Fluorescence properties of organic ligands for copper(II) in Lake Biwa and its rivers

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Organic ligands for copper(II) were isolated from lake waters in Lake Biwa and its river waters by immobilized metal ion affinity chromatography (IMAC), and were characterized by three-dimensional excitation/emission matrix spectroscopy (3DEEM) and fluorescence quenching titration. The results show that the contribution of total organic ligands was 0.63–4.68% of the bulk dissolved organic matter (DOM), in terms of UV absorbance, in lake and river waters. Three characteristic excitation/emission (Ex/Em) fluorescence peaks were identified in organic ligands from both lake and river waters, at Ex/Em 310–330/374–434 nm (Peak A), 250/414–454 nm (Peak B), and 260–270/306–330 nm (Peak C). Peaks A and B were referred to as humic-like fluorescence, Peak C as protein-like fluorescence. All Ex/Em maxima of organic ligands in lake waters were shifted towards shorter wavelength, and the fluorescence intensities were higher than those in river waters. The results of fluorescence quenching titration show that the IMAC ligands were weak ligands, with conditional stability constants (log $K'_{\text{CuL}}$) around 7.27 for river ligands, and 7.84–9.23 for lake ligands. The differences of fluorescence properties indicate the variability of fluorescent ligands between river terrestrial and lake aquatic environments.

INTRODUCTION

Naturally occurring organic ligands have been extensively studied in terms of metal speciation in aquatic environments during the past decades (Tanoue and Midorikawa, 1995). In oceanic waters, copper speciation is controlled by complexation with organic ligands (Donat and Bruland, 1995). In freshwaters, there are at least three ligand classes with logK values ranging from 15 to 8.6 (Xue and Sunda, 1997), which may control the bioavailability, toxicity and speciation of copper. Organic ligands were also reported to be responsible for other trace metal speciation (e.g., Hg) in freshwater (Mantoura et al., 1978), and play a significant role in the mobilization and transport of nutrients and pollutants (As, Zn, Cd, Pb, and Ce), and soil weathering in terrestrial ecosystems (Tegen and Dorr, 1996; Li and Shuman, 1997; Kalbitz and Wennrich, 1998). However, most previous studies have only provided concentrations and conditional stability constants of organic ligands, the fundamentals pertaining to the properties of organic ligands are poorly understood since they have never been isolated (Gordon et al., 1996; Midorikawa and Tanoue, 1996, 1998).

Immobilized metal ion affinity chromatography (IMAC) has been successfully applied to isolate organic ligands for copper(II) from estuarine, coastal and oceanic waters (Gordon, 1992; Gordon...
et al., 1996; Midorikawa and Tanoue, 1996, 1998; Donat et al., 1997). 3DEEM and UV-VIS spectroscopy have been demonstrated to be useful methods for characterizing DOM in natural waters (Coble et al., 1990; Mopper and Schultz, 1993; Coble, 1996; Smith and Kramer, 1999; Del Castillo et al., 1999). Absorbing and fluorescing complexes such as humic substances and proteins were expected to be good candidates for organic ligands for copper(II) because of their known binding abilities with trace metals (Ryan and Weber, 1982; Senesi, 1990; Luster et al., 1996; Smith and Kramer, 1999). Midorikawa and Tanoue (1996, 1998) reported chromophoric properties of dissolved organic ligands for copper(II) in oceanic waters. To our knowledge, little is known about the characteristics of organic ligands in freshwaters.

In this study, by a combination of IMAC, 3DEEM and fluorescence quenching titration, we report here the contribution of organic ligands relative to the bulk DOM, and their fluorescence and absorbance properties and binding abilities. The differences between river and lake ligands are also discussed.

**MATERIALS AND METHODS**

**Sampling**

Lake Biwa is the largest lake and the greatest freshwater resource in Japan with a surface area of 670 km², and a maximum depth of 103.6 m. There are more than 20 main rivers flowing into the lake.

Sampling sites are shown in Fig. 1. Lake water was collected at Stn 17B and 15A in Lake Biwa in April and June, 1999. River samples (Azusa, Egadani, Kiryu, and Daido) were taken in the upper streams in the lake watershed. Water samples were filtered through GF/F glass-fiber filter (Whatman, Maidstone, UK) immediately after sampling, and were kept in frozen until analysis.

