Competing ligand exchange-solid phase extraction method for the determination of the complexation of dissolved inorganic mercury (II) in natural waters

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Abstract

A method employing dual competitive ligand exchange followed by solid phase extraction (CLE-SPE) for characterizing the complexation of inorganic Hg(II) in natural waters is described. This method employs parallel use of two competing ligands: diethyldithiocarbamate (DEDC), which forms hydrophobic complexes with Hg(II), and thiosalicylic acid (TSA), which forms hydrophilic complexes with Hg(II). Inorganic mercury complexed by natural and competing ligands are separated based on hydrophobicity using C18 solid phase extraction columns.

Data modeling allows for the calculation of the concentration and conditional stability constants of natural ligands capable of complexing Hg(II) in both the operationally defined hydrophilic and hydrophobic fractions. The use of multiple ligand concentrations, and thus multiple analytical windows, to characterize different ligand classes within both of these two fractions is described. Studies of the kinetics of the ligand exchange involved, potential for changes in the stability of natural ligands during freezing and thawing, potential breakthrough during solid phase extraction, as well as the method’s precision and estimation of error, are presented and discussed.

Results from the application of the method to natural freshwaters demonstrated that in the limited samples collected over 99.99% of the ambient inorganic mercury is strongly complexed by ligands with conditional stability constants (K_{cond} HgL, Hg^{2+}) on the order of 10^{30}, values similar to that of reduced sulfur ligands. At ambient conditions 85–90% of the mercury exists in hydrophobic complexes in these freshwaters, but strong Hg-binding ligands exist in both the hydrophobic and hydrophilic fractions.

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1. Introduction

Mercury is a toxic heavy metal and global pollutant originating from natural and, increasingly, anthropogenic sources [1,2]. Like other trace metals, mercury’s bioavailability and toxicity are a function of its chemical speciation. Knowing merely the total dissolved concentration of inorganic mercury, Hg(II), provides only limited insight into its possible uptake at the base of the food chain and by microbes, including those responsible for the methylation of mercury to its more toxic form, monomethyl mercury [3–6]. To better understand and predict the biogeochemical cycling of mercury in aquatic environments requires knowledge of the chemical forms and species of mercury present.

The uptake of trace metals by phytoplankton and microbes has been shown to follow the biological ligand model in many cases [7–12]. This model assumes that the hydrated free ion and inorganic complexes with rapid exchange kinetics, relative to binding by membrane transporters, are the forms which are most bioavailable and involved in uptake; this has not proved to be the case for mercury. Instead, neutrally charged hydrophobic inorganic complexes, such as Hg(Cl)_{2} and HgS^{0}, have been shown to determine the rate of uptake of inorganic mercury via passive diffusion under some conditions by phytoplankton, as well as by sulfate reducing bacteria responsible for the methylation of mercury [4,5]. However, more recent studies have suggested that under other conditions microbial uptake of Hg(II) likely also involves facilitated and active transport into the cell [13–15].
While uncertainty remains as to exactly how inorganic mercury is taken up across cell membranes and which chemical species are most important in this transport, there is little doubt as to the key role which complexation with both organic and inorganic ligands and chemical speciation play. As a result, methods which are able to characterize the speciation of Hg(II) in solution and provide information on the nature of the ligands complexing Hg(II) at ambient mercury levels in natural waters are highly desirable. Due to the ability of hydrophobic complexes of Hg(II) to passively diffuse through the phospholipid bilayer of cell membranes [4,5], information on the distribution of hydrophobic and hydrophilic natural complexes of Hg(II) in aquatic environments is also valuable.

Unfortunately, detailed studies of the complexation and speciation of mercury are relatively few in number compared to the abundance of such studies involving other trace metals. While this is due largely to analytical constraints precluding such mercury speciation studies until relatively recently, this is likely also due in part to the strong complexation of mercury by the inorganic ligands OH\(^-\) (log\(K_1\) = 10.6; log\(\beta_2\) = 21.2; log\(\beta_3\) = 20.9; [16]) and Cl\(^-\) (log\(K_1\) = 7.3, log\(\beta_2\) = 14.0, log\(\beta_3\) = 15.0; log\(\beta_4\) = 15.6; [16]) relative to other trace metals. Based on this information alone mercury was predicted to exist as hydroxide and mixed hydroxide–chloride complexes in oxygenated freshwaters at near neutral pHs, and as chloride complexes in brackish and saline waters [17]. Later studies demonstrated the importance of organic ligands associated with dissolved organic matter, especially those containing thiol functional groups, in binding mercury [18–20]. These have been substantiated by recent investigations of the complexation of dissolved Hg(II) that have employed competing ligand exchange, liquid–liquid extraction [21,22], competing ligand exchange with equilibrium dialysis [23], a reducible mercury method to determine “labile” Hg(II) [24], and voltammetry to directly assess electrochemically active [Hg(II)] [25].

The method described in this paper employs a dual competitive ligand exchange with solid phase extraction approach based on that of Hsu and Sedlak [26]. A number of important modifications and adjustments are described to optimize this method, and a novel method of data analysis is presented which allows for the simultaneous calculation of the concentration and conditional stability constants for natural ligands forming both hydrophobic and hydrophilic complexes with Hg(II), which has not previously been reported. We also describe the use of multiple ligand concentrations to widen the analytical window [27–29] over which information on different ligand classes within each of the two operationally defined fractions can be ascertained.

2. Experimental

2.1. Theory of competitive ligand exchange and data analysis

The theoretical basis for much of the data analysis and ligand fitting of the competing ligand exchange-solid phase extraction (CLE-SPE) results used for this study have been described elsewhere [30–32]. In a natural water sample the total speciation using the operationally defined hydrophobic and hydrophilic fractions relevant to this study can be described by the equation:

\[
[Hg]_T = [Hg^{2+}] + \Sigma [HgL_{\text{hpi}}] + \Sigma [HgL_{\text{hpo}}] + \Sigma [HgLk_{\text{hpi}}] + \Sigma [HgLk_{\text{hpo}}] + \Sigma [HgTSAl_{\text{ad}}^0] \]

(1)

where \([Hg]_T\) is the total dissolved Hg(II), \([Hg^{2+}]\) is the free aqua Hg(II) ion, \(\Sigma [HgL_{\text{hpi}}]\) is the sum of hydrophilic complexes of Hg(II) with known inorganic ligands, such as chloride and hydroxide, and \(\Sigma [HgL_{\text{hpo}}]\) is the sum of hydrophobic complexes of Hg(II) with known inorganic ligands, such as chloride and hydroxide: \(\Sigma [HgLk_{\text{hpi}}]\) is the sum of hydrophilic complexes of Hg(II) with unknown inorganic or organic natural ligands, and \(\Sigma [HgLk_{\text{hpo}}]\) is the sum of hydrophobic complexes of Hg(II) with unknown natural ligands. The use of the terms hydrophilic and hydrophobic refer to the operationally defined conditions of this study (i.e., hydrophobic species are retained by a C18 column, while hydrophilic species are not).

The reaction forming a hydrophilic natural complex involving ligand \(L_k\), and assuming a 1:1 stoichiometry, with unknown charges omitted for simplicity can be represented by the equation:

\[
Hg^{2+} + L_k \leftrightarrow HgLk_{\text{hpi}}
\]

(2)

which at equilibrium is described by the conditional stability constant:

\[
K_{\text{cond}}^{\text{HgLk}} = \frac{[HgLk_{\text{hpi}}]}{[Hg^{2+}][L_k]}
\]

(3)

where \([L_k]\) is the natural ligand not bound to Hg, and \(K_{\text{cond}}^{\text{HgLk}}\) is the ionic strength corrected conditional stability constant for the complex \([HgLk_{\text{hpi}}]\). This stability constant is conditional to the pH and concentration of all components in the system responsible for side reactions with the species of interest. After the addition of the competing ligand thiosalicylic acid (TSA), which forms predominately anionic bis hydrophilic complexes with Hg(II) under the conditions used, the expression above for total Hg(II) becomes:

\[
[Hg]_T = [Hg^{2+}] + \Sigma [HgL_{\text{hpi}}] + \Sigma [HgL_{\text{hpo}}] + \Sigma [HgLk_{\text{hpi}}] + \Sigma [HgLk_{\text{hpo}}] + \Sigma [HgTSAl_{\text{ad}}^0] \]

(4)

The stability constants for the complexes of Hg(II) with TSA\(^{2–}\) are

\[
\beta_1 = \frac{[HgTSAl_{\text{ad}}^0]}{[Hg^{2+}][TSA^{2–}]} \]

(5)

and

\[
\beta_2 = \frac{[Hg(TSA)_{2–}]}{[Hg^{2+}][TSA^{2–}]^2}
\]

(6)

