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Fluorescence and amino acid characteristics of molecular size fractions of DOM in the waters of Lake Biwa

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Abstract. Dissolved organic matter (DOM) in the waters from Lake Biwa, Japan was fractionated using tangential flow ultrafiltration, and subsequently characterized by fluorescence properties and amino acids. While major dissolved organic carbon (DOC), UV absorbance (Abs), humic-like fluorescence (Flu) and total hydrolyzed amino acids (THAA) occurred in the less than 5 kDa molecular size fraction, they were not evenly distributed among various molecular size fractions. Flu/Abs ratios increased, and THAA/DOC ratios decreased with decreasing molecular size. Humic-like fluorescence occurred in all molecular size fractions, but protein-like fluorescence only occurred in the 0.1 μm-GF/F fraction. Subtle differences in amino acid compositions (both individuals and functional groups) were observed between various molecular size fractions, this may indicate the occurrence of DOM degradation from higher to lower molecular weight. The results reported here have significance for further understanding the sources and nature of DOM in aquatic environments.

Introduction

In aquatic environments, molecular mass distribution and characteristics of DOM have been reported in terms of elemental (C, N) and isotopic (^{13}C and ^{14}C) composition, fluorescence and absorbance (Smith 1976; Stewart and Wetzel 1980; Carlson et al. 1985; Hart et al. 1992; Guo et al. 1994; Martin et al. 1995; Guo and Santschi 1996; Mopper et al. 1996; Guo and Santschi 1997). These studies showed that although major DOC was mainly in the less than 5 kDa molecular size fractions, and minor DOC in the >0.1 μm fractions, the chromophoric properties and chemical compositions of DOM were not evenly distributed among different molecular size fractions. Recent studies on metal binding characterization (Salbu et al. 1987; Orlandini et al. 1990; Hart et al. 1992; Guo et al. 1994; Martin et al. 1995; Wu et al. 2001; Wu and Tanoue 2001a) also indicated that there did exist evident

differences in the properties and nature of DOM in different molecular size fractions.

Recently, three dimensional excitation/emission (Ex/Em) matrix spectroscopy (3DEEM) has been used successfully to probe the chemical structure of DOM due to its ability to distinguish different classes of organic matter (Coble et al. 1990; Senesi 1990; Mopper and Schultz 1993; Del Castillo et al. 1999; Mayer et al. 1999). HPLC has been also used to detect amino acid compositions in studies of DOM biogeochemical cycling in aquatic environments (Robertson et al. 1987; Berdie et al. 1995; Colombo et al. 1998; Wu and Tanoue 2001b). These methods, however, have never been used to investigate various molecular size fractions of DOM.

In this paper, an initial investigation was carried out to explore fluorescence and amino acid characteristics, and the possible interrelationship of various molecular size fractions of DOM. 3DEEM and HPLC, coupled with both acid and alkaline hydrolysis, were used. Tangential flow ultrafiltration was used to fractionate DOM, which has been demonstrated to be a promising fractionation method for DOM (Carlson et al. 1985; Guo et al. 1994; Guo and Santschi 1996). Water samples from Lake Biwa were chosen as a case study.

Materials and methods

Sampling

We selected two sampling stations in the north basin of Lake Biwa; Stations A (70 m depth), and B (40 m depth), located in the northeast and southwest regions of the basin, respectively. Lake Biwa (35°00′–35°30′ N, 135°50′–136°15′ E) is the largest freshwater source in Japan with a surface area of 674 km^2, a maximum depth of 104 m and a mean depth of 41 m. It is composed of two basins; the large, deep and mesotrophic North Basin and the small, shallow and eutrophic South Basin. Seasonal stratification usually occurs from April to January in Lake Biwa (Miyajima et al. 1997). Water samples were collected in June 1999, and were filtered through glass-fiber filters (GF/F, Whatman, Maidstone, UK) immediately after sampling, and stored at 2 °C. The GF/F filtrate was then fractionated using a tangential flow ultrafiltration system (Minitan II system, Millipore Co. Ltd) with Durapore (0.1 μm pore size) and Biomax (cutoff membrane, molecular size 5 kDa) membranes successively. About 15–20 l of original water was concentrated to 200–400 ml in each fraction, namely, 0.1 μm-GF/F and 5 kDa-0.1 μm. The <5 kDa fraction was not concentrated. The fractionation was carried out within 2 days after GF/F filtration, and the fractions were then kept frozen until further analysis. The system was carefully pre-cleaned following the manufacturer's instructions.