**The IMAC isolation procedure**

Filtered water samples were subjected to IMAC following the procedure previously reported elsewhere (Midorikawa and Tanoue, 1996, 1998). Briefly, 20 ml Chelating Sepharose® Fast Flow gel (Pharmacia, Uppsala, Sweden) was used in a column (20 cm × 16 mm i.d., Pharmacia) as the solid matrix for IMAC. The column was charged with 0.02 M copper solution. After copper ions were loaded, the column was rinsed with 0.1 M borate buffer (pH = 8.20). Samples were then loaded at a flow rate of 90 ml·cm⁻²·h⁻¹, followed by washing the column with the borate buffer again. The organic ligands were then eluted with 0.01 M HCl + 0.1 M NaCl solution at a flow rate of 20 ml·cm⁻²·h⁻¹. In order to avoid the possible effect of pH on chemical properties of organic ligand fractions, all fractions were adjusted to pH = 8.15 before monitored by UV absorbance. The UV absorbance was recorded with a spectrophotometer (Shimadzu, MPS-2400, UV-VIS multipurpose). The column was regenerated every run to maintain reproducible condition according to the manufacturer’s instructions.

**3DEEM analysis**

3DEEM was measured with a fluorescence spectrophotometer (Hitachi, Model F-4500). Wavelength ranged from 230 nm to 400 nm for
Fluorescence of organic ligands in lake and river excitation (5 nm bandwidth), from 250 nm to 600 nm for emission (2 nm bandwidth). The instrument was corrected according to the manufacturer’s instruction. A procedural blank was prepared by passing Milli-Q water through a blank copper-loaded column, the blank 3DEEM was subtracted to eliminate possible column contamination and water Raman scattering of samples. The surface and contour plots of 3DEEM were plotted using the Matlab™ program, in which Ex/Em fluorescence maxima can be identified. Fluorescence intensity was expressed in terms of quinine sulfate units (QSU), 1 QSU = 1 µg L⁻¹ of quinine sulfate monohydrate in the solution of 0.05 M H₂SO₄ at Ex/Em 350/450 nm.

Fluorescence quenching titration was performed to determine the complexing abilities of major fluorescence maxima of organic ligands. Standardized copper(II) nitrate solution was added incrementally to reach the final concentration of C_Cu = approximately 40 µM in about 1 ml of ligand fractions at an ionic strength of 0.1 M, pH = 8.15 at 25°C. Changes in fluorescence intensity were essentially modeled by the Ryan-Weber equation (Ryan and Weber, 1982). Quantitative treatment, described in Appendices A and B, was used to evaluate complexing characteristics, and binding parameters were calculated.

**RESULTS**

**IMAC chromatograms**

IMAC chromatograms from lake and river waters generally exhibited one major and resolved peak, as determined by UV absorbance at 254 nm and 280 nm. Examples for river and lake waters are illustrated in Fig. 2. The absorbance peaks occurred at the elution volume of approximately 35 mL for both river and lake waters.

No obvious peak was observed in the copper-loaded column without sample loading (Fig. 2), and no significant change in the absorbance at 254 nm was detected when water samples were loaded without copper loading (data not shown). This indicates that the eluted organic ligands were interacting with copper immobilized on the column, not from non-selective adsorption onto the solid matrix of the IMAC gel, nor contamination from the IMAC gel.

DOM is the most significant contributor to the total UV absorbance in natural water, particularly in the 230–400 nm region (Armstrong and Boalch, 1961; Ogura and Hanya, 1966). There was a broad absorbance shoulder between around 280 and 250 nm in the UV spectra for both the IMAC ligands and original water (Fig. 3). This suggests that the absorbance at 254 nm and 280 nm wavelength
would be appropriate to quantitatively monitor the IMAC ligand fractions.

**The contribution of organic ligands relative to the bulk DOM**

Table 1 shows the absorbance of organic ligands, expressed as in original water. The absorbance value ranged from 3.77 to $14.04 \times 10^{-5}$ cm$^{-1}$ for river waters, and from 21.0 to $32.1 \times 10^{-5}$ cm$^{-1}$ for lake waters. Thurman (1985) reported that about 5–15% of the total DOM was isolated as “humic substance” by adsorption onto hydrophobic resins. However, our results show that the UV-adsorption by the organic ligands was less than 2.2% of the adsorption by total DOM (except for Azusa River, 4.7%) (Table 1). The contribution of organic ligands relative to the bulk DOM in original water was higher for lake waters (1.50–2.2%) than that for river waters (0.63–0.74%, except for Azusa River).

