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Research Paper

A critical look at the calculation of the binding characteristics and concentration of iron complexing ligands in seawater with suggested improvements

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Environmental context. The low concentration of iron in the oceans limits growth of phytoplankton. Dissolved organic molecules, called ligands, naturally present in seawater, bind iron thereby increasing its solubility and, consequently, its availability for biological uptake by phytoplankton. The characteristics of these ligands are determined indirectly with various mathematical solutions; we critically evaluate the underlying method and calculations used in these determinations.

Abstract. The determination of the thermodynamic characteristics of organic Fe binding ligands, total ligand concentration ($[L_t]$) and conditional binding constant (K'), by means of titration of natural ligands with Fe in the presence of an added known competing ligand, is an indirect method. The analysis of the titration data including the determination of the sensitivity (S) and underlying model of ligand exchange is discussed and subjected to a critical evaluation of its underlying assumptions. Large datasets collected during the International Polar Year, were used to quantify the error propagation along the determination procedure. A new and easy to handle non-linear model written in *R* to calculate the ligand characteristics is used. The quality of the results strongly depends on the amount of titration points or Fe additions in a titration. At least four titration points per distinguished ligand group, together with a minimum of four titration points where the ligands are saturated, are necessary to obtain statistically reliable estimates of S, K' and $[L_t]$. As a result estimating the individual concentration of two ligands, although perhaps present, might not always be justified.

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Introduction

Fe is an essential micronutrient for marine phytoplankton growth; however, dissolved Fe concentrations are extremely low in ocean water relative to biological demand. High nutrient–low chlorophyll (HNLC) regions in the oceans are often caused by a lack of Fe.[1–3] Seasonal Fe limitation of primary production in the Iceland Basin,[4] and control of nitrogen fixation in the North Atlantic Ocean[5] show that Fe also regulates primary productivity outside the classic HNLC regions.

Since 1994 it has been known that ~99 % of dissolved Fe in seawater is bound to organic molecules (ligands) with high affinity for Fe.[6] The binding by dissolved organic ligands prevents or at least retards precipitation of Fe as insoluble oxides and may play an important role in the dissolution of Fe from dust[7] and in keeping Fe from glacier melt water[8,9] and hydrothermal sources in the dissolved phase.[10,11] Organic complexation influences the photochemistry and bioavailability of Fe.[12–14] To allow biological uptake of Fe, part of the organically complexed Fe pool must be biologically available for phytoplankton. It is still not clear which part of the organically complexed Fe pool is available and how it is assimilated by organisms.[15–17] It is also not exactly known which organic molecules in the marine environment bind Fe. The term 'ligand soup' is probably a good description of the reality in seawater: a continuum of different ligands in very low concentrations over a wide spectrum of size classes and Fe-binding functional groups.[18,19] Some components contributing to the natural ligand pool have been investigated. Siderophores are known to bind Fe strongly and have been detected in natural seawater.[6,20–22] Laglera et al.[23,24] investigated the role of the weaker binding humic substances. Hassler et al.[12,25] discovered the contribution of sugars to the ligand pool as relatively weak Fe-binding compounds, but present at relatively high concentrations.[25]

Most methods use adsorptive cathodic stripping voltammetry (adCSV) with a hanging mercury drop electrode based on ligand exchange.[6,26–28] This is an indirect method because the natural ligands themselves are not measured. The characteristics of the natural ligands, the total ligand concentration ($[L_t]$) and the conditional stability constant (K', commonly expressed as $\log K'$), are mostly calculated according to the Langmuir isotherm model.[29–31] Using this model, it is assumed that the added ligand (AL) competes with one or two different natural ligand groups present in the sample. This is a well known oversimplification.

The simplicity and strength of the Langmuir model has popularised this model since 1982.[30–32] To estimate the ligand characteristics K' and $[L_t]$, a series of observations is needed to solve the Langmuir equation. Therefore, the ligands are titrated

with Fe, typically resulting in 10 titration points. Although different linear[30,31,33] and non-linear[34–37] mathematical solutions have been developed using the Langmuir model, they all estimate the unknown parameters by fitting solutions to the Langmuir equation through the observations. Improvements and more sophisticated mathematical approaches to calculate the binding characteristics have been published.[35,37–39] An increasing number of publications on organic Fe speciation present data in which two ligand groups are distinguished[40] with one strong ligand (L_1) having K'_1 between 10^{21} and 10^{24}, and one relatively weak ligand (L_2) with K'_2 between $10^{19.8}$ and 10^{22}. Global models use this concept of two ligands to fit the measured dissolved Fe concentrations.[41,42] However, calculating the ligand characteristics of these two ligand groups in a correct way from the results of a titration, including standard deviations of the estimated values, is mathematically difficult.[37,38]

Inspired by the review paper of Gledhill and Buck[40] on Fe speciation in oceans we discuss the relationship between the quality of the analytical data and the interpretation of these results. After a short explanation of the principle of the ligand exchange method we discuss the consequences when the assumptions of the applied ligand exchange or Langmuir model are not fulfilled. We relate these consequences to the quality of the results and the data interpretation.

To interpret the data we use a straightforward and simple method in which the sensitivity (S), the conditional stability constant (K') and the total ligand concentration ($[L_t]$) are estimated using non-linear regression. By including S as an unknown parameter in the non-linear regression we allow for the possibility that the visually linear part of the titration curve is still affected by unsaturated natural ligands. The large datasets from IPY-GEOTRACES[43,44] from the International Polar Year program and unpublished measurements from the Western Atlantic Ocean are used to calculate the effects of S on Fe speciation results as well as the influence of the number of data points in a titration and the saturation with Fe of the natural ligands on results of Fe complexation in natural samples. In addition, we discuss whether a distinction of the ligand pool in two groups is warranted by the data quality. Although we do not doubt the existence of more than one ligand group, we propose a more critical data interpretation.

The ligand exchange method

Definitions

The prime in the stability constants K' and β' is used to indicate the conditional nature of the sample matrix to express the dependency on the conditions, differing from ideal conditions in temperature, pressure, pH, major ion composition, concentration of competing ligands and the ionic strength or salinity.[45] In this paper the values of K' and β' are given with respect to free Fe (Fe′). Fe′ corresponds to the sum of the fractions of dissolved Fe that are ionic Fe^{3+} and bound by inorganic complexes.

Principle

Voltammetry is a well suited method to measure low metal concentrations at a constant pH and at a high ionic strength as found in the marine environment. Ligand exchange is based on the addition of a well characterised electro-active ligand that competes with the natural unknown ligands for Fe. The concentration of Fe bound to this well characterised ligand can be measured. Its concentration reflects the equilibrium after competition with the natural ligands for Fe. The natural ligands are titrated by adding increasing concentrations of Fe to subsamples. At low added Fe concentrations both the natural and the added known ligands (hereafter added ligand, AL) compete for the added Fe. At high added Fe concentrations almost all natural ligands bind Fe and the measurable concentration of known ALs increases almost linearly with added Fe (Fig. 1a). We use this 'linear' part of the titration to calculate the concentration of non-Fe bound or 'free' natural ligands not bound to Fe and S that describes the sensitivity of our analytical signal for the addition of Fe. The sensitivity is used to translate the current I in nA as peak height into Fe concentration in nM. If any Fe_xAL_y complex is formed that is not electro-active an error is introduced overestimating the Fe complexation: until now no information has been known about this for the methods used for Fe.

The Langmuir equation combines the equilibrium expression and the mass balance of the natural ligands. The equilibrium expression is

$$K' = [FeL]/[Fe'][L'] \quad (1)$$

where K' is the conditional stability constant, often expressed as $\log K'$, and $[Fe']$ and $[L']$ the concentrations of free Fe and natural ligand L, whereas $[FeL]$ is the concentration of Fe that is complexed by natural ligand L. The mass balance of the natural ligands is given by

$$[L_t] = [FeL] + [L'] \quad (2)$$

in which $[L_t]$ is the total ligand concentration. Inserting $[L']$ from Eqn 2 into 1 gives the Langmuir equation:

$$[FeL] = K'[L_t][Fe']/(K'[Fe'] + 1) \quad (3)$$

The competing ligand method is an indirect method with a limited range defined by the detection window (D), which is the product of the non-Fe bound known AL concentration ($[AL']$) and conditional binding strength (β') of the AL.[45,46] The AL should have such a high concentration that any decrease in $[AL']$ by binding of Fe is insignificant. Consequently $[AL]$ is assumed to be equal to $[AL']$, implying $D = [AL]\beta'$ (Eqn 4). The product of the non-Fe bound ligand ($[L']$) and conditional binding strength (K') of the natural ligands that can be detected is in the range of one order of magnitude above and below D, according to Apte et al.[45] and van den Berg et al.[46] However, the range used is a little wider, by up to two orders of magnitude.[40]

A known amount of a well characterised competing ligand is added to the sample at a desired pH. After equilibrium is established, the Fe complexed by the AL can be adsorbed at a certain negative or neutral potential on the mercury drop: scanning in the more negative direction will result in the dissociation of Fe from the AL and a reduction of Fe^{III} to Fe^{II}, which can be measured as a current. The instrument signal can be translated into a concentration by S. Four different added Fe-binding ligands are currently used in adCSV: 1-nitroso-2-napthol (1N2N), later abbreviated as NN,[6] salicylaldoxime (SA),[26] 2-(2-thiazolylazo)-*p*-cresol (TAC)[27] and dihydroxynaphthalene (DHN).[28] The original method of Gledhill and van den Berg[6] was based on the added ligand NN, using a solution pH of 6.9.[47,48] Because a change in pH may change the chemistry of Fe, we restrict ourselves to the later NN work from 2001 onwards when a solution pH of 8.05 was used,[49] which is

