Article

Characterization of Dissolved Organic Matter in River Water Flowing through Temperate and Tropical Peatlands Based on Size Exclusion Chromatography and Fluorescence Spectrometry

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Abstract

River water flowing through wetlands is a rich source of dissolved organic matter (DOM) in estuarine and coastal zones. To evaluate the composition and structural characteristics of DOM that is found in distinct climates, the molecular size distribution and fluorescence characteristics of ultrafiltered DOM (UDOM; >1 kDa) preparations from two rivers in Sarawak, Malaysia (tropical), and three rivers in eastern Hokkaido, Japan (cool temperate), were investigated using high-performance size exclusion chromatography (HPSEC) and excitation-emission matrix (EEM) in combination with statistical analysis using the parallel factor model, respectively. Saccharide and phenol concentrations, the ratio of absorbance at 400 nm to that at 250 nm (A_{400}/A_{250}), and EEM for size fractions obtained by HPSEC were also determined. A higher heterogeneity in molecular size was observed in the Hokkaido samples compared to the Sarawak samples. The Hokkaido UDOM was characterized by the presence of larger size fractions that eluted near the void volume and contained darker colored HS, with a higher concentration of saccharides in the autumn (November), and middle size fractions with greater phenol concentrations and HS-like fluorescence peaks as the major fractions. The Sarawak UDOM was characterized by smaller variations in the composition and the A_{400}/A_{250} ratio of the different size fractions, in which the saccharides were possibly protected by humic substances (HS). It was also suggested that and fluorescent components were minor contributors, except for the soil humic acid-like component. EEM analysis of the size fractions also suggested that the relative contributions of microbial HS-like fluorescent component and the protein-like fluorescent component increased with decreasing molecular size in all sample and in the Sarawak UDOM, respectively.

Key words: Dissolved organic matter, Hokkaido, PARAFAC analysis, Sarawak, Size exclusion chromatography.

Introduction

Dissolved organic matter (DOM) in river water has diverse functions, including the dissolution and transport of trace elements via complex formation, supplying nutrients to aquatic biota and microbes, and the storage and transport of carbon (C), as well as contributing to the biogeochemical processes in local ecosystems and the global C cycle (Findlay and Sinsabaugh, 2003; Boyer et al., 2006; Worrall et al., 2012; Muller and Cuscov, 2017). These functions of DOM depend on their composition and structure,

which can vary according to the natural environment where DOM is generated (Estevens et al., 2009; Jaffé et al., 2012). This fact represents a driving force for investigating the chemical structures and dynamics of DOM in river systems in different biomes.

Wetlands are a large source of DOM in river water, in particular when peat soil is present in the watershed. Since climatic conditions also affect the quality of DOM supplied from wetlands (Fong and Mohamed, 2007), it is important to understand its similarity/variability among wetlands distributed in different climates. However, such information is

limited, particularly regarding DOM in wetland-associated rivers in tropical areas (Yamashita et al., 2010; Dalmangro et al., 2017). Gandois et al. (2014) suggested that the C and N contents, isotopic signatures, and composition of lignin phenols obtained after CuO oxidation were similar between river water DOM and pore water DOM from peatlands in Brunei and both of them consist mostly of recently assimilated C regardless of peat dome history of more than 2000 years. Watanabe et al. (2014) characterized ultrafiltered DOM (UDOM; Repeta et al., 2002; Maie et al., 2006) in wetland associated rivers located in tropical (Sarawak, Malaysia), subtropical (Everglades, Florida), and cool temperate (east Hokkaido, Japan) regions with a focus on nitrogen-containing components (DON). They found that the composition and structure of the bulk DON are strongly related to the difference in the ratio of humic substances (HS) and non-HS (NHS) among the three regions as was reported by Watanabe et al. (2012).

Because DOM is, by nature, a mixture, various fractionation techniques, including an adsorption chromatography for separating HS from NHS, have been applied to characterize it (Schwede-Thomas et al., 2005; Huguet et al., 2010; Cawley et al., 2016). High performance size exclusion chromatography (HPSEC), a tool for examining molecular size distribution or estimating the average molecular weight (MW) of natural organic matter (NOM) including DOM (Maie et al., 2004; Tsuda et al., 2010; Dalzell et al., 2009) and soil organic matter (Watanabe and Kuwatsuka, 1992; Karim and Aoyama, 2013), is one such technique. It is difficult to obtain an accurate average MW of a NOM sample using this method, because the apparent molecular size of NOM changes with pH or ionic strength (Tsutsuki and Kuwatsuka, 1989; Minor et al., 2002) and an appropriate standard with a known MW is not available. Molecular weight distribution based on HPSEC is frequently larger than the m/z distribution obtained by mass spectrometry (MS), including atmospheric chemical ionization MS (Pfeifer et al., 2001), laser desorption/ionization MS (Peña-Méndez et al., 2007), electrospray ionization (ESI) MS (Dalzell et al., 2009), and Fourier-transformed ion cyclotron MS (Ikeya et al., 2012; Wagner et al., 2015). Interaction between NOM molecules (Peuravuori and Pihlaja, 2007; Asakawa et al., 2008)

in a solution may be the cause of this difference. However, the m/z distribution may also not be equal to the MW distribution because of the limited number of NOM molecules that can be detected by MS, unexpected fragmentation, the existence of multiply charged parent ions, etc.. Nevertheless, Dalzell et al. (2009) reported a significant correlation between the weight average MW obtained by HPSEC and that by ESI MS for estuarine DOM samples. The m/z distribution of fulvic acids (FAs) obtained from lake water in ESI MS also shifted in parallel with molecular size determined by HPSEC (Pfeifer et al., 2001).