The pH of water samples may influence the extraction of the IMAC ligands, e.g., higher extraction efficiency was observed with increasing pH (Gordon et al., 1996; Midorikawa and Tanoue, 1996). However, absorbance value of organic

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**Table 1. The absorbance of organic ligands, and their contribution to the bulk DOM, as determined by UV absorbance**

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>Samples</th>
<th>DOC</th>
<th>pH</th>
<th>Absorbance at 254 nm ($10^{-5}$ cm$^{-1}$)*</th>
<th>The proportion of ligands relative to bulk DOM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(µM C)</td>
<td></td>
<td>Bulk DOM</td>
<td>Organic ligands</td>
</tr>
<tr>
<td><strong>River</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>April, 1999</td>
<td>Azusa</td>
<td>13.3</td>
<td>8.2</td>
<td>100</td>
<td>4.68</td>
</tr>
<tr>
<td></td>
<td>Egadani</td>
<td>26.5</td>
<td>8.1</td>
<td>600</td>
<td>3.77</td>
</tr>
<tr>
<td></td>
<td>Kiryu</td>
<td>60.6</td>
<td>6.8</td>
<td>1800</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>Daido</td>
<td>62.8</td>
<td>7.5</td>
<td>1900</td>
<td>14.04</td>
</tr>
<tr>
<td>June, 1999</td>
<td>Kiryu</td>
<td>75.5</td>
<td>7.9</td>
<td>1100</td>
<td>7.20</td>
</tr>
<tr>
<td><strong>Lake</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stn 17B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>April, 1999</td>
<td>2.5 m</td>
<td>90.0</td>
<td>7.94</td>
<td>1300</td>
<td>28.48</td>
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<tr>
<td></td>
<td>70 m</td>
<td>84.0</td>
<td>7.84</td>
<td>1300</td>
<td>23.54</td>
</tr>
<tr>
<td>June, 1999</td>
<td>2.5 m</td>
<td>106.1</td>
<td>8.43</td>
<td>1600</td>
<td>32.10</td>
</tr>
<tr>
<td></td>
<td>70 m</td>
<td>91.0</td>
<td>7.63</td>
<td>1400</td>
<td>21.00</td>
</tr>
<tr>
<td>Stn 15A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>April, 1999</td>
<td>2.5 m</td>
<td>89.0</td>
<td>8.2</td>
<td>1400</td>
<td>28.97</td>
</tr>
</tbody>
</table>

*The value was given as that in the original water.*
Fig. 4. The surface and contour plots of 3DEEMs of the IMAC ligands isolated from Lake Biwa and its rivers. Samples were collected in April, 1999. (a) 2.5 m depth water, Stn 17B, Lake Biwa; (b) 70 m depth water, Stn 17B, Lake Biwa; (c) 2.5 m depth water, Stn 15A, Lake Biwa; (d) Azusa River; (e) Kiryu River; (f) Daido River.
ligands, and their contribution to the bulk DOM did not vary significantly with pH for river and lake waters (Table 1). This indicates that extraction efficiency of the IMAC ligands was not greatly influenced by pH changes within 7.5–8.4 range in our samples.

Table 2. Ex/Em maxima of EEMs and ratios of relative fluorescence to absorbance (at 280 nm) of the IMAC ligands from Lake Biwa and its rivers*

