Analysis of Methyl Mercury Binding to Freshwater Humic and Fulvic Acids by Gel Permeation Chromatography/Hydride Generation ICP-MS

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This study examined variation in M(ethyl mercury)(III) binding to humic and fulvic acids using GPC/HG-ICP-MS (gel permeation chromatography/hydride generation-inductively coupled plasma-mass spectrometry). Binding capacities of extracted fulvic acids for M(ethyl mercury) ranged between 6.65 × 10^{-11} and 2.37 × 10^{-10} mol/mg of DOC (dissolved organic carbon) as compared to between 2.96 × 10^{-10} and 9.59 × 10^{-10} mol/mg of DOC for extracted humic acids (i.e., 2–14 times higher). M(ethyl mercury) binding to fulvic acids increased at higher molecular sizes, but binding to humic acids was not related to molecular size. In the past, some mercury cycling models have used total sulfur as a proxy for thiol groups (the principal sites for M(ethyl mercury) binding). We show that total sulfur is not a good estimate of M(ethyl mercury–DOC binding and that its use would result in underestimates of mercury biomagnification in food chains.

Introduction

The determination of methyl mercury binding by dissolved organic carbon (DOC) is important for several reasons. Methyl mercury (MeHg) is one of the most toxic species of mercury; it is widely distributed in the environment, and it has the ability to bioaccumulate and biomagnify in ecosystems (1–2). Regulating bodies are limited in their ability to perform risk assessment for MeHg, in part, due to the lack of accurate DOC binding constants and capacities (3). Current models of mercury cycling are weak in areas that involve the binding of MeHg to DOC because of limitations in our ability to accurately measure free versus DOC-bound MeHg. Since it has been shown that MeHg–DOC binding does occur, there may be only a small portion of total MeHg available for uptake by organisms (4). Binding of MeHg by DOC may ultimately be the most important factor in determining how MeHg will partition between water and aquatic organisms in a natural system.

DOC is derived from a wide array of carbon-containing molecules present in freshwater systems. A portion of these molecules (50–80% of DOC) are collectively referred to as humic substances (5). Humic substances are operationally defined as the hydrophobic material in natural water that will bind to an XAD-8 column when acidified to a pH of 2.0 and that is released at a pH of 8.0 (6). Humic substances contain many acidic functional groups that are capable of binding cations, such as carboxylic acids, phenolic, keto, and thiol groups (5). Humic substances are operationally divided into humic acid, fulvic acid, and humin based on solubility (6). Humic acid is the precipitate that forms at a pH of 2.0 while fulvic acid is the fraction that remains soluble at all pH values. Humin is the portion of humic substances that is insoluble at any pH value (5, 7).

In terms of metal binding ability, the humic and fulvic acid fractions are believed to be the most important (8). The formation of humic– and fulvic–MeHg complexes may be a critical factor in the transport and bioaccumulation of MeHg in aquatic systems (9–11). There are very few published values of MeHg binding capacities for humic and fulvic acids in the literature. One recent study using equilibrium dialysis found values ranging from 0.2 to 1.2 ng of MeHg/mg of humic acid (12) and in a later study 0.2–72 ng of MeHg/mg of humic substance (13). One characteristic of humic and fulvic acids that have been studied in the past is their size distribution. This has been accomplished by utilizing a variety of methods such as ultrafiltration, ultracentrifugation, dialysis, and liquid chromatography (14, 12, 15). Although there have been many recent studies on humic metal interactions, the relationship between DOC, molecular size, and MeHg binding is still unclear. Extended knowledge of MeHg binding to humic substances will be useful for predicting transport of MeHg through watersheds, lake inputs from wetlands, and the biological fate of the MeHg.

Materials and Methods

Sampling. A single 150–200-L sample was collected from each freshwater site within a relatively short time frame (June–August 1997) to minimize changes in DOC composition. Sites were chosen based on published data in order to reflect a variety of molecular weight distributions in the DOC (16). The sites consisted of five lakes within a 200-km radius of Peterborough, Ontario: Cavan Bog, Dickie Lake, Buck Lake, Brandy Lake, and Long Lake. Sampling was conducted near the shore in the littoral zone, below the surface of the lake. The water was collected in acid-washed 25-L Nalgene carboys and stored in a cold dark room until the extraction procedure was complete (3–6 days). The extraction procedure followed the methods for XAD-8 extraction as published by Thurman and Malcolm (6). Exceptions to the method include the use of 0.45-μm cellulose acetate membranes in place of silver filter membranes and the air-drying of samples in place of freeze-drying. A sample of each dried humic and fulvic acid was redissovled and analyzed for total metal content by ICP-MS.

