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Environ. Sci. Technol., **Just Accepted Manuscript** • Publication Date (Web): 27 Jun 2013Downloaded from <http://pubs.acs.org> on June 27, 2013**Just Accepted**

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1 Pb(II) binding to humic substances: an equilibrium and
2 spectroscopic study

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8 BRIEF: Pb(II) is proposed to bind to humic substances in two ways: a relatively strong multiligand
9 complex leading to humic aggregation, and a weaker 1:1 complex.

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15

16 ABSTRACT

17 The binding of Pb(II) to humic acids is studied through an approach combining equilibrium and
18 spectroscopic measurements. The methods employed are potentiometric and fluorometric titrations,
19 fluorescence excitation-emission matrices (EEM) and IR spectroscopy. Potentiometric titration curves
20 are analyzed using the NICA equations and an electrostatic model treating the humic particles as an
21 elastic polyelectrolyte network. EEMs are analyzed using parallel factor analysis, decomposing the
22 signal in its independent components and finding their dependence on Pb(II) activity. Potentiometric
23 results are consistent with bimodal affinity distributions for Pb(II) binding, whereas fluorometric
24 titrations are explained by monomodal distributions. EEM analysis is consistent with three independent
25 components in the humic fluorescence response, which are assigned to moieties with different degree of
26 aromaticity. All three components show a similar quenching behavior upon Pb(II) binding, saturating at
27 relatively low Pb(II) concentrations. This is attributed to metal ion induced aggregation of humic
28 molecules, resulting in the interaction between the aromatic groups responsible for fluorescence; this is
29 also consistent with IR spectroscopy results. The observed behavior is interpreted considering that initial
30 metal binding (observed as strongly binding sites), correspond to bi- or multidentate complexation to
31 carboxylate groups, including binding between groups of different humic molecules, promoting
32 aggregation; further metal ions (observed as weakly binding sites) bind to single ligand groups.

33 KEYWORDS Potentiometric titration; fluorescence spectroscopy; IR spectroscopy; metal-humic
34 binding; affinity spectra.

35

36 **Introduction**

37 Humic substances (HS) are important components of natural organic matter (NOM) in groundwater
38 and soils^{1,2} HS interact with other chemical species in the natural environment, in particular binding
39 heavy metal cations.³⁻⁵ Among different experimental studies, equilibrium binding data, usually titration
40 curves, have been reported and modeled.^{3,6-8} Recently, the electrostatic behavior of HS was treated
41 through the elastic polyelectrolyte network model (EPN)⁹ which allows for expansion and shrinking as
42 the HS charge changes; it can be combined with the NICA¹⁰ intrinsic isotherm, resulting in the NICA-
43 EPN model which has been applied to proton binding to HS.⁹ Fluorescence spectroscopy is an useful
44 tool in the study of metal binding to HS.^{4,11} In the last years, excitation-emission matrix (EEM)
45 fluorescence spectroscopy with parallel factor analysis (PARAFAC) was successfully applied for the
46 identification and characterization of NOM.¹²⁻¹⁶ For simple mixtures, PARAFAC components are
47 identified with individual fluorophores present; for HS, the identified components represent
48 fluorescence phenomena, which may be individual fluorophores or approximations to the effects of local
49 processes such as intramolecular charge transfer.

50 Even after many research efforts, the exact nature of HS and the way in which they interact with metal
51 cations remains unclear. With the aim of gaining insight in this problem, the binding of Pb(II) to humic
52 acids (HA) is studied here by a combined analysis of titration and spectroscopic experiments.
53 Equilibrium curves are obtained through potentiometric titrations and analyzed with the NICA-EPN
54 model for metal binding to obtain intrinsic binding parameters; also, fluorometric titrations are
55 performed to compare the binding affinities. EEM measurements¹⁷ with PARAFAC analysis^{13,18} are
56 conducted to study the behavior of the different fluorophores with Pb(II) additions. Finally, IR-ATR
57 spectra are recorded to study the Pb-HA interactions.

