were adjusted to 7.5 by phosphate buffer. The ionic strength was adjusted to 20 mM for all working solutions.

2.3. Spectra Recording Method

Absorption spectra: The absorption spectra were recorded with an ultraviolet (UV)-visible spectrometer (UV 2550; Shimadzu, Tokyo, Japan) at 25 ± 0.5 °C. The optical path length of the cuvette was 1 cm.

Steady-state fluorescence spectra of CDOM solutions: Steady-state fluorescence spectra of CDOM were recorded with either an Edinburgh FS-5 or a Horiba FluoroLog-3 fluorimeter. The excitation (Ex) and emission (Em) wavelength range for the excitation—emission matrix (EEM) fluorescence spectra of CDOM in the absence and presence of Ca^{2+} and Mg^{2+} were 250–600 nm and 270–750 nm, respectively. The step size was 5 nm and 2 nm for Ex wavelength and Em wavelength, respectively. The slit size was 5 nm. The dwell time was 0.100 s. Fluorescence intensity was corrected using factory-measured correction files. Ultrapure water was used for background and scatter subtraction, and the inner-filter effect was also corrected.

To get a more precise fluorescence quenching efficiency, the single emission profile (400–800 nm, increments of 1 nm, dwell time was 0.100 s) was recorded at excitation wavelengths of 375, 440, and 550 nm, and the intensity correction followed the same protocol for EEM fluorescence spectra.

Time-resolved fluorescence spectra of CDOM solutions: Time-resolved fluorescence spectra of CDOM were measured with FluoTime 200 equipment (PicoQuant GmbH, Berlin, Germany) at 25 ± 1 °C. The excitation source was a picosecond laser diode (LDH-P-C-375B for 375 nm and LDH-P-C-440B for 440 nm). The fluorescence decay of CDOM was detected using a PMA hybrid 07 detector and recorded in a Picoharp 300 time-correlated single-photon counter. The maximum intensity of CDOM was 10,000 counts, and the counting rate was less than 1% of the excitation rate to avoid pile-up problems. The instrument response function (IRF) was recorded using a Ludox solution by recording the scattered light at 375 nm or 440 nm. Lifetime distribution analysis was applied to model emission decays of CDOM with the FAST program (Version 3.5, Edinburgh, UK). Detailed procedures for data analysis and fitting criteria were reported in a previous study [23].

Hydrodynamic diameters of CDOM solutions: Hydrodynamic diameters of CDOM solutions (ESHA: 60 mg C/L; PPHA: 100 mg C/L) in the presence and absence of Ca^{2+} and Mg^{2+} were determined using a Malvern Zen 3700 Zetasizer (Worcestershire, UK).

3. Results

3.1. Changes of CDOM's Absorption Spectra with Ca^{2+} and Mg^{2+}

As shown in Supplementary Material Figure S1, the absorption spectra of ESHA and PPHA did not change much in the presence of Ca^{2+} or Mg^{2+} while an inconspicuous but discernible change of absorption spectra was reported for the binding of Ca^{2+} to Suwannee River humic acid (SRHA) [21]. It is worth noting that light scattering became notable for ESHA in the presence of high Ca^{2+} (>20 mg/L). The increase of light scattering was in line with the change of hydrodynamic size with Ca^{2+} and Mg^{2+} (Supplementary Material Figure S2). In the presence of Ca^{2+} and Mg^{2+} , the hydrodynamic size of ESHA and PPHA became larger, particularly for ESHA. Similar results were also reported for fulvic acid after binding Cu^{2+} , Pb^{2+} , or Cd^{2+} [24], which resulted from cation bridge effects [25].

3.2. Changes of CDOM's Emission Spectra with Ca^{2+} and Mg^{2+}

Compared with the slight change of absorption spectra, the steady-state fluorescence intensity of ESHA and PPHA significantly decreased with an increase of Ca^{2+} and Mg^{2+} concentration in the whole conducted spectra range (Figure 1). As presented in Supplementary Material Figure S3, 50 mg/L Cl⁻ or SO₄²⁻ exhibited a marginal (approximately 5%) inhibition effect on ESHA's fluorescence intensity, indicating the fluorescence quenching of ESHA (Figure 1) and PPHA (Figure S4, Supplementary Material) resulting from the presence of Mg²⁺ and Ca²⁺. By comparison, it was found that the inhibition effect of Ca²⁺ was more evident than Mg²⁺, while the decrease of ESHA's fluorescence intensity was more profound than that of PPHA (Supplementary Material Figure S4).