Conditions for the Analysis of MeHg Bound to Fulvic and Humic Acids. Sephadex G-25 medium grade was employed as the gel permeation matrix for the chromatography of fulvic acids, while Sephadex G-75 was used for the humic acids. This choice was based on the fact that humic acids are generally of larger molecular weight than fulvic acids. Sephadex G-25 has a fractionation range of 1000–5000 MW for peptides and globular proteins and 100–5000 MW for dextrins. Sephadex G-75 has a fractionation range of 3000–80 000 MW for peptides and globular proteins and 1000–50 000 MW for dextrins (17). The columns were calibrated using an appropriate range of polystyrene sulfonate and globular protein standards. The line of best fit through these standards was used as the calibration curve.
A total of 40 mg of dried humic or fulvic acid from each lake was dissolved in 8 mL of 0.01 M Tris-HCl buffer with 0.1 M NaSO₄ adjusted to pH 8.0 using NaOH and trace metal grade HCl. Initial testing of various MeHg concentrations indicated higher binding of MeHg to the humic acids than the fulvic acids. Therefore, appropriate MeHg concentrations were chosen in order to saturate the DOC binding sites but to reduce the amount of free MeHg available in solution. This was done to reduce the large peaks of free MeHg that would overlap with fulvic-bound MeHg on the chromatogram if large concentrations of unbound MeHg were present. The fulvic solutions contained 50 ng/mL of MeHgCl while the humic solutions contained 500–600 ng/mL of MeHgCl. This solution was allowed to equilibrate in the dark at room temperature for 24 h.

After 24 h, 2 mL of the equilibrated fulvic acid solution was applied to the Sephadex G-25 column with buffer solution at a rate of 1 mL/min, and 5–mL fractions were collected. The fractions were then acidified using 1 M HCl and refrigerated in 15-mL polypropylene centrifuge tubes until analysis. The Sephadex column was then washed with with 4–5 bed volumes of Tris-HCl buffer. The procedure was the same for the humic solutions with the exception that only 1 mL of the equilibrated humic solution was applied to the Sephadex G-75.

**Hydride Generation ICP-MS Analysis.** The acidified fractions were analyzed using hydride generation ICP-MS, employing an AS 90 autosampler and a FIAS 400 (flow injection autosampler system) with a 200-μL sample loop. A Perkin-Elmer ELAN 5000 equipped with a hydride generation system was used for the analyses. NaBH₄ (0.05%) was used as the reductant, and 3% HNO₃ was used as the carrier in the analysis. A 3-s wash was performed between samples replicates. Three replicates of each humic and fulvic acid from a lake site were fractionated and analyzed for Hg content; as well, each individual sample was analyzed three times and the average value taken. The recovery of mercury was determined to be 100% for the hydride generation system. The %RSD between replicates was found to be less than 10% in all cases, and the detection limit for the method was found to be 0.01 ppb. Flocculation of humic acids once acidified was minimized by periodic agitation of the solutions to keep the sample homogeneous. The signal profile was integrated and smoothed to ensure greater reproducibility between sample replicates.

**Dissolved Organic Carbon Analysis.** The Tris-HCl buffer used was found to provide reproducible results, with minimal losses of humic substances to the Sephadex gels. Due to the fact that there was a high background of carbon in each fraction from the presence of the Tris-HCl buffer and the breakdown of the Sephadex gels, a method was developed to determine the amount of carbon present due to humic substances.

All samples were diluted 1:20 using double-distilled H₂O and later corrected for dilution due to HCl acidification in the hydride generation analysis. This dilution served to reduce the acid concentration and background signal to levels that were within the range of the TOC analyzer. Triplicates of each sample were analyzed such that the coefficient of variation for three measurements was less than 1%. DOC was calculated as the difference between the total carbon measurements and the inorganic carbon measurements.

The average DOC background was calculated from the average of the first four column fractions and the last five fractions, which were determined to be before the void volume of the column and after the salt volume of the column, respectively. Therefore, they represent the carbon concentration that is present once the column had returned to baseline. Carbon measurements were corrected for baseline and dilution, and the sum of the recovered carbon was compared with expected values.