DOC, absorbance and fluorescence analysis

All fractions were analyzed for DOC, fluorescence and absorbance. DOC concentration was measured by a high temperature catalytic oxidation method using potassium hydrogen phthalate as a standard; After the water sample was acidified with HNO_3, and the DIC was removed by bubbling with pure air for 15 minutes, 200 μl of sample was injected into TOC analyzer (TOC 5000A, Shimadzu Co. Ltd) (Wu et al. 2001). System and pure water (Milli-Q TOC, Millipore Co. Ltd) blanks were, on the average, 2–4 μM C and 6 μM C, respectively.

Fluorescence was measured with 3DEEM using a fluorescence spectrophotometer (Hitachi, Model F-4500). The excitation wavelength ranged from 240 nm to 400 nm (5 nm bandwidth), and the emission from 250 nm to 600 nm (2 nm bandwidth). Each sample was scanned three times, and the resulting spectra were smoothed and averaged. The spectra were subsequently normalized to water Raman Scattering area, and Matlab™ was used to obtain the normalized 3DEEM surface and contour plots, in which Ex/Em maxima can be identified. Instrumental correction was made according to the manufacturer's instructions. UV absorbance of samples was measured at wavelength 254 nm using a spectrophotometer (Shimadzu, MPS-2400, UV-vis multipurpose) equipped with a 2 cm quartz cell.

Amino acid analysis

Individual amino acid concentrations were determined by pre-column o-phthaldialdehyde (OPA) derivatization and separation of the components by HPLC and fluorescence detection (Lindroth and Mopper 1979). For acid hydrolyzable amino acids, 2 ml water samples were hydrolyzed at 110 °C for 22 h in 6N HCl. The hydrolysate was then diluted, neutralized with cooled 2N NaOH, and reacted with an OPA fluorescent tag for HPLC analysis. A reference mixture of 17 standard amino acids including aspartic acid (Asp), glutamic acid (Glu), serine (Ser), histidine (His), glycine (Gly), threonine (Thr), arginine (Arg), tyrosine (Tyr), alanine (Ala), methionine (Met), valine (Val), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), ornithine (Orn), lysine (Lys) and proline (Pro) was used to assign the identities. Due to the nonlinear response, Orn, Lys and Pro were ignored. Since tryptophan (Trp) was not stable in the acid hydrolysis, alkaline hydrolysis was applied before HPLC analysis. For alkaline hydrolysis, ascorbic acid was added as antioxidant, and water samples were first hydrolyzed in 4.2N NaOH at 110 °C for 16 h; The hydrolysate was then diluted, and neutralized to pH = 9 with cooled 2N HCl, reacted with a fluorescent tag and analyzed for tryptophan in a similar manner (Wu and Tanoue 2001b). The recovery of tryptophan was 91±3.3% (n = 4). The analytical precision expressed as standard deviation from multiple standard injections of 25 μl was less than 0.8% for Val, Met, Ile, Phe and Leu, 1.2 – 1.9% for Ser, His and Gly, 2.0 – 4.7% for Glu, Thr, Ala, Arg, Trp and Tyr, and 9.9% for Asp (Wu and Tanoue 2001b).