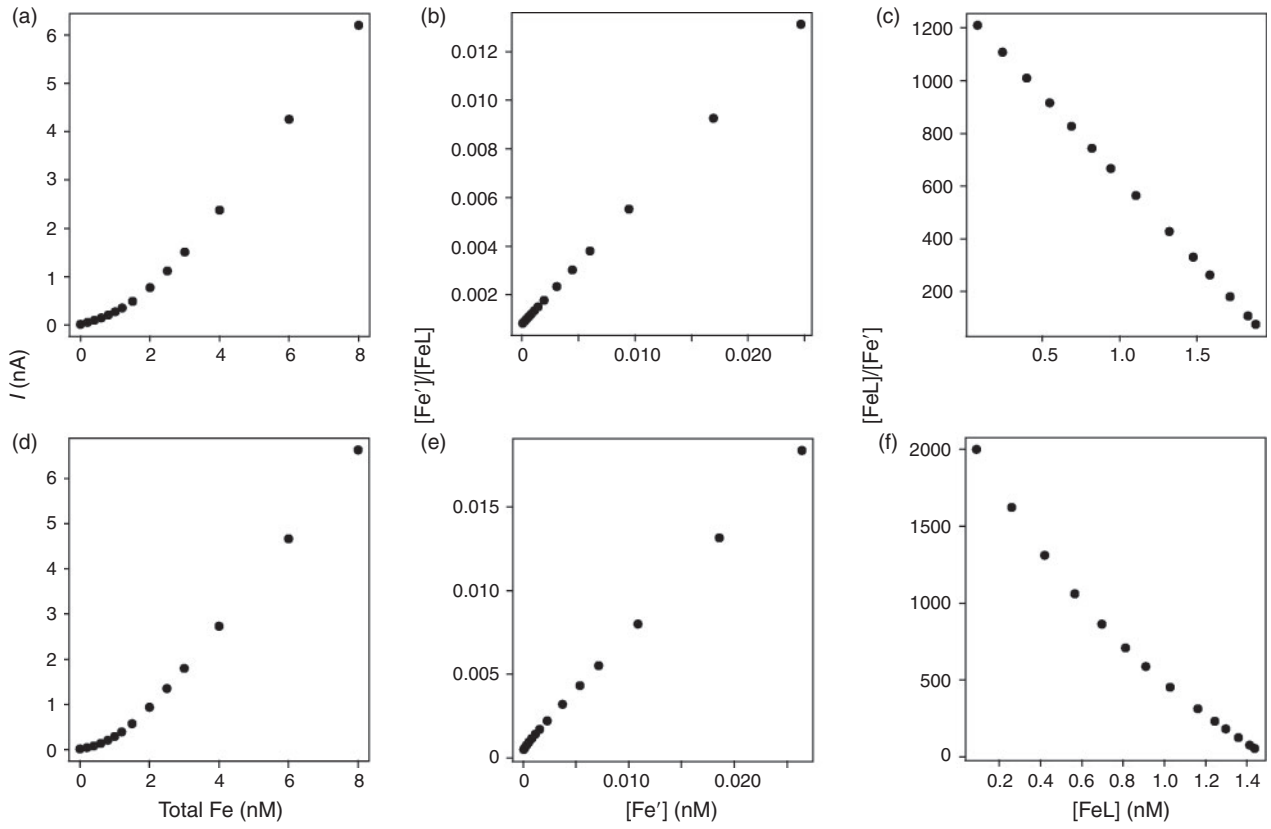

Fig. 1. Examples of treatment of (theoretical) data with different forms of the Langmuir equation for one ligand (a–c) and for two ligands (d–f). (a) The titration of one ligand with measured current in peak heights (nA) on the *y*-axis representing the Fe bound by the known added competing ligand and on the *x*-axis the total dissolved Fe ([Fe_{dis}] + added Fe) (nM). (b) The van den Berg–Ružić linearised form of the data in (a), in which the quotient of the concentration of free Fe (Fe′) and Fe complexed by natural ligand L (i.e. [Fe′]/[FeL]) is on the *y*-axis and [Fe′] (nM) (Eqn 7) is on the *x*-axis. (c) The Scatchard linearisation of the data in (a); in which the quotient of [FeL] and [Fe′] is on the *y*-axis and [FeL] (nM) on the *x*-axis (Eqn 8). (d) As in (a) but now two ligands are present in the sample. (e) As in (b) but now two ligands are present in the sample. (f) As in (c) but now two ligands are present in the sample. (a–c) Theoretical data: [Fe_{dis}] = 0.1 nM, log K' = 11.8, [L_t] = 2 nEq of M Fe. (d–F) Theoretical data: [Fe_{dis}] = 0.1 nM, log K'_1 = 12.5, [L_{t1}] = 0.5 nEq of M Fe, log K'_2 = 11.8, [L_{t2}] = 2 nEq of M Fe.

similar to the ambient pH in the open ocean. The used detection window (D) varied between 87.7 and 640.[7,24,27,28,43,44,49–55]

All these applications give comparable results.[56] K' varies between $10^{9.5}$ and 10^{14} with respect to Fe′ and $10^{19.5}$ and 10^{24} with respect to Fe^{3+}.

The calculation of K′ and [L_t], using the Langmuir equation

Applying the Langmuir equation to estimate K' and [L_t] has been described elsewhere[30,34] and we only briefly report the essentials. The concentration of Fe bound to the AL ([FeAL]) reflects the result of competition with the natural ligands. The concentration FeAL can be written as:

$$[\mathrm{FeAL}] = \beta_{\mathrm{FeAL}}[\mathrm{AL}][\mathrm{Fe}'] = D[\mathrm{Fe}'] \quad (4)$$

with D, the detection window, as the product of the concentration of AL not bound to Fe ([AL′]) and β_{FeAL}. Because the concentration of the AL is orders of magnitude larger than that of dissolved Fe, [AL] is used in Eqn 4 instead of [AL′], and [Fe′], the concentration of Fe not bound to the added and natural ligands can be calculated. Because we assume equilibrium in the sample solution, Eqn 1 can be rearranged to give,

$$[\mathrm{FeL}] = K'[\mathrm{L}'][\mathrm{Fe}'] \quad (5)$$

With the mass balance of Fe,

$$[\mathrm{Fe_{dis}}] = [\mathrm{FeAL}] + [\mathrm{FeL}] + [\mathrm{Fe}'] \sim [\mathrm{FeAL}] + [\mathrm{FeL}] \quad (6)$$

In this mass balance we assume that [AL] is sufficiently high that the detection window of the added ligand (D_{FeAL}) is >>100, therefore [FeAL] is large compared to the concentration of inorganic Fe species ([Fe′]), which is lower than 1 %. [Fe′] can therefore be removed from the mass balance (second part of Eqn 6). [FeL] can thus be calculated using the measured [FeAL] and the dissolved Fe concentration ([Fe_{dis}]), which needs to be known. The [Fe′] species are predominantly Fe hydroxides. At a fixed pH, this fraction of dissolved inorganic Fe can be calculated. At pH 8, [Fe′] = 10^{10} × [Fe^{3+}].[57] In Eqn 6 [Fe_{dis}] is the natural dissolved concentration of the sample, increasing to the total dissolved Fe concentration after Fe additions. In Eqn 5 two unknowns remain, [L′] and K'. We need a sufficient number of data points, hereafter called titration points, to estimate [L′] and K' with regression (Fig. 1a). The titration points are obtained by Fe additions, thus for a titration, the sample is typically subdivided into 6 to 15 subsamples with increasing Fe concentration.

To obtain [L_t] and K' the Langmuir equation (Eqn 3) is fitted to the measured [FeAL] as a function of added Fe

concentrations. Linearised forms of the Langmuir equation (Eqn 3) are also used, e.g. the van den Berg–Ružić linearisation[30,31] (Fig. 1b),

$$\frac{[Fe']}{[FeL]} = \frac{[Fe']}{[L_t]} + \frac{1}{K'L_t} \quad (7)$$

and the Scatchard linearisation[33] (Fig. 1c)

$$\frac{[FeL]}{[Fe']} = -K'[FeL] + K'[L_t] \quad (8)$$

The left hand sides of Eqns 7 and 8 (respectively [Fe′]/[FeL] and [FeL]/[Fe′]) are seen as *independent* variables, whereas it is clear from their expressions that they still depend on the dependent variables on their right hand sides (i.e. [Fe′] and [FeL]). Although the Scatchard linearisation (Eqn 8) most times doesn't fit the data properly,[45,58] it is a good method to reveal the presence of two ligand groups as indicated by two distinctive linear regions (Fig. 1f) instead of one if only one ligand group is present (Fig. 1d). Because [Fe′] is in the denominator of the function of [FeL] it magnifies the relationship between [FeL] and [Fe′] in the region of low FeL concentrations enabling distinction between two ligand groups[34,45,59] (Fig. 1f).

If *two* ligand groups, L_1 and L_2, are assumed to exist,

$$K'_1 = [FeL_1]/[Fe'][L'_1] \quad (9)$$

$$K'_2 = [FeL_2]/[Fe'][L'_2] \quad (10)$$

Inserting these into the total ligand concentration equation:

$$\sum [FeL] = [FeL_1] + [FeL_2] \quad (11)$$

one obtains:

$$\sum [FeL] = \frac{(K'_1[L_{t1}][Fe'])}{(K'_1[Fe'] + 1)} + \frac{(K'_2[L_{t2}][Fe'])}{(K'_2[Fe'] + 1)} \quad (12)$$

The calculation of four parameters (K'_1, K'_2, $[L_{t1}]$ and $[L_{t2}]$) using 10 titration points needs data with little noise, otherwise the error in the estimates by least-squares regression will be very large. For the moment we will restrict ourselves to the assumption that one ligand exists as given in Eqn 3. This is still one equation with two unknowns, K' and $[L_t]$. The greater Fe additions are meant to fully occupy all natural ligand sites resulting in a linear relationship between the instrumental signal, I (nA), and the Fe concentration, giving $S = I/[FeAL]$. The linear part is typically determined by visual inspection of the titration curve. S is the third unknown and this was not acknowledged for a long time. There are two reasons why the estimation of S by visual inspection has been criticised[35,36]: first the determination is very subjective, second the so-called straight part is not straight but still slightly curving. This error can be restored using an iterative process[36] or by using non-linear regression of all three parameters.[35]

The assumptions

The Langmuir equation[29] is often used to calculate ligand characteristics from titration data. Several assumptions need to be made to be able to use the Langmuir equation. Applying the Langmuir equation to titration data is based on the equilibrium expression (Eqn 1) and the mass balance of the natural ligands (Eqn 2). Therefore the first and most important assumption is that the system under observation is in equilibrium or at least in a steady-state. The second assumption is that all binding sites of L with Fe are equal and do not influence each other. In the above equations a 1 : 1 coordination is used, meaning that the FeL complex consists of one Fe and one natural ligand, but any coordination can be assumed to exist between L and Fe. The Langmuir equation (Eqn 3) can be extended to more than one ligand and the assumption that the sites are equal to each other within the distinguished groups remains.

The third assumption, related to Eqn 2, is that no other elements other than Fe influence [L′].

The fourth assumption, related to the first assumption of equilibrium, is that natural ligands bind Fe reversibly, which is inherent to the analysing method of ligand exchange. In other words, Fe bound to natural ligands can be released to an AL within the time limits of the method. This is defined by the association and dissociation kinetics of the natural and added ligands.

Assumption 1: equilibrium

Equilibrium, or at least a steady-state, is assumed to exist in the open ocean, although it is a natural environment in which processes occur continuously. However, the photic zone is subject to fast processes like photo-reduction and, on a longer timescale, biological processes such as phytoplankton blooms and degradation of fresh organic matter. Phytoplankton take up Fe and excrete dissolved organic molecules that might act as Fe binding ligands. Mineralisation below the photic zone releases Fe and organic molecules. Natural ligands are produced and altered by biological and physical processes that can lead to exchange between particles and solution. Somewhere in this environment a sample is taken. The result is a snapshot, as is every measurement. However, the sample is filtered and the effect of filtration, the removal of the particulate phase, may affect the natural equilibrium conditions and therefore measurement of dissolved organic ligands. Ligands keep Fe in the dissolved phase whereas scavenging removes Fe from the dissolved phase. Filtration removes one phase and sampling adds another competitor for Fe: the bottle wall.[60,61] The ligands might therefore be more under saturated than they were under natural conditions, and we might overestimate the non-Fe bound ligand concentration. Comparison between the dissolved Fe concentration immediately after filtering and after being present for a day in a sample bottle at natural pH indeed shows a reduction of 13 % (data not shown; $n = 130$, Fe content is 86.8 % ± 17.6 s.d. of the $[Fe_{dis}]$ measured in immediately acidified samples; GEOTRACES West Atlantic data). It is important to carefully describe when and under which conditions samples for dissolved Fe were taken, and it is advisable to sample for dissolved Fe from the same sample bottle as that in which the sample for organic speciation is kept and to sample just before organic speciation is measured. Another possibility is to condition sample bottles for speciation analysis with the sample before sampling, this is already routine with bottles for the subsamples of the titration. Free or inorganic Fe and complexed Fe adsorbs on the bottle wall. The added ligand TAC is known to be able to colour low density poly(ethylene) (LDPE) bottles and thus is clearly sticking to these bottles. If complexed Fe is adsorbing, the consequence is that the total ligand concentration is underestimated but not the excess ligand concentration [L′]. When the

non-Fe bound ligands adsorb, the total ligand concentration and [L′] will be underestimated.

We also can not exclude interactions between the AL and the natural ligands. Such interactions might exist between TAC and humic substances, masking complexation of Fe by humics.[24]

The consequence of not fulfilling assumption 1, thus when the system under study is not in equilibrium, is that the results are only valid for the moment the sample is measured. To apply the Langmuir model, equilibrium is necessary during the measurement but this also implies equilibrium in the sample before analysis. The formation of a new equilibrium after addition of the AL and increasing Fe concentrations has been tested extensively by the authors of the methods in use; we assume that the assumption of equilibrium during analysis is fulfilled. However, Town and van Leeuwen[62] wrote a critical note on this assumption, arguing that the kinetics of the reactions guaranteed a non-equilibrium situation during the measurement itself. Although this was rebutted by Hunter[63] and van den Berg,[64] it is still under debate whether such strong complexes, assumed to exist between Fe and marine organic complexes, can react within the time span of the measurement.[65] Recent kinetic data from the open ocean[66] proves that the time frame to reach equilibrium is ~12–48 h.