Present study was aimed to characterize Sarawak and east Hokkaido UDOM samples in terms of the molecular size distribution, using HPSEC, within a framework of the studies on key structural properties that are used to characterize the functionality and reactivity of UDOM in wetland-associated rivers in various climatic regions. Size fractions were obtained and analyzed using colorimetry and UV-visible and fluorescent spectroscopies. Through these analyses, it was expected that components that contribute to different sizes of UDOM molecules and the factors that determine the similarity/difference in molecular size distribution between UDOM from cool temperate and tropic regions could be determined. For example, a large molecular size in combination with a high concentration of NHS components may indicate new DOM derived from biological or biochemical processes, while that in combination with a dark color and fluorescent peak possibly related to soil-derived HS might be attributed to the progression of humification. Excitation-emission matrix (EEM)-parallel factor analysis (PARAFAC) was also applied to bulk UDOM samples in an attempt to compare the locational and seasonal variations in DOM quality.

Materials and Methods

Sampling sites

Tropical river water samples, 50–60 L, were collected from two streams along the Mukah River in Sarawak, Malaysia, during the dry season (December 2007) and the wet season (August 2008). One site was the Lebang River (N2° 51.118', E112° 09.990') flowing through a relatively unaffected peat swamp forest, and another site was the Bakong River (N2° 46.200', E112° 08.139') flowing through a newly

cleared peat swamp forest developed for use as an oil palm plantation. River water samples from cool temperate climates, 20 L, were collected from three wetlands in eastern Hokkaido, Japan, namely, the Kimonto (Kimonto River; N42° 37.173', E143° 28.698'; Taiki, Hiroo), the Kiritappu (Dei River; N43° 05.092', E145° 03.565'; the Dei River, Hamanaka, Akkeshi), and the Bekanbeushi (Chiraikaribetu River, abbreviated as 'Chirai'; N43° 06.458', E144° 53.526'; Akkeshi, Akkeshi), in July and November 2007. Additional samples were also collected in April (Kimonto), June (Dei and Chirai), and September (Kimonto and Chirai) 2008. pH and EC recorded for untreated water samples are presented in Table 1. The DOC concentration in the water samples, which was determined after filtration through a PTFE membrane (nominal pore size, 0.40 μm; ADVANTEC, Tokyo, Japan), pH adjustment at 3 with 6 M H_2SO_4, and CO_2 exclusion by sparging with N_2, using a dissolved carbon analyzer (TOC-V_{CPH}, Shimadzu, Kyoto, Japan), is also shown in Table 1.

Preparation of UDOM samples

The river water samples were filtered through a pre-combusted glass fiber filter (nominal pore size, 0.4 μm; GB-140, ADVANTEC), followed by hydrophilic polyvinylidene difluoride membranes

Table 1 pH, EC, and DOC concentration of river water samples

Sample	pH	EC (μS cm^{-1})	DOC (mg L^{-1})
Kimonto (Aug.)	6.7	63.7	13.0
Kimonto (Nov.)	7.3	61.9	3.2
Kimonto (Apr.)	5.4	50.3	3.8
Dei (Aug.)	N.D.	N.D.	15.6
Dei (Nov.)	7.3	61.9	3.3
Dei (Jun.)	6.3	83.7	8.2
Chirai (Aug.)	7.1	137	3.7
Chirai (Nov.)	7.7	156	2.9
Chirau (Jun.)	6.4	115	7.8
Chirai (Sep.)	6.3	121	5.1
Leban (Dec.)	9.2	10.6	54.0
Leban (Aug.)	N.D.	N.D.	48.9
Bakong (Dec.)	9.6	11.8	42.9
Bakong (Aug.)	N.D.	N.D.	42.8

N.D., Not determined.

(pore size, 0.22 μm) mounted in a tangential flow filtration system, Pellicon-2 Mini (Millipore, Billerica, MA, USA), to remove colloidal particles. The UDOM was then simultaneously concentrated and desalted by removing materials with < 1 kD using a Pellicon-2 Mini equipped with regenerated cellulose membranes that have the smallest pores among available membranes. The UDOM samples were purified by 3 repetitions of the dilution with ultrapure water and ultrafiltration with membranes having a 1 kD MW cutoff. The desalted samples were filtered with a PTFE membrane (pore size, 0.45 μm; ADVANTEC) and freeze-dried to obtain powder samples. The proportion of DOC that was recovered as UDOM ranged approximately from 48–93%. C and N contents and ash content in the UDOM samples were measured using an elemental analyzer (Micro-Corder JM10, J-Science Group, Tokyo) and by the combustion method, respectively, after a 1-week period of drying under a vacuum on P_4O_{10}.

High performance size exclusion chromatography

Sarawak UDOM samples collected in December 2007 and eastern Hokkaido UDOM samples collected in August and November 2007 were used. Each UDOM sample (10 mg) was dissolved in 10 mL of eluent, 0.033 M borate buffer (pH, 9.2; ionic strength, 0.1), and passed through a short column packed with Chelex 100 resin (Bio-Rad, Hercules, CA, USA) to remove metals bound to the UDOM constituents. The 1-mL aliquot was then loaded on a Toyopearl HW-50F column (inner diameter, 10 mm; length, 450 mm; MW range, 500–80,000 Da) and eluted with 0.033 M borate buffer at a flow rate of 1.0 mL min^{-1}. The absorbance at 250 nm (A_{250}) of the eluate was recorded using a flow cell UV detector unit (UV-1, Amersham Biosciences, Amersham, UK). Toyopearl HW-65S column (inner diameter, 10 mm; length, 450 mm; MW range, 40–5,000 kDa; Tosoh Bioscience, Tokyo) was also used under the same operating conditions to confirm the variation in the molecular size of the void fraction on the HW-50F column. The eluent was used after filtration with a membrane filter (< 0.45 μm) and evacuation for degassing. Calibration of MW was accomplished using a SIGMA MW-GF-70 kit (Sigma-Aldrich, St. Louis, MO, USA) that included blue dextran (MW >2.0 ×10^6), albumin (MW 66,000), carbonic anhydrase (MW 29,000), cytochrome (MW 12,400), and

vitamin B_{12} (MW 1,355). Folic acid (MW 441) and tryptophan (MW 204) were also used to cover the low molecular weight range. The void volume (V_0) and inner volume were 30.4 and 62.1 mL for the HW-65S column and 28.9 and 87.2 mL for the HW-50F column.