**Elemental Analysis of Humic and Fulvic Acids.** Total percentage carbon in each sample was determined by dissolving a known mass of each humic or fulvic sample in 10 mL of double distilled water. The mass of each sample was determined by weighing on a Sartorius Supermicro balance. The carbon concentration of each sample was then determined using a Shimadzu TOC-5000 analyzer. From these measurements the percentage carbon could be calculated. Analysis of samples for sulfur was performed by LECO Analytical Labs. A sample of mass 10 mg or greater was analyzed by combustion in an induction furnace under oxygen and detected by IR (infrared) absorbance.

**Results and Discussion**

Initial method development indicated that the technique provided accurate measurements of MeHg binding to humic and fulvic acids without interference of unbound MeHg. Preliminary analysis of mass balances accounted for all MeHg applied to samples, while some interaction with the Sephadex column resulted in losses of unbound MeHg to the Gel. No losses of DOC-bound MeHg were observed. The unbound MeHg was found to bind to Sephadex G-25 and G-75 in small quantities, while bound MeHg and DOC recovery was unaffected (18).

Table 1 displays the %C and the %S for each of the humic and fulvic samples analyzed. Also shown are the S/C ratios for each sample so that comparisons may be made between DOC-normalized sulfur content and DOC-normalized MeHg binding.

**Analysis of MeHg Binding to Lake Water Isolates.** Initial analysis of the extracted humic and fulvic acids by ICP-MS indicated naturally present metal contents that were below detection limits in all cases. To examine trends in MeHg binding over the separation range of each column, the MeHg data were normalized to the amount of DOC present in each fraction. To ensure that there would be no interference from unbound MeHg, samples were analyzed before the salt volume of the column. This corresponds to >1000 MW on the G-25 column and >3000 MW on the G-75 column. The inclusion of post-salt volume data in this calculation was found to result in negligible changes to the calculated values. However, to avoid error due to unbound MeHg, post-salt volume data were not included in the presented results.

The results of the fulvic-bound MeHg data normalized for DOC content are shown in Figure 1. It can be seen that between 1000 and 4000 MW there are no prevalent trends in the MeHg binding. It is apparent that the Dickie Lake fulvic sample is binding more MeHg over all the size fractions, although the reason for this is unknown. After 4000 MW a clear trend can be seen for increased MeHg binding to larger molecular sizes. As these last samples are approaching the void volume of the column, the molecular weight may not
be precise. This is due to the fact that larger molecules (> 5000 MW) will elute at the same volume. This will not affect the overall trend, which increases as much as a factor of 5 over this molecular weight distribution. The results of the humic-bound MeHg data normalized for DOC content were quite different. It was observed that while the samples exhibit individual trends in MeHg binding, there are no common trends in the MeHg binding over the fractionation range (3000–80 000 MW) of the column (as shown in Figure 2).

The binding capacities range from 100 to approximately 300 ng of Hg/mg of C for any humic fraction. The fulvic fractions are mostly between 10 and 20 ng of Hg/mg of C for the lower molecular weights and then increase to 30–70 ng of Hg/mg of C toward the void volume (Figure 1). This indicates that molecules > 5000 MW are most important for the binding of MeHg. It may be that the largest molecules of the fulvic fraction share some common characteristics with the humic fraction as the binding capacities become similar with increasing molecular weight.

Another important aspect of MeHg binding that could be quantified from the data was the total amount of bound MeHg/DOC in each sample (Table 2). The average MeHg binding to the fulvic acids was calculated to be 23.88 ng/mg, and the average MeHg binding to the humic acids was calculated to be 137.73 ng/mg. It was observed from these results that the humic acids bind more MeHg than the fulvic acids when normalized for carbon content. The results for the binding of MeHg to humic acids range from 1.9 to 14.4 times higher than the fulvic acid binding. This was established by a t-test of the DOC-normalized humic acid means versus the DOC-normalized fulvic acid means. A two-sample t-test assuming unequal variances was performed. The F-test confirmed that the variances were not equal.