Results and discussion

Molecular size distribution of DOC, absorbance and fluorescence

The distribution of DOC, absorbance and fluorescence of the fractionated DOM is shown in Table 1. As determined by DOC concentration, the relative abundance of the <5 kDa fraction ranged from 55 to 69% of the total DOC, 5 kDa-0.1 μm fraction from 30 to 43%, and 0.1 μm-GF/F fraction from 1 to 2% (Table 1), indicating that most DOC was in the <5 kDa fraction. Relative DOC abundance in the <1 kDa fraction ranged from 50 to 78% in oceanic environments (Carlson et al. 1985; Guo et al. (1994, 1995); Guo and Santschi 1996). For freshwater, Martin et al. (1995) reported that only 43% of total DOC was in the <10 kDa fraction in Lena River. These results suggest that the relative abundance of DOC in the lower molecular size fractions varied among different environments. In terms of UV absorbance at 254 nm, the <5 kDa fraction accounted for 57–85% of the total DOM, which was slightly higher than those (55–69%) determined by DOC. For humic-like fluorescence, the <5 kDa molecular size fraction was also dominant, accounting for 74–88% of the total fluorescence. The recoveries for DOC, absorbance and fluorescence ranged from 84% to 121%, which are similar to the previous reports on DOC (80–118%, Carlson et al. (1985) and Guo et al. (1994, 1995), Guo and Santschi (1996)). Ultrafiltration systems that gave good fluorescence or absorbance balances may have poor DOC mass balances since fluorescence and absorbance techniques are more selective and thus may miss contaminants (Buesseler et al. 1996; Mopper et al. 1996). Good balances from all fluorescence, absorbance, DOC and THAA in this study indicate the validity of the ultrafiltration system used.

As seen in Table 1, Flu/Abs abundance ratios increased from 0.1 μm-GF/F to 5 kDa-0.1 μm, to the <5 kDa molecular size fraction, suggesting that the distribution was shifted towards the lower molecular fractions for humic-like fluorescence, as compared to UV absorbance. This is in agreement with previous reports that fluorescence efficiency or Flu/Abs ratios of DOM increased with reducing molecular weight (Stewart and Wetzel 1980; Ewald et al. 1988; Senesi 1990). The difference also implies that fluorescing and absorbing DOM was not evenly distributed over various molecular weights in freshwater.

3DEEM fluorescence characteristics of molecular size fractions

The normalized 3DEEM surface and contour plots of the fractionated DOM are shown in Figure 1. Two general Ex/Em maxima can be observed in these plots: Peak A with Ex/Em 320–350/430–460 nm, and Peak B with Ex/Em 230–250/430–470 nm. Part of the Peak B fluorescence was obscured by water Raman Scattering. Peaks A and B were similar to previous reports for DOM fluorescence in aquatic environments, and were usually referred to as humic-like fluorescence (Mopper and Schultz 1993; Coble 1996; Del Castillo et al. 1999; Wu et al. 2001).

It is interesting to note that an additional Peak C with Ex/Em 260–290/330–350 nm was obvious only in the 0.1 μm-GF/F fractions and GF/F filtrates. Fluorescence

Table 1. Mass balances of DOC, absorbance, fluorescence and THAA of molecular size fractions of DOM in Lake Biwa.

Samples	Fractions	DOC	Absorbance [a]	Fluorescence [a]	THAA [d]	Ratios	
		μ M C [a]	at 254 nm(10^{-4} cm^{-1})	Ex/Em 330/434 nm [c]	Concentration (nM) [a]	Flu/Abs [e]	THAA/DOC [e]
Station B							
2.5 m	GF/F filtrate	104	160	7.1	1106		(1.1)
	0.1 μm-GF/F	2 (2)	10 (7)	0.4 (5)	69 (7)	0.6 (0.04)	3.5 (3.3)
	5 kDa-0.1 μm	40 (43)	50 (36)	1.8 (21)	411 (42)	0.6 (0.04)	1.0 (1.0)
	< 5 kDa	51 (55)	80 (57)	6.4 (74)	500 (51)	1.3 (0.08)	0.9 (1.0)
	Sum of 3 fractions	93 (100)	140 (100)	8.6 (100)	980 (100)		
	Recovery (%) [b]	89	88	121	89		
70 m	GF/F filtrate	89	132	8.4	975		(1.1)
	0.1 μm-GF/F	1.0 (1)	4 (3)	0.2 (2)	83 (8)	0.6 (0.05)	8.0 (8.0)
	5 kDa-0.1 μm	28 (30)	18 (12)	1.0 (10)	302 (29)	0.8 (0.06)	1.0 (1.1)
	< 5 kDa	65 (69)	125 (85)	8.4 (88)	655 (63)	1.0 (0.07)	0.9 (1.0)
	Sum of 3 fractions	94 (100)	147 (100)	9.6 (100)	1042 (100)		
	Recovery (%) [b]	106	111	114	107		
Station A							
2.5 m	GF/F filtrate	107	190	9.8	1530		(1.4)
	0.1 μm-GF/F	2 (2)	10 (6)	0.5 (5)	156 (11)	0.8 (0.05)	5.5 (7.8)
	5 kDa-0.1 μm	47 (40)	30 (19)	1.9 (18)	540 (38)	0.9 (0.06)	1.0 (1.1)
	< 5 kDa	67 (58)	120 (75)	8.1 (77)	724 (51)	1.0 (0.07)	0.9 (1.1)
	Sum of 3 fractions	116(100)	160 (100)	10.5 (100)	1420 (100)		
	Recovery (%) [b]	108	84	107	93		