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>Samples</th>
<th>Peak A</th>
<th>Peak B</th>
<th>Peak C</th>
<th>A/B ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake Biwa, Stn 15A</td>
<td>April, 1999</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 m</td>
<td>315/382 (8.97)</td>
<td>250/430 (6.91)</td>
<td>260/322 (4.55)</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>70 m</td>
<td>320/376 (16.92)</td>
<td>250/432 (11.10)</td>
<td>265/318 (5.45)</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>June, 1999</td>
<td>2.5 m</td>
<td>310/384 (7.94)</td>
<td>250/430 (7.62)</td>
<td>270/306 (1.52)</td>
<td>1.0</td>
</tr>
<tr>
<td>10 m</td>
<td>315/386 (6.89)</td>
<td>250/422 (6.61)</td>
<td>270/308 (1.83)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>20 m</td>
<td>315/380 (13.61)</td>
<td>250/438 (6.11)</td>
<td>—</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>40 m</td>
<td>320/378 (15.02)</td>
<td>250/434 (7.39)</td>
<td>—</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>50 m</td>
<td>315/378 (12.20)</td>
<td>250/432 (7.69)</td>
<td>—</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>60 m</td>
<td>315/380 (14.61)</td>
<td>250/430 (8.22)</td>
<td>—</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>70 m</td>
<td>315/380 (16.27)</td>
<td>250/414 (8.01)</td>
<td>—</td>
<td>2.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rivers</th>
<th>April, 1999</th>
<th>Peak A</th>
<th>Peak B</th>
<th>Peak C</th>
<th>A/B ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azusa</td>
<td>330/428 (2.61)</td>
<td>250/442 (5.68)</td>
<td>265/320 (2.66)</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Egadani</td>
<td>315/428 (2.51)</td>
<td>250/454 (4.27)</td>
<td>265/326 (1.40)</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Kiryu</td>
<td>315/426 (4.70)</td>
<td>250/452 (12.40)</td>
<td>265/330 (3.23)</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Daido</td>
<td>320/434 (3.31)</td>
<td>250/452 (6.31)</td>
<td>265/326 (1.55)</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>June, 1999</td>
<td>Kiryu</td>
<td>320/432 (2.91)</td>
<td>250/444 (4.23)</td>
<td>270/322 (0.47)</td>
<td>0.7</td>
</tr>
<tr>
<td>Average</td>
<td>315–330/426–434 (3.21)</td>
<td>250/442–454 (6.57)</td>
<td>265–270/320–330 (1.86)</td>
<td>0.56</td>
<td></td>
</tr>
</tbody>
</table>

*Ex/Em maxima are indicated by Ex/Em wavelength (nm), values in parentheses represent the ratios of relative intensity to the UV absorbance at 280 nm.
—: not detected.
Fluorescence of organic ligands in lake and river

Fig. 5. Fluorescence quenching titration of organic ligands and their fitting curves using Eq. (B3) in Appendix B. Quenching ratio represents a proportion of fluorescence relative to that before copper addition.

Fig. 6. The quenching titration plotting of $C_{Cu}((1 - X)/X)$ vs. $(1 - X)$ (Appendix A) of Peaks A (●) and B (○) in the IMAC ligands, showing their binding characteristics. A: Azusa River; B: 2.5 m depth water at Stn 17B, Lake Biwa.

hereafter), the second from excitation in the UV-B region and emission in the visible region (Peak B, hereafter), and the third from both excitation and emission in the UV-B region (Peak C, hereafter). For Peak A, there was no obvious shift in the Em maximum at increasingly longer or shorter Ex wavelength (Figs. 4a, b and c), indicating that Peak A may come from a single fluorophore. This is different from previous reports of DOM (Donard et al., 1989; Coble et al., 1990; De souza Sierra et al., 1994; Coble, 1996). They reported fluorescence maxima in this similar region in oceanic waters, but the Em maximum depended on chosen Ex. The contour plots for Peak B, however, were in the elliptical shape, and Em maximum increased with Ex wavelength, indicating that Peak B was from a mixture of fluorophores or single exciplex type. Part of Peak B was obscured by neighbor water Raman scattering. Em maximum at Ex = 250 nm was measured for Peak B in this study. Peak C was the smallest among these three peaks.

The Ex/Em maxima in all lake ligands are summarized in Table 2. The results show that Peak A had fluorescence maxima at Ex/Em 310–320/376–386 nm and Peak C at 265–270/306–318 nm. Em maximum of Peak B ranged from 414 nm to 438 nm at Ex 250 nm.

Comparison of 3DEEMs between lake and river ligands

The patterns of 3DEEMs of all river ligands were similar to those of lake ligands, as shown in examples in Figs. 4d, e and f for ligands from Azusa, Kiryu and Daido rivers. The three similar maxima were Peak A at Ex/Em 315–330/426–434 nm, Peak B at 250/442–454 nm, and Peak C at 265–270/320–330 nm. Comparison between lake and river ligands (Table 2 and Fig. 4), however, shows that all three maxima for lake ligands were shifted towards shorter wavelength compared to those for river ligands. Ex/Em wavelength, on the average, differed by up to 7/48 nm for Peak A, 0/19 nm for Peak B, and 0/13 nm for Peak C, respectively. Another distinguishing difference between river and lake ligands is that lake ligands had higher fluorescence intensity than river
ligands for all Peaks A, B and C, particularly Peak A (Table 2). Among the three peaks, Peak A was the most pronounced in lake ligands as indicated by the intensity ratio of Peak A to B (Table 2) whereas Peak B was the most obvious in river ligands. The fluorescence ratios of Peak A to B in lake ligands were 1.0–2.4, higher than those in river ligands (0.4–0.7) (Table 2).