Assumption 2: equal binding sites

It is clear that assumption 2, all binding sites between L and Fe are equal to each other, is not fulfilled in natural samples. The results from the Langmuir model gives conveniently convincing results, but the existence of one or two ligands is a much too simple reflection of reality. We know that the ligands binding Fe consist of a variety of groups like siderophores, sugars and humic substances, but predominantly contain unidentified substances in different size fractions (Gledhill and Buck[40] and references therein). The variation in characteristics of metal binding ligands might be endless as discussed extensively by Buffle and co-workers forming a kind of continuum of binding strengths that can be modelled by affinity spectra.[67,68] The data resulting from application of a simple 1 or 2 ligand model is that the obtained [L_t] is from the most positive point of view the sum of all binding sites present.[50] Modelling a pool of ligands as if it is a single ligand results in a conditional stability constant K' as a kind of weighted mean, weighted by especially the strength and to a lesser extent by the concentration of the separate ligand (group)s. However, the influence of the different ligand (group)s on K' is restricted by the detection window (D) of the method, ligands with α-values ($\alpha = K'[L']$) outside D will interfere less. The magnitude and kind of this interference also depend on the α-value being at the upper or lower limit of D.

Sources of ligands and processes of degradation will be reflected in variations in ligand characteristics with depth (source relation) or time (production and degradation of ligands). The distinction into one or two ligand groups depends on this variability. The saturation with Fe of one group of ligands with time or depth will influence the interpretation of the binding characteristics of the sample.

Assumption 3: no competition by other metals

The binding strength between Fe and the organic ligands is so strong that the chance of competition is fairly small. Competition by divalent metals is unlikely. Although research on humic substances showed that the conditional binding constants of Zn, Co and Cu in seawater are only two orders of magnitude lower,[69] competition with Fe for humics might occur in a coastal environment with elevated Cu and Zn concentrations. Little is known about competition of rare earth metals and because their concentration is low, it is probably of less importance. If a competition does exist, both K' and [L_t] are underestimated. [L_t] is underestimated by the concentration occupied by the competing metal. The explanation of the underestimation of K' is that K' was calculated for a smaller [L_t] than present in the sample and a ligand with an α-value larger than assumed and so [L′] is smaller than assumed and consequently K' must be larger (Eqn 1). Competition falls under the conditional nature of the binding constant. However, it does disturb our interpretation if the unknown competing element or compound changes in concentration with time and place (depth), as then an observed increase in ligand concentration might in reality be only a decrease in the competing element. The extent of the error made in interpreting such a change will depend on the change in the concentration of the competing metal.

Assumption 4: reversibility

We here define Fe to be bound reversibly to a natural ligand when the AL is able to compete with this natural ligand for Fe and to bind Fe in a concentration related to the detection window of the AL, which is thus related to the concentration and the binding strength of the AL. For Cu in coastal waters it was shown that up to 60 % of the dissolved Cu was irreversibly bound.[70] If assumption 4 is not fulfilled and irreversibly bound Fe is present, as shown for Cu, the added ligand will not be able to bind Fe. Eqn 6 is then incomplete. The interpretation of the data will result in an overestimation of the concentration of natural ligands binding reversibly within the detection window.[40,55] The overestimation will amount to the concentration of irreversible bound Fe. [L_t] is overestimated, as well as K'.[40,55] K' is overestimated because it is calculated with an overestimated [L_t] (Eqn 6), and only [L′] is estimated correctly.[55] The effect of the concentration of Fe bound irreversibly on the obtained ligand characteristics can be illustrated by the data from Thuróczy et al.[55] They measured Fe binding organic complexes in filtered and unfiltered samples. In the unfiltered samples part of the Fe was bound in particles not available for complexation by an AL or in another way made inert for ligand exchange. Although particulate Fe not available for complexation is not the same as irreversibly bound Fe in the dissolved phase, the study of Thuróczy et al.[55] can be used to illustrate the effect of inert dissolved Fe that does not take part in the ligand exchange. In the unfiltered samples it was unknown which part of the measured total dissolvable Fe (unfiltered samples acidified at pH 2 and measured after 0.5 year) was bound reversibly to dissolved ligands. Therefore the parameters [L_t] and K' were calculated twice, with the total dissolvable Fe concentration as a maximum for the concentration of Fe bound reversibly by organic ligands and with the dissolved Fe concentration as an estimate of the minimum of the Fe concentration bound reversibly by organic ligands. Here we add that if the concentration of Fe bound irreversibly is large, fitting the model through the data might become difficult, therefore resulting in large errors in [L_t] and K', and thus also a large error in [L′]. The [L′] concentrations were the same for both calculations (0.92, s.d. = 0.24 and 0.957, s.d. = 0.31, $n = 10$). The difference between the Fe concentrations influenced the calculated K' and α-values according to the linear relationships shown in Fig. 2.

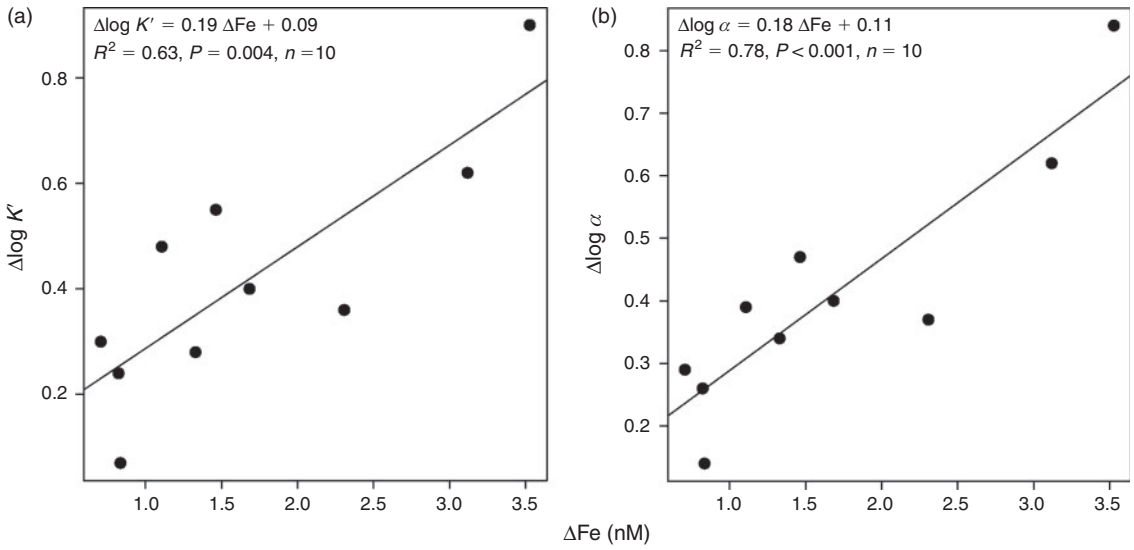

Fig. 2. The relationship between the Fe concentration used as input data and the resulting K' and α-values. On the horizontal axis the difference in Fe concentration (nM) between two calculations, on the vertical axis the difference in resulting K' and α. The data are from Thuroczy et al.[55] $\Delta Fe = [Fe_{UNF}] - [Fe_{dis}]$, $\Delta \log K' = \log K'_{UNF} - \log K'_{dis}$, Δlog α equals log α, derived from unfiltered Fe, minus log α, derived from dissolved Fe. Fe_{UNF} is Fe measured in unfiltered samples after conservation at pH = 2 for at least half a year.

Non-linear fitting of $[L_t]$, K' and S

Description of method used

In the present section we derive a non-linear model relating measured current (I) to the total dissolved iron concentration, x = added Fe + $[Fe_{dis}]$. Observations are used to estimate unknown parameters ($[L_t]$, K' and S) by fitting the non-linear model for a single ligand to the data using non-linear regression. We also show how the method can be extended when two (or more) ligands are present. In that case the model is instead, more easily (and exactly) cast in inverse form, i.e. it is namely one that relates total dissolved iron (x) non-linearly to measured current (I).

Fitting programs generally work better when data are of order one. Therefore the current I is given in nanoamperes and concentrations in nanomoles per litre instead of amperes and moles per litre, and the value of K' is divided by D to make the number of order one. The Langmuir model is rewritten in the following way.

Using the mass balance equation (Eqn 2) $[FeL] = [L_t] - [L']$, $[FeAL] = D[Fe']$ and the measured peak height (nA) $I = S$ [FeAL], with S the sensitivity of the analytical method, using Eqn 4 we find

$$I = SD[Fe'] \tag{13}$$

Using Eqn 1, the current is rewritten as

$$I = SD\frac{[FeL]}{K'[L']} \tag{14}$$

With $[Fe_{dis}]$ and added Fe expressed as x, we can rewrite the mass balance of Fe as

$$D[Fe'] = x - [FeL] - [Fe'] \tag{15}$$

From Eqn 1 we get

$$[FeL] = [Fe']\,K'[L'] \tag{16}$$

From Eqn 15 and 16 we get

$$(1 + D + K'[L'])[Fe'] = x \tag{17}$$

From Eqn 1, we replace $[Fe']$ by $[FeL]/K'\,[L']$. Using Eqn 2, we then replace [FeL] by [Lt] – $[L']$ and, moving the last term on the left-hand side of Eqn 17 to the right hand side, we get

$$(1 + D)([L_t] - [L']) = K'[L'](x + [L'] - [L_t]) \tag{18}$$

We define

$$K' = (1 + D)k \tag{19}$$

which introduces a normalised stability constant, k.

Inserting Eqn 19 into Eqn 18, we thus need to solve a quadratic in $[L']$, as also shown by Wu and Jin,[37]

$$[L_t] - [L'] = k[L'](x + [L'] - [L_t]) \tag{20}$$

Because $k[L'] > 0$, the positive root is used

$$2k[L'] = -(1 + k(x - [L_t])) + \sqrt{(1 + k(x - [L_t]))^2 + 4k[L_t]} \tag{21}$$

Defining a normalised sensitivity (s):

$$s = \frac{SD}{1 + D} \tag{22}$$

from Eqns 13 and 17 we find

$$I = \frac{sx}{1 + k[L']} \tag{23}$$

Inserting Eqn 21 in the denominator, finally leads to a relation between measured current (I) and total dissolved Fe (x):

$$I = \frac{2sx}{(1 + k([L_t] - x) + \sqrt{(1 + k(x - [L_t]))^2 + 4k[L_t]})} \tag{24}$$

Fitting this Eqn 24 to I (measured currents) *v.* x (total dissolved Fe), results in s, k and $[L_t]$. From Eqn 22 it follows that S is nearly equal to s because $D/(1+D) \approx 1$ (in the case of 10 µM TAC, $D = 10^{2.4}$ with respect to Fe′). From Eqn 19, K' is calculated by multiplying k with $1 + D \approx D$ and, when expressed in moles per litre, with 10^9 (because nanomoles were used instead of moles).

To extend the method to two ligands makes the mathematics complicated. A possibility is to use the one ligand fit to estimate S and then continue with the non-linear method, fitting Eqn 12[34,71] with only four parameters instead of five (because S is then known). Alternatively, as we propose to do here, we can exchange the role of the dependent and independent variables, I and x. For this, we can rewrite Eqn 20 as

$$\frac{x}{1+k[L']} = \left(\frac{[L_t]}{[L']} - 1\right)\frac{1}{k} \tag{25}$$

With Eqn 23, we infer

$$\frac{[L_t]}{[L']} = 1 + k\frac{I}{s} \tag{26}$$

and get a relation between $[L']$ and current I:

$$[L'] = \frac{[L_t]}{1 + \frac{kI}{s}} \tag{27}$$

Inserting Eqn 27 into Eqn 23, we obtain the inverse relation between x and I:

$$x = I\left(\frac{1}{s} + \frac{k[L_t]}{s + kI}\right) \tag{28}$$

This expression is interesting because it contains two linearisations in the limits that I is very small ($I \to 0$) or very large ($I \to \infty$). The former limit, $I \to 0$, can only be obtained in samples that have a very large $[L']$. In this case, it reduces to

$$x \to I(1 + k[L_t])/s \tag{29}$$

or, inversely,

$$I \to sx/(1 + k[L_t]) \tag{30}$$

which is similar to Eqn 23 except for a replacement of $[L']$ by $[L_t]$ (red dotted line in Fig. 3). This implies small yet non-zero [FeL], because $[FeL] \ll [L']$ is assumed, allowing for the approximation $[FeL] = K'\,[L'][Fe'] \sim K'\,[Lt][Fe']$.