Combining and rearrangement of the above equations give

\[
\Sigma [HgTSAl_{\text{ad}}^{2–}] = [Hg^{2+}] \Sigma (\beta_{TSA}[TSA^{2–}]^n)
\]

(7)

As described above, TSA can bind to Hg(II) to form both a neutrally charged hydrophobic monocomplex, HgTSA\(_{\text{ad}}^0\),
as well as an anionic hydrophilic bis complex, Hg(TSA)_2^2—

However, the hydrophilic bis complex was dominant under
the conditions used during this study. Hg(TSA)_2^2— represents
99.8% of the Hg—TSA complexes even at the lowest concentra-
tion of TSA employed (4 μM TSA) at a pH of 7.7. As a result,
we can simplify the term \( \sum [\text{HgTSA}_2^{2—}] \):

\[
\Sigma [\text{HgTSA}_2^{2—}] = [\text{Hg(TSA)}_2^{2—}] = \beta_{2\text{TSA}}[\text{Hg}^{2+}][\text{TSA}^{2—}]^2
\]  

(8)

A side reaction coefficient, \( \alpha \), is introduced such that

\[
\alpha_{\text{TSA}} = \frac{[\text{Hg(TSA)}_2^{2—}][\text{Hg}^{2+}]}{\Sigma [\text{HgTSA}_2^{2—}]}
\]  

(9)

where \( \alpha_{\text{TSA}} \) is the side reaction coefficient for TSA with Hg(II).

In most freshwater systems complexation of Hg(II) by OH^- alone requires that \( \alpha_{\text{OH}^-} \) be at least as great as \( \alpha_{\text{TSA}} \), which at a
pH of 7.7 is roughly 10^0.2 due to the formation of Hg(OH)_2^0(aq). If
this condition is not met, OH^- alone will out compete TSA
for binding of Hg(II).

It is now necessary to define a new term [Hg]_nat as the sum of
all the natural Hg(II) species after equilibrium has been reached
following the addition of the competing ligand TSA, such that

\[
[\text{Hg}]_{\text{nat}} = [\text{Hg}^{2+}] + \Sigma [\text{HgL}_i]_{\text{hpi}} + \Sigma [\text{HgL}_j]_{\text{hpo}}
\]

\[+ \Sigma [\text{HgL}_k]_{\text{hpi}} + \Sigma [\text{HgL}_l]_{\text{hpo}}
\]  

(11)

and

\[
[\text{Hg}]_{\text{nat}} = [\text{HgL}_1]_{\text{hpi}} - [\text{Hg(TSA)}_2^{2—}]
\]  

(12)

Substituting from above gives

\[
[\text{Hg}]_{\text{nat}} = [\text{HgL}_1]_{\text{hpi}} - [\text{Hg}^{2+}] \alpha_{\text{TSA}}
\]  

(13)
or

\[
[\text{Hg}]_{\text{nat}} = [\text{HgL}_1]_{\text{hpi}} (1 + \alpha_{l_i} \text{ hpi} + \alpha_{l_j} \text{ hpo} + \alpha_{l_k} \text{ hpi} + \alpha_{l_j} \text{ hpo})
\]  

(14)

It is useful to further simplify Eq. (4), which describes the system
after the addition of the competing ligand TSA. Speciation
calculations for freshwaters at pH ~ 7.7 with chloride concentra-
tions of less than 5 mM allow for the assumption that [Hg^{2+}] is
negligible and that the dominant known inorganic complexes are
Hg(OH)_2^0(aq), HgClOH^0(aq), and Hg(Cl)_2^0(aq), all of which
are retained by the C18 column. At lower pH’s or more brack-
ish and saline waters the hydrophilic complexes Hg(Cl)_2^0 and
Hg(ClI)_2^2— must also be included in these calculations as they
become important, and are easily incorporated here with the use
of the relevant stability constants. The above assumptions pro-
vide the basis for the prediction of the partitioning of each term
above during SPE based upon hydrophobicity, described by the
simplified equations:

\[
[\text{HgL}_1]_{\text{hpi}} = [\text{HgL}_{k}]_{\text{hpi}} + [\text{HgL}_{l}]_{\text{hpo}}
\]  

(15)

\[
[\text{HgL}_1]_{\text{hpo}} = [\text{HgL}_{k}]_{\text{hpi}} + [\text{HgL}_{l}]_{\text{hpo}}
\]

\[(\text{16})\]

\[
[\text{HgL}_1]_{\text{hpo}} = [\text{HgL}_{k}]_{\text{hpi}} + [\text{HgL}_{l}]_{\text{hpo}}
\]

\[(\text{17})\]

where [HgL]_hpi represents the fraction of Hg(II) measured in the
column passing fraction, referred to as the hydrophilic Hg, and
[HgL]_hpo represents the fraction of Hg(II) retained by the C18
column, referred to as the hydrophobic Hg.

Ideally, each of the above equations for [HgL]_hpi and [HgL]_hpo
would have only one unknown, allowing for a direct solution.
Unfortunately, this is not the case, and it is not possible to set up
a competition purely between the competing ligand TSA, which
forms hydrophilic complexes with Hg(II), and natural ligands
which form hydrophobic complexes with Hg(II), which would
allow for the separation of the Hg(II) complexes of the added
competing ligand and those of the natural ligands of interest.
Instead, the hydrophilic fraction includes Hg(II) complexes of
both TSA and the unknown natural ligands which are both com-
peting with a second unknown class of natural ligands that form
hydrophobic complexes with Hg(II), resulting in the inability to
distinguish between the different complexes in the hydrophilic
fraction with the method described.

Previous studies using CLE liquid–liquid extraction have cir-
cumvented this obstacle by assuming, or demonstrating, that
the natural ligand dominating the speciation of the metal of interest
were contained in either the hydrophilic or hydrophobic fraction
[31,33]. In these studies, they subsequently ignored one of the
natural ligand complex terms in the equations above. However,
the nature of ligands capable of complexing Hg(II) in different
natural water systems has not been well defined.

Previous studies of wastewater effluent [26] and estuarine
waters [22] have shown the presence of ligands that form both
hydrophobic and hydrophilic complexes with Hg(II). In addi-
tion, a study of surface waters in the Atlantic and Pacific Oceans
revealed strong complexation of both Cu and Zn based upon voltammetry measurements and calculations, while extraction
of those same metals by C18 columns varied from 10 to 40% [34],
demonstrating that organic complexes of these metals were found
in both the hydrophilic and hydrophobic fractions based
upon C18 extraction. In light of these findings, the assumptions
described above were not made for this study regarding Hg(II)
complexes in one of the operationally defined hydrophobic or
hydrophilic fractions being negligible. This constraint necessi-
tated a modified approach to modeling the CLE-SPE titration
data based upon traditional methods previously described for
CLE-SPE, CLE-liquid–liquid extraction, CLE-anodic stripping
voltammetry, and CLE-cathodic stripping voltammetry [30–32].

We used an iterative approach to permit the modeling of the
ligand concentrations and binding strengths of natural ligands
capable of complexing Hg(II) in each of the two operationally
defined fractions. This approach begins with the assumption
that for the first round of iteration the \( \Sigma [\text{HgL}_k]_{\text{hpi}} \) term can be
ignored, and thus under the freshwater conditions of this study
Eq. (16) above can be simplified to

\[
[\text{HgL}]_{\text{hpi}} = [\text{HgL}_{k}]_{\text{hpi}}
\]  

(18)
This assumption also allows \([\text{Hg}^{2+}]\) to be calculated as

\[
[\text{Hg}^{2+}] = \frac{[\text{Hg}_{\text{hpi}}]}{1 + \alpha_{\text{TSA}}} \tag{19}
\]

where \([\text{Hg}_{\text{hpi}}]\) is measured after SPE, and \(\alpha_{\text{TSA}}\) is directly calculated from the concentration of TSA added, pH, stability constants taken from the literature \([16,35]\), and the knowledge that TSA is in great excess of Hg(II). The term \(\sum[\text{HgL}_l]_{\text{hpo}}\) can be calculated as

\[
\Sigma[\text{HgL}_l]_{\text{hpo}} = [\text{Hg}]_{\text{hpo}} - [\text{Hg}^{2+}](\alpha_{\text{OH}} + \alpha_{\text{Cl}} + \alpha_{\text{ClOH}}) \tag{20}
\]

where \(\alpha_{\text{OH}}\), \(\alpha_{\text{ClOH}}\), and \(\alpha_{\text{Cl}}\) are the low and high stability constants for the two competing ligands, where the low and high concentration of TSA and DEDC were chosen such that the system with no added competing ligand can be described as above using Eqs. (15)–(17). A freshwater system at near neutral pH under the same simplifying assumptions already described with TSA added can be described as above using Eqs. (15)–(17):

\[
[\text{Hg}]_{\text{hpi}} = \Sigma[\text{HgL}_l]_{\text{hpo}} \tag{23}
\]

\[
[\text{Hg}]_{\text{hpo}} = \Sigma[\text{HgL}_l]_{\text{hpo}} + [\text{Hg(OH)}^2_0] + [\text{HgClOH}^0] + [\text{Hg(Cl)}^0] + [\text{Hg(DEDC)}^0] \tag{24}
\]

where speciation calculations at pH 7.7 demonstrate that \(\Sigma[\text{HgL}_l]_{\text{hpo}}\) is the dominate DEDC complex formed with Hg(II), and thus \(\Sigma[\text{HgL}_l]_{\text{hpo}}\) has been substituted for \(\Sigma[\text{HgL}_l]_{\text{hpo}}\) for simplicity. A value for \(\alpha_{\text{hpo}}\) can be calculated for each data point, and modeling of the data set yields estimates of \(\Sigma[\text{HgL}_l]_{\text{hpo}}\) and \([\text{HgL}_l]_{\text{hpo}}\), as described above.