**Complexing abilities of the IMAC ligands**

In the absence of Cu, the 3DEEM of the IMAC ligands from both river and lake waters consisted of three major maxima (Fig. 4). The addition of Cu led the fluorescence intensity decrease during copper quenching titration (Fig. 5). Since Peak C was the smallest peak, only binding abilities of Peaks A and B were investigated in this study.

In the calculation of complexing abilities, the plot of $C_{Cu}((1 – X)/X)$ vs. $(1 – X)$ (Appendix A) is usually applied for one metal and one ligand model. For the possible multi-site system in the IMAC ligands, the plot was used to evaluate their binding properties by applying for these peaks individually. Examples of diagrams are shown in Fig. 6. The results show that the diagrams for both Peaks A and B in river ligands were not linear, but two similar trend curves, indicating that Peaks A and B fluorescence may be from at least two different binding sites. The ratio of the stability constant of Peak A to B was calculated by Eq. (B4) (Appendix B). An example of the ratio calculation is shown in Fig. 7a for Azusa River. The results (Table 3) show that the stability constants between Peaks A and B in all river ligands were very similar, the difference between log$K'_{CuL}$ values was within the 0.009–0.071 range. It is highly possible that Peaks A and B represented two individual binding sites with very similar binding strength.

Whereas for lake ligands, the plot of $C_{Cu}((1 – X)/X)$ vs. $(1 – X)$ (Appendix A) shows that all diagrams for Peak A were linear, which were different from Peak B, a non-linear curve, as shown in Fig. 6b. It is considered that the binding site for Peak A was predominant, Peak B was minor. The ratios of the stability constants of Peak A to B support this suggestion, log$(K_A/K_B)$ ranged from 0.17 to 0.9 in lake ligands (Table 3).

Therefore, 1:1 metal:ligand discrete model and parameter fitting program (Eq. (B3), Appendix B) was applied for Peak A in river ligands to get the average binding capacity and stability constant. For lake ligands, the fitting program was applied for Peak A to approximately obtain complexing abilities of the predominant binding site, the stability constant of Peak B was determined after the ratio of Peak A to B was independently calculated (Eq. (B4), Appendix B). Examples of the fitting curves are shown in Fig. 5. The fitting results of all river and lake ligands are summarized in Table 3. The stability constants and binding capacities of fluorescence Peaks A and B from lake ligands were higher than those from river ones. For river ligands, the average stability constants (log$K'_{CuL}$)
Fluorescence of organic ligands in lake and river

Table 3. Calculated stability constants ($\log K_{\text{CuL}}'$) and binding capacities ($C_L$) of Peaks A and B of the IMAC ligands from river and lake waters

<table>
<thead>
<tr>
<th>Samples</th>
<th>Binding sites</th>
<th>$C_L$ (10$^{-6}$ M)</th>
<th>$\log K'_{\text{CuL}}$</th>
<th>$\log(K_{A}/K_{B})^*$</th>
<th>$C_L/\text{At}$ ($\mu$M cm)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rivers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>April, 1999</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azusa</td>
<td>Average</td>
<td>0.64</td>
<td>7.29</td>
<td>0.009</td>
<td>10.0</td>
</tr>
<tr>
<td>Egadani</td>
<td>Average</td>
<td>1.04</td>
<td>7.28</td>
<td>0.045</td>
<td>9.9</td>
</tr>
<tr>
<td>Kiryu</td>
<td>Average</td>
<td>1.26</td>
<td>7.31</td>
<td>0.071</td>
<td>23.3</td>
</tr>
<tr>
<td>Daido</td>
<td>Average</td>
<td>0.75</td>
<td>7.21</td>
<td>0.056</td>
<td>10.3</td>
</tr>
<tr>
<td>Lake</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>April, 1999</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17B, 2.5 m</td>
<td>A</td>
<td>4.99</td>
<td>7.84</td>
<td>0.17</td>
<td>56.7</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>—</td>
<td>7.67</td>
<td></td>
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</tr>
<tr>
<td>17B, 70 m</td>
<td>A</td>
<td>4.59</td>
<td>9.23</td>
<td>0.54</td>
<td>101.8</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>—</td>
<td>8.69</td>
<td></td>
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</tr>
<tr>
<td>15A, 2.5 m</td>
<td>A</td>
<td>4.47</td>
<td>7.96</td>
<td>0.90</td>
<td>72.3</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>—</td>
<td>7.06</td>
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</table>

*$\log(K_{A}/K_{B})$ was calculated from Eq. (B4) in Appendix B.  
**$\log(K_{A}/K_{B})$ was calculated from Eq. (B4) in Appendix B.

and binding capacities ($C_L$) were 7.21–7.31, 0.64–1.26 $\times 10^{-6}$ M, respectively. While $\log K'_{\text{CuL}}$ and $C_L$ for lake ligands were 7.84–9.23, and 4.47–4.99 $\times 10^{-6}$ M, respectively.