As demonstrated in Figure 2, under the excitation of 375, 440, and 550 nm, the fluorescence intensity of ESHA gradually decreased with Ca^{2+} concentration, increasing from 0 to 20 mg/L and Mg²⁺ concentration rising from 0 to 50 mg/L, where the decrease of intensity was much smaller at higher Ca^{2+} (>20 mg/L) and Mg²⁺ concentrations (>50 mg/L). The change of PPHA's fluorescence intensity with Ca^{2+} and Mg²⁺ was analogous to that of ESHA, but to a less extent (Figure S5, Supplementary Material).

The modified Ryan–Weber equation was applied to calculate the binding constant (*K*) between metal ions and CDOM (Figure 3) [26]. As listed in Table 1, lg*K* was excitation and emission wavelength-dependent, which was larger at longer excitation and emission wavelengths. For instance, under the excitation of 375 nm, *K* for binding of Mg²⁺ and Ca²⁺ to ESHA at emission 650 nm was 1.36 and 1.92 times than for emission at 550 nm, respectively. *K* for binding of Mg²⁺ to ESHA and Ca²⁺ to ESHA increased from 1.39×10^4



to 1.72×10^4 L/mol and from 9.02×10^4 to 12.38×10^4 L/mol with excitation wavelength rising from 375 nm to 550 nm (Em = 650 nm).

Figure 1. Effects of Mg^{2+} and Ca^{2+} concentration on the EEM spectra of ESHA (10 mg C/L) aqueous solution.

Table 1. Binding constants (K, 10⁴ L/mol) of Ca²⁺ and Mg²⁺ to ESHA and PPHA.

Ex (nm)	Em (nm) -	ESHA		РРНА	
		Ca ²⁺	Mg ²⁺	Ca ²⁺	Mg ²⁺
	550	4.69	1.02	2.85	0.83
375	600	6.89	1.15	2.98	0.90
	650	9.02	1.39	3.49	1.13
440	650	6.17	1.61	3.43	0.86
550	650	12.38	1.72	3.65	1.36



Figure 2. Effects of Mg^{2+} and Ca^{2+} concentration on the steady-state fluorescence spectra of ESHA (10 mg C/L) aqueous solution at the excitation of 375, 440, and 550 nm.



Figure 3. Changes of steady-state fluorescence intensity of ESHA (10 mg C/L) and PPHA (10 mg C/L) with Mg^{2+} and Ca^{2+} concentration. Curves represent experimental data fitted with the modified Ryan–Weber equation [26].

3.3. Changes of CDOM's Emission Decays with Ca²⁺ and Mg²⁺ Concentration

Compared to the large decrease of steady-state fluorescence intensity, emission decays of ESHA became slightly faster in the presence of Ca^{2+} and Mg^{2+} (Figures 4–6). The change of emission decays was also cation concentration-dependent and wavelength-dependent. Analogous results were found in the case of PPHA (Figure S6, Supplementary Material). Since it was proved that discrete lifetimes recovering from multi-exponential analysis of fluorescence decays had no physical meaning [23], lifetime distribution analysis was applied to quantify the effects of Ca^{2+} and Mg^{2+} on the deactivation of CDOM excited states.



Figure 4. Effects of Mg²⁺ (10 mg/L) and Ca²⁺ (5 mg/L) on the time-resolved fluorescence spectra of ESHA (10 mg C/L) aqueous solution at excitation of 375 and 440 nm.

As demonstrated in Figure 5, compared with the lifetime distribution of ESHA in the absence of cations, the lifetime for CDOM excited states shifted to the smaller value side regardless of excitation wavelength. The result demonstrated that the formation of shorter-lived species in the presence of Ca^{2+} and Mg^{2+} suggested the change of CDOM excited states composition. Moreover, the changes of fluorescence decays and reduction of fluorescence lifetimes were more evident in the presence of 20 mg/L Ca^{2+} or 40 mg/L Mg^{2+} (Figure 6). On the other side, the deactivation kinetics of PPHA excited states slightly accelerated in the presence of Ca^{2+} or Mg^{2+} , which was consistent with its smaller change of steady-state fluorescence relative to ESHA. Therefore, the lifetime distribution of PPHA barely changed in the presence of cations (Figures S7 and S8, Supplementary Material).



Figure 5. Lifetime distributions for emission decays of ESHA (10 mg C/L) aqueous solution at excitation of 375 and 440 nm, Mg^{2+} (10 mg/L) and Ca^{2+} (5 mg/L).