The model is now refined by carrying out the calculations and ligand fitting of \(\Sigma[\text{HgL}_l]_{\text{hpo}}\) and \([\text{HgL}_l]_{\text{hpo}}\) a second time. This time the initial assumption that there were not natural ligands present forming hydrophilic complexes with Hg(II) is discarded. This is done by including the calculated values of \(\Sigma[\text{HgL}_l]_{\text{hpi}}\) and \([\text{HgL}_l]_{\text{hpi}}\) which could be thought of as a second hydrophilic competing ligand in addition to the added TSA. Once this second iteration has been completed, an approximation of the ambient free \(\text{Hg}^{2+}\) ion concentration can be calculated using the fitted conditional stability constants and free natural ligand concentrations for the operationally defined \(\text{L}_{\text{hpi}}\) and \(\text{L}_{\text{hpo}}\) along with the pH, concentration of major inorganic ions in solution, and their related thermodynamic data.

A second more straightforward method for modeling the two competitive ligand exchange systems simultaneously was also used. This second method involved using two concentrations of each of the two competing ligands, where the low and high concentration of TSA and DEDC were chosen such that the side reaction coefficient, \(\alpha\), for the two competing ligands was the same under the conditions used. Under these conditions the system with no added competing ligand can be described as

\[
[\text{Hg}]_{\text{T}} = [\text{Hg}]_{\text{hpo}} + [\text{Hg}]_{\text{hpi}} \tag{25}
\]

A freshwater system at near neutral pH under the same simplifying assumptions already described with TSA added can be described as above using Eqs. (15)–(17):

\[
[\text{Hg}]_{\text{T}} = [\text{Hg}]_{\text{hpi}} + [\text{Hg}]_{\text{hpo}} \tag{15}
\]

\[
[Hg]_{\text{hpi}} = [Hg(TSA)]^2 + [\Sigma[HgL_{\text{l}}]_{\text{hpi}}] \tag{16}
\]

\[
[Hg]_{\text{hpo}} = [Hg(OH)]^2 + [HgClOH] + [Hg(Cl)]^0 + [Hg(DEDC)]^0 + [\Sigma[HgL_{\text{l}}]_{\text{hpo}}] \tag{17}
\]

where the amount of \([\text{Hg}]_{\text{hpi}}\) in the presence of TSA will be greater than \([\text{Hg}]_{\text{hpi}}\) with no TSA added. However, the amount of Hg(II) complexed by TSA cannot be measured directly at this point since the Hg complexed by TSA came at the expense of not only natural hydrophobic Hg complexes, but also Hg which initially existed in natural hydrophilic complexes. This latter fraction of Hg changed complexes during the competition, but not hydrophobicity, and thus remained in the same fraction during SPE. While the shift in Hg from hydrophobic natural complexes to TSA complexes can be easily calculated by difference in the \([\text{Hg}]_{\text{hpo}}\) with and without TSA added, the amount of Hg which was initially complexed by natural hydrophilic complexes that subsequently became bound in hydrophilic TSA complexes after the addition of TSA cannot be direct measured.
However, this value can be calculated from the system when DEDC is used as the competing ligand if the $\alpha$ of DEDC is the same as the $\alpha$ of the TSA. In this case the system with DEDC added can be described as

$$[\text{Hg}]_T = [\text{Hg}]_{hpo} + [\text{Hg}]_{hpi}$$  \hspace{1cm} (15)  
$$[\text{Hg}]_{hpi} = \Sigma [\text{HgL}_k]_{hpi}$$  \hspace{1cm} (23)  
$$[\text{Hg}]_{hpo} = \Sigma [\text{HgL}_l]_{hpo} + [\text{Hg(OH)}_2]^0_{(aq)} + [\text{HgClOH}^0_{(aq)}]$$  
$$+ [\text{Hg(Cl)}_2]^0_{(aq)} + [\text{Hg(DEDC)}_2]^0_{(aq)}$$  \hspace{1cm} (24)  

where the amount of $[\text{Hg}]_{hpi}$ in the presence of DEDC will be less than $[\text{Hg}]_{hpi}$ with no competing ligand as a result of the formation of hydrophobic $\text{Hg(DEDC)}_2^0$ at the expense of the natural hydrophilic complexes.

The three systems can therefore be combined to calculate the amount of Hg complexed by either competing ligand at any given Hg concentration when $\alpha_{\text{DED}} = \alpha_{\text{TSA}}$ using the equation:

$$[\text{Hg}]_{\text{CL}} = \Delta [\text{Hg}]_{hpi} + \Delta [\text{Hg}]_{hpo}$$  \hspace{1cm} (27)  
where

$$\Delta [\text{Hg}]_{hpi} = [\text{Hg}]_{hpi \text{ no added ligand}} - [\text{Hg}]_{hpi \text{ DEDC added}}$$  \hspace{1cm} (28)  
$$\Delta [\text{Hg}]_{hpo} = [\text{Hg}]_{hpo \text{ no added ligand}} - [\text{Hg}]_{hpo \text{ TSA added}}$$  \hspace{1cm} (29)  

and where $[\text{Hg}]_{\text{CL}}$ represents the total amount of Hg complexed by a competing ligand, either TSA or DEDC, which will be equal when the concentrations of each competing ligand are such that the $\alpha$ for TSA and DEDC are the same. Values of $[\text{Hg}]_{\text{nat}}$ for each Hg concentration are now calculated simply as

$$[\text{Hg}]_{\text{nat}} = [\text{Hg}]_T - [\text{Hg}]_{\text{CL}}$$  \hspace{1cm} (30)  

and $[\text{Hg}^{2+}]$ can be calculated from $[\text{Hg}]_{\text{CL}}$ using the side reaction coefficient of the competing ligands. This process of calculations was done for two concentrations of each competing ligand, a low $\alpha$ and a high $\alpha$ system, for each water sample, allowing for a wider analytical working window than possible if only one concentration of each competing ligand had been employed [27–29,31].

When comparing results of the ligand fitting employing the two data analysis methods using the same raw data, we found them to be very similar. The two methods always yielded ligand concentrations that were equivalent within the analytical error of the method. In general, the first method, the iterative approach, yielded conditional stability constants for natural ligand binding that were one to three orders of magnitude greater than the second method employing equal competing ligand $\alpha$’s. Since the conditional stability constants calculated with both methods were extremely high ($\sim 10^{30}$), this difference does not substantially affect our interpretation nor the extent of complexation at any of the sites. Because the earlier samples were not characterized using TSA and DEDC concentrations yielding the same $\alpha$’s for the two competing ligands, only the first data analysis method can be applied to all samples collected to date. Because of this, all ligand-fitting results reported here were calculated using the iterative approach.

### 2.2. Multiple working windows and assumptions of CLE-SPE

Due to the heterogeneous nature of natural ligands, the side reaction coefficients $\alpha_{\text{hpo}}$ and $\alpha_{\text{hpi}}$ can be expected to vary as the $[\text{Hg(II)}]$:ligand ratio varies over the range of the $\text{Hg(II)}$ titration. As a result, the side reaction coefficients of natural ligands which will most accurately describe the ambient complexation of $\text{Hg(II)}$ are those determined when the titration range involves $\text{Hg(II)}$ concentrations similar to ambient levels of dissolved $\text{Hg(II)}$. At higher $\text{Hg(II)}$ concentrations the strongest binding sites, generally assumed to be present at low levels, will be saturated and the results will usually yield higher natural ligand concentrations and lower conditional stability constants for those ligands most responsible for $\text{Hg(II)}$ complexation at ambient $\text{Hg(II)}$ levels. As such, any method’s working window is intimately tied to the results the method can potentially yield. Because concentrations of operationally defined dissolved $\text{Hg(II)}$ in oxic freshwaters generally range from low picomolar at background sites not contaminated with mercury to high picomolar levels at polluted sites [39–42], the $\text{Hg(II)}$ titrations chosen for this study span the range from 10 pM to 11 nM. This range allowed for characterization of Hg complexation near ambient Hg levels, while also using Hg concentrations sufficiently great to saturate the dominant complexing ligands and allow for an accurate estimate of their concentrations.