**DISCUSSION**

The typical 3DEEM features of natural DOM

The reported Ex/Em fluorescence maxima of DOM in aquatic environments, extract of litter and coal reef are summarized in Fig. 8. The results show that natural fluorophores can be clearly grouped into at least three general classes: Class I (Ex about 290–390 nm, Em about 340–490 nm), Class II (Ex about 220–270 nm, and Em about 400–490 nm), Class III (Ex about 255–290 nm, and Em about 290–410 nm). The data of Ex/Em maxima in Fig. 8 were obtained from recent literature, in which most instruments were corrected and 3DEEM was measured. It is suggested that three unique classes of fluorophores occurred in a variety of environments.

Fluorescence in Classes I and II was referred to as humic-like fluorescence (Mopper and Schultz, 1993; De souza Sierra et al., 1994; Coble, 1996), and probably involved conjugated unsatu-
rated bond systems bearing carbonyl and carboxyl groups (Senesi, 1990; Midorikawa and Tanoue, 1998). Among many naturally occurring fluorophores in Class I, simple phenol, coumarins and alkaloids with some solubility in water (Wolfbeis, 1985) are the most likely contributors to such types of fluorophores in aquatic environments. Fluorescence in Class II was always reported in oceanic waters (Donard et al., 1987; Mopper and Schultz, 1993; De souza Sierra et al., 1994), but there were only a few reports in freshwater (Blaser et al., 1999). The chemical identity of Class II is unknown. Fluorescence in Class III was reported to be due to the presence of dissolved proteins, derivatives of amino acids, and other heterocyclic compounds containing the indole group, and was referred to as protein-like fluorescence (Traganza, 1969; Mopper and Schultz, 1993; Determann et al., 1994; Coble, 1996).

All fluorescence peaks of river and lake IMAC ligands fell into these three Classes (Fig. 8), indicating general similarity of natural organic molecules or structures with the IMAC ligands. This is the first fluorescence data for copper-complexing ligands in natural environments.

River vs. lake IMAC ligands

pH may be one factor that could result in 3DEEM differences between river and lake ligands. pH varied in the range of 6.8–8.2 in river samples, 7.63–8.43 in lake samples. The pH of the ligand fractions was adjusted to 8.15. In order to avoid the effect of sample loading volume on the IMAC ligand-adsorption efficiency, all sample volumes were controlled around 4–6 L, in which adsorption efficiency in the IMAC column was independent of sample volume (Midorikawa and Tanoue, 1996). The interaction with copper did not affect 3DEEM patterns of the IMAC ligands. Coble (1996) reported that C18 Sep-Paks isolation and XAD extraction procedure did not significantly affect DOM fluorescence properties. In terms of absorbance values, 3DEEM patterns, relative intensity and binding strength, there were excellent similarity between lake ligands, and between river ligands as well (Tables 1, 2 and 3). It is highly possible that other factors rather than pH and IMAC procedure were mainly responsible for the differences between lake and river IMAC ligands.

The observed similarity in fluorescence properties between lake ligands and between river ligands indicates that fluorescence of the IMAC ligands was derived from a mixture of very similar fluorophores from original water. DOC concentrations of Azusa, Egadani, Kiryu and Daido river waters in this study represented four different DOC levels among the 23 major rivers in the watershed of Lake Biwa, and the bulk DOM in all rivers had similar chromophoric properties (Konohira, personal communication). The four selected rivers may represent, to some extent, the terrestrial ecosystem in the watershed because all river samples were taken in the upstream. The differences of fluorescence properties between lake and river ligands (Tables 1, 2, and 3) may reflect the variability of organic ligands between river terrestrial and lake aquatic ecosystems.