The eluate was further fractionated into 2.5-mL fractions using a fraction collector, and the saccharide and phenol concentrations were determined by colorimetric methods. The Folin method was used to determine the phenol concentration (Tsutsuki and Kuwatsuka, 1979). A 1 mL aliquot of each fraction mixed with 50 μL Folin-Ciocarteu's reagent (Kanto Kagaku, Tokyo) was reacted at 30°C for 30 min. A 100 μL volume of a saturated Na_2CO_3 solution was added and the resulting solution allowed to stand for 30 min at 30°C. The absorbance at 760 nm of the reacted solution was then determined. A calibration curve was prepared using phenol solutions.

Saccharide concentrations were determined using the phenol-sulfuric acid method (Dubos et al., 1956). An 800 μL aliquot of each fraction was mixed with 20 μL of 80% phenol and 2 mL of conc. sulfuric acid with stirring using a touch mixer and then heated at 100°C for 10 min. After cooling to room temperature, the absorbance at 490 nm was measured (UV1240, Shimadzu, Kyoto, Japan). Calibration curve was obtained using glucose solutions.

Absorbances at 250 and 400 nm without any reaction were also measured using a 1.0 cm quartz cell (UV mini-1240, Shimadzu) and their ratio (A_{400}/A_{250}) was calculated for estimating the depth of dark color.

EEM-PARAFAC analysis

The EEM of all of the bulk UDOM samples and the size fractions from the Sarawak UDOM samples collected in December and the Hokkaido UDOM samples collected in November were measured using a spectrofluorometer (FluoroMax-4, Horiba Jobin Yvon, Longjumeau, France) equipped with a 150-W continuous output xenon arc lamp. For EEM measurements, 106 emission scans were obtained at an excitation (Ex) wavelength between 240–550 nm at increments of 5 nm. Emission (Em) wavelength were scanned between 290–600 nm at increments of 2 nm. All fluorescence spectra were acquired in the ratio mode, whereby the sample (emission signal, S) and reference (excitation lamp output, R) signals

were collected and the (S/R) ratio was calculated (Abe et al., 2011). The inner filter effect was corrected according to McKnight et al. (2001), and each EEM sample was corrected for Raman scattering and background fluorescence by subtracting the spectra of a Milli-Q water. The intensity of the EEM spectra was normalized using quinine sulfate and expressed as quinine sulfate units (QSU; 1 QSU=1 ng L^{-1} of quinine sulfate monohydroxide). EEM was decomposed statistically using the PARAFAC model with the DOMFluor toolbox (Stedmon and Bro, 2008; Maie et al., 2014) on MATLAB software ver. 7.7 (MathWorks, Inc., Natick, MA, USA). PARAFAC analysis was conducted using 82 EEMs consisting of the Malaysian ($n = 24$) and Hokkaido river water samples ($n = 58$). The spectral region of EEMs used in the analysis employed an Ex wavelength of 260–450 nm and Em wavelengths of 290–518 nm.

Results

Molecular size distribution determined by HPSEC

Figures 1a, 1c, and 1e show the elution curves of the UDOM samples on HW-50F recorded by A_{250}. All of the Hokkaido UDOM samples (Figs. 1a and 1c) showed an elution peak at V_0 (>80 kDa), which was largest for the Kimonto August sample (Fig. 1a). According to the calibration curve, the elution maximum at 41.6–43.6 mL corresponded to a MW in the range of 14,900–19,100. There were no differences in the MW of the elution maximum between the August (Fig. 1a) and November (Fig. 1c) samples common to the three rivers. The elution patterns for the Leban and Bakong DOM samples (Fig. 1e) were similar and no void fraction was found. The MW at the elution maximum (38.2–38.7 mL) was 26,400–27,900.

Figures 1b, 1d, and 1f show the elution curves of the UDOM samples on HW-65S. The Kimonto August sample (Fig. 1b) showed three peaks at V_0 (>5,000 kDa), 49.5 (MW, 113,000), and 60.0 mL (MW, 22,200). The Chirai August sample also showed elution at V_0, whereas it was scarce and a shoulder was observed at around 51 mL (MW, 89,300) for the Dei August sample. The Hokkaido November samples (Fig. 1d) showed a minor elution between V_0 and an elution maxima at 60.0 mL. The elution maximum for the Sarawak UDOM samples

(Fig. 1f) was earlier than that of the Hokkaido UDOM, similar to the elution curves on HW-50F.

Saccharide and phenol concentrations in size fractions

Figures 2a–2d show the phenol and saccharide concentrations in size fractions from the Hokkaido UDOM. The phenol concentration in the void fraction on HW-50F was low but was higher in the August sample than in the November sample in the Kimonto UDOM (Fig. 2a), while it was slightly higher in the November sample than in the August sample in the Dei and Chirai UDOM (Figs. 2c and 2d). The highest concentration was consistently observed in the fraction corresponding to elution maximum recorded at A_{250} (Fig. 1).

The saccharide concentration in the void fraction

on HW-50F was larger than the phenol concentration in all samples. In the Kimonto UDOM, the distribution of saccharides into the void fraction was larger in August than in November (Fig. 2a). The saccharide concentration in the fractions from just after the V_0 to the shoulder at around 50 mL on HW-65S was also higher in the August sample (Fig. 2b). In all the three rivers, the overall saccharide concentration was higher in the November samples compared to the August samples (Figs. 2a–2d). The highest concentration was frequently in the same fraction as the highest phenol concentration.