In addition to the concentrations of $\text{Hg(II)}$ used for the CLE titrations, the other factor controlling the method’s analytical window is the side reaction coefficient, $\alpha$, of the competing ligand employed. Ideally, the competing ligand side reaction coefficient should be within an order of magnitude of that of the natural ligands being characterized [32,43]. If the $\alpha$ of the competing ligand is substantially lower than that of the natural ligands, the complexes formed with the added ligand will not be measurable, and the system will not have been perturbed sufficiently to be modeled. Conversely, if the $\alpha$ of the competing ligand is substantially greater than that of the natural ligands the added ligand will out compete the natural ligands at all $\text{Hg(II)}$ concentrations, and the resulting data cannot be modeled with the accuracy required to yield the desired information. In general, the concentration of the competing ligand should be in substantial excess of the $\text{Hg(II)}$ added to solutions so that the concentration of the free competing ligand is constant across the $\text{Hg(II)}$ titration. As a consequence, the concentration of added ligand should be substantially greater than that of the natural complexing ligands, and have a lower conditional stability constant.

The formation of $\text{Hg(OH)}_2^0$ in freshwater systems, and at appreciable chloride levels $\text{HgClOH}^0_{(aq)}$ and $\text{Hg(Cl)}_2^0_{(aq)}$, immediately place a lower limit on the $\alpha$ of any natural ligands which could be hypothesized to be important in the complexation of $\text{Hg(II)}$ in natural waters, as well as on the selection of an appropriate competing ligand. In order for any natural organic or inorganic ligand to play a substantial role in the complexation of $\text{Hg(II)}$ in freshwaters it at least has to out compete hydroxide. At a pH of 7.7 $\alpha_{\text{OH}^-}$ is approximately $10^{9.2}$, which requires both that any natural ligand have an $\alpha$ greater than or nearly equal
to this, which in turn necessitates that the conditional stability constant and concentration of the competing ligand chosen also combine to result in a side reaction coefficient greater than this.

All of the above considerations were provided for in this study. In addition, two concentrations of each competing ligand, TSA and DEDC, were chosen to allow for the characterization of ligand classes with low concentrations and high binding affinities for Hg(II), as well as weaker ligands which might become more important at higher Hg(II) levels once the stronger binding sites have become saturated. Essentially, the use of multiple concentrations of the competing ligands creates a larger analytical working window of [Hg]_{nat} versus [Hg^{2+}] over which to gain information on the complexation capacity and natural ligands at each site [27–29,44].

Metal speciation studies employing CLE methods and ligand fitting techniques rely upon a number of noteworthy assumptions. One is that the ligand exchange kinetics involved be rapid relative to the equilibration times used to ensure that the new thermodynamic equilibrium is established before separation of complexes. A second is that no change in speciation or ligand stability results from sample collection, storage, or processing. A third assumption during ligand fitting is generally that only 1:1 metal to ligand complexes need be considered. Obviously, these assumptions may or may not be valid under different experimental conditions, and as a result experiments examining these under the conditions used in this study were conducted. These included studies of ligand exchange kinetics and changes in Hg(II) complexion due to freezing and thawing samples.

2.3. CLE-SPE procedure

Two parallel competitions were established with natural complexing ligands using TSA, which at micromolar concentrations and near neutral pH forms predominately Hg(TSA)$_2$$^{2-}$, an anionic hydrophilic complex with Hg(II), and DEDC, which at near neutral pHs forms predominately Hg(DEDC)$_2$$_0^0$, a neutral hydrophobic complex [26,35,45]. Solid phase extraction using end-capped C$_{18}$ columns (Supelclean ENVI-18, 0.5 or 2.0 g resin) were used to separate the Hg(II) complexed by the natural and added ligands based on hydrophobicity. Mercury concentrations in each fraction were then determined by oxidation with BrCl, reduction with SnCl$_2$, gold trap amalgamation, and quantification by cold vapor atomic fluorescence spectrometry (CVAFS) [46,47].

Bulk solutions for CLE-SPE were amended in the lab after thawing to 5.0 mM 3-morpholinopropane sulfonic acid (MOPS) buffer and a site averaged pH of 7.8 with the use of trace metal grade NH$_4$OH. The CLE was then carried out on separate aliquots of samples in 250 mL Teflon bottles which were spiked with between 10 pM and 11 nM Hg(II), in addition to aliquots which had no Hg(II) added. Solutions were allowed to equilibrate for 12 h in the dark at room temperature (~25 °C), and then had competing ligands typically added to concentrations of 1.0 μM DEDC (α$_{DEDC} = 10^{21.5}$), 25 μM DEDC (α$_{DEDC} = 10^{24.3}$), 4 μM TSA (α$_{TSA} = 10^{21.5}$), and 105 μM TSA (α$_{TSA} = 10^{24.3}$), in addition to solutions with no added ligand. The exact concentration of competing ligands used varied some-what from these values as we attempted to find the optimal levels. The solutions were then allowed to equilibrate for an additional 6 h in the dark at room temperature, at which time they underwent solid phase extraction using end capped C$_{18}$ SPE columns at flow rates between 4 and 7 mL s$^{-1}$. The C$_{18}$ columns had been wetted and preconditioned prior to use by the passage of 10 mL methanol (Optima grade) followed by 20 mL high purity 18.2 MΩ cm$^{-1}$ water (Milli-Q) at a flow rate of 5 mL s$^{-1}$. Aliquots of the column passing fraction were taken for measurements of pH, which would be used in speciation calculations. The Hg(II) retained by the column, referred to as hydrophobic Hg, was then eluted with 2% HCl (trace metal grade). Both the column passing fraction and the 2% HCl eluent were amended to 0.5% BrCl and analyzed for total mercury within 72 h using the established CVAFS techniques described above [46,47].

Because the amount of inorganic Hg(II) in each fraction was measured as total mercury, the presence of other forms of mercury, including monomethyl mercury (MMHg), the next most common form of mercury in freshwaters, could prove to be an interference to determining the complexation of inorganic Hg(II). However, in practice this is not the case since MMHg generally represents less than 10% of the total dissolved mercury pool in freshwater systems [39,41,42], and because as samples are titrated with inorganic Hg(II) the percentage of the total mercury which will exist as MMHg quickly becomes insignificant, and will not affect the results appreciably. However, this assumption should be checked for any given water system where this method is applied.

2.4. Ligand fitting

Once values of [Hg]$_{nat}$ and [Hg$^{2+}$] had been calculated for each point in the Hg titration curve thermodynamic modeling of each of the two competitive ligand exchange systems was done by ligand fitting with the program FITHEQL [37,38], which employs nonlinear least squares optimization. This ligand fitting enabled the calculation of the concentrations and conditional stability constants of natural ligands in both the operationally defined hydrophilic and hydrophobic fractions of each water sample. These results, along with the pH, major cation and anion concentration data were used in equilibrium speciation calculations to back calculate the original speciation of Hg(II) in each water sample.

Again, while much of the approach described above is based upon the recent work by Hsu and Sedlak [26], their method has been modified to optimize various parameters and adapt it to suit the requirements of this study. Modifications include the substitution of TSA for glutathione as the ligand forming hydrophilic complexes with Hg(II), the addition of two concentrations of each competing ligand, titrating samples with a range of Hg concentrations rather than adding one concentration of Hg and titrating samples with a range of competing ligands, and the inclusion of lower concentrations of Hg(II) in titrations which are more environmentally relevant. Additional modifications have involved the use of longer equilibration times, as that can affect both ligand stabilities and the extent to which equilibrium is achieved [31,32], which in turn lead to our sub-
stitution of TSA for glutathione due to its increased stability over time.

2.5. Estimation of error associated with CLE-SPE ligand fitting results

The desired quantitative comparison of ligand fitting results, namely ligand concentration and conditional stability constants with respect to binding of free Hg(II), from one site or date to another using parametric statistics requires an estimation of error terms for each data set, such as a standard deviation or standard error. However, these values are not easily calculated given the nature of the ligand fitting, and we therefore estimated them using slightly non-traditional means. One reason for this difficulty is that because of the relatively long time required to process a single sample by CLE-SPE it was not feasible to analyze multiple replicates for all samples. The second reason is that when triplicates were run, the ligand fitting results for each replicate yields a ligand concentration and conditional stability constant. However, these values are not truly independent of each other, and a variation of one during any ligand fitting procedure only comes at the expense of a change in the value of the other due to the inverse relationship between the two. Thus, a simultaneous estimate of the error associated with both the fitted ligand concentration and conditional stability constant is not as straight forward as merely calculating the standard deviation of the fitted ligand concentration for sample replicates, then calculating independently the standard deviation of the fitted conditional stability constants of the same sample replicates.