58 59 **Modeling of proton and metal binding data: the NICA-EPN model**

60 Titration data was analyzed using the NICA isotherm for the intrinsic affinity⁷ and the Elastic
61 Polyelectrolyte Network (EPN) submodel⁹ for the electrostatic contribution. In the NICA-EPN model,

62 the amount bound of species i per unity of mass in the presence of species j is given by

$$63 \quad Q_i = g_f \sum_{k=1}^M \frac{n_{i,k}}{n_{1,k}} Q_{\max,k} \mathcal{G}_{i,k}(a_1^{\text{int}}, \dots, a_j^{\text{int}}, \dots, a_n^{\text{int}}) + (1 - g_f) \sum_{k=1}^M \frac{n_{i,k}}{n_{1,k}} Q_{\max,k} \mathcal{G}_{i,k}(a_1, \dots, a_j, \dots, a_n) + Q_{i,el} \quad (1)$$

64 where $\mathcal{G}_{i,k}(a_i)$ is the fraction of type k sites occupied by species i , given in the NICA isotherm by

$$65 \quad \mathcal{G}_{i,k}(a_1, \dots, a_j, \dots, a_n) = \frac{(\bar{K}_{i,k} a_i)^{n_{i,k}}}{\sum_{j=1}^n (\bar{K}_{j,k} a_j)^{n_{j,k}}} \times \frac{\left[\sum_{j=1}^N (\bar{K}_{j,k} a_j)^{n_{j,k}} \right]^{p_k}}{1 + \left[\sum_{j=1}^N (\bar{K}_{j,k} a_j)^{n_{j,k}} \right]^{p_k}} \quad (2)$$

66 In eqs 1 and 2, N is the number of species binding to the HA, M the number of sites, Q_{\max} the total
 67 number of sites per unity of mass, $Q_{\max,k}$ the same for type k sites (assuming 1:1 binding), q_k the fraction
 68 of type k sites ($Q_{\max,k} = q_k Q_{\max}$), $n_{i,k}$ the nonideality parameter for species i on type k sites, ($n_{1,k}$ being for
 69 H^+), p_k is the heterogeneity parameter of type k sites and $\bar{K}_{i,k}$ is the average binding constant of species i
 70 on type k sites. Two site types are considered: low affinity ($k = 1$, attributed to carboxylic groups) and
 71 high affinity ($k = 2$, usually attributed to phenolic groups),^{7,19} so $M = 2$, and for binding of a metal cation
 72 in the presence of protons $N = 2$.

73 As it is widely accepted, HS are actually composed of relatively small molecules and molecular
 74 fragments (as opposed to a macromolecular/polymeric view) which are entangled and/or bound by
 75 diverse forces, like hydrogen bonding, metal ion bridging, van der Waals interactions, etc. forming
 76 particles of supramolecular nature which can shrink and swell.^{20,21} In the EPN model,⁹ these HS
 77 particles are treated, from a purely mechano-statistical point of view, as an elastic network which can
 78 exchange ions and solvent with the surrounding solution; further, the particles are considered as divided
 79 in two fractions: an inner fraction g_f , which behaves as a gel in Donnan equilibrium with the bulk
 80 solution, and an external fraction $1 - g_f$. The humic binding sites outside the gel are in equilibrium with
 81 the bulk solution, whereas those inside the gel are in equilibrium at the internal ionic activity, a_j^{int} ,
 82 which is given by

$$83 \quad a_j^{\text{int}} = a_j \exp(-F\psi_D / RT) \quad (3)$$

84 where ψ_D is the Donnan potential, a_j is the activity in the bulk and F , R and T have their usual meaning.