Figure 6. Effects of Mg^{2+} and Ca^{2+} concentrations on the lifetime distributions for emission decay of ESHA (10 mg C/L) aqueous solution at excitation of 375 and 440 nm.

4. Discussion

4.1. Binding of Ca^{2+} and Mg^{2+} to CDOM

Consistent with previous reports for other heavy metal ions [27], the binding affinity of Ca²⁺ and Mg²⁺ to terrestrial source CDOM reference samples (ESHA and PPHA) was large. Similar results were also reported for fluorescence quenching of CDOM by other organic quenchers [28], in which fluorescence of CDOM or its fractions with larger molecular size were more evidently quenched. The preferential loss of long-wavelength emission resulted in blue-shifted spectra. The results were in line with the fluorescence quenching of CDOM by Fe³⁺, Eu³⁺, and other heavy metal ions [29,30]. The dependence of quenching efficiency on wavelength also suggested that the composition of excited states was excitation wavelength-dependent. Moreover, these excited states contributed differently at each emission wavelength.

4.2. Comparison of the Binding Ability of Ca^{2+} , Mg^{2+} , and Cu^{2+} to CDOM

The binding constant of Ca²⁺ to CDOM was smaller than that of Cu²⁺ to CDOM [9,18–20]. Notably, CDOM used in this study differed from previous studies. To exclude the effect of CDOM structure on the binding affinity, quenching of ESHA by Cu²⁺ was further investigated. As shown in Supplementary Material Figure S9, the fluorescence quenching efficiency for ESHA at emission wavelength longer than 600 nm by 10 mg/L Ca²⁺ was approximately 1.5 times that by 0.3 mg/L Cu²⁺, while the quenching efficiency for ESHA by 0.3 mg/L Cu²⁺ was comparable to that by 50 mg/L Mg²⁺. These results supported the finding of a smaller lg*K* for Cu²⁺ to the organic matter in higher salinity water [9], particularly when organic matter was dominated by terrestrial sources. Because the diverse water hardness and other chemical components would influence fluorescence intensity, the accuracy of quantifying CDOM or NOM concentrations by in-situ fluorescence spectrometry alone is suspect.

4.3. Process for Fluorescence Quenching of CDOM by Ca^{2+} and Mg^{2+}

Fluorescence decays of fluorophore would not change in a typical static quenching process [31]. Under the scenario of CDOM, the situation could be complex. Fluorescence decays became faster in the presence of cations, particularly in the case of Ca^{2+} , leading to a decreased amplitude-weighted average lifetime (τ_a). The decrease of τ_a could result from several possibilities. (1) Ca^{2+} or Mg^{2+} quenched CDOM excited states. (2) Compared to fluorophores with a relatively short lifetime, Ca^{2+} or Mg^{2+} was more likely to form a complex with fluorophores with a relatively long lifetime. (3) Larger CDOM aggregates formed in the presence of Ca^{2+} or Mg^{2+} , which could increase light scattering to some extent.

Under the first scenario, fluorophores with the longest lifetime were more likely to be involved in collisional quenching. However, as demonstrated in Figures 5 and 6, except for a decreased amplitude, the values for the long lifetime barely shifted. For the component with a lifetime smaller than 1 ns, collisional quenching was negligible when taking a millimolar Ca²⁺ and Mg²⁺ concentration and a diffusion-controlled rate constant into account. Therefore, collision quenching was not the dominant reason for the decreased steady-state fluorescence intensity and the accelerated emission decay.

The second scenario requires fluorophores within CDOM not equally quenched, which was supported by the dependence of quenching efficiency on excitation and emission wavelengths (Table 1, Figure S8, Supplementary Material). Moreover, as shown in inset Figure 6, the amplitude for fluorophores with a lifetime longer than approximately 1 ns decreased with Ca^{2+} larger than 5 mg/L. Therefore, the overall decay of CDOM became faster in the presence of Ca^{2+} and Mg^{2+} . Notably, the distribution of fluorophore lifetimes was more isolated in the presence of cations than in the absence of cations, indicating certain fluorophores were fully quenched and/or the interaction between fluorophores was weakened by Ca^{2+} and Mg^{2+} .

The third scenario was supported by the different responses of steady-state fluorescence intensity and emission decay to Ca^{2+} and Mg^{2+} concentrations. For instance, the steady-state fluorescence intensity of ESHA barely changed when Ca^{2+} concentration was larger than 20 mg/L or Mg^{2+} concentration was larger than 50 mg/L (Figure 3), respectively. This result suggested that the composition or concentration of light-emitting fluorophores did not change, which conflicted with the further acceleration of emission decays. As shown in Figures S1 and S2, larger aggregates formed in the presence of a high concentration of Ca^{2+} and Mg^{2+} , which would increase light scattering that contributed to the fast decay component (Figure 6).