In the HPSEC of the Sarawak UDOM on HW-50F, the elution curves for the saccharide concentration were nearly overlapped with those for the phenol concentration (Figs. 2e and 2f). Saccharides and phenols were faintly detected in the void fraction,
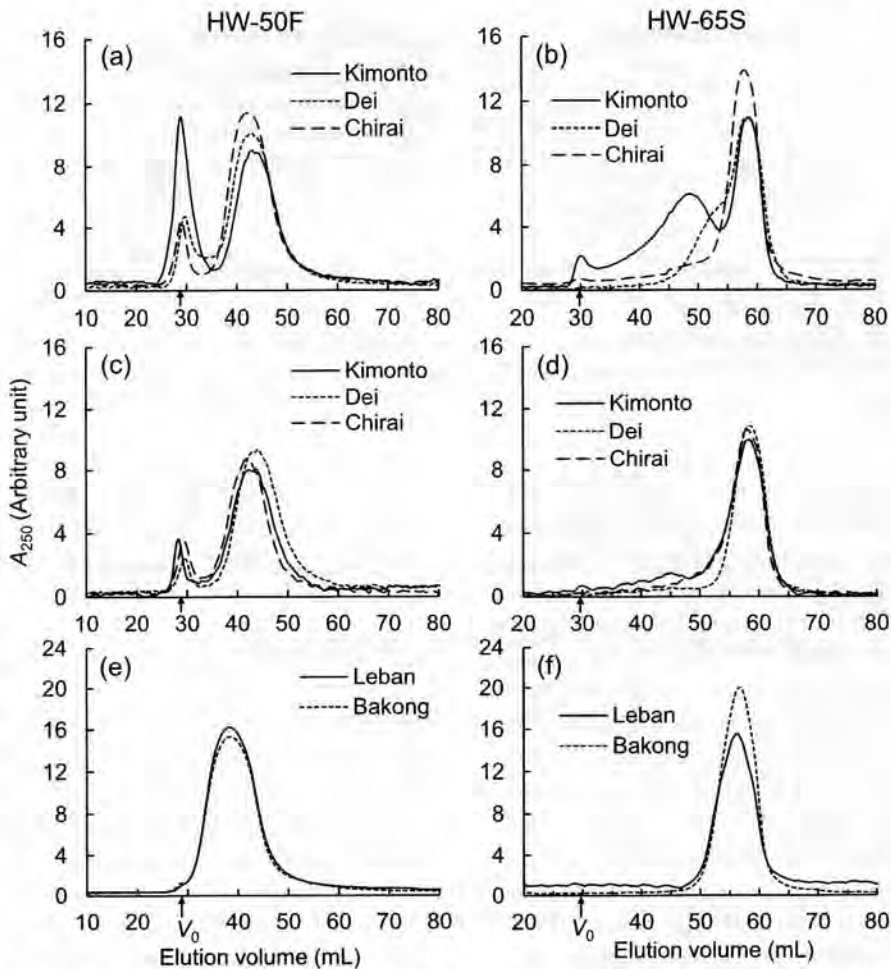

Fig. 1. Elution curves for the east Hokkaido UDOM in August (a, b) and November (c, d) and the Sarawak UDOM (e, f) on Toyopearl HW-50F (a, c, e) or Toyopearl HW-65S (b, d, f).

and their elution pattern was close to the normal distribution. The highest concentrations were recorded earlier (40 mL) than that in the Hokkaido UDOM (42.5–45 mL).

The percentages of saccharide C and phenol C in the total DOC were calculated from the amount of DOC loaded into the HW-50F column and the sum of the amounts of saccharides and phenols in the size fractions (Table 2). The % saccharide C in the Kimonto and two Sarawak UDOM samples was similar to each other, at 9–10%, and smaller than that in the Dei UDOM, 13–16%. The % phenol C was larger in the Sarawak UDOM samples, at 11–12%, compared to the Hokkaido UDOM, 4–6%, which may be related to the higher %HS in the Sarawak UDOM than in the Hokkaido UDOM (Table 2).

Optical property A_{400}/A_{250} of size fractions

Figure 3 shows the transition in A_{400}/A_{250}, which was expected to be higher when the color of UDOM is darker, along with molecular size for the Hokkaido and Sarawak UDOM samples in December on HW-50F. The darker color is possibly related to the presence of a larger amount of or more developed conjugated systems in the aromatic components (Watanabe and Kuwatsuka, 1991b). The results for the fractions eluted before the V_0 and after 60 mL were excluded because the A_{400} value was negligible. In the Hokkaido UDOM samples, the A_{400}/A_{250} was higher in the larger size fractions, 0.16–0.23, and showed a steep decrease from 35–40 mL to 45–47.5 mL (0.08–0.11), followed by an increase up to 0.12–0.16 after the peak had nearly completely eluted (Fig. 1c). The A_{400}/A_{250} of the larger size fractions from the
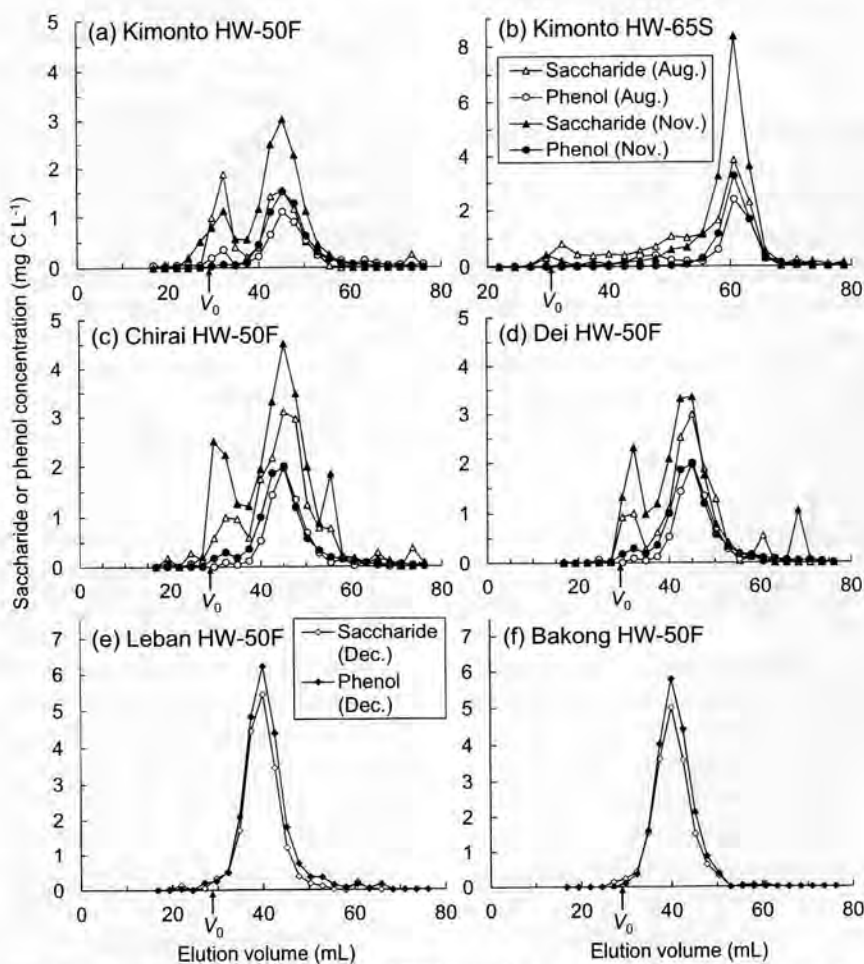