[a]The value was estimated as that in the original water. The value in the parentheses denoted that of each fraction as a percentage of the sum for all fractions. [b]Recovery was expressed as a proportion of the sum of all fractions relative to the value for the GF/F filtrate. [c]The unit was arbitrary. [d]THAA denoted total hydrolyzed amino acids. [e]Ratios were based on their relative abundance, and the values in the parentheses were based on their contribution.

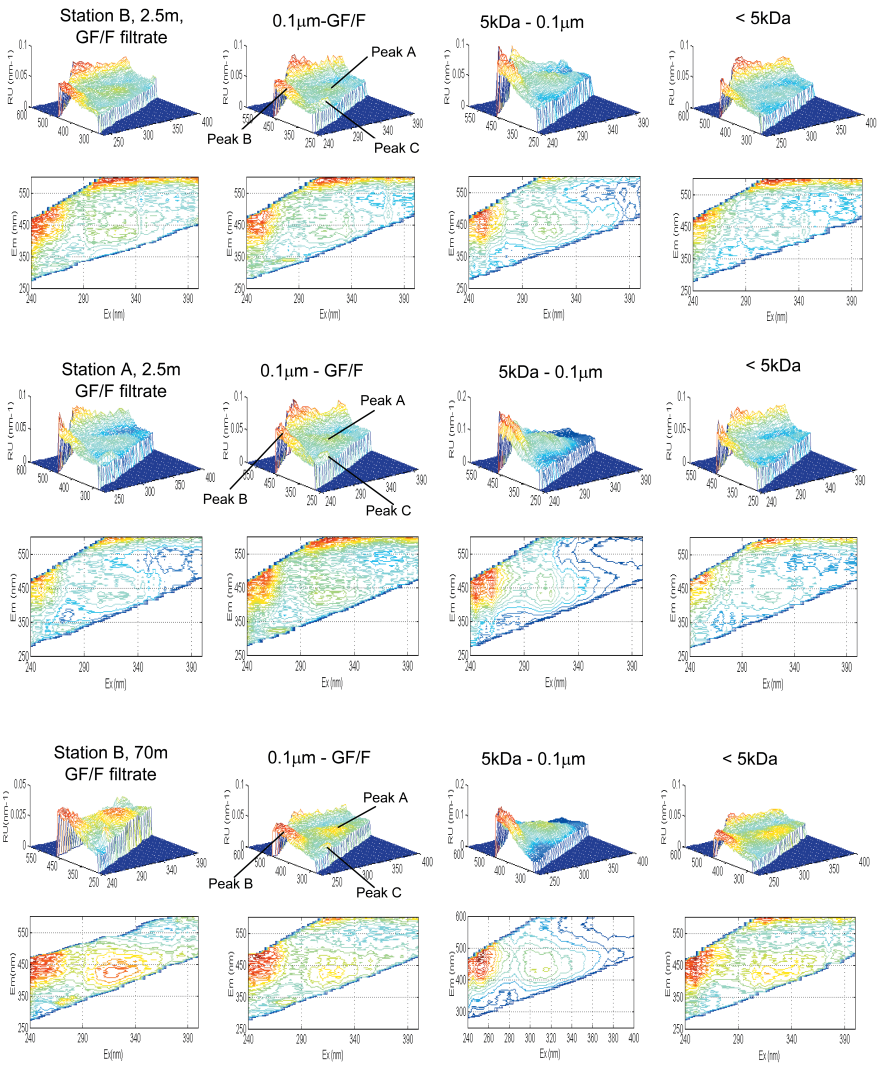

Figure 1. 3DEEM surface and contour plots of molecular size fractions of DOM in Lake Biwa. The fluorescence was calibrated by water Raman scattering. The fractions were the same as those in Table 1.