It is widely assumed in the literature that sulfur-containing functional groups are the main source of MeHg binding to humic substances (14). If this is the case, these results would indicate a similar amount of sulfur-containing functional groups in the larger-sized fulvic molecules as there are in the humic molecules. The smallest fulvic acid molecules would also seem to contain fewer sulfur-containing binding sites relative to the larger molecules. It would also indicate that

![FIGURE 1. DOC-normalized MeHg binding results for fulvic acid samples after separation by Sephadex G-25.](image1)

![FIGURE 2. DOC-normalized MeHg binding results for humic acid samples after separation by Sephadex G-75.](image2)

<table>
<thead>
<tr>
<th>site</th>
<th>ng of MeHg/mg of C</th>
<th>SD</th>
<th>ng of MeHg/mg of C</th>
<th>SD</th>
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</thead>
<tbody>
<tr>
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<td>2.09</td>
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<td>17.10</td>
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<td>9.10</td>
<td>95.55</td>
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<td>Long Lake</td>
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<td>1.71</td>
<td>59.31</td>
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<td>2.09</td>
<td>191.94</td>
<td>31.36</td>
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<td>13.68</td>
<td>137.73</td>
<td>57.17</td>
</tr>
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</table>

*SD refers to method replicates of one isolate.*

14.4 times higher than the fulvic acid binding. This was established by a t-test of the DOC-normalized humic acid means versus the DOC-normalized fulvic acid means. A two-sample t-test assuming unequal variances was performed. The F-test confirmed that the variances were not equal.

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the number of sulfur-containing functional groups in the humic acids studied do not change with molecular weight in any consistent manner among sampling sites.

Since the samples were collected from the littoral zones of each lake site during the same season, it was assumed that there was negligible variation in the sample composition due to seasonal fluctuations. Differences in DOC composition and binding capacity between sample sites were assumed to be due to variations in site-specific factors that control DOC composition. Although variations in oxygen functional group content have been observed in humic substances extracted from various freshwater sites, no reliable data are available for thiol content between sites (19).

The total moles of MeHg binding per milligram of carbon was compared with the moles of sulfur per milligram of carbon as determined by elemental analysis (Table 3). It can be seen that the number of moles of sulfur present is much higher than the number of moles of MeHg bound in each case. Since only a portion of the MeHg added was bound to the humic material, much of the sulfur present is contained in nonbinding functional groups. This indicates that sulfur is not a good indicator of the MeHg binding capacity.

While the origin of sulfur in DOC is still unknown, there are two commonly cited possibilities. Sulfur may be derived from sulfur-containing amino acids in humus formation, or originate from microorganisms during diagenesis in sediments (20). There are several possibilities that may account for a portion of the sulfur content not being involved in MeHg binding. One possibility is that some of the sulfur is contained in organic structures such as sulfide bridges or cyclical sulfur compounds, as opposed to having all the sulfur present as thiols. It was suggested by Bruchert (1998), that sulfur in sedimentary organic material is initially added by nucleophilic addition of polysulfides and replacement of terminal alcohols and carboxyl groups with sulfides. Subsequently sulfide bridges may form between thiols and result in cross linking of reactive organic compounds (21). This may explain the larger difference observed in MeHg binding to humic acids and sulfur content as compared to fulvic acids. It has been suggested that sulfidization may result in a gradual increase in the molecular weight of sedimentary organic materials and that large molecules with extensive sulfur cross linking may be protected from enzymatic decomposition (21). Therefore, this might suggest that the humic acids studied have a larger portion of sulfur present in cross linkages than do the fulvic acids studied. A better technique for the analysis of MeHg binding sites would be a quantitative and functional site-specific technique. Nuclear magnetic resonance is one such technique that has proven useful in the analysis of humic substance functional groups and may have the potential to speciate sulfur (22).

**Implications for Mercury Modeling.** Current models involving mercury cycling are hindered by the lack of DOC binding data to calculate input parameters. Some models attempt to overcome this problem by assuming that mercury species will only interact with thiol sites in the humic substances and use total sulfur as an indicator. This is undoubtedly a very rough estimate due to the limitations of sulfur analysis. Another limitation is the fact that not all of the sulfur will be contained in thiol sites, and it may not be evenly distributed between DOC fractions.

Since significant differences are observed between humic and fulvic acid binding of MeHg, the use of a single DOC parameter to estimate the binding of Hg species in a mercury cycling model is questionable. This study indicates that the composition of DOC in terms of percentage humic and fulvic acids should be characterized in order to calculate reliable input parameters. Since the composition of DOC varies both spatially and temporally within a lake system, a more detailed analysis of DOC cycling may be required in order to model MeHg binding over time (19). The results presented in this study have simplified matters by sampling the littoral zone of several lakes with differences in DOC size distributions within the same season.