We have attempted to address this problem using the following method. Sample triplicates underwent CLE-SPE and the subsequent ligand fitting were performed independently, yielding three sets of corresponding fitted ligand concentrations and conditional stability constants. An average of the ligand concentrations was then calculated from these three sets. The ligand fitting for all three data sets was then repeated with FITEQL, this time setting the averaged ligand concentration as a constant, leaving only the conditional stability constant as the fitted variable. This yielded three independent values of the conditional stability constant for the sample, which were then used to calculate the standard deviation in the conditional stability constant for the ligand fitting. This error could be taken as an estimate of the total error associated in the ligand fitting, expressed solely as a function of the conditional stability constants initially calculated from all sample replicates in independent ligand fitting. This average value was set as a constant in FITEQL and used to recalculate the fitted ligand concentration for each sample replicate. These multiple ligand concentrations were then used to calculate the standard deviation, which would be taken as an estimate of the total error associated in the ligand fitting, expressed solely as a function of the ligand concentration.

All values of the fitted ligand concentrations and conditional stability constants are reported to within one standard deviation, calculated as just described, in which the errors introduced during the CLE-SPE and ligand fitting process were estimated in terms of both the ligand concentration and conditional stability constant. It should be noted that this method will tend to overestimate the errors involved if one standard deviation of both the conditional stability constant and ligand concentrations are reported at the same time when each was calculated such that it provided an estimate of the total error involved as each term.

We have decided to use this reporting method in order to report an error term for both values at the same time.

2.6. Sample collection and analysis

Surface water samples were collected using trace metal clean techniques with a peristaltic pump using a Teflon sampling line with C-Flex tubing in the pump head, which had been cleaned as described below for sample bottles. Filtered water samples were collected using acid cleaned 0.45 μm polypropylene cartridge filter (Osmonics) fitted to the end of the sample line.

Measurements of pH, major cations, anions and trace metals were made using established techniques. Samples for trace metals, cations, and anions were collected in polyethylene bottles cleaned by a 1 week soak in Micro detergent, followed by through rinsing with deionized water, a 1 week soak in 3N HCl (trace metal grade), followed by through rinsing with Milli-Q water, a 1 week soak in 3N HNO3 (trace metal grade), followed by a final through rinsing with Milli-Q water. Cleaned bottles were then double bagged in polyethylene zip-lock bags until used.

Bottles used for collecting and storing samples for Hg(II) studies were made of Teflon, and were cleaned as described above, except that the order of the HCl and HNO3 cleaning steps was reversed so that the final acid cleaning was in trace metal grade HCl. Water samples were double bagged and immediately frozen with dry ice or stored in coolers until transported back to the lab.

Total mercury concentrations were determined by oxidation with BrCl, reduction with SnCl2, gold trap amalgamation, and quantification by CV AFS.

2.7. Reagents

All chemicals used were purchased from Fisher Scientific, Sigma–Aldrich, or Fluka, and were of the highest quality available unless otherwise noted.

2.8. Field sites

Panoche Creek is a seasonal stream located on the eastern side of the central California coastal range. A tributary of Panoche Creek upstream of the sites sampled in this study is San Carlos Creek, which drains the New Idria mercury mine site. Surface water samples were collected from Panoche Creek on 7 July 2004.

Alamitos Creek is a freshwater stream in the eastern Santa Cruz Mountains of central California which drains a portion of the historic New Almaden mercury mining district. It is a tributary of the Guadalupe River, which drains into Alviso
Slough and the southern reach of San Francisco Bay. Surface water samples were collected from Alamitos Creek (Table 2) on 16 January 2005 and 6 November 2005.

2.9. Method validation

Method validation experiments for the CLE-SPE of Hg(II) were performed using model waters with the ligand EDTA to access the method’s accuracy and recovery of Hg(II) and ligands. EDTA is a well characterized ligand which primarily forms the hydrophilic complex HgEDTA$^{2-}$ at near neutral pHs and low millimolar concentrations of EDTA, with the stability constants involved being well known [16]. Therefore, EDTA was used as the reference ligand in model water titrations to determine the formation constant ($\beta_2$) for Hg(DEDCA)$^{2-}$. Experimental conditions of 200 pM Hg(II), 1.0 mM EDTA, 2.0 mM MOPS buffer at pH 7.4 were used for a titration of 0–33 nM DEDC. Table 1 contains the relevant formation constants [16,35,45,50–52] used for calculations throughout this study.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$\log K$ or $\beta$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hg$^{2+}$ + EDTA$^{3-}$ ↔ HgEDTA$^{2-}$</td>
<td>23.24</td>
<td>[16]</td>
</tr>
<tr>
<td>Hg$^{2+}$ + H$^+$ + EDTA$^{4-}$ ↔ HgHEDTA$^{-}$</td>
<td>26.87</td>
<td>[16]</td>
</tr>
<tr>
<td>Hg$^{2+}$ + 2H$^+$ + EDTA$^{4-}$ ↔ Hg$_2$EDTA$^{3-}$(aq)</td>
<td>29.17</td>
<td>[16]</td>
</tr>
<tr>
<td>EDTA$^{4-}$ + H$^+$ ↔ HEDTA$^{3-}$</td>
<td>10.95</td>
<td>[16]</td>
</tr>
<tr>
<td>EDTA$^{4-}$ + 2H$^+$ ↔ H$_2$EDTA$^{2-}$</td>
<td>17.22</td>
<td>[16]</td>
</tr>
<tr>
<td>EDTA$^{4-}$ + 3H$^+$ ↔ H$_3$EDTA$^{-}$</td>
<td>20.34</td>
<td>[16]</td>
</tr>
<tr>
<td>EDTA$^{4-}$ + 4H$^+$ ↔ H$_4$EDTA$^{0}$(aq)</td>
<td>22.55</td>
<td>[16]</td>
</tr>
<tr>
<td>EDTA$^{4-}$ + 5H$^+$ ↔ H$_5$EDTA$^+$</td>
<td>24.05</td>
<td>[16]</td>
</tr>
<tr>
<td>Hg$^{2+}$ + TSA$^{2-}$ ↔ HgTSA$^{0}$(aq)</td>
<td>24.84</td>
<td>[35]</td>
</tr>
<tr>
<td>Hg$^{2+}$ + 2TSA$^{2-}$ ↔ Hg(TSA)$_2^{2-}$</td>
<td>33.48</td>
<td>[35]</td>
</tr>
<tr>
<td>TSA$^{3-}$ + H$^+$ ↔ HTSA$^{-}$</td>
<td>8.2</td>
<td>[50]</td>
</tr>
<tr>
<td>TSA$^{2-}$ + 2H$^+$ ↔ H$_2$TSA$^{0}$(aq)</td>
<td>11.8</td>
<td>[50]</td>
</tr>
<tr>
<td>Hg$^{2+}$ + 2DDEC$^{-}$ ↔ Hg(DDEC)$_2^{0}$(aq)</td>
<td>33.92</td>
<td>Average of [45,51]</td>
</tr>
<tr>
<td>DDEC$^{-}$ + H$^+$ ↔ HDDEC$^{0}$(aq)</td>
<td>3.38</td>
<td>[52]</td>
</tr>
<tr>
<td>Hg$^{2+}$ + OH$^-$ ↔ HgOH$^+$</td>
<td>10.6</td>
<td>[16]</td>
</tr>
<tr>
<td>Hg$^{2+}$ + 2OH$^-$ ↔ Hg(OH)$_2^{0}$(aq)</td>
<td>21.2</td>
<td>[16]</td>
</tr>
<tr>
<td>Hg$^{2+}$ + 3OH$^-$ ↔ Hg(OH)$_3^{0}$(aq)</td>
<td>20.9</td>
<td>[16]</td>
</tr>
<tr>
<td>Hg$^{2+}$ + Cl$^-$ + OH$^-$ ↔ HgClOH$_2$$(aq)$</td>
<td>18.25</td>
<td>[16]</td>
</tr>
<tr>
<td>Hg$^{2+}$ + Cl$^-$ ↔ HgCl$^+$</td>
<td>7.3</td>
<td>[16]</td>
</tr>
<tr>
<td>Hg$^{2+}$ + 2Cl$^-$ ↔ Hg(Cl)$_2^{0}$(aq)</td>
<td>14.0</td>
<td>[16]</td>
</tr>
<tr>
<td>Hg$^{2+}$ + 3Cl$^-$ ↔ Hg(Cl)$_3^{0}$(aq)</td>
<td>15.0</td>
<td>[16]</td>
</tr>
<tr>
<td>Hg$^{2+}$ + 4Cl$^-$ ↔ Hg(Cl)$_4^{0}$(aq)</td>
<td>15.6</td>
<td>[16]</td>
</tr>
</tbody>
</table>

The stability constant for Hg(TSA)$_2^{2-}$ was then estimated by using DEDC as the reference ligand and a range of TSA concentrations from 0.5 to 8.0 μM in model water solutions buffered to pH 7.6 by 5.0 mM MOPS, containing 200 pM Hg(II).