similar to Peak C in this study was also reported in other natural waters (280/325–335 nm, Coble et al. (1990); 270/320 nm, Mopper and Schultz (1993); 270/320 nm, Determann et al. (1994) and Wu et al. (2001)), and was usually referred to as protein-like fluorescence. Determann et al. (1994, 1998) reported that some phytoplankton, picoplankton and bacteria were the major sources of protein-like fluorescence in natural aquatic environments. Our results are consistent with those reports since the 0.1 μm-GF/F fraction may possibly include small phytoplankton and bacteria particles.

Thus, differences in fluorescence properties were evident for different molecular size fractions of DOM. The humic-like and protein-like fluorescence was shifted to the lower and higher molecular size fractions, respectively.

Amino acids in various molecular size fractions, and their relationship with fluorescence characteristics

Total hydrolyzed amino acids (THAA) included total acid hydrolyzed amino acids and alkaline hydrolyzed tryptophan. THAA concentrations and composition in various molecular size fractions of DOM are shown in Tables 1 and 2. The mass balance ranged 89–107%, and was again satisfactory. The <5 kDa molecular size fraction accounted for 51–63% of total THAA, indicating that major THAA were in the <5 kDa fraction. This result is similar to fluorescence and absorbance distribution discussed earlier. However, THAA/DOC ratios increased with increasing molecular size (Table 1), showing that THAA were more heavily weighted in the higher molecular size fractions than in the lower molecular fractions.

Molar percent ratios of individual amino acids in THAA in different molecular size fractions were analyzed to identify differences in composition (Figure 2, Table 2). It is shown that the most abundant species were Ala, Asp, Glu, Gly and Ser in all molecular size fractions, accounting for 53.4 – 58.4% of total THAA.

Amino acids in different molecular size fractions have not been well documented in aquatic environments. At present, only a few studies have been done in freshwaters, and these have mainly focused on comparison between particulate and dissolved fractions (Mayers et al. 1984; Coffin 1989; Berdie et al. 1995). Amino acids have been extensively studied in bulk DOM, sediments and particulate in aquatic environments (Siezen and Mague 1978; Lee and Cronin 1984; Steinberg et al. 1987; Burdige and Martens 1988; Colombo et al. 1998; Dauwe and Middelburg 1998). These previous studies demonstrated that despite overall similarity of amino acid composition in sediments, particulate and setting particle in the water column, evident differences (composition and concentration) with depth existed, indicating organic matter degradation. It was also reported that glutamic acid, aromatic tyrosine and phenylalanine were labile, while glycine, serine and threonine were selectively preserved in degradation. Our data (Table 2 anf Figure 2a) show that the relative abundance of aspartic acid and glutamic acid decreased with reducing molecular size (from 0.1 μm-GF/F, to 5 kDa-0.1 μm, to the <5 kDa fractions), while that of glycine and serine increased. This finding may imply that amino acids were good biomarkers for DOM degradation, possibly tracing the occurrence of organic matter degradation from higher to lower molecular weight. This is strongly supported by the higher nitrogen abundance and lability of higher molecular size fractions of DOM in recent studies (Hollibaugh and Azam 1983; Harvey et al. 1995), and is also consistent with the fact that THAA contribution to total DOC decreased from the higher to lower molecular size fractions (Table 1), as this may suggest the preferential removal of THAA relative to organic carbon during degradation. The fluorescence results discussed in earlier sections also support our suggestion since the <0.1 μm fractions were dominated by humic-like fluorescence, which was report-