Fig. 2. Variations in saccharide and phenol concentrations in UDOM samples from the Kimonto (a, b), Chirai (c), Dei (d), Leban (e), and Bakong (f) Rivers with molecular size, as determined by HPSEC using Toyopearl HW-50F (a, c, d, e, and f) or Toyopearl HW-65S (b).

Table 2 Chemical characteristics of the DOM samples used in this study

Sample	Sum of saccharide C in size fractions[†] (% total C)	Sum of phenol C in size fractions[†] (% total C)	Total C[‡] (mg g^{-1})	Total N[‡] (mg g^{-1})	HS:NHS ratio[‡]
Kimonto (Aug.)	9.2	5.4	458	47.1	N.D.
Kimonto (Nov.)	9.9	4.0	455	50.0	76:24
Dei (Aug.)	12.5	4.9	450	16.2	N.D.
Dei (Nov.)	15.9	5.2	464	20.1	71:21
Chirai (Aug.)	9.4	4.8	440	20.0	N.D.
Chirai (Nov.)	13.4	6.1	451	23.0	80:20
Leban (Dec.)	9.2	10.6	485	10.4	98:2
Bakong (Dec.)	9.6	11.8	491	10.6	95:5

[†]Fractionated on HW-50F.
[‡]As reported by Watanabe et al. (2014), except for the Hokkaido August samples (ash-free basis).
HS, Humic substances; NHS, non-humic substances. N.D., Not determined.
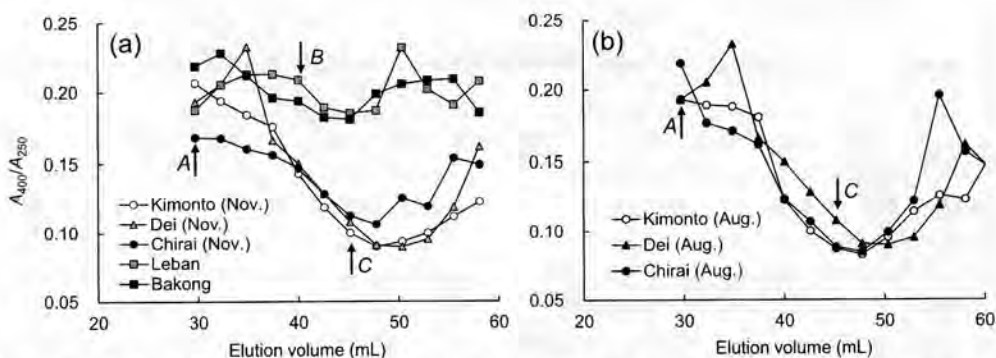
Fig. 3. Variations in the A_{400}/A_{250} ratio for the Hokkaido UDOM (November) and Sarawak UDOM (December) samples (a) and for the Hokkaido UDOM (August) (b) with molecular size, as determined by HPSEC using Toyopearl HW-50F. Arrows A–C indicate V_0 and elution maximum of the Sarawak and Hokkaido and UDOM recorded by A_{250}, respectively.

Leban and Bakong samples, 0.19–0.23, was similar to that for the Dei and Kimonto samples and fluctuated within the same range throughout the size fractions.

EEM-PARAFAC analysis of UDOM and size fractions

Figure 4a shows representative EEM data of the Hokkaido (Kimonto August) and Sarawak (Leban August) UDOM. The Kimonto August sample showed a shoulder peak at around 315 nm/442 nm (excitation wavelength/emission wavelength), while the Leban August sample showed ashoulder peak at 360 nm/494 nm. Assuming that these differences were derived from the different composition of common fluorophores, PARAFAC analysis were conducted. EEM were decomposed into validated 5

PARAFAC components (Fig. 4b). According to previous data, 4 peaks were assigned to soil FA-like (C1), soil humic acid (HA)-like (C2), microbial HS-like (C3), and photorefractory or photo-degraded HS-like (C4) peaks, and another one was assigned to a protein-like material (C5) (Table 3). Fluorescence intensity of the C1, C3, C4, and C5 peaks was larger in the Hokkaido UDOM than in the Sarawak UDOM, while that for C2 was similar between the two regions, possibly slightly smaller in the Hokkaido UDOM (Fig. 5).