2.10. Kinetics study

Initial experiments of mercury complexation using CLE-SPE in this study were conducted by adding the Hg(II) to solutions to form a mercury titration, waiting 1 h to allow the added mercury to equilibrate with natural ligands, then adding the competing ligand and allowing an additional 12–15 h for the system to re-equilibrate before the solid phase extraction was carried out. These equilibration times were based on those reported by Hsu and Sedlak [26].

However, one of the assumptions inherent in methods employing competitive ligand exchange is that the system has been allowed to reach thermodynamic equilibrium prior to the final separation or determination of the amount of the metal of interest complexed by the natural and competing ligands. The use of excessively long equilibration times can allow for changes in solution chemistry, degradation of the natural ligands being characterized, as well as degradation of the competing ligands used. This was of special concern in this study since the competing ligands employed were thiols which may be susceptible to oxidation in the aerated waters being characterized. As such, a kinetics study was conducted to determine the optimal equilibration times for use with the CLE-SPE extraction method.

For this kinetic experiment of ligand exchange and ligand stability we collected filtered (0.45 μm) water samples from Alamitos Creek site B on 16 January 2005. After transport on ice back to the lab they were buffered to pH 7.7 with 2.0 mM MOPS and were spiked with 500 pM Hg(II). We varied the equilibration time between when the Hg(II) spike was added and when the competing ligand was added by adding TSA at 2–72 h after the Hg(II) additions. For each of these initial equilibration times the second equilibration time involved, between when the competing ligand was added until when the solid phase extraction was carried out, was also varied from 2 to 48 h.

Experiments of the stability of a 5.0 mM stock solution of the competing ligand TSA were also conducted. Milli-Q water buffered to pH 7.7 with 2.0 mM MOPS was amended to 500 pM Hg(II) and 40 μM TSA. These were allowed to equilibrate for between 2 and 24 h before solid phase extraction. One of the TSA stock solutions used for TSA additions was made 1 h prior to the start of the experiment, while the other TSA solution had been made up 5 days earlier and had been kept in the dark at 4 °C until used. Both 5.0 mM stock solutions of TSA were made in Milli-Q deoxygenated by purging for at least 20 min with high purity nitrogen which had been passed though an oxygen scrubber and gold trap.

2.11. Freeze–thaw study

The time-consuming nature of the CLE-SPE method prevents the simultaneous processing of more than one sample due
to material, space, and time constraints. As a consequence, the collection of samples from multiple locations in a single day, such as a stream transect, required that samples be frozen in the field and later thawed for CLE-SPE. No previous studies have reported investigating the effect of freeze–thaw cycles on mercury complexation in natural aqueous samples. We were therefore concerned that freezing and thawing samples may affect the stability of natural Hg binding ligands or complexes, and as a consequence undertook experiments to determine if freezing samples in the field for future processing introduced any biases or errors into the CLE-SPE results.

For these studies samples were collected in triplicate in three sets of 2 L Teflon bottles. One set was kept cool and in the dark until transported back to the lab where the CLE-SPE was begun that same evening, while the other two sets of sample were frozen in the field with dry ice immediately after collection and were kept frozen and in the dark until thawed and processed 2–6 weeks later.

2.12. SPE breakthrough experiments

Experiments were conducted to quantify any column breakthrough of Hg during solid phase extraction. For selected samples collected from Alamitos Creek Sites A and B on 16 November 2005 the column passing fraction of various treatments in the Hg titration curve was collected in either three or four sequential aliquots over the course of the solid phase extraction and were analyzed for total Hg separately. This was done using end capped C18 columns with 0.5 g of packing material.

3. Results and discussion

3.1. Method validation

Results of the method validation experiments for the CLE-SPE of Hg(II) using the reference ligand EDTA in model water titrations to determine the $\beta_2$ for Hg(DEDC)$_2$ is shown in Fig. 1. When no DEDC was present 100% of the Hg existed as the hydrophilic complex HgEDTA$^{2-}$, which was found in the column passing fraction after SPE. As the amount of DEDC was increased the amount of Hg(II) in the hydrophilic column passing fraction complexed by EDTA decreased, while the amount of Hg(II) found in the hydrophobic fraction retained by the C18 column increased due to the formation of Hg(DEDC)$_2$ (Fig. 1).

Thermodynamic modeling of these competitive ligand exchange data and ligand fitting using the program FITEQL yielded a log $\beta_2$ of 33.6 ± 0.3. This value was remarkably similar to the log $\beta_{2Hg(DEDC)}$ of 33.4 reported by Hsu and Sedlak [26] that was derived under similar conditions using the same method. Other values reported in the literature for log $\beta_{2Hg(DEDC)}$ include an averaged value of 32.1 [51] and 33.8 [45]. The favorable comparison of the results from this study with others in the literature demonstrates the method’s ability to accurately determine the conditional stability constants of mercury with complexing ligands.

The stability constant for Hg(TSA)$_2^{2-}$ was then estimated by using DEDC as the reference ligand and a range of TSA concentrations in model water solutions. The log $\beta_2$ Hg(TSA)$_2^{2-}$ was estimated from this titration to be 31.6 ± 0.5. This value compares relatively favorably with an ionic strength corrected value of 33.9 calculated from Koul and Dubey [35].

The recovery of mercury during the course of these experiments was calculated using the sum of Hg measured in the column passing fraction plus that eluted off the C18 column with 2% HCl. The average percent recovery of mercury was 100.9%, with a standard deviation of 7.7.

3.2. Kinetics study

Kinetic experiments carried out used a crossed design which allowed for an interpretation of both the ligand exchange involved and natural and competing ligand stability. The first phase of the competing ligand exchange involved addition of an Hg(II) spike to the natural waters, then waiting to allow this added Hg(II) to be taken up by the natural ligands before the competing ligand was added. As the natural ligands are quite likely initially bound to some other metal or cation, the optimal equilibration interval will be determined by both the kinetics and pathway by which the natural ligands exchange the cation initially being complexed for the newly added Hg(II) [53,54]. This equilibration time can be exceptionally long if the exchange kinetics of the natural ligands which would dominate complexation of Hg(II) at thermodynamic equilibrium are slow. When this occurs weaker Hg(II) binding ligands with faster kinetics may quickly complex the newly added Hg(II), which would then require a double ligand exchange before a new thermodynamic equilibrium is reached [54]. However, the optimization of this first equilibration of the natural ligands with the added Hg(II) also requires that an excessive amount of time not be permitted before added the competing ligand, as this could allow for degradation of the natural ligands. Thus, employing an equilibration time too short to allow for a new equilibrium be reached with the added Hg(II) will tend to underestimate complexation, as will
Fig. 2. Results of kinetics experiments of ligand exchange and ligand stability using 0.45 μM filtered water samples collected on 16 January 2005 from Alamitos Creek Site B. Samples already contained 79 pM Hg, and were buffered to pH 7.7 and spiked with an additional 500 pM Hg. The equilibration time between when the Hg(II) spike was added and the addition of TSA was varied from 2 to 72 h, while the equilibration time between when the TSA was added until when the solid phase extraction was carried out was varied from 2 to 48 h.

The idea that the natural ligands eventually begin to degrade to some extent over time appears to be supported by the treatment group to which an Hg(II) spike was added, and then the SPE performed without a competing ligand being added. This treatment allowed for a rough measure of the natural ligand stability, as a major shift in the complexation over time would be due solely to ligand exchange within the natural ligand pool and the relative stability of the natural ligands forming hydrophilic or hydrophobic complexes. This treatment with no competing ligand had Hg(II) added, and then underwent SPE after 2, 6, 9, 12, 16, and 24 h. While the distribution of mercury during the SPE did not drastically change over time, there was a significant decrease ($p < 0.01$, ANOVA) in the amount of mercury found in the hydrophilic fraction after equilibration (Fig. 2). This decline could be due either to the rapid initial uptake of mercury by weak hydrophilic ligands, which over time are out competed by stronger ligands forming hydrophobic complexes with Hg(II) as equilibrium is reached. Another possibility is that the kinetics involved were sufficiently rapid to allow for equilibrium to be reached in the first 2 h, and the shift in distribution of Hg(II) during SPE was due to the relatively greater breakdown of ligands forming hydrophilic complexes with Hg(II), which resulted in more of the mercury ending up in hydrophobic complexes over time. It is also quite possible that both processes were at work, but further work would be required to resolve the importance of each. In any case, the overall change in the distribution of mercury among the natural ligands was relatively small, and there was no significant change ($p > 0.05$) after the first 12 h.