4.4. The Residues of CDOM Fluorescence in the Presence of Ca^{2+} and Mg^{2+}

Results obtained in this study showed that CDOM maintained at least 10% of its original steady-state fluorescence intensity among the conducted wavelength regions. There are two sources for the residual fluorescence; the first source is the unquenched fluorophore, and the second source is the metal–CDOM complex.

The first source requires that some fluorophores be not accessible to metal ions. Recently, the lack of classical solvatochromism phenomena in CDOM optical spectra challenged the validation of the charge-transfer model in interpreting the photophysical properties of CDOM [32], which put forward the argument that whether fluorophore responding for locally-excited states or charge-transfer complex was freely diffusing and fully accessible [33,34]. As shown in Figures 5 and 6, fluorophores with a long lifetime still presented in CDOM solution at high Ca²⁺ and Mg²⁺ concentrations, which clearly showed that certain fluorophores were not accessible to these cations.

Notably, the distribution of ESHA's fluorescence lifetime shifted to the small value side in the presence of 5 mg/L Ca²⁺. However, the shortest lifetime was still longer than 100 ps, which might not result from stray light. Therefore, the metal–CDOM complex (second source) was also likely to contribute to residual fluorescence. Nevertheless, their contribution was minor because of their large deactivation rate constant.

5. Conclusions

Based on the change of optical spectra and hydrodynamic size of CDOM with Ca^{2+} and Mg^{2+} , the current findings showed that both Ca^{2+} and Mg^{2+} had a high binding constant to terrestrial source CDOM. The formation of metal–CDOM complex quenched CDOM fluorescence, whereas collisional quenching did not occur. Moreover, static quenching was more evident for fluorophores with a relatively long lifetime. The excitation and emission wavelength-dependent quenching efficiency suggested that fluorophores were quenched to different extents. Fluorophores with a long lifetime were not fully quenched by Ca^{2+} or Mg^{2+} , which suggested that the metal–CDOM complex was not the sole contributor to the unquenched fluorescence.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10 .3390/w13162182/s1, Figure S1: Absorption spectra of ESHA and PPHA (10 mg C/L) in the presence of Mg²⁺ and Ca²⁺, Figure S2: Effects of Ca²⁺ or Mg²⁺ on the hydrodynamic size of ESHA (60 mg C/L) and PPHA (100 mg C/L) aqueous solution (pH 7.5), Figure S3: The ratio of fluorescence intensity in the presence of Cl⁻ (FCl) or SO₄²⁻ (FSO₄) to the fluorescence intensity in the absence of anionic ions (F). Figure S4: Effects of Mg²⁺ and Ca²⁺ on the EEM spectra of PPHA (10 mg C/L) aqueous solution, Figure S5: Effects of Mg²⁺ and Ca²⁺ on the steady-state fluorescence spectra of PPHA (10 mg C/L) aqueous solution at excitation of 375, 440, and 550 nm, Figure S6: Effects of Mg²⁺ (10 mg/L) or Ca²⁺ (10 mg/L) on the time-resolved fluorescence spectra of PPHA (10 mg C/L) aqueous solution at excitation of 375 and 440 nm, Figure S7: Lifetime distributions for emission decays of PPHA (10 mg C/L) aqueous solution at excitation of 375 and 440 nm, Mg²⁺ (10 mg/L), Ca²⁺ (10 mg/L), Figure S8: Effects of Mg²⁺ or Ca²⁺ concentrations on the lifetime distributions for emission decays of PPHA (10 mg C/L) aqueous solution at excitation of 375 nm, Figure S9: Effects of excitation and emission wavelength on the fluorescence quenching of ESHA (10 mg C/L) aqueous solution by Mg²⁺, Ca²⁺, and Cu²⁺.

Author Contributions: Conceptualization, X.Z.; methodology, J.L., X.Z.; formal analysis, J.L., R.Z., X.Z.; investigation, J.L.; data curation, R.Z., J.L., X.Z.; writing—original draft preparation, X.Z., J.L., R.Z.; writing—review and editing, X.Z., R.Z., J.L.; funding acquisition, X.Z., J.L. and R.Z. made equal contribution to this work. They are the co-first authors. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China, Grant No. 22076148.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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