In the Hokkaido UDOM, the C4 peak tended to be larger in the summer (June, August, and September) than in the autumn (November) and spring (April), while such a seasonal trend common to the three rivers was not observed in the cases of the C5 peak and other HS-related peaks. No seasonal variations
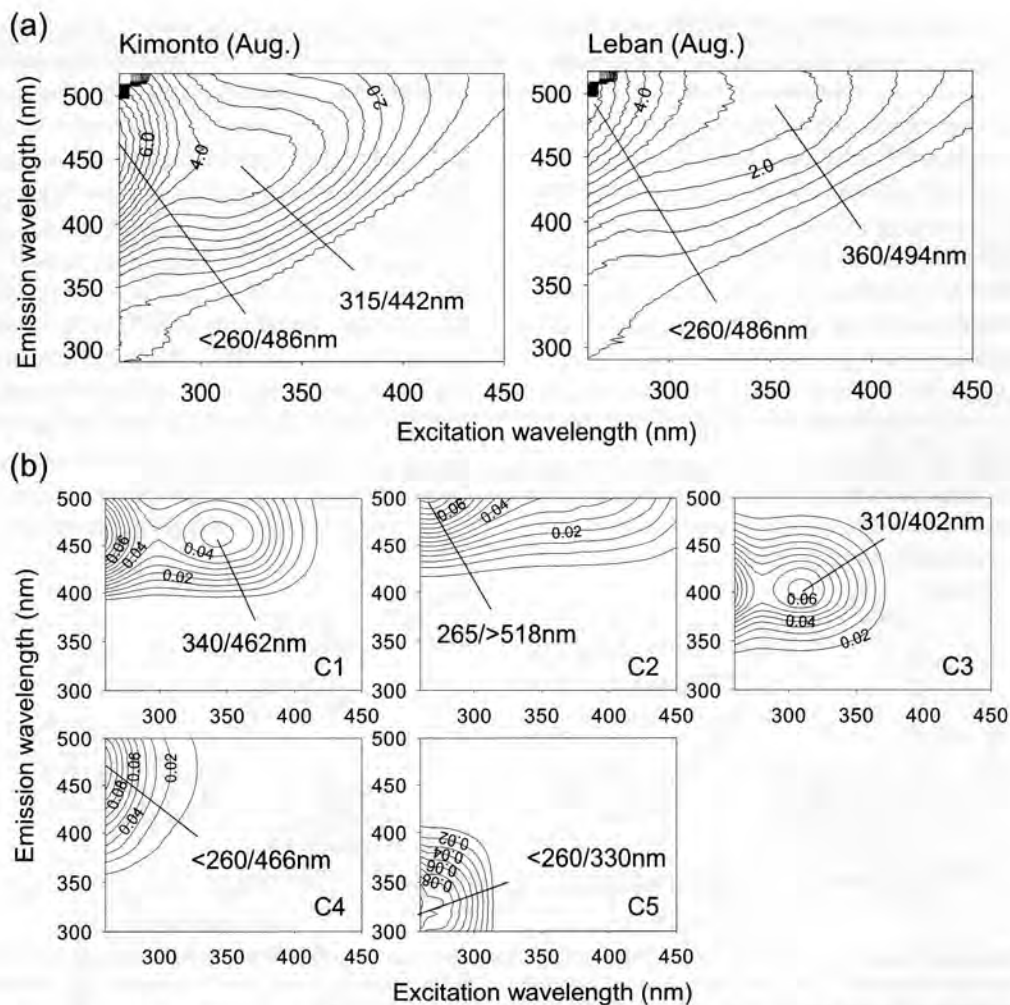
Fig. 4. Representative EEM of Hokkaido and Sarawak UDOM (a) and contour plots of five fluorescence components decomposed by the PARAFAC analysis (b). Unit of contour line is QSU.

Table 3 PARAFAC end members in the present study

Code	Potential source	Excitation / emission maxima (nm)	Reference
C1	Soil fulvic acid-like substances	260/460, 340/462	Stedman and Makager (2005)
C2	Soil humic acid-like substances	265/518	Maie et al. (unpublished data)
C3	Microbial HS-like substances	310/402	Cory and McKnight (2005)
C4	Photorefractory or photodegraded HS-like substances	<260/466	Stedmon et al. (2007)
C5	Protein-like materials including tannin	<260/330	Yamashita and Tanoue (2003)

HS, Humic substances.
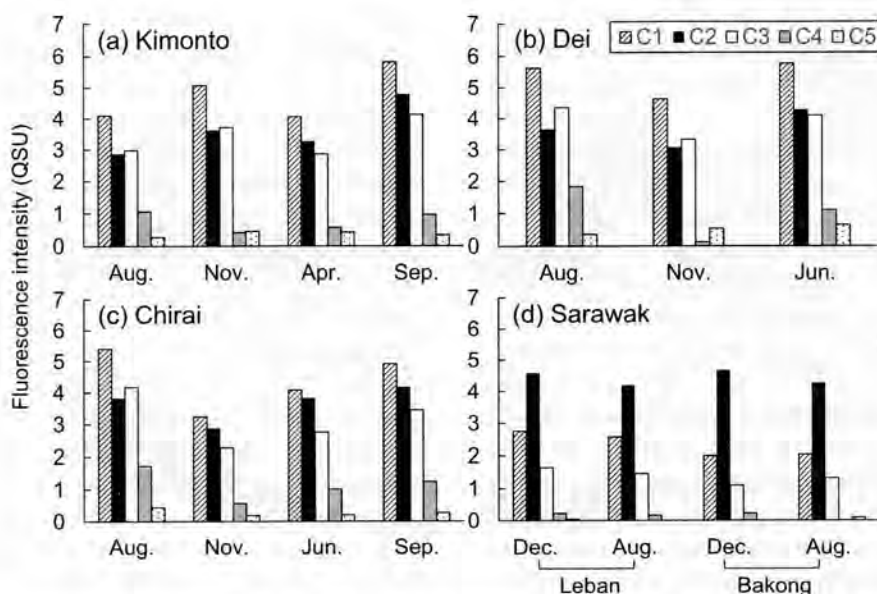
Fig. 5. Fluorescence intensity of five EEM-PARAFAC components in Kimonto (a), Dei (b), Chirai (c), and two Sarawak (d) UDOM samples.

were observed in the EEM for the Sarawak UDOM.

Figures 6a and 6b show the fluorescent intensity of the C1 and C5 components in the size fractions, respectively. The EEM for the fractions eluted before the V_0 and after 55 mL (Sarawak samples only) were too weak to permit an analysis to be carried out. The patterns of the elution peaks for the C2, C3, and C4 components were basically similar to the C1 component (data not shown), but the relative proportion changed among size fractions as described below. The highest intensity of those 4 HS-like peaks was typically observed at 42.5 (Leban) or at 45–47.5 (the others) mL. The highest intensity of the C5 component in the Hokkaido UDOM was observed at 45 (Chirai) or at 47.5 (Dei and Kimonto) mL. The Kimonto sample showed an additional peak for a C5 component at around the V_0, while the C5 peak intensity in the size fractions of the Sarawak UDOM was consistently low.