In the latter limit, $I \to \infty$, the ligands are saturated with Fe, $[Fe']$ increases and Eqn 26 simply becomes

$$x \to [L_t] + I/s \tag{31}$$

or

$$I \to s(x - [L_t]) \tag{32}$$

represented as a blue dotted line in Fig. 3. This applies when [FeL] is negligible compared to $(1 + D)[Fe']$. It also implies that in samples with small $[L']$, samples with saturated ligands, k can hardly be estimated.

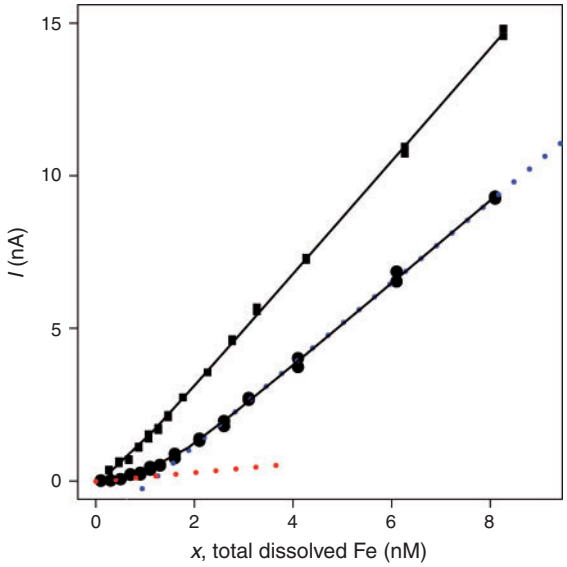

Fig. 3. The titration and non-linear fit of the Langmuir model (the fitted line discussed in this paper) of sample M (circles) and P (squares). Measured current in peak heights (nA) on the y-axis representing the Fe bound by the known added competing ligand and on the x-axis total dissolved Fe (x) the natural dissolved Fe concentration $[Fe_{diss}]$ + added Fe (nM). For sample M the red dotted line represents Eqn 30, the linearisation of Eqn 28 where $I \to 0$. The blue dotted line represents Eqn 32, the linearisation of Eqn 28 where $I \to \infty$ (see text). M and P are stations form the W Atlantic GEOTRACES cruises (2010–2011) (see text).

Slopes and x-intercept of these two asymptotes give us estimates for s, $[L_t]$ and the product $k[L_t]$, from which we retrieve k and hence K'. As mentioned in the section *The ligand exchange method*, the van den Berg–Ružić and Scatchard linearisation methods also provide estimates for $[L_t]$ and K', but do so at the expense of treating the ratio of two unknown variables as an independently measured quantity, without determining S. Eqn 28 shows that in reality there will be a smooth transition between these two limits (Fig. 3). Non-linear fitting allows determination of all three unknown parameters, even in cases where the two asymptotes are not strictly reached in the data.

The method employed in the two subsequent sections is an easy-to-perform non-linear regression, with either least-squares regression using the computer program *R* or any other fitting program or simple regression.[72] It calculates the standard deviations (s.d.) of the fitted parameters. It is well suited for the estimation of three or (if two ligands are assumed) five parameters out of a fairly small number of observations. Just as in Hudson et al.,[35] peak current heights I (nA), and concentrations of x (nM), i.e. total dissolved Fe present in the sub-samples (= added Fe + $[Fe_{dis}]$) are entered into the regression without any previous data handling. Programs like *Matlab*, *R*, *Systat* and *Scientist* can easily handle the fitting and supply the fitted parameters with standard deviations of the fit. In the appendix the method is given for *R* (v2.10.1, www.Rproject.org). Apart from the non-linear fit, the calculation and graphical representation of the two linearised forms, Eqn 7 and 8, are also programmed with the main reason to facilitate the detection of the presence of two ligands. Comparison with the linearised forms facilitates the detection of a possible overestimation of S due to among others the presence of nearly saturated ligands.[73]

Model for two or more ligands

In this inverse form, the determination of more than one ligand is possible as the relation in Eqn 26 can be extended. To see this, consider the iron balance, expressing total dissolved iron, x = added Fe + [Fe_{dis}], as

$$\begin{aligned} x &= [FeAL] + [Fe'] + [FeL_1] + [FeL_2] + \ldots \\ &= [Fe'](1 + D + K'_1[L'_2] + K'_2[L'_2] + \ldots) \end{aligned} \quad (33)$$

where, in the second expression, we made use of Eqn 4 and of the equilibrium expressions for individual ligands, labelled by subscripts $i = 1, 2, \ldots$

$$K'_i = \frac{[FeL_i]}{[Fe'][L'_i]} \quad (34)$$

Normalising each of $K'_i = (1 + D)k_i$, as before, allows us to split off a common factor $(1 + D)$:

$$x = (1 + D)[Fe']\left(1 + \sum k_i[L'_i]\right) = \left(\frac{I}{s}\right)\left(1 + \sum k_i[L'_i]\right) \quad (35)$$

where we used Eqn 13 and 22 to rewrite $(1 + D)[Fe'] = I/s$, and where Σ is summation over i.

We now write the [L'_i] in terms of current I. To do so, we use the equilibrium expressions in Eqn 34,

$$[L'_i] = \frac{[FeL_i]}{[Fe']K'_i} = \frac{s[FeL_i]}{Ik_i} = \frac{s}{Ik_i}([L_{ti}] - [L'_i]) \quad (36)$$

which we rewrote in the penultimate expression using Eqn 13, and where in the last expression we use ligand mass balances, $[L_{ti}] = [L'_i] + [FeL_i]$.

Solving Eqn 36 for [L'_i],

$$[L'_i] = s/(s + Ik_i)[L_{ti}] \quad (37)$$

We finally rewrite Eqn 35 as

$$x = I\left(\frac{1}{s} + \frac{k_1[L_{t1}]}{s + k_1 I} + \frac{k_2[L_{t2}]}{s + k_2 I} + \ldots\right) \quad (38)$$

adding similar terms when more ligands are present. This indicates that dissolved iron (x) may be expected to show $N+1$ linear regimes, when N different ligands are present and when stability constants and ligand concentrations differ sufficiently. However, the x *v.* I plot will never be as revealing as the Scatchard plot to study by visual inspection the heterogeneity of the ligands in titration results.

The number of titration points

The non-linear fitting has to estimate three parameters (S, K' and [L_t]) if one ligand is present. If two ligands are present, five parameters (S, K'_1 and [L_{t1}], K'_2 and [L_{t2}]) have to be estimated using the available titration points. We did a sensitivity study on the influence of the number of titration points, irrespective of the other sources of errors using GEOTRACES data from a cruise in the West Atlantic (2010–2011). These titrations contained 14 titration points measured in duplicate to give a total of 28 observations, of the following 14 Fe additions: 0, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.5, 2, 2.5, 3, 4, 6 and 8 nM Fe. We selected two samples:

1. Sample M that fitted the Langmuir model closely (station 11 bottle number 22, 49-m depth: $S = 1.361 \pm 0.017$, $\log K' = 12.22 \pm 0.11$, [L_t] = 1.28 ± 0.06 nEq of M Fe, [Fe_{dis}] = 0.103 ± 0.004 nM), containing a fairly large [L'] = 1.18 nEq of M of Fe,
2. Sample P that fitted the Langmuir model closely but contained almost saturated ligands (station 24 bottle number 16, 302-m depth: $S = 1.85 \pm 0.017$, $\log K' = 11.99 \pm 0.27$, [L_t] = 0.36 ± 0.06 nEq of M of Fe, [Fe_{dis}] = 0.269 ± 0.005 nM) with [L'] of 0.09 nEq of M of Fe.

For these samples the fitting program randomly removed Fe additions, starting with removing 1, up to 10 titration points (thus reducing the number Fe additions from 14 to 4, which measured in duplicate reduced the number of observations from 28 to 8) and applied the non-linear regression to see how the size of the titration data affects the obtained value. Subsequent analysis for S, K' and [L_t] was repeated 30 times for each number of titration points that was randomly removed (from 1–10). For some titrations after removal of a certain number of data points the model could not converge. This influences the number of estimated values being ≤30 times (Fig. 4a–d).

For data that are closely fitted by the Langmuir model, either with high or low [L'], the standard deviation of the estimated parameters [L_t] and K' is directly linked to the number of observations. The median of the estimates does not show a large change with increasing removal of titration points (Fig. 4). A difference between the mean value and the median in the sample indicates the presence of outliers. In sample P, with almost saturated ligands, the estimates of [L_t] (Fig. 4c) and to a lesser extent also of $\log K'$ (Fig. 4d) are apparently more susceptible for outliers, especially after more than three points have been removed ($n \leq 10$).

Sample P has few observations where K' can be determined. K' is determined where competition between the ALs and natural ligands occurs, thus by the data points lying in the curved part of the titration near the red dotted line in Fig. 3 (described by Eqn 30). [L_t] is determined by the position of the straight part of the titration near the blue dotted line in Fig. 3 (Eqn 32). We expect that the number of observations in those different parts (curved and straight) of the titration curve have consequences for the reliability of K' and [L_t]. We therefore estimated the effect of removal of titration points in the straight part and in the curved part of the titration. We divided the data points of each sample M and P in two groups, data points where the natural ligands were unsaturated to just saturated and data points where the natural ligands were saturated with Fe. This division was done by visual inspection and was supported by the intercept of Eqns 30 and 32 for the data of both samples. We removed titration points until $n = 2$ (Fig. 5). The results showed for both curved and straight parts that removal increased the standard deviation of the estimates. [L_t] is hardly sensitive to the number of titration points in the curved part, but is sensitive to the number of titration points in the straight part (Fig. 5c, d). The standard deviation of the estimates of K' is especially sensitive to the number of titration points for the sample with the almost saturated ligands where the number of titration points in the curved part is already small (maximum of five Fe additions) (Fig. 5a, c).

If two ligands are present the sum of both ligands has the same reliability as [L_t], indicated by the straight part. However, three parameters ([L_{t1}], K'_1 and K'_2) now have to be estimated by the number of titration points in the curved part.