85 For a 1:1 electrolyte, ψ_D results to be, from the electroneutrality condition,⁹

$$86 \quad \psi_D = \frac{RT}{F} \operatorname{arcsinh} \left(\frac{g_f Q \delta \phi_2}{2I(1-\phi_2)} \right) \quad (4)$$

87 where Q is the net charge of the humic particle, δ the density of the dry humic substance (following

88 Dinar²² $\delta = 1.5 \text{ g cm}^{-3}$), I the ionic strength, and ϕ_2 is the volume fraction of the HS in the gel, obtained

89 from the swelling equilibrium condition:⁹

$$90 \quad \frac{1}{v_1} [\chi \phi_2^2 + \phi_2 + \ln(1-\phi_2)] + \frac{1}{v_2} \left(\phi_2^{1/3} - \frac{2\phi_2}{f_P} \right) + 2I \left[1 - \sqrt{1 + \left(\frac{g_f Q \delta \phi_2}{2I(1-\phi_2)} \right)^2} \right] = 0 \quad (5)$$

91 where v_1 is the water molar volume, v_2 is the average molar volume of a humic equivalent chain, χ is a

92 Van Laar heat of mixing parameter and f_P is the Flory functionality,²³ which was set equal to 3. $Q_{i,el}$ is

93 the excess amount of species i electrostatically bound in the gel phase:

$$94 \quad Q_{i,el} = (c_i^{\text{int}} - c_i) \frac{g_f}{\delta \phi_2} \quad (6)$$

95 where c_i^{int} and c_i are the concentrations of species i in the gel and the bulk solution, respectively. The

96 Davies equation was employed to calculate activity coefficients throughout; it was verified that the ionic

97 strength inside the gel was always within its validity limit ($I < 0.3 \text{ M}$).

98 As it is well known,⁷ eq 2 reduces to a Langmuir-Freundlich isotherm for non competitive (H^+) binding:

$$99 \quad \vartheta_{H,k}(a_H) = \frac{(\bar{K}_{H,k} a_H)^{m_{H,k}}}{1 + (\bar{K}_{H,k} a_H)^{m_{H,k}}} \quad (7)$$

100 where $m_{H,k} = n_{H,k} p_k$.

101 Also, conditional affinity distributions can be obtained using constrained regularization methods.²⁴ If the

102 proton affinity spectrum $g(\log K_H)$ is known, a conditional metal affinity distribution $f(\log K_M)$ can be

103 estimated solving numerically the equation (assuming uncorrelated proton and metal affinity spectra):²⁴

$$Q_M = Q_{\max} \int_{-\infty}^{\infty} \left[\int_{-\infty}^{\infty} \frac{K_M a_M}{1 + K_H a_H + K_M a_M} g(\log K_H) d \log K_H \right] f(\log K_M) d \log K_M \quad (8)$$

105 where Q_M is the amount of metal bound.

106

107 **Materials and methods**

108 The HAs employed were a purified Fluka humic acid (FHA) and IHSS standard Elliot soil humic acid
109 (EHA); these HS have been characterized in the literature, as presented in Table S1, Supporting
110 Information. Acid-base potentiometric titrations were performed following usual procedures²⁵ at 1 g L⁻¹
111 HA concentration and different ionic strengths in NaClO₄. Pb(II)-humic potentiometric titrations also
112 followed known procedures¹⁰ at pHs of 4.0 and 5.5, and ionic strengths (NaClO₄) of 0.1 M and 0.02 M.
113 Fluorometric titrations were performed, at a HA concentration of 20 mg L⁻¹. In order to enable
114 comparisons, binding curves for this concentration were calculated using the fitted model parameters;
115 some potentiometric measurements were also done, but no processed due to the low reliability of ISE
116 measurements at low HA concentrations;²⁶ however, they reflected the same general tendency.
117 Fluorescence EEMs of 20 mg L⁻¹ solutions of HA alone and with increasing amounts of Pb(II) were
118 recorded. UV-Vis spectra were also recorded to check the onset of humic flocculation. IR-ATR
119 spectroscopy measurements of HA-Pb complexes were performed following usual techniques,^{27,28} using
120 the electrolyte spectrum as reference to obtain the difference spectra. Experimental details are given in
121 Appendix A, Supporting Information.