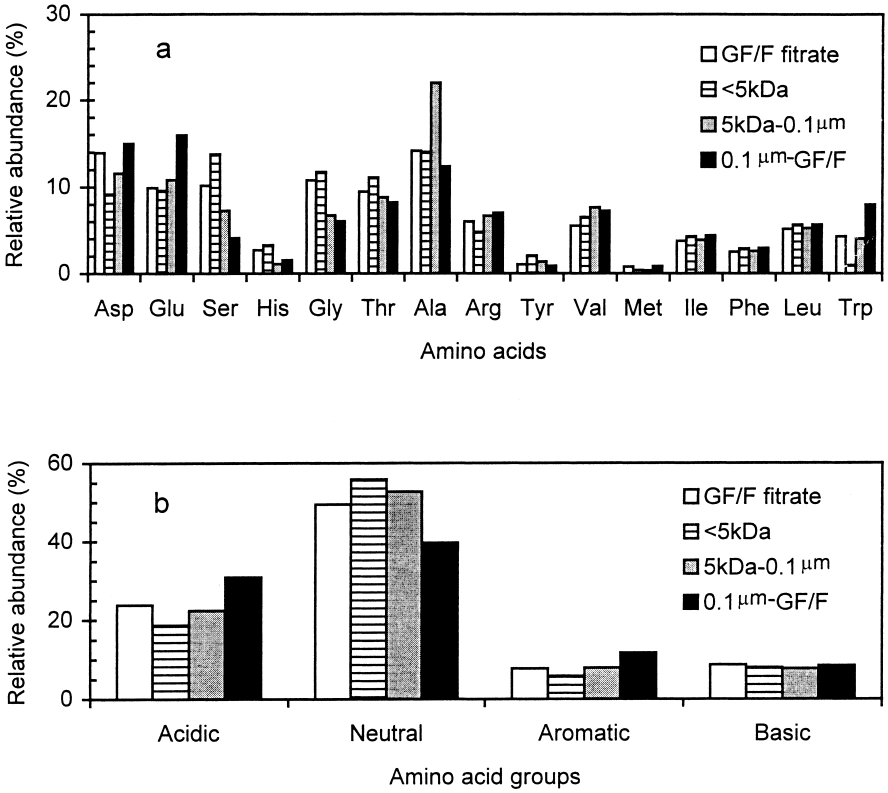

Figure 2. The average relative abundances of amino acids and their functional groups in molecular size fractions of DOM in Lake Biwa.

edly resulted from refractory organic materials (Mopper and Schultz 1993; Determann et al. 1994); only the 0.1 μm-GF/F fraction had the protein-like fluorescence, which was linked to recent biological origin (e.g. Traganza (1969) and Mopper and Schultz (1993)). Moran et al. (2000) observed that protein-like fluorescence was increased during DOM biological degradation experiments, suggesting that biological degradation would not be possibly responsible for the DOM degradation from higher to lower molecular weight. Photochemical degradation may be the most likely process as this has been widely reported to play key roles in controlling the degradation of DOM in aquatic environments (e.g. Zepp (1998) and Moran et al. (2000)).

In terms of amino acid functional groups, there also existed subtle differences (Figure 2b). Acidic (Asp and Glu) and aromatic (Tyr, Phe and Trp) species decreased from 0.1 μm-GF/F, to 5 kDa-0.1 μm, to the <5 kDa molecular size fraction, while neutral species (Gly, Thr, Ala, Val and Ile) increased. This may suggest preferential decomposition of acidic and aromatic amino acids relative to neutral amino acids during degradation. Similar results were also reported in sediments (Maita et al. 1982; Gonzalez et al. 1983; Steinberg et al. 1987; Burdige and Mar-

Table 2. Molar abundances (%) of individual amino acids relative to THAA in molecular size fractions of DOM in Lake Biwa.