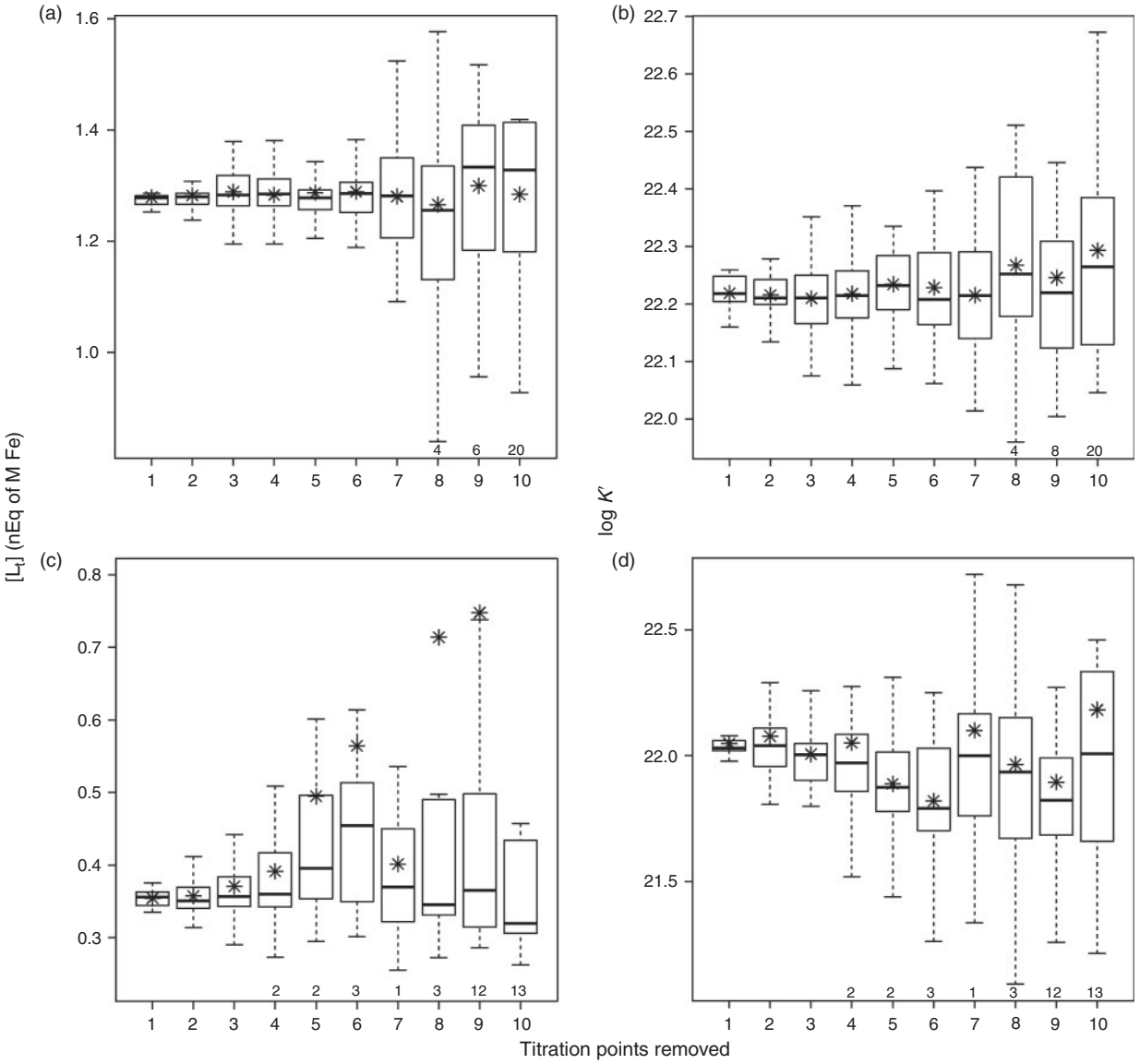

Fig. 4. Values of total ligand concentration ([L_t]) (a, c) and log conditional binding constant (log K') (b, d) for two different samples, M (a, b) and P (c, d), are shown in relation to the number of removed titration points. A titration point is equal to an Fe addition, which is equal to two observations (duplicate measurements). The total amount of titration points or Fe additions was 14. The mean of the 30 ensembles of estimates for [L_t] and log K' is shown as a black star, the median as a vertical line within the box, the box represents the interquartile range, the whiskers extend to the 5th and 95th percentile values and outliers are not shown. Above the x-axis are the number of fits that did not converge. M, with a large concentration of free ligand L ([L′]) = 1.18 nEq of M Fe, and P, with a small [L′] = 0.09 nEq of M Fe (see text).

Comparison of the old non-linear fit with the new non-linear fit determining three parameters, S, K′ and [L_t] assuming one ligand

Thuróczy et al.[43,44] produced large datasets of organic speciation of dissolved Fe over the whole water column in the Arctic (Arc) and the Atlantic sector of the Southern Ocean (ANT). These were obtained by voltammetry using TAC as an added ligand.[27] Their data were subjected to non-linear regression after estimation of S by linear regression to the last points of the titration[34] and to the new method presented here. Note that [L′] is calculated not by Eqn 2, but by repeated calculations of Eqns 4, 5 and 6, including the inorganic species here with the inorganic α-value of 10^{10}, ($[Fe_{dis}] = [Fe^{3+}](1 + 10^{10} + \beta_{FeAL}[AL] + K'[L'])$, using Newton's algorithm.[75]

The application of the non-linear fit of the solution of the Langmuir equation to obtain K', [L_t] and S results in larger S values as observed earlier for Cu.[35,36] For the Arc samples[44] the corrected S is 1.097 times higher than the uncorrected S and consequently [L_t] is 1.27 times higher, log K' 0.995 times ($n = 126$), whereas in the ANT samples[43] the corrected S is 1.017 larger, [L_t] 1.09 higher and no difference in log K' was observed ($n = 147$). This difference in the change in S between the Arc and ANT samples is related to the concentration of [L′] (Fig. 6). The S becomes larger with increasing [L′]. This can be explained by the competition between natural ligands and AL, expressed by comparing α to D. The higher [L′], the larger is the competition with the AL and thus the correction of S is larger.

The mean [L′] is 0.695 nEq of M Fe (s.d. = 0.301 $n = 147$) for ANT and 1.564 (s.d. = 0.906, $n = 126$) for Arc. The[43,44] datasets contain three different size fractions, unfiltered samples, <0.2 µm and <1000 kDa over the whole water column. The ratio of corrected S over uncorrected S (S_{cor}/S_{old}) was

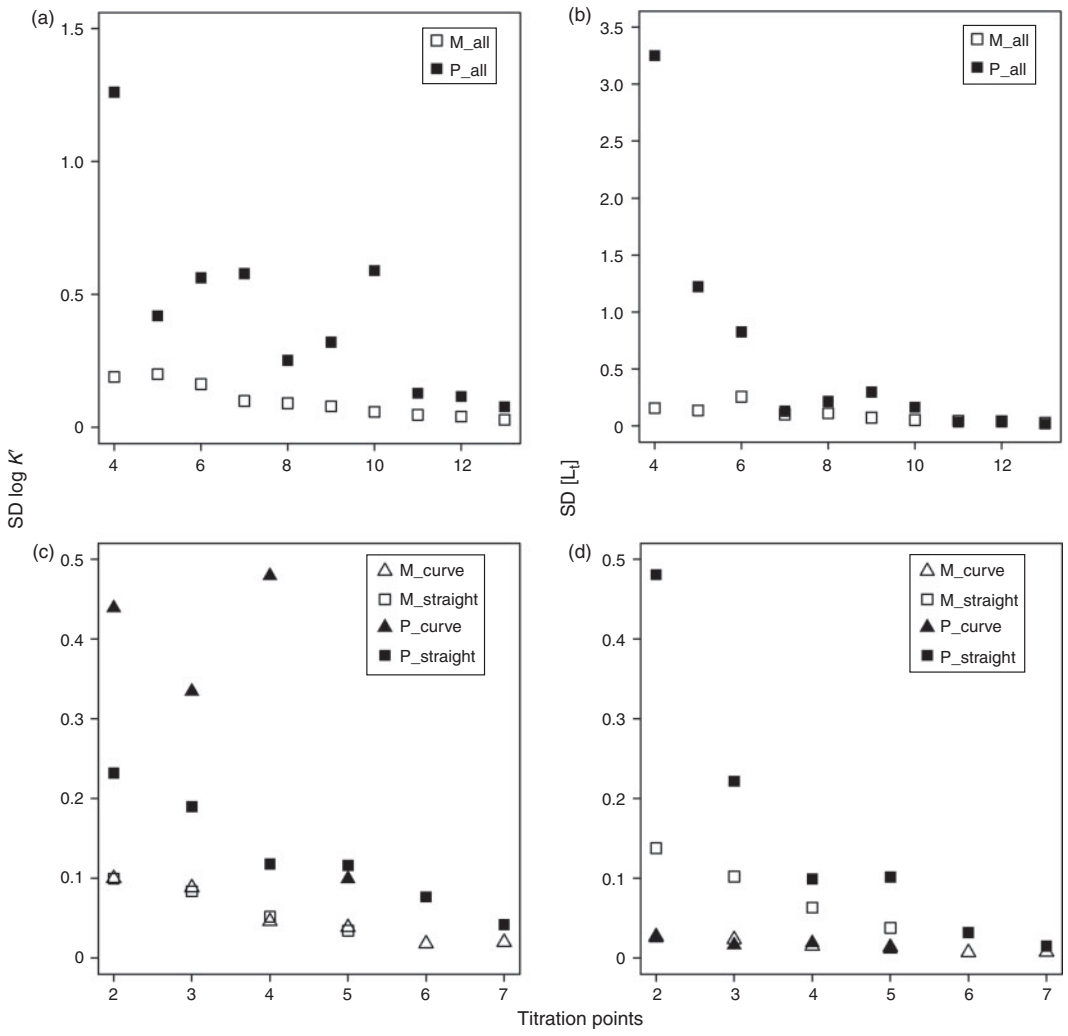

Fig. 5. The standard deviations (s.d.) of the non-linear estimates of log conditional binding constant (log K') and total ligand concentration ($[L_t]$) *v*. the number of titration points for two samples, M (with a large concentration of free ligand L ($[L']$) = 1.18 nEq of M Fe) and P (with a small $[L']$ = 0.09 nEq of M Fe). Parts (a) and (b) show the results of the standard deviations of the 30 times fitted log K' (a) and $[L_t]$ (b) with decreasing numbers of titration points for samples M (open symbol) and P (filled symbol). A titration point is equal to an Fe addition (total $n = 14$), which is equal to two observations (28 duplicate measurements). Titration points for every non-linear fit were removed at random until $n = 4$ titration points. Parts (c) and (d) show the results of the standard deviations of 30 times fitted log K' (c) and $[L_t]$ (d) per number of titration points after random removal in two separate parts of the titration curve: the curved part (triangles) and the linear part (squares). As in parts (a) and (b) the samples were M (open symbols) and P (closed symbols). Within these parts the titration points were removed until $n = 2$ titration points.

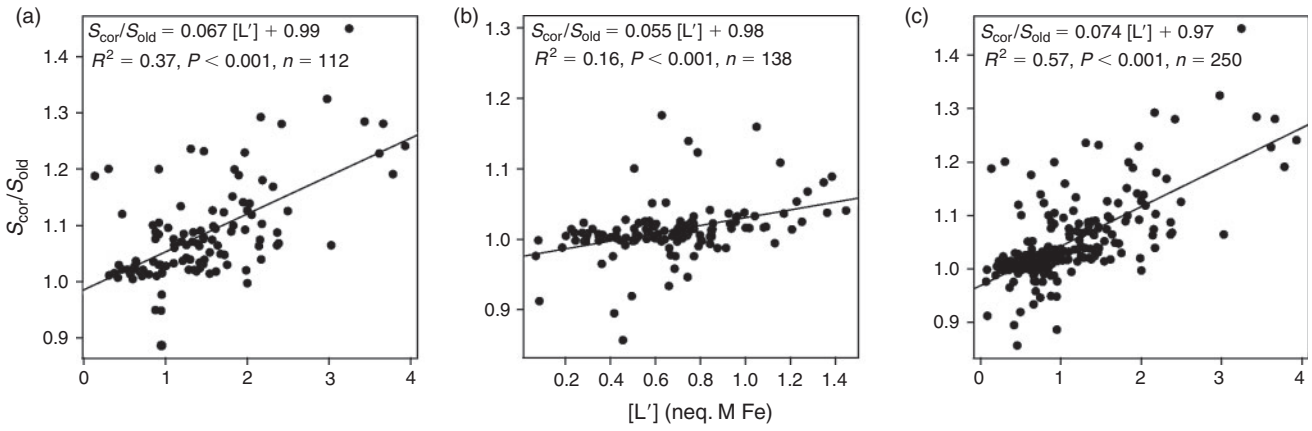

Fig. 6. The correction factor expressed as ratio of the corrected S (S_{cor}) and S obtained by the conventional method of linear regression over the last, straight part of the titration (S_{old}), *v*. the concentration of free ligand L ($[L']$). (a) Data from the Arctic Ocean,[44] from unfiltered samples, <0.2 m and <1000 kDa. (b) Data from the Atlantic section of the Southern Ocean[43] from the fractions <0.2 m and <1000 kDa. (c) Data from (a) and (b) combined.