The second equilibration time optimized during the CLE experiment was the interval after the addition of the competing ligand and before the SPE is conducted. Again, this period must be long enough for the aqueous system to reach a new thermodynamic equilibrium after the perturbation caused by the addition of the competing ligand, while not being any longer than necessary to minimize the degradation of the competing or natural ligands. For all groups, regardless of the equilibration time used between the addition of the mercury spike and the addition of
the TSA, the percentage of mercury in the hydrophilic column passing fraction initially increased with time, reaching a peak between 6 and 12 h, then decreased with additional time (Fig. 2).

One interpretation of these data is that the exchange kinetics between the natural ligand initially complexing the mercury and the added TSA were very rapid, and the initial increase in hydrophilic Hg during the first 6–12 h reflects the degradation of natural hydrophobic Hg complexes, which lead to a shift to more hydrophilic Hg during the first 12 h. The subsequent shift back to hydrophobic complexes could be the result of partial degradation of the TSA, which forms hydrophilic complexes with Hg(II), after the initial 12 h. However, this possibility seems unlikely and is contradicted by the data from the natural water with no competing ligand, which as discussed above, shows only a slight shift to more hydrophobic complexes of Hg within that time period.

A more plausible interpretation is that the increase in the percentage of hydrophilic mercury during the first 6–12 h is due to the slow shift of Hg between natural hydrophobic Hg complexes and hydrophilic TSA–Hg complexes as the system reaches a new dynamic equilibrium. The decrease in the percentage of Hg in the hydrophilic column passing fraction after 12 h can be explained by the partial degradation of both natural ligands forming hydrophilic complexes, which is supported by the data of natural water with no added ligand, as well as the eventual degradation of TSA over time, which is supported by the data of TSA in aerated Milli-Q, to which 25 μM DEDC was added 15 min before extraction.

In light of these kinetics data and their possible interpretations, we decided to use an initial equilibration time of 12 h between Hg(II) addition and the addition of the competing ligand, followed by an equilibration period of 6 h between the addition of the competing ligand and SPE. These conditions allow for the majority of ligand exchange to occur within the natural ligand pool, but minimize any possibility of natural and competing ligand oxidation or degradation.

3.3. Freeze–thaw experiments

Sample triplicates were collected at Panoche Creek on 7 July 2004, from Alamitos Creek Site B on 16 January 2005, and from Alamitos Creek Site B on 6 November 2005. One of each of these samples was kept cold and in the dark until the CLE-SPE was performed the same day as sampling while the other two sample replicates were frozen in the field with dry ice and thawed out for CLE-SPE 2–6 weeks later. While these data sets are small and do not allow for a rigorous statistical comparison of the samples processed immediately without freezing compared to those samples frozen in the field, the average relative standard deviation within the three sets of pooled fitted ligand concentrations was 29.5%, while the average relative standard deviation for pooled values calculated for $K_{\text{HgL}}^{\text{cond}}$ was 1.1%. These variations are within the estimated overall error associated with the method, based upon both the results of the method validation experiments and method triplicates for individual aliquots run through the CLE-SPE randomly from each field sample set. Thus, there appears to be no major difference in the ligand fitting results when comparing the two sample storage and possessing methods.

We therefore took this consistency as an indication that freezing samples in the field for future processing did not introduce a substantial error into our results. This conclusion is in line with studies of the complexation of other trace metals in natural waters which have reported freezing samples after collection without any apparent effect on ligand stability [31,55,56]. Because there was no noticeable difference in the results from the frozen and non-frozen samples, we treated these as replicates to be pooled when reporting results. The average value and standard deviation of these three replicate samples for the fitted ligand concentration and $K_{\text{HgL}}^{\text{cond}}$ are reported in Table 3.

These preliminary data do not preclude the possibility that the sample collection, storage during transportation to the lab, or sample processing common to both samples do not alter the specification of Hg(II) in a significant manner. Nonetheless, because of the demands in terms of both time and trace metal clean supplies required for the CLE-SPE of each individual sample, freezing samples in the field for later processing was the only practical means available which would allow for more than one sample to be collected and analyzed at a time by this method.

3.4. Evaluation of column breakthrough during SPE

Typical results from experiments conducted to quantify any column breakthrough of mercury during solid phase extraction are shown in Fig. 3. While the concentration of mercury in the column passing fraction was not constant and did vary between the multiple aliquots collected over the course of the extractions (average % relative standard deviation of 13% ± 4, n = 18 with three to four aliquots collected for each during SPE), there was no evidence of a systematic change in the Hg passing through the column with time. Thus, there was no indication of column breakthrough nor of a change in the interaction of mercury complexes with natural organic matter which had become associated with the C18 column as more hydrophobic material became adsorbed over the course of the extraction for any of the sites, ligand concentrations, or mercury concentrations tested.

3.5. Ancillary parameters

Ancillary parameters from water samples collected for this study are summarized in Table 2, along with filtered and unfiltered total mercury concentrations. The average daily detection limit for total mercury analyses (n = 27 days), calculated as three times the standard deviation of blank values, was 0.55 ± 0.75 pM. Spike recoveries (n = 46) of total mercury ranged from 85.3% to 112.6%, with an average spike recovery of 100.5% ± 6.1.

3.6. CLE-SPE

Comparing the results of SPE in natural water samples with no added ligand to those of the CLE-SPE with DEDC and TSA added demonstrated that the system qualitatively behaved as expected with respect to shifts in the amount of hydrophobic
mercury retained by the C18 column. The addition of a low concentration (1 μM) of DEDC resulted in a decrease in the amount of Hg in the hydrophilic fraction (Fig. 4) due to the formation of the hydrophobic complex Hg(DEDCC)20, and the addition of 25 μM DEDC led to an even greater decrease in the amount of Hg in the hydrophilic fraction. Conversely, the addition of 4 μM TSA resulted in an increase in the amount of Hg in the hydrophilic fraction (Fig. 4) due to the formation of the hydrophilic complex Hg(TSA)22−. As hypothesized, the addition of 105 μM TSA led to an even greater increase in the amount of hydrophilic Hg.

At the highest Hg(II) concentrations in the titration curve, when TSA was the competing ligand, the curves approached a slope of one when plotted as total mercury versus hydrophilic mercury (Fig. 4), denoting that the strongest natural hydrophobic ligands had been saturated and additional added mercury was complexed by the TSA in hydrophilic complexes. Whereas at the highest Hg concentrations in the titration curve when DEDC was added as the competing ligand the slope of the curves approached zero when plotted as total mercury versus hydrophilic mercury as the strongest hydrophilic natural ligands became saturated and additional Hg became complexed in hydrophobic DEDC complexes.

These CLE-SPE results were then used to generate plots of [Hg]nat versus [Hg2+] for each sample, and these data were used for ligand fitting of the natural ligands complexing Hg(II) as already described. Results from Alamitos Creek Site A sampled 6 November 2005 with CLE-SPE conducted using two concentrations of both TSA and DEDC are shown in Fig. 5. Whenever possible the complexion was modeled assuming a 1:1 complex of Hg(II):L1, with one ligand class used to describe the complexion within the hydrophilic and one ligand class in the hydrophobic fractions (Fig. 5).

However, some data sets indicated the presence of two or more classes of ligand within the hydrophilic fraction as determined from Scatchard plots [56,57]. The need to incorporate the use of multiple ligand classes when modeling the complexion observed was further increased when data were pooled from both concentrations of DEDC and the analytical window was expanded. At times it was found that a one ligand model, which could accurately describe the data from only one concentration of the competing ligand DEDC, failed to describe the behavior of the system across the entire range of combined values of [Hg]nat. In these cases the data were modeled with the use of a stronger binding class, L1, which dominated complexation at lower Hg levels, and a weaker binding ligand class, L2, which becomes important in complexation at higher Hg levels as L1 becomes saturated. This provided for an improved depiction of the system’s behavior across all Hg(II) titrations.