Figures 6c–e show the ratio of the peak intensity for the C2, C3 or C4 peaks to that for C1 in the size fractions, respectively, to express the difference in the distribution of fluorescent components to each size fraction. Large variations in the C2/C1 ratio were observed in the Sarawak UDOM with the maximum value at 37.5 or 40.0 mL (Fig. 6c), suggesting that a component having a soil HA-like fluorescence

contributed to the dominant fraction. The C3/C1 ratio was smallest at around V_0 and increased with decreasing molecular size in all of the samples (Fig. 6d), suggesting that the microbial derived HS-like component contributed more to the smaller size fraction relative to soil derived HS-like component. The distribution of C4/C1 ratio was considerably normal and the maximum value was recorded at 45.0 mL for all the samples (Fig. 6e), indicating that C4 components are more homogeneous in terms of molecular size compared to C1 components.

Discussion

The molecular size of the Hokkaido UDOM samples was distributed over a wider range than that of the Sarawak UDOM (Fig. 1), suggesting a larger heterogeneity of molecular composition in the Hokkaido UDOM. This could be related to the larger relative abundance of NHS in the Hokkaido UDOM samples than in the Sarawak UDOM (Table 2). The presence of an elution peak at V_0 in all the Hokkaido UDOM samples on HW-50F (Fig. 1) was strikingly different from the Sarawak UDOM samples, although the molecular size distribution of the void fraction varied among the samples from the three rivers (Fig. 1b). Larger size fractions that eluted at around V_0
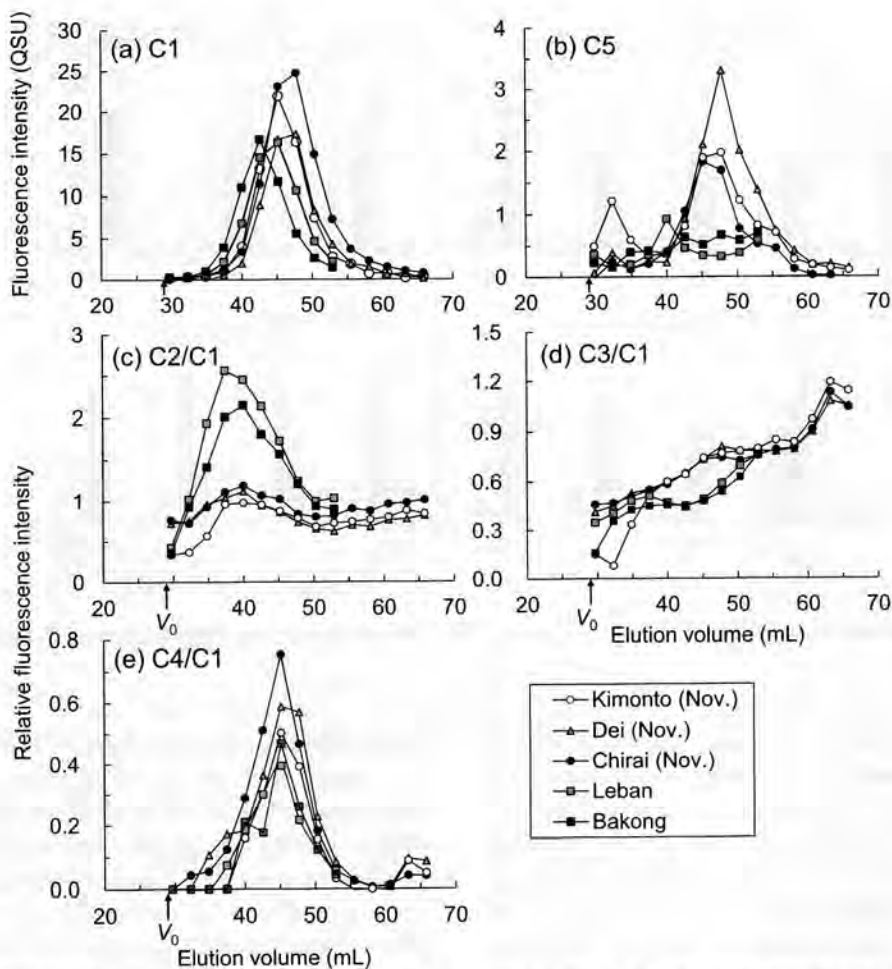
Fig. 6. Variations in fluorescence intensity for the C1 (a) and C5 (b) components and the fluorescence intensity ratio of C2/C1 (c), C3/C1 (d), and C4/C1 (e) in the Hokkaido UDOM (November) and Sarawak UDOM (December) samples, as determined by HPSEC using Toyopearl HW-50F.

consisted of darker colored HS (Fig. 3), which contain a smaller phenolic C content (Fig. 2) and a very weak fluorescence (Fig. 6), and polysaccahrides (Fig. 2). The C5 fluorescent peak did not confirm that protein-like materials were a regular component of the void fraction (Fig. 6). Although neither the presence of interactions between polysaccharides and HS nor the existence of saccharide moieties in HS molecules could be confirmed for the Hokkaido UDOM samples, a similar relationship between the molecular size and the depth of the dark color has also been reported for soil HAs, in which free polysaccharides are not present (Tsutsuki and Kuwatsuka, 1984). The larger relative abundance of carbohydrate C in the largest size fraction obtained by HPSEC was also reported for a lake UDOM (Peuravuori, 2005).