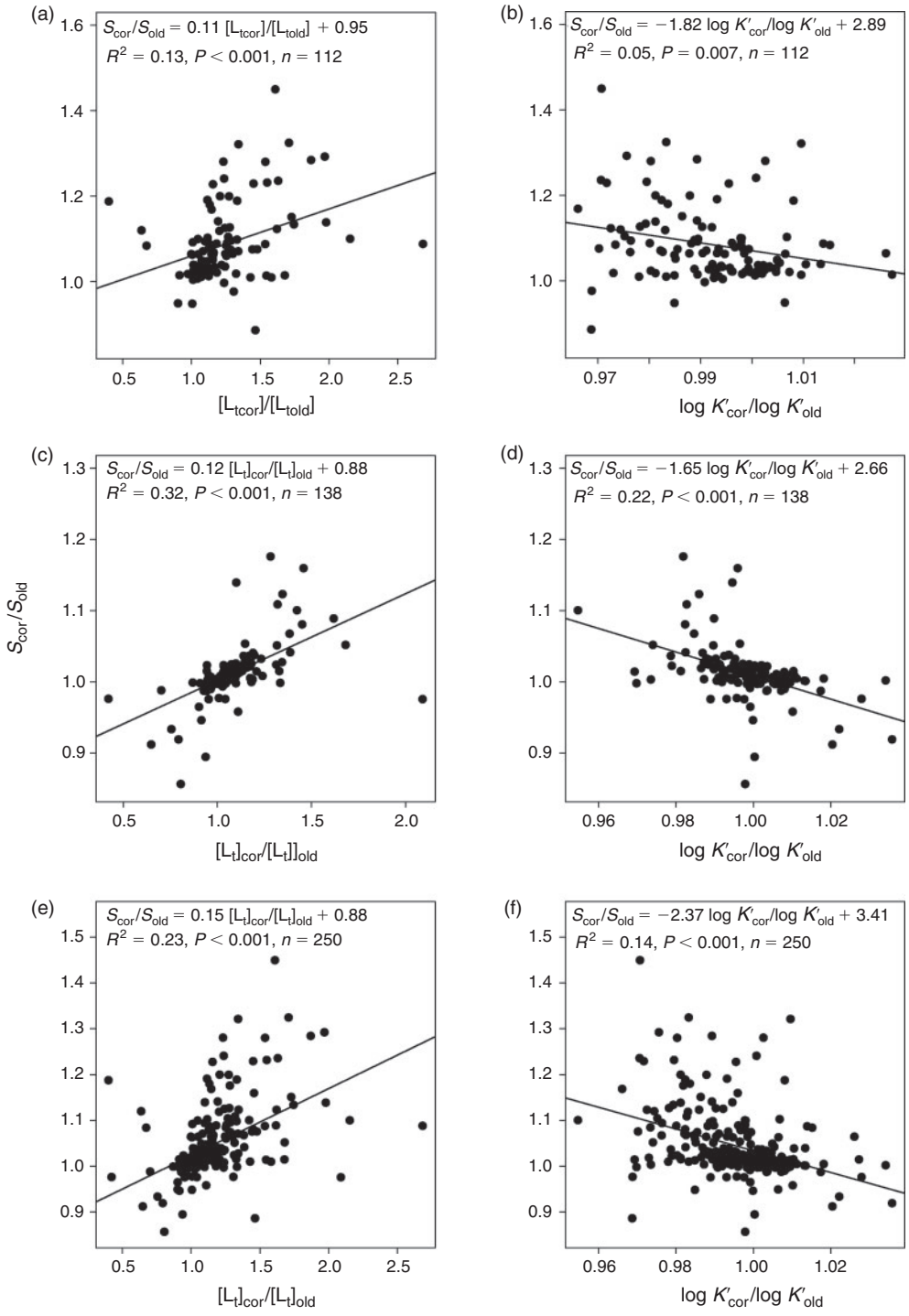

Fig. 7. The correction factor as a ratio of S_{cor}/S_{old} of the corrected S (S_{cor}) and S obtained by the conventional method of linear regression of the last straight part of the titration (S_{old}), *v*. the ratio of the results in total ligand concentration ($[L_t]$) and log conditional binding constant (log K') calculated with both S values.[43,44] (a) S_{cor}/S_{old} *v*. $[L_{tcor}]/[L_{told}]$ for the Arctic data; (b) S_{cor}/S_{old} *v*. log K'_{cor}/log K'_{old} for the Arctic data; (c) S_{cor}/S_{old} *v*. $[L_{tcor}]/[L_{told}]$ for the Antarctic data; (d) S_{cor}/S_{old} *v*. log K'_{cor}/log K'_{old} for the Antarctic data; (e) S_{cor}/S_{old} *v*. $[L_{tcor}]/[L_{told}]$ for the combined data of (a) and (c); (f) S_{cor}/S_{old} *v*. log K'_{cor}/log K'_{old} for the combined data of (b) and (d).

slightly lower for the fraction <1000 kDa compared to the other fractions. This relates directly to the smaller [L′] in this fraction. Moreover, this correction ratio was larger in the surface samples and again this is explained by the larger [L′] there.

As a consequence, changes due to the correction factor are larger in the surface and the total dissolvable fractions (as an illustration: in the surface ≤200 m of Arc the mean S_{cor}/S_{old} varies between 1.291 and 1.0771 as mean of deep samples, >200 m).

We calculated the relationship between S_{cor}/S_{old} and the change in the results for $[L_t]$ and log K' using the ratio of the values obtained with the corrected S and the conventionally obtained S (Fig. 7). We found, as can be expected, a significant

Table 1. Published data in which two ligands (L_1 and L_2) could be distinguished
After Gledhill and Buck.[40] (NN, 1-nitroso-2-napthol; SA, salicylaldoxime; TAC, 2-(2-thiazolylazo)-*p*-cresol)

Range in $\log K_1$	Range in $\log K_2$	Source and added ligand used	Characterisation L_1	Characterisation L_2	Location
12.3–13.6	11–12	Ibisanmi et al.[38] TAC	25–200-m depth	L_2 is characterised as the sum of the weaker ligands ΣL	Southern Ocean
11.8–13.9	10.7–11.8	Buck et al.[54] SA	River origin, surface	Marine origin	Columbia River estuary, San Francisco Bay
11.1–12.0	9.8–10.8	Buck and Bruland[74] SA	Regulating Fe concentrations, present in whole water column	Shelf sediments	Bering Sea
12.4–13.1	11.4–11.9	Cullen et al.[53] TAC	Only in surface in two size fractions	Whole water column in two size fractions	Atlantic Ocean
12.4–12.5	11–11.1	Nolting et al.[71] NN	At a depth of 300–800 m	Whole water column	Pacific Southern ocean
12.1–13	11.1–11.9	Rue and Bruland[76] SA	Produced by phytoplankton	Whole water column	Equatorial Pacific
12.7–13.2	11.3–11.8	Rue and Bruland[26] SA	First detection of L_1; only present in top 300 m, where $Fe < L_1$	Present whole water column	Central N Pacific

positive relation with the change in $[L_t]$ and a negative relation with the change in $\log K'$. The relationship with $\log K'$ is less significant than with $[L_t]$ and the slope is small, $\log K'$ is less sensitive to the correction.

The distinction between two ligand groups

Recent papers[35,37,38] from the last decade describe quite complicated ways to calculate the ligand characteristics of two ligands from titration data. Although we do not doubt the existence of more than one ligand group we will discuss whether a sophisticated calculation is warranted considering the combination of interferences discussed above combined with the few titration points, generally only 10. If we look at published data, the characteristics of the ligand groups that have so far been distinguished (Table 1, adapted from Gledhill and Buck[40]) hardly make a distinction into two groups possible. The data show a large overlap, which is not attributable to the different methods used (Table 1).

The distinction of more than one ligand group requires more titration points, as five parameters have to be estimated (S, K'_1, K'_2, $[L_{t1}]$ and $[L_{t2}]$). This is difficult, if not impossible, when the ligands are near saturation. The curved part in Fig. 3 is enlarged in Fig. 1c, the Scatchard plot: if two ligands exist, two linear regions with different slopes can be recognised showing the increasing saturation of two ligand groups.[45,59] To speak of two linear regions intersecting at an angle in the Scatchard plot is a simplification, because they are not linear, instead there is a relationship with two linear regions separated by a curved part where both ligand groups compete. The α values (α_i) of the two ligand groups reflect the competition for the added Fe and with decreasing $[L'_i]$, α_1 of the strong ligand becomes smaller and the weaker ligand becomes more competitive.

Similar to what Ibisanmi et al.[38] showed theoretically as a matter of illustration, a ligand concentration of 0.2 nM is difficult to estimate if additions with Fe are done in concentrations larger than 0.2 nM. Fig. 5a shows that under our experimental conditions, in order to be able to calculate the ligand characteristics of a ligand group with whatever method with a certain amount of precision (degrees of freedom), a minimum of 11 data points is necessary, below this number an unpredictable relationship exists between the number of data points and the standard deviation of $\log K'$. Fig. 5c shows that under our experimental conditions at least five points are necessary in the curved part to give a reliable relationship between the number of data points and the standard deviation of $\log K'$ and Fig. 5d shows that under our experimental conditions, below four data points in the straight part of the titration, a steep negative linear relationship exists between the number of data points and the standard deviation of the ligand concentration. This is valid for the determination of the ligand characteristics of one ligand group, therefore it is advisable to use at least 12 data points to obtain proper estimates of *S*, $[L_t]$ and $\log K'$ and a possible identification of a second ligand group. Critical simple data evaluation using the raw titration data and a Scatchard linearisation plot can detect whether the data quality is high enough. This means that if after analysis and data interpretation the strongest ligand is only determined by one data point, the sample needs to be analysed for a second time with an adapted titration scheme to improve the precision of the determination of the strongest ligand. When the ligands in a sample are saturated or almost saturated with Fe, only the total ligand concentration can be calculated because no curvature, hence no competition, is measured. The non-linear regression will indeed not be able to give a value for K' or will give a value with a large standard deviation. The linearised form of the van den Berg–Ružić algorithm will give values for K' with incorrect small standard deviations (if noise in the data is low).

In deeper water, the ligands are more saturated with Fe and this may explain why two ligand groups are predominantly found in surface waters. It might not be true that they do not exist in deep waters, but because the strong ligands are saturated there and because the weaker ligand group competes too, competition with the added ligand does not reveal its presence. In surface waters the dissolved Fe concentrations are lowest and $[L']$ is highest enabling detection of more than one ligand.

Conclusions

We devised a new and easy way to calculate ligand characteristics.

For one ligand group, the ligand exchange method using the Langmuir equation works well, although the decrease in Fe as a result of sampling and underestimations of $[L_t]$ and *K* attributable to unknown irreversible bound Fe concentrations has to be acknowledged. Competition for the same ligand by metals other than Fe might give an interference with the results that we are not aware of. The correction of *S* for incomplete saturation of the ligands during a titration is advised especially in samples with high excess L (i.e. high $[L']$). $[L']$ is high in the surface ocean,

where Fe consumption and ligand production and also (photo) oxidation and reduction occur. Correction increases $[L_t]$ and decreases K'.

As previously pointed out,[7,40,55] $[L']$ is a proper parameter to characterise the ligand concentration because it does not depend on the concentration of dissolved Fe. The concentration $[L']$ also affects the precision of the calculated ligand characteristics. If the concentration of $[L']$ is low the $[L']$ will be saturated after only a few Fe additions. The precision in the calculation of the ligand characteristics will decrease with a decreasing amount of titration steps before $[L']$ saturation. Therefore it is advisable to include information about the amount and concentrations of the Fe additions used for a titration. It is important to carefully describe when and under which conditions samples for dissolved Fe were taken, it is advisable to sample for dissolved Fe from the same sample bottle as where the sample for organic speciation is kept and to sample just before organic speciation is measured. For the calculation of two different Fe binding organic ligands a critical data evaluation is essential, and it might be necessary to repeat the analysis with a different Fe addition scheme in order to be able to calculate the parameters with better statistical precision. If a repeated analysis is not possible, added information on how the titration was executed as advised above is essential to allow better estimations with future tools and knowledge.