Table 2
Summary of sample dates, locations, ancillary parameters, and filtered and unfiltered total mercury concentrations

<table>
<thead>
<tr>
<th>Date</th>
<th>Location</th>
<th>Latitude/longitude</th>
<th>pH</th>
<th>Water temperature (°C)</th>
<th>Unfiltered [Hg]T (pM)</th>
<th>Filtered [Hg]T (pM)</th>
<th>[Cl−] (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 July 2004</td>
<td>Panoche Creek</td>
<td>36°35′41.98″N/120°45′29.68″W</td>
<td>7.8</td>
<td>21.7</td>
<td>4.5 ± 2</td>
<td>3.0 ± 2</td>
<td>3.1</td>
</tr>
<tr>
<td>16 January 2005</td>
<td>Alamitos Creek Site B</td>
<td>37°12′04.62″N/121°49′44.18″W</td>
<td>8.3</td>
<td>10.7</td>
<td>718</td>
<td>83</td>
<td>2.0</td>
</tr>
<tr>
<td>6 November 2005</td>
<td>Alamitos Creek Site A</td>
<td>37°10′25.41″N/121°49′28.68″W</td>
<td>7.3</td>
<td>15.8</td>
<td>232</td>
<td>30</td>
<td>1.4</td>
</tr>
<tr>
<td>6 November 2005</td>
<td>Alamitos Creek Site B</td>
<td>37°12′04.62″N/121°49′44.18″W</td>
<td>8.4</td>
<td>14.4</td>
<td>285</td>
<td>63</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Values are reported to within one standard deviation of field triplicates. The average daily detection limit (n = 27 days) for total mercury analyses, calculated as three times the standard deviation of blank values, was 0.55 pM.
Fig. 4. Hydrophilic, column passing Hg(II) after CLE-SPE using TSA or DEDC across Hg(II) titration for Alamitos Creek Site A sampled 6 November 2005, pH was buffered to 7.8 with 5 mM MOPS. Closed symbols represent data for which DEDC was the competing ligand, open symbols represent data for which TSA was the competing ligand, and the solid line represents a slope of 1.

The assignment of an L1 and L2 does not imply that there exist two discrete ligands, or even two discrete classes of ligands, responsible for the complexation observed. Instead, the natural system more likely consists of a continuum of binding sites and concentrations [22,58], the combined behavior of which across a range of Hg(II) is modeled here for practical purposes as two ligands. However, the concentrations and conditional stability constants calculated for L1 and L2 may prove valuable in inferring what types of naturals ligands are likely responsible for the complexation of Hg(II) in these oxic freshwaters, as discussed below.

Values for the conditional stability constants and natural ligand concentrations after ligand fitting for both the hydrophilic and hydrophobic fractions for samples collected for CLE-SPE are reported in Table 3. Natural ligands capable of complexing Hg(II) in the aquatic environments studied ranged in concentrations from 22 pM to 11 nM, while values of log $K_{\text{HgL}}^{\text{cond}}$ ranged from 29.9 to 33.5.

While ligand concentrations and metal–ligand formation constants provide useful information for inferring the nature of ligands responsible for, and the extent of, Hg(II) complexation in these waters, it is difficult to directly compare different sites within the same study. It is even more difficult to compare these data in a meaningful way across different studies due to the large influence of both the analytical working window and the technique employed for ligand fitting.

However, a graphical means by which the complexation capacity from different sites can easily be qualitatively compared is a plot of [Hg]nat versus [Hg$^{2+}$] (Fig. 5). When using this method of visualization data sets which are found to the bottom or the right side of the plot have stronger complexation than those towards the top or left side if the plot. This is demon-

Fig. 5. Calculated CLE-SPE and ligand fitting results for both hydrophilic and hydrophobic natural ligands, Alamitos Creek Site A collected 6 November 2005, 5 mM MOPS, pH 7.8. Closed symbols represent data for which DEDC was the competing ligand, open symbols represent data for which TSA was the competing ligand. The dashed line represents the one ligand model fit for the natural ligands forming hydrophilic complexes with Hg where $[L]_{\text{Hpi}} = 9.2$ nM, log $K_{\text{HgLhpi}}^{\text{cond}} = 30.8$. The solid line represents the one ligand model fit for the natural ligands forming hydrophobic complexes with Hg where $[L]_{\text{Hpo}} = 5.4$ nM, log $K_{\text{HgLhpo}}^{\text{cond}} = 32.2$. 

stratified in Fig. 5, which shows there is stronger complexation in the natural pool of ligands forming hydrophobic complexes with Hg(II) than those forming hydrophilic Hg(II) complexes.

3.7. Hydrophobic versus hydrophilic natural Hg complexes

When comparing the CLE-SPE results for the four natural water samples collected it was apparent that the strongest, or kinetically inert, natural ligands resulted in complexation in which inorganic Hg(II) at ambient levels was predominantly in hydrophobic complexes. This is demonstrated for Alamitos Creek Site A sampled 3 November 2006 in Fig. 4, which shows that the percentage of the total Hg in hydrophobic complexes is 86% at near ambient Hg levels in the absence of any competing ligands. There is a shift to more hydrophilic complexes with increasing mercury concentrations, and the percentage of total mercury in hydrophobic complexes decreases to 58% after the addition of 11 nM Hg(II). A similar trend in complexation was seen at all four sites.

This partitioning of mercury between the hydrophilic and hydrophobic fractions during CLE across an Hg titration provides an additional means to qualitatively evaluate the quality of the ligand fitting. This is done by comparing the raw data from the CLE-SPE of a sample when no competing ligand was added to the predicted partitioning of Hg between hydrophobic and hydrophilic complexes using the calculated values for ligand concentrations and conditional stability constants for natural ligands forming both hydrophobic and hydrophilic complexes with the use of a chemical equilibrium modeling program, such as VMINTEQ. An example of this type of comparison is shown in Fig. 6 for Alamitos Creek Site A sampled 6 November 2005. The percentage of the total Hg(II) which exists in hydrophilic Hg(II) complexes in the absence of a competing ligand increases with increasing Hg(II) added. This trend is also predicted when using VMINTEQ to calculate the distribution of Hg(II) between hydrophobic and hydrophilic complexes using ligand concentration and conditional stability constants calculated using the CLE-SPE data from the TSA and DEDC competitions.

3.8. Strength of natural ligand binding to Hg(II)

The strength of Hg(II) binding, as evaluated by values of log $K_{cond}^{HgL}$, and the complexation capacity, as evaluated by the $\alpha ([L] \log K_{HgL}^{cond})$, was very strong in all four samples (Table 3). The natural ligands capable of forming strong complexes with Hg(II) were always in excess of the total mercury, resulting in the calculation that at equilibrium over 99.99% of the inorganic Hg(II) in all systems would be complexed by the natural ligands characterized. It is clear that the notion of free Hg$^{2+}$ simply does not apply to these natural waters.

3.9. Comparison to other studies

Table 4 includes results from other studies of Hg(II) speciation and complexation in aquatic environments, as well as the results from this study. The relative analytical window must be considered when comparing results across multiple complexation studies as this is intimately tied to, and ultimately determines, the potential range of ligand concentrations and stability constants which will be reported, if any complexation exists [27,28,58]. A comparison of results from this study to other studies with similar detection windows in Table 4, such as Hsu and Sedlak [26], Haitzer et al. [23], and Han and Gill [22] demonstrates that the values reported for $K_{HgL}^{cond}$ in different aquatic systems and DOC isolates are within a couple orders of magnitude of each other. Conversely, a comparison of these results to studies with lower analytical detection windows

![Fig. 6. Distribution of Hg(II) in hydrophilic complexes across Hg(II) titration range for Alamitos Creek Site A collected 6 November 2005. Closed symbols represent raw SPE data with no competing ligand. Solid line represents predicted concentration of hydrophilic Hg complexes across total Hg(II) range calculated using VMINTEQ and ligand fitting results from CLE-SPE with yielded ligand concentrations and conditional stability constants for Hg binding of [L]hpo = 5.4 nM, log $K_{HgL}^{cond} = 32.2$, and [L]hpi = 9.2 nM, log $K_{HgL}^{cond} = 30.8$.](image)
[21,24,25], where higher concentrations of Hg(II) were added to solutions or weaker or no competing ligands were used, shows that those studies found Hg(II) complexation to be dominated by weaker ligands with much lower formation constants. While part of this disparity is due to the different conditions under which the values of $K_{\text{HgL}}^{\text{cond}}$ were measured, this contrast is consistent with the idea that at the higher concentrations of Hg(II) used the stronger ligands had already been saturated, and at the lower side reaction coefficients of the competing ligands used only competition with weaker ligands could accurately be measured and modeled.

4. Conclusions

The competing ligand exchange method described here is capable of not only characterizing the complexation and speciation of Hg(II) in aquatic environments, but has the additional advantage of providing information on the distribution of mercury between hydrophilic and hydrophobic natural complexes, while also discerning different ligand classes present in both the hydrophilic and hydrophobic fractions. The use of multiple analytical windows allows for the examination both of the complexation of Hg(II) at ambient mercury levels, as well as information on how the chemical speciation and distribution of mercury between hydrophilic and hydrophobic complexes changes at higher mercury concentrations. While this method has only been applied to freshwater systems to date due to the lack of information regarding the side reaction coefficients of TSA with major cations, this method could be accurately applied to brackish and saline waters once these values have been determined, this contrast is consistent with the idea that at the higher concentrations of Hg(II) used the stronger ligands had already been saturated, and at the lower side reaction coefficients of the competing ligands used only competition with weaker ligands could accurately be measured and modeled.

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