The observed Ex/Em maximum shift between lake and river IMAC ligands (Table 2 and Figs. 4 and 8) was similar to those of previous reports on the bulk DOM fluorescence. Senesi (1990) reported an increase in relative intensity, which was associated with shift towards shorter Em maximum and the main Ex maxima, when passing from river humic acids to dissolved aquatic fulvic acids. Similar blue-shift of DOM Em wavelength at Ex larger than 300 nm was reported in various freshwater, estuarine and marine environments (Donard et al., 1989; De souza Sierra et al., 1994; Coble, 1996). The results are the first report that all three major maxima in lake ligands were shifted towards shorter wavelength compared those in river ligands.

Changes in fluorescence Ex maximum are attributed to chemical differences of fluorescence compositions (Coble, 1996). Fluorescence Em maximum shift towards to shorter wavelength can be caused by (a) the decrease in the number of aromatic rings condensed in a straight chain, and conjugated double bonds (Senesi, 1990), (b) the branching of aromatic system (Coble, 1996), and
Fluorescence of organic ligands in lake and river

(c) more localization of π-electron system (Hayase and Tsubota, 1985). Recent studies on natural DOM have suggested that the shifts of Ex maxima and Ex/Em maxima, and fluorescence intensity were closely associated with the molecular weight and functional group concentrations. Ex maximum of natural DOM was shifted towards shorter wavelength with decreasing molecular weight, accompanied by an apparent increase in fluorescence efficiency (Hall and Lee, 1974; Ewald et al., 1988). The Ex/Em fluorescence intensity was reported to increase with decreasing molecular weight in soil, sedimentary and aquatic fulvic and humic substances (Levesque, 1972; Hall and Lee, 1974; McCreary and Snoeyink, 1980; Visser, 1984; Hayase and Tsubota, 1985), and natural organic ligands (Midorikawa and Tanoue, 1998). Buffle (1988) and Midorikawa and Tanoue (1998) pointed out that lower molecular mass fraction was linked to higher concentrations of carboxyl groups in freshwater. Functional groups (such as hydroxyl, methoxyl and carboxyl groups) can greatly enhance fluorescence intensity by increasing the transition probability between the singlet and ground states (Hall and Lee, 1974). Similarly, the shorter Ex/Em maxima and higher fluorescence intensity for lake ligands, may suggest that lake organic ligands were composed of higher proportion of low molecular weight masses with higher concentration of functional groups than river organic ligands. This may partially explain the higher stability constants of lake ligands than those of river ligands. If this is the case, degradation of organic ligand molecules in aquatic environment may be the possible factor responsible for the variability in fluorescence properties between river and lake ligands.

CONCLUSIONS

This study demonstrated the advantage of combined IMAC and 3DEEM technique in characterizing copper-complexing organic ligands. Simple mathematic and quantitative treatment of multi-fluorophore quenching system was developed to obtain major binding parameters. The overall fluorescence maxima including humic-like and protein-like fluorescence in lake ligands were shifted towards shorter wavelength compared to those in river ligands. The IMAC ligands from lake waters fluoresced intensively and contained higher copper-complexing capacities with higher stability constants than those from river waters. The observed differences of fluorescence properties between lake and river ligands may reflect the variability between river terrestrial and lake aquatic ecosystems, probably resulting from differences in molecular and functional groups.

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REFERENCES


Appendix A

For 1:1 stoichiometry for copper(II) complexation with organic ligands, the complexing reactions that fit the quenching titration data can be described by the linear regression program in the following equation (Midorikawa and Tanoue, 1998).

\[ C_{Cu} \cdot \left( \frac{1 - X}{X} \right) = C_L \cdot (1 - X) + \frac{\alpha_{Cu}}{K'_{CuL}} \]  \hspace{1cm} (AI)

where \( C_{Cu} \) is the total concentrations of all inorganic forms of copper; \( C_L \) is ligand binding capacity;

\[ X = \frac{[CuL]}{C_L} = \frac{FL_{\text{init}} - FL_{\text{end}}}{FL_{\text{init}} - FL_{\text{end}}} \, , \]

the quantity \([CuL]/C_L\) is the fraction of the ligand bound to copper(II), \( CuL \), expressed in terms of the measured fluorescence intensity. \( FL_{\text{init}} \) and \( FL_{\text{end}} \) are the limiting intensities before and after copper(II) titration and correspond to those when all ligand is free and bound, respectively. The stability constant \( K'_{CuL} \) is defined as follows:

\[ K'_{CuL} = \frac{[CuL_i]}{[Cu][L_i]} = \frac{[Cu][L_i]_{\text{init}}}{[Cu][L_i]_{\text{end}}} \]

where \( K_{CuL_i} \) is the constant with regard to the concentration of free copper(II) ion, \([Cu]; [Cu']\) and \([L_i']\) are the total concentrations of all inorganic forms of copper(II) and the ligand \( L_i \) bound to copper(II), respectively; \( \alpha_{Cu} \) is the inorganic side-reaction coefficient for copper(II).