We can conclude that for data that follow the model of the Langmuir model the standard deviation of the estimated parameters $[L_t]$ and K' is directly linked to the number of observations. Under our experimental conditions our sensitivity study on the effect of the number of titration points on the standard deviations of the estimated parameters showed that at least four titration points are needed per ligand in the curved part and also four titration points are needed in the linear part after the ligands are saturated with the added Fe.

Acknowledgements

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Appendix 1.

Script file to calculate Metal-ligands characteristics using non-linear Gerringa–Maas, linear van den Berg–Ruzic and linear Scatchard. To use it copy paste the below code into a R script file and follow the instructions. R code lines are in bold. You activate them by Ctrl R.
Warning: check when copy–pasting this text that all symbols are correctly pasted in your R script files. Problems may arise with symbols such as: "smart quotes", =, <–, +, >, ~.
For updates contact Micha Rijkenberg (micha.rijkenberg@nioz.nl).

```
# SCRIPT
#  Script file for the calculation of metal ligand characteristics for 1 ligand: Micha J.A. Rijkenberg & Loes J.A. Gerringa
#  Some code lines can only be used in R in a Microsoft Windows environment
#  Version 22 Mar 2013 (made in R version 2.10.1)

#  The database that you use needs minimal three columns:
#  column 1 with heading "statbot" contains the sample code, e.g. st11b24
#  column 2 with heading "tMe" contains the total metal concentration for each titration point.
#  column 3 with heading "peak" contains the measured response I (nA)
#  Example of database (if you want to try, transfer these titrations in an excel sheet in the working directory you set below, remove the # in front of
#  every line and save as tab delimited txt file and call it "Example.txt".)
#statbot	tMe	peak
#st11b22	0.102	0.01973
#st11b22	0.102	0.02419
#st11b22	0.102	0.008
#st11b22	0.302	0.02407
#st11b22	0.302	0.01664
#st11b22	0.502	0.05885
#st11b22	0.502	0.06315
#st11b22	0.702	0.1993
#st11b22	0.702	0.2168
#st11b22	0.902	0.2114
#st11b22	0.902	0.268
#st11b22	1.102	0.3683
#st11b22	1.102	0.4577
#st11b22	1.302	0.5055
#st11b22	1.302	0.5267
#st11b22	1.602	0.7499
#st11b22	1.602	0.8883
#st11b22	2.102	1.315
#st11b22	2.102	1.386
#st11b22	2.602	1.799
#st11b22	2.602	1.964
#st11b22	3.102	2.721
#st11b22	3.102	2.659
#st11b22	4.102	3.734
#st11b22	4.102	4.023
#st11b22	6.102	6.534
#st11b22	6.102	6.86
#st11b22	8.102	9.25
#st11b22	8.102	9.315
#st24b16	0.269	0.339
#st24b16	0.269	0.357
#st24b16	0.269	0.377
#st24b16	0.469	0.578
#st24b16	0.469	0.647
#st24b16	0.669	0.678
#st24b16	0.669	0.714
#st24b16	0.869	1.129
#st24b16	0.869	1.085
#st24b16	1.069	1.380
#st24b16	1.069	1.536
#st24b16	1.269	1.743
#st24b16	1.269	1.660
#st24b16	1.469	2.086
#st24b16	1.469	2.164
#st24b16	1.769	2.734
```

```
#st24b16    1.769    2.746
#st24b16    2.269    3.560
#st24b16    2.269    3.561
#st24b16    2.769    4.574
#st24b16    2.769    4.646
#st24b16    3.269    5.688
#st24b16    3.269    5.557
#st24b16    4.269    7.241
#st24b16    4.269    7.321
#st24b16    6.269    10.720
#st24b16    6.269    10.960
#st24b16    8.269    14.820
#st24b16    8.269    14.570
#  You can add multiple titrations to one database. The samples will be selected via "statbot".
#  to clear the workspace (i.e. remove defined data sets from working directory etc.)
rm(list=ls())
#  to set your working directory fill in the address of your working folder here below; use "/" instead of "\" in the address!!
setwd("E:/address of working directory")
#  Libraries necessary to perform the calculations you can download them using the coding e..g.:
#  install.packages("nlme", dependencies = TRUE)
library(nlme)
library(MASS)
#  The code or the source file with the function "MeLcalc" can be found in Appendix B.
source("Library_MeLcalc.r")
#  To open your database fill in your filename, open the txt file with data "filename.txt"
ligand <- read.table("Example.txt",header=T)
names(ligand)
table(ligand$statbot)

################################################
#  tot get the initial metal values used: just activate (Ctrl R) all code lines here below until "######"
#  minMe = minimum Me concentration in titration
#  maxMe = maximum Me concentration in titration
vec <- as.vector(unique(ligand$statbot))
inM <- matrix(round(99,0),ncol=3,nrow=length(vec))
colnames(inM) <- c("statbot","minMe","maxMe")
for(i in 1:length(vec)){
                        inM[i,1] <- vec[i]
                        xM <- subset(ligand, statbot == vec[i])
                        inM[i,2] <- min(xM$tMe)
                        inM[i,3] <- max(xM$tMe)
                        }
inM <- as.data.frame(inM)
inM
################################################
#########################################################################################################################
#  Explanation of the parameters that you have to fill in here below
#  data:                the dataframe used
#  sample:              the sample used, name of the sample, e.g."st11b24"
#  Me:                     the concentration metal (Me) of the sample
#  linMe:                  the tMe concentration above which the titration curve becomes linear
#  alphaLig:               you give an alpha of a competing ligand, e.g. alphaLig = K'Fe(TAC)2*[TAC]^2,
#                          if you give a value for alphaLig than CLig will not be included in the calculation.
#                          or you indicate that you work with TAC and Fe (pH = 8.05), e.g. alphaLig = "TAC_Fe",
#                          or you indicate that you work with SA and Cu (pH = 8.05), e.g. alphaLig = "SA_Cu",
#                          or you indicate that you work with NN and Fe (pH = 8.05), e.g. alphaLig = "NN_Fe"
#                          if you use "TAC_Fe" or "SA_Cu" you have to include the concentration
#                          of the competing ligand, e.g. CLig = 10^-6
#  CLig:                the concentration of the added known ligand in moles per litre.
#  alphaMe:                the inorganic side reaction coefficient for Me'
#########################################################################################################################
```

```
# provides you with the information about the samples and the minimum and maximum Me concentrations
# the minimum Me concentration is most likely the concentration in your 0 nM Me addition sample and
# represents therefore the Me concentration of your sample that you have to fill in here below
table(ligand$statbot)
inM

# Here you have to fill in the information for the titration data that you now work with. You don't have
# to do that within the the function MeLcalc here below. Explanation for what to fill in see here above.

data = ligand
sample = "st11b22"
Me = 0.102
linMe = 2
alphaLig = "TAC_Fe"
CLig = 10^-5
alphaMe = 10^10

# Activate (Ctrl R) this function here below to calculate the ligand characteristics. It will use the data as filled in here just above.
# After activation you see the results in the R console and you can just go like this (without copying) to an excel sheet and paste (Ctrl V) the results in Excel.
# The S calculated from the non-linear Gerringa–Maas fit (S auto) is used for the van den Berg–Ruzic and Scratchard linearisation.
# If the Gerringa–Maas fit does not work it will use the slope of the linear part of the titration (as defined by linMe) as S (S manual).

MeLcalc(data=data, sample=sample, Me = Me, linMe = linMe, alphaLig = alphaLig, CLig = CLig , alphaMe = alphaMe)

#### If you have to remove datapoints from your titration curve go further here below #####
# to remove outliers
# the titration you work with is already selected just use Ctrl R to activate the coding here below
lig2 <- subset(ligand, statbot == sample);lig2

# If you want to copy_paste the data of your full titration curve to an excel sheet activate below coding line,
# go to excel file, use ctrl V to paste in excel file
write.table(lig2,"clipboard",sep="\t",col.names=T,row.names=T)

# create plot and identify outliers by moving you mouse over the points in the graph and clicking on the data that you want to remove
# select the line of code here below and activate (Ctrl R).
windows(10,5); par(mfrow=c(1,2)); plot(lig2$peak ~ lig2$tMe, las=1, pch=19); tmp <-identify(lig2$peak ~ lig2$tMe, pch=19)
# if you finish selecting datapoints use your right mouse click on the graph and select stop, otherwise you can't go further
# Don't close the graph yet, leave it
# go further here below

# remove data and create new database by just selecting the code line here below and activate (Ctrl R),
# the adapted graph will appear next to the original one
lig <- lig2[-c(tmp),]; plot(lig$peak ~ lig$tMe, las=1, pch=19)

# Analyse your titration curve again but now without the datapoints that you removed,
# just activate the below code line, no further changes necessary, use ctrl V to paste the new data in your excel sheet
MeLcalc(lig, sample=sample, Me = Me, linMe = linMe, alphaLig = alphaLig, CLig = CLig , alphaMe = alphaMe)

# If you want to save the adapted dataset of this titration activate below code line and paste in excel sheet
write.table(lig,"clipboard",sep="\t",col.names=T,row.names=T)

### END SCRIPT
```

Appendix 2.