Differences in the molecular size distribution of the Hokkaido UDOM between August and November were mainly observed in the relative abundance of the larger size fractions (Figs. 1 and 2). In samples from the Dei and Chirai Rivers, the saccharide concentration in the larger size fractions tended to be higher in November than in August. For example, the sum of saccharides eluted by 35 mL on HW-50F that covers the void fraction was equivalent to 3.2% vs 1.5% (Chirai) or 3.8% vs 2.2% (Dei) when the total C content was regarded to be 100%. Saccharide concentrations in the middle size fractions, including the elution peak, of the Kimonto UDOM was also higher in November than in August. These results suggest more intensive biological saccharide consumption during the summer season and/or a larger saccharide supply from senescent plant

materials in the autumn season. The renewal of DON after the summer was also suggested by the amino acid composition (Watanabe et al., 2014). An exception was the larger size fractions of the Kimonto August sample, in which the sum of saccharides eluted by 35 mL on HW-50F, 3.3% of total C, tended to be greater than that in November, 2.2%. In the larger size fractions of the Kimonto August sample, A_{250} (Fig. 1) and A_{400} (data not shown) were much larger compared to the November sample. As such, interactions with or incorporation into HS might have inhibited the decomposition of saccharides. Note that the close relationship between HS and polysaccharides throughout the fractions with MW 30,000 (just before the elution of the largest peak) to >2×10^6 (V_o) in the Kimonto August sample was confirmed in HPSEC using HW-65S (Fig. 1b).

The dominant middle size fractions of the Hokkaido UDOM samples were characterized by a lower A_{400}/A_{250} ratio (Fig. 3), a lower phenol concentration (Fig. 2), and a higher C5 intensity (Fig. 6b) compared to the Sarawak UDOM. The same trend was also observed for the intensities of the C3 and C4 peaks (data not shown). Sources of these three peaks (Table 3) could contribute to the lower A_{400}/A_{250}, since those would be expected to have a lighter color than the source of the C2 peak, substances having a soil HA-like fluorescence (not actually HAs but FAs based on solubility). Only the C4 peak showed a seasonal variation common to the three Hokkaido rivers in the EEM-PARAFAC analysis (Fig. 5), which can be attributed to the degradation of photo-sensitive moieties in HS under the conditions of higher irradiation in the summer season. No seasonal variation in the C4 intensity was observed in the samples from the Leban and Bakong Rivers, which had a very high HS concentration, possibly due to the high light absorption capacity of surface water.

The molecular size distributions of the Leban and Bakong UDOM samples were similar, as was the case of the other chemical characteristics reported by Watanabe et al. (2014). Although in the Hokkaido UDOM a lower A_{400}/A_{250} ratio and a lower phenol concentration were observed simultaneously, in the Sarawak UDOM a greater phenol concentration than the Hokkaido UDOM was not accompanied by a lower A_{400}/A_{250}. These observations suggest that a lighter color is not attributed to a lower phenol

content. Since the %HS of the Sarawak UDOM samples was 95% or more (Table 2), a considerable portion of the saccharides would be included in the HS fraction. Furthermore, the saccharide and phenol concentrations did not appear to vary significantly with molecular size. Although direct binding between saccharides and phenols has not been confirmed, the incorporation of saccharides in HS could affect their decomposition rate. The distribution of proteinaceous materials in the Sarawak UDOM samples was likely to differ from that of saccharides in the same samples and that of proteinaceous materials in the Hokkaido UDOM samples, based on the larger relative intensity of the protein-like fluorescent component (C5) in the smaller size fractions (Fig. 6). However, the C5 peak data should be interpreted carefully, because it may not be derived solely from proteinaceous materials (Maie et al., 2007) and the proportionality of fluorescent intensity to the concentration was not confirmed. This behavior of the C5 component in HPSEC is consistent with findings reported by Watanabe et al. (2014), who estimated that the average molecular weight of peptides was smaller in the Sarawak UDOM samples than in the Hokkaido UDOM samples, as evidenced by X-ray photoelectron spectroscopy data. The highest elution of four HS-like fluorescent components was also observed later (42.5–45 mL) than the elution maximum recorded at A_{250} (38–39 mL). A similar observation was reported for the neutral phosphate buffer-extractable organic matter in various mineral soils (Aoyama, 2006) and coastal DOM (Shimotori et al., 2016). These results also suggest that the HS molecules related to the four HS-like fluorescence peaks are not the main HS in the Sarawak UDOM samples.

Slight differences in the behavior of each fluorescent component in HPSEC could be visualized by obtaining the ratio of two components in the size fractions (Figs. 6c–e). The relatively greater contribution of the microbial HS-like component (C3) to the smaller size fractions compared to the soil FA-like component (C1) might reflect the difference in the molecular size of their major origins, e.g., pigments synthesized by microorganisms versus lignin. In the Sarawak UDOM samples, the contribution of the soil HA-like component (C2) to the larger size fractions was higher than the C1 component (Fig. 6c). Since peat soils are generally

rich in HAs and poor in FAs (Watanabe and Kuwatsuka, 1991a; Kawahigashi and Sumida, 2006), colored DOM generated as the result of the oxidative degradation of HAs might have a C2 peak and a larger molecular size than the other fluorescence components. The narrower C4/C1 distribution may be related to a higher susceptibility of fluorescence components with a larger molecular size to undergo photodegradation (Scully et al., 2004; Wagner and Jaffé, 2015). C4 peak is produced and C1 peak is consumed during photodegradation of DOM (Stedmon et al. 2007), resulting in a higher C4/C1 in the middle size DOM fractions. As such the investigation of component analyses in combination with molecular size-based fractionation can serve valuable information to make up the stories on DOM dynamics.

Conclusions

The molecular size and the composition of size fractions of the Sarawak UDOM were more homogeneous compared to those of the Hokkaido UDOM. These may be related to the higher HS/NHS ratio in the Sarawak samples, probably due to a higher rate of NHS degradation under a tropical climate. On the other hand, the similar elution pattern for saccharides and phenol in the Sarawak UDOM suggest that HS inhibited the degradation of saccharides. Same mechanism seems to be operative in the case of the larger size fractions in the Kimonto August UDOM. The contribution of fluorescent components to HS was significant in the lighter colored HS in the Hokkaido UDOM while it was quite small to HS in the Sarawak DOM. In the Hokkaido rivers, polysaccharides in the larger size UDOM may have been decomposed more actively while the levels of photodegradation products derived from HS in UDOM may increase in the summer season. Thus, through HPSEC and EEM-PARAFAC analyses, differences/similarities in the composition/structure of UDOM that influence the function and dynamics (e.g., generation and decomposition) of UDOM, in Hokkaido and Sarawak rivers are indicated.

Acknowledgements

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