For organic ligands with only one binding site, by combining Eqs. (B1) and (B2), conditional stability constant \( K'_{CuL_i} \) and binding capacity \( C_L \) of the binding site can be quantitatively determined by nonlinear fitting of a plot \( FL \) vs. \( C_{Cu} \) using the following equation:

\[ C_{Cu} \cdot \left( \frac{(1 - X)/X} \right) \, \text{vs.} \, (1 - X). \, \text{If nonlinearity of the diagram is observed, there are at least two ligand classes.} \]

Appendix B

In fluorescence quenching titration experiment, the relationship between measured fluorescence intensity and complexation can be described by the following equation (Midorikawa and Tanoue, 1998):

\[ X_i = \frac{[CuL_i]}{C_{L_i}} = \frac{FL_{\text{init}}^i - FL_{\text{end}}^i}{FL_{\text{init}}^i - FL_{\text{end}}^i} \]  \hspace{1cm} (B1)

where \( C_{L_i} \) is the binding capacity of binding site \( i \), the ratio of \([CuL_i]\) to \( C_{L_i} \), \( X_i \) is the fraction of binding site \( i \), \( L_i \), bound to copper(II), \( CuL_i \), expressed in terms of the fluorescence intensity. \( FL_{\text{init}}^i \) and \( FL_{\text{end}}^i \) are the limiting intensities before and after copper(II) titration and correspond to those when the binding site \( i \) is free and bound, respectively.

The conditional stability constants \( K'_{CuL_i} \) of copper(II) complexes of binding site, \( i \), is defined as follows:

\[ K'_{CuL_i} = \frac{[CuL_i]}{[Cu][L_i]} = \frac{[CuL_i][\alpha_{Cu}]}{[Cu][L_i]_{\text{end}} - [CuL_i]} \]  \hspace{1cm} (B2)

where \( K_{CuL_i} \) is the constant with regard to the concentration of free copper(II) ion, \([Cu]; [Cu']\) and \([L_i']\) are the total concentrations of all inorganic forms of copper(II) and the ligand \( L_i \), respectively; \( \alpha_{Cu} \) is the inorganic side-reaction coefficient for copper(II).
FL = FL\textsubscript{init} - (FL\textsubscript{init} - FL\textsubscript{end}) \cdot \alpha\textsubscript{Cu} \cdot \frac{K \cdot C_L + 1 + K \cdot C\textsubscript{Cu}}{2 \cdot C_L \cdot K} \cdot \frac{\alpha\textsubscript{Cu}}{\alpha\textsubscript{Cu} + 1 + \frac{K \cdot C\textsubscript{Cu}}{\alpha\textsubscript{Cu}}} \cdot \frac{\left(\frac{K \cdot C_L}{\alpha\textsubscript{Cu} + 1} \cdot \frac{K \cdot C\textsubscript{Cu}}{\alpha\textsubscript{Cu}}\right)^2 - 4 \cdot \left(\frac{K \cdot C\textsubscript{Cu}}{\alpha\textsubscript{Cu}}\right) \cdot C\textsubscript{Cu} \cdot C_L}{\left(\frac{K \cdot C\textsubscript{Cu}}{\alpha\textsubscript{Cu}}\right) \cdot C\textsubscript{Cu} \cdot C_L}.

For multi binding sites, substituting for

\[ [\text{CuL}_i] = C_i \cdot X_i, \]

the ratio, $\beta_{ij}$ of stability constant
for two different binding sites, $i$ and $j$, can be written as:

\[
\beta_{ij} = \frac{K'\text{CuL}_{ij}}{K'\text{CuL}_{ji}} = \frac{X_j \cdot (1 - X_j)}{X_j \cdot (1 - X_j)} = \frac{1}{X_j - 1}.
\]

then

\[
\left(\frac{1}{X_j - 1}\right) = \beta_{ij} \cdot \left(\frac{1}{X_i - 1}\right).
\]

For the plotting of $(1/X_i - 1)$ vs. $(1/X_j - 1)$, the least-square regression gave the best fitting value of $\beta_{ij}$ from the slope. Thus the relative binding strength of any two binding sites for copper(II) in organic ligands can be accurately calculated without consideration of equilibrium in the multi-binding site system.