Source code for the function "MeLcalc" to calculate Metal-ligands characteristics using non-linear Gerringa–Maas, linear van den Berg–Ruzic and linear Scatchard. To use the source code copy paste the below code into a R script file and save as "Library_MeLcalc.r" in your working directory. R code lines are in bold.

```
# START SOURCE CODE
# To calculate MeL characteristics made by Micha J.A. Rijkenberg & Loes J.A. Gerringa
# The S calculated from the non-linear Gerringa–Maas fit is used for the van den Berg–Ruzic and Scratchard linearisation.
# If the Gerringa–Maas fit does not work it will use the slope of the linear part of the titration (as defined by linMe) as S.
# The log K of the metal-ligand binding is given with respect to the metal ion.

# FUNCTION

# Explanation of the parameters that you have to fill in here below
```

```
# data:              the dataframe used
# sample:            the sample used, name of the sample, e.g."st11b24"
# Me:                the concentration metal (Me) of the sample
# linMe:             the tMe concentration above which the titration curve becomes linear
# alphaLig:            you give an alpha of a competing ligand, e.g. alphaLig = K'Fe(TAC)2*[TAC]^2,
#                      if you give a vlaue for alphaLig than CLig will not be included in the calculation.
#                      or you indicate that you work with TAC and Fe (pH = 8.05), e.g. alphaLig = "TAC_Fe",
#                      or you indicate that you work with SA and Cu (pH = 8.05), e.g. alphaLig = "SA_Cu",
#                      or you indicate that you work with NN and Fe (pH = 8.05), e.g. alphaLig = "NN_Fe"
#                      if you use "TAC_Fe" or "SA_Cu" you have to include the concentration
#                      of the competing ligand, e.g. CLig = 10^-6
# CLig:              the concentration of the added known ligand in moles per litre.
# alphaMe:             the inorganic side reaction coefficient for Me'

# For example: MeLcalc(data, sample="st11b24", Me = 0.6, linMe =2.5, alphaLig = "TAC", CLig = 10^-5, alphaMe = 10^10)

MeLcalc <- function(data, sample, Me, linMe, alphaLig, CLig, alphaMe){

#######################################
# 1) Calculation of alpha

# if a value for alpha is give this value will be used throughout the coding
alphaLig2 = alphaLig
# if "TAC_Fe" was chosen the alpha would be calculated from the logBFeTAC2 and the TAC concentration CLig
if(alphaLig == "TAC_Fe") {alphaLig2 = 10^12.4*CLig^2}
# if "SA_Cu" was chosen the alpha would be caluclated from the logBCuSA1, logBCuSA2 and the SA concentration CLig
if(alphaLig == "SA_Cu") {alphaLig2 = (10^9.57*CLig)+ (10^14.57*CLig^2)}
# if "NN_Fe" was chosen the alpha would be caluclated from the logBFeNN3 and the NN concentration CLig
if(alphaLig == "NN_Fe") {alphaLig2 = (5.12*10^16.2)*CLig^3}
# the K'Fe3+(NN)3 of 5.12 10^26 is for a pH of 7.90 and a alpha Fe' = 9.8 therefore the K'Fe'(NN)3 is 5.1210^16.2 (J. A. Hawkes, M. Gledhill,
# D. P. Connelly, E. P. Achterberg, Characterisation of iron binding ligands in seawater by reverse titration. Analytica Chimica Acta 2013, 766, 53.
# doi:10.1016/j.aca.2012.12.048)
alphaT <- alphaLig2 * alphaMe

# to terminate all warning messages
options(warn=-1)
#########################################################################################################################################
# 2) Fill in the station.bottle in which you are interested, e.g. station 11 bottle 24 is "st11b24"!

station.bottle = sample

#################################################
lig = subset(data, statbot == station.bottle)
attach(lig)

#########################################################################################################################################
# 3) Determine S for van den Berg/Ruzic and Scatchard methods

# concentration tMe (nM) above which linear part of the curve exists, you can change this value according to the individual samples and
# run the graph part here below to see the effect of the change

above.tMe = linMe

# Graph
sublig = subset(lig, tMe > above.tMe)
x11(12,10)
layout(matrix(c(1,2,3,4), 2, 2, byrow = TRUE), heights=c(1,1))
par(mar=c(5,8.5,4,2)+0.1) #c(bottom, left, top, right)
mx <- max(lig$tMe)+1
plot(lig$peak ~ lig$tMe, las=1,xlab="total Me (nM)", ylab = "I (nA)",pch=16, col="red",main=station.bottle,xlim=c(0,mx))
points(sublig$peak ~ sublig$tMe, pch=16, col="blue")
abline(lm(sublig$peak ~ sublig$tMe))
sens=lm(sublig$peak ~ sublig$tMe)
mtext(paste("R^2 =",round(summary(sens)[9][[1]],4)), side=3, lin=-1.5, adj=0.05, cex=0.7)
mtext(paste("S manual =",round(summary(sens)[4][[1]][[2]],4)), side=3, lin=-3, adj=0.05, cex=0.7)
legend("bottomright", legend = c("non linear data","defined linear data"), col=c("red","blue"),pch=c(16,16),cex=0.8, bty="n")

# S to be used for van den Berg–Ruzic and Scatchard method when the non-linear fit fails
# takes automatically the S as determined by linear regression here above

Sbrs = round(summary(sens)[4][[1]][[2]],4)
```

```
################################################
#  Method: Nonlinear Gerringa_Maas
## Nonlinear fit 1 ligand, S, K, L automatically estimated
#  create progress bar, a vector with initial values for S, K and L
vec <- seq(from=0.5, to=15, by=2)
pb <- winProgressBar(title = "progress bar", min = 0, max = length(vec), width = 300)
Sys.sleep(0.5)
#  sets the stringency of the gnls non linear fit
gnlsControl(maxIter=100, nlsMaxIter=100, msMaxIter=100)
for(i in 1:length(vec)){
                        S=vec[i]
                        # Progress bar
                        Sys.sleep(0.1)
                        setWinProgressBar(pb, i, title=paste( round(i/length(vec)*100, 0),
                          "% of vector for K,L and S explored for 1 ligand"))
    for(j in 1:length(vec)){
                            K=vec[j]
                        for(k in 1:length(vec)){
                                    L=vec[k]
model = try(gnls(peak ~(2*S*tMe)/((1+K*(L-tMe))+sqrt(((1+K*(tMe-L))^2)+4*K*L)), data=lig, start = list(S = S,K = K,L = L)),
silent=TRUE)
#  if the model ends up in an error it goes to the next round of S, K and L in the given vector
if(summary(model)[2][[1]] == "try-error") next
#  terminates the loop
if(summary(model)[4][[1]][[2]] > 0) break
}
if(summary(model)[2][[1]] == "try-error") next
if(summary(model)[4][[1]][[2]] > 0) break
}
if(summary(model)[2][[1]] == "try-error") next
if(summary(model)[4][[1]][[2]] > 0) break
}
Sys.sleep(1)
close(pb)    # to close the progress bar
ModelGood <- 0
if(summary(model)[2][[1]] == "try-error"){ Sbrsaut <- Sbrs} else {
ModelGood <- 1
#  calculation log K,
#10^21.4 = 10^12.4 * 10^9, 10^9 is from nmol to mol, 10^12.4 is alpha TAC = 10^22.4 * (10^-5)^2
Knl = summary(model)[4][[1]][[2]]*10^9*alphaT
logKnl = log10(Knl)
#  calculation logK.SE, the standard error of log K,: logK.SE = ((log10(K)-log10(K-SE))+(log10(K+SE)-log10(K)))/2
logKal = log10(summary(model)[4][[1]][[2]])
logKmse = log10(summary(model)[4][[1]][[2]]-summary(model)[20][[1]][[5]])
logKpse = log10(summary(model)[4][[1]][[2]]+summary(model)[20][[1]][[5]])
logK.SE = ((logKal - logKmse)+(logKpse-logKal))/2
#Sbrsaut = summary(model)[4][[1]][[1]]*((1+(alphaLig2+alphaMe))/(alphaLig2+alphaMe))
Sbrsaut = summary(model)[4][[1]][[1]]
mtext(paste("S auto =", round(summary(model)[4][[1]][[1]],4), sep= ""), side=3, lin=-4.5, adj=0.05, cex=0.7)
}
```

```
################################################
#  Method: van den Berg–Ruzic
#  [Fe3+]/[FeL] = [Fe3+]/[Lt] + 1/[Lt]*KFeL

Me3pplus = lig$peak/(Sbrsaut*alphaT)
MelabL = Me3pplus/(tMe-(lig$peak/Sbrsaut))

#  Linear regression
bergruzic = lm(MelabL ~ Me3pplus)
summary(bergruzic)

#  Calculation total L (FeL+L-)
totalL = 1/summary(bergruzic)[4][[1]][[2]]

#  Calculation log K
logKMeL = log10(summary(bergruzic)[4][[1]][[2]]*10^9/summary(bergruzic)[4][[1]][[1]])

################################################
#  Method: Scatchard
#  FeL/Fe3+ = - K*FeL + K*Lt

Me3plus = (lig$peak/(Sbrsaut*alphaT)*10^-9)
MeL = (tMe - (lig$peak/Sbrsaut))*10^-9
MeLlab = MeL/Me3plus

#  Linear regression
scatchard = lm(MeLlab ~ MeL)
summary(scatchard)

#  calculation total L
#  x-intercept = [Lt]
stotalL = (-1*summary(scatchard)[4][[1]][[1]])/summary(scatchard)[4][[1]][[2]]

#  calculation log KMeL
#  y-intercept = KMeL * [Lt]
slogKMeL = log10(summary(scatchard)[4][[1]][[1]]/stotalL)

################################################
#  Graphs

plot(lig$peak ~ lig$tMe, las=1, xlab="total Me (nM)", ylab = "I (nA)", pch=16, col="red", main=station.bottle,xlim=c(0,mx))
mtext("Method: Nonlinear Gerringa–Maas", side=3, lin=0.5, adj=0.0, cex=0.7)
if(ModelGood == 0) {
mtext("Model did not work!", side=3, lin=-1.5, adj=0.5, cex=1, col="red")
mtext("S manual was used for", side=3, lin=-3, adj=0.5, cex=0.8, col="red")
mtext("Berg–Ruzic & Scatchard!", side=3, lin=-4, adj=0.5, cex=0.8, col="red")

}
if(ModelGood == 1) {
MyData1 <- data.frame(tMe=seq(from=min(lig$tMe),to=max(lig$tMe),length=10))
P1 <- predict(model, newdata = MyData1)
lines(MyData1$tMe, P1, lty=1,col=1)
}

plot(Me3pplus, MelabL, las=1, xlab=expression(Me^"x+"~(M)), ylab = "" , pch=16, col="red", main=station.bottle)
title(ylab=expression(Me^"x+"~ "/ "~MeL), line=5.5)
mtext("Method: van den Berg–Ruzic", side=3, lin=0.5, adj=0.0, cex=0.7)
mtext(paste("R^2 =",round(summary(bergruzic)[9][[1]],4)), side=3, lin=-1, adj=0.05, cex=0.7)
if(summary(bergruzic)[4][[1]][[2]] > 0){
mtext(paste("y =", round(summary(bergruzic)[4][[1]][[2]],2),"x +",round(summary(bergruzic)[4][[1]][[2]],1), sep=" "), side=3,
lin=-2, adj=0.05, cex =0.7, col="black")
} else {
mtext(paste("y =", round(summary(bergruzic)[4][[1]][[2]],2),"x -",-1*round(summary(bergruzic)[4][[1]][[2]],1), sep=" "), side=3,
lin=-2, adj=0.05, cex =0.7, col="black")
}
abline(bergruzic)
```

```
plot(MeL, MeLlab, las=1, xlab="MeL (M)", ylab = "", pch=16, col="red", main=station.bottle)
mtext("Method: Scatchard", side=3, lin=0.5, adj=0.0, cex=0.7)
mtext(paste("R^2 =",round(summary(scatchard)[9][[1]],4)), side=3, lin=-1, adj=0.05, cex=0.7)
title(ylab=expression(MeL~"/"~Me^"x+"), line=5.5)
abline(scatchard)

#####################################################
# Table

results <- matrix(99,ncol=13,nrow=3)
results = as.data.frame(results, row.names=c("Gerringa_Maas", "vandenBerg_Ruzic","Scatchard") )
colnames(results) <- paste(c("station.bottle","S auto","S.SE","S.p", "logK", "logK.SE","LogK.p",
                             "totalL","totalL.SE","totalL.p", "non Fe bound.L", "Me", "S manual"))
results[1,1] <- station.bottle
if(ModelGood == 1) {
results[1,2] <- Sbrsaut
results[1,3] <- summary(model)[20][[1]][[4]]
results[1,4] <- summary(model)[20][[1]][[10]]
results[1,5] <- logKnl
results[1,6] <- logK.SE
results[1,7] <- summary(model)[20][[1]][[11]]
results[1,8] <- summary(model)[4][[1]][[3]]
results[1,9] <- summary(model)[20][[1]][[6]]
results[1,10] <- summary(model)[20][[1]][[12]]
results[1,11] <- summary(model)[4][[1]][[3]]-Me
results[1,12] <- Me
results[1,13] <- NA
} else {
results[1,2] <- NA
results[1,3] <- NA
results[1,4] <- NA
results[1,5] <- NA
results[1,6] <- NA
results[1,7] <- NA
results[1,8] <- NA
results[1,9] <- NA
results[1,10] <- NA
results[1,11] <- NA
results[1,12] <- NA
results[1,13] <- NA
}

results[2,1] <- station.bottle
results[2,2] <- Sbrsaut
results[2,3] <- NA
results[2,4] <- NA
results[2,5] <- logKMeL
results[2,6] <- NA
results[2,7] <- NA
results[2,8] <- totalL
results[2,9] <- NA
results[2,10] <- NA
results[2,11] <- totalL-Me
results[2,12] <- Me
results[2,13] <- Sbrs

results[3,1] <- station.bottle
results[3,2] <- Sbrsaut
results[3,3] <- NA
results[3,4] <- NA
results[3,5] <- slogKMeL
results[3,6] <- NA
results[3,7] <- NA
```

```
results[3,8] <- stotalL*10^9
results[3,9] <- NA
results[3,10] <- NA
results[3,11] <- (stotalL*10^9)-Me
results[3,12] <- Me
results[3,13] <- Sbrs

write.table(results,“clipboard”,sep=“\t”,col.names=T,row.names=T)

results
}

#  END SOURCE CODE
```