The amount of sulfur contained in humic and fulvic acids has been examined in many different experiments. Although there is no consistent answer, the results seem to range from 0 and 1% for XAD extracted humic and fulvic acids (23, 19). If all of this sulfur were in the form of ionized thiol groups, the MeHg binding capacity would be approximately $0.3 \times 10^{-7}$ mol/mg of carbon. The results of this experiment show the binding capacities to range between $6.5 \times 10^{-11}$ and $9.57 \times 10^{-10}$ mol/mg of carbon. This is substantially less than what is accounted for in the current models. Binding capacities similar to those calculated in this study were obtained by Hintelmann et al. (13) through the use of membrane dialysis for MeHg–Humic mixtures. It was found by Hintelmann et al. (13) that extracted freshwater humic and fulvic acids had binding capacities between $0.2$ and $13$ ng of MeHg(II)/mg of HS for strong sites and between $1.2$ and $72$ ng of MeHg(II)/mg of HS for weaker sites (13). This translates to a range of $9.22 \times 10^{-12}$ to $3.32 \times 10^{-10}$ mol/mg of carbon. Another study observed that the cysteine content of extracted humic and fulvic acids may be as large as $2 \times 10^{-10}$ and $7 \times 10^{-10}$ mol/mg of HS, respectively (23). This result seems to compare well with the binding capacities determined in this analysis and lends support to the idea that MeHg is predominantly bound by sulfide sites (such as in cysteine) in the humic and fulvic acid molecules.

The concentration of total Hg in most freshwater is thought to be an average of 1 ng/L; of this, between 20 and 30% is thought to be MeHg (24). At this level of MeHg, the calculated binding capacities are far greater than the amount of MeHg available for binding. For example, if we assume a high MeHg concentration of 1 ng/L for a given lake site, all of this could be bound to humic acids (with the calculated binding capacities and experimental conditions) at a DOC concentration of 7.26 ng/mL. This is much lower than the DOC concentration present in most lakes. This number may vary in natural conditions as the binding capacities presented here are calculated under ideal binding conditions (i.e., high pH, absence of competing metals). In natural systems, effects such as the competition of other cations for binding sites should be expected. Assuming that there are a limited number of sites available for MeHg binding on humic substances, the competition for MeHg binding sites by a metal such as iron or copper may result in increased free MeHg in the water column. The potential exists that iron- and copper-contaminated sites may be significant areas of MeHg uptake due to this effect. This hypothesis is one that is now testable as results may be compared to MeHg–DOC binding capacities calculated in this study.

While this method does suffer from the fact that high DOC concentrations are required in order to perform an

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**TABLE 3. Comparison of mol of S/mg of C with mol of MeHg Bound/mg of C for Lake Isolates**

<table>
<thead>
<tr>
<th>lake isolate</th>
<th>mol of S/mg of C</th>
<th>mol of MeHg/mg of C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cavan humic acid</td>
<td>4.94E–04</td>
<td>8.56E–10</td>
</tr>
<tr>
<td>Buck humic acid</td>
<td>4.61E–04</td>
<td>8.48E–10</td>
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<tr>
<td>Dickie humic acid</td>
<td>4.12E–04</td>
<td>4.76E–10</td>
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<tr>
<td>Long humic acid</td>
<td>1.21E–05</td>
<td>2.96E–10</td>
</tr>
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<td>Brandy humic acid</td>
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<tr>
<td>Cavan fulvic acid</td>
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</tr>
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<td>Buck fulvic acid</td>
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<td>Dickie fulvic acid</td>
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<td>Long fulvic acid</td>
<td>1.70E–04</td>
<td>8.39E–11</td>
</tr>
<tr>
<td>Brandy fulvic acid</td>
<td>2.09E–04</td>
<td>9.38E–11</td>
</tr>
</tbody>
</table>
analysis, the method does allow for DOC analysis to be performed on the fractions. Also, while some aggregation may occur the overall trend of MeHg binding to larger molecular weights will still hold true.

The results indicate several important areas for future research. Future studies should focus on humic and fulvic acid binding capacities and the effects of competing cations, pH, and other chemical parameters that may change the amount of MeHg bound. It is obvious from this study that a more detailed understanding of the speciation of sulfur in humic and fulvic acids is required. The type of functional sites involved in MeHg binding should also be characterized. Another area of research should focus on seasonal and spatial variability in DOC–MeHg binding, as this factor was not explored in this study. Finally the remaining fractions of DOC present in surface waters should be characterized in terms of MeHg binding importance. Once these parameters are adequately explored, accurate measurements of humic- and fulvic-bound MeHg can be estimated from basic chemical analysis of lake sites.

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Received for review February 2, 2000. Revised manuscript received June 15, 2000. Accepted June 21, 2000.