THAA	Station B, 2.5 m				Station B, 70 m				Station A, 2.5 m			
	GF/F filtrate	<5 kDa	5 kDa-0.1 μm	0.1 μm-GF/F	GF/F filtrate	<5 kDa	5 kDa-0.1	0.1 μm-GF/F	GF/F filtrate	<5 kDa	5 kDa-0.1 μm	0.1 μm-GF/F
acidic												
Asp	15.7	9.1	11.6	14.4	15.4	8.9	11.4	13.6	10.6	9.5	11.7	17.0
Glu	9.4	8.9	10.5	15.5	9.2	8.7	10.2	14.6	11.0	11.1	11.8	17.8
aromtaic												
Tyr	0.5	2.0	1.0	0.6	0.5	1.9	1.0	0.5	2.1	2.3	2.2	1.5
Phe	2.3	2.4	2.6	2.8	2.3	2.4	2.6	2.6	2.9	3.7	2.6	3.4
Trp	4.1	0.2	3.4	7.3	6.1	2.3	5.4	12.6	2.5	0.4	3.2	4.0
neutral												
Gly	8.9	11.6	6.5	5.2	8.8	11.4	6.4	4.9	14.6	12.2	7.3	7.9
Thr	10.8	13.7	8.0	9.7	10.6	13.4	7.8	9.2	7.1	6.2	10.6	5.8
Ala	14.0	14.1	24.3	13.7	13.7	13.8	23.8	13.0	14.7	14.2	18.0	10.4
Val	5.1	5.1	7.8	6.8	5.0	5.0	7.6	6.5	6.4	9.4	7.6	8.4
Leu	4.8	4.7	5.2	5.0	4.7	4.6	5.1	4.7	5.9	7.5	5.3	7.1
Ile	3.5	3.6	3.9	4.0	3.4	3.5	3.8	3.8	4.3	5.6	3.8	5.2
basic												
Arg	8.0	6.0	7.0	8.9	7.9	5.9	6.9	8.4	2.1	2.4	6.0	3.8
His	3.4	3.8	1.2	1.8	3.2	3.7	1.2	1.7	1.7	2.3	0.9	1.1
other												
Ser	8.4	14.5	6.7	3.3	8.3	14.2	6.5	3.1	13.8	12.7	8.7	5.8
Met	1.1	0.3	0.3	0.9	1.0	0.3	0.3	0.8	0.2	0.6	0.5	0.8

tens 1988). There was no observable trend for basic species (Arg and His) in different molecular size fractions.

It is noteworthy that tryptophan concentrations in DOM increased with increasing molecular size (Table 2 and Figure 2). There are no comparable data at present since tryptophan analysis has been ignored due to its lability in the acid hydrolysis used in most recent studies (Mayers et al. 1984; Robertson et al. 1987; Berdie et al. 1995). Fluorescence similar to protein-like fluorescence in both freshwater and oceanic waters has been widely assumed to be due to the aromatic amino acids since only three aromatic amino acids (tryptophan, tyrosine, phenylalanine) among all amino acid species were reported to fluoresce (e.g. Wolfbeis (1985)). Our observations show that the 0.1 μm-GF/F molecular size fraction containing protein-like fluorescence not only had abundant amino acids (relative to DOC), but also had a higher proportion of aromatic amino acids (relative to total THAA) than the lower molecular size fraction without protein-like fluorescence (Table 1, Figure 1 and Figure 2). Thus our results reconfirm the previous assumed relationship between the protein-like fluorescence and aromatic amino acids in DOM. Since phenylalanine usually has lowest fluorescence efficiency among the three aromatic amino acids, tryptophan fluoresces much more strongly than tyrosine when bound (Wolfbeis 1985; Determann et al. 1998), the protein-like fluorescence is likely due to the presence of tryptophan in DOM. The decrease of relative tryptophan abundance from the higher to lower molecular size fractions may imply that tryptophan was labile, and may be used as a new proxy for DOM degradation from higher to lower molecular weight.

Summary

At present, little is known about the relationships between the physical and chemical characteristics and the molecular size of DOM. Our observations (Table 1, Table 2, Figure 1 and Figure 2) provide some new insight on the relationship between fluorescence and amino acid composition. The results show that the 0.1 μm-GF/F molecular size fraction had a higher relative abundance of amino acids (Table 1), and a higher proportion of acidic and aromatic species (Figure 2, Table 2). Differences in relative abundances of individual amino acids were evident among different molecular size fractions (Table 2 and Figure 2). All fractions had similar humic-like fluorescence (Peaks A and B), but unique protein-like fluorescence was found in the 0.1 μm-GF/F fraction. Based on these results, it is likely that the 0.1 μm-GF/F fraction may be of more recent biological origin, representing newly produced organic materials, and the lower molecular size fractions may have been considerably degraded.

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