122 The data treatment was, briefly, as follows. First from acid-base titration data the humic charge as a
123 function of pH was computed and fitted to the NICA-EPN model, using a constrained Levenberg-
124 Marquardt algorithm,²⁹ obtaining the corresponding NICA parameters as well as the EPN ones, namely
125 χ , g_f and ν_2 . Then, curves of Pb(II) binding as a function of Pb(II) activity were obtained from titration
126 data, and also fitted to the NICA-EPN model, taking the proton binding and EPN parameters from the
127 proton experiments.

128 Fluorometric titration data was converted to relative fluorescence quenching, θ_F , defined as:

$$\theta_F = \frac{I_F^0 - I_F}{I_F^0 - I_F^\infty} \quad (9)$$

129 Here, I_F^0 is the fluorescence emission intensity in the absence of Pb(II), I_F^∞ is the intensity observed at
130 maximum quenching and I_F is the intensity at each point. This intensity values can be taken as the
131 intensity at the wavelength of maximum emission or as the integrated emission spectra. Both ways led to
132 the same results. Fluorescence EEMs were converted to Raman units following Stedmon et al.,¹⁸
133 processed with PARAFAC,^{12,13} yielding the number of independent fluorescent phenomena, their
134 absorption and emission spectra, and their relative concentrations (scores) in each sample. The data
135 treatment procedures are detailed in Appendix B, Supporting information.

137

138 **Results and discussion**

139

140 **Potentiometric titrations.** Acid-base titration curve fitting to the NICA-EPN model shows very good
141 agreement (Figure S1, Supporting Information). The intrinsic NICA parameters for proton binding are
142 collected in Table 1, falling in the ranges reported in the literature;^{19,30} also included are the total sites
143 (Q_{\max}) and the electrostatic parameters g_f , χ and v_2 . The proton binding constants found for low affinity
144 sites are close to typical values for isolated carboxylic groups. For the high affinity type sites our results
145 are close to the pK_a for phenol (9.8); it has been reported that in HS most phenolic OH groups are
146 isolated^{31,32} which is consistent with these results.

147 The electrostatic parameters g_f and χ are similar to those reported previously;⁹ the first one represents
148 the part of the humic particle where the electrostatic potential is on average equal to ψ_D , being about half
149 of the binding sites, whereas a positive value for χ corresponds to a repulsive heat of mixing humic-
150 water, which is expected because HS have a significant fraction of non polar groups. There are
151 significant differences in v_2 , quite low for EHA and high for FHA; this parameter is the molar volume of
152 the equivalent chain segments in a network representing the humic particle, thus the results found would
153 indicate that FHA is relatively less entangled whereas EHA would be highly entangled.

154

155 **Table 1.** NICA-EPN model fitting parameters

| Proton binding | log $\bar{K}_{H,1}$ | log $\bar{K}_{H,2}$ | $Q_{\max}/$ mol kg ⁻¹ | q_1 | q_2 | $m_{H,1}$ | $m_{H,2}$ | g_f | χ | $v_2/$ L mol ⁻¹ |
|----------------|-------------------------|-------------------------|-------------------------------------|------------|-------|-----------|-----------|---------|---------|-------------------------------|
| Elliot HA | 4.42 | 9.43 | 5.0 | 0.52 | 0.48 | 0.51 | 0.20 | 0.48 | 0.47 | 1.7 |
| Fluka HA | 4.44 | 9.91 | 5.2 | 0.56 | 0.44 | 0.39 | 0.23 | 0.47 | 0.51 | 10.2 |
| Pb(II) binding | log $\bar{K}_{Pb,1}$ | log $\bar{K}_{Pb,2}$ | $n_{Pb,1}$ | $n_{Pb,2}$ | p_1 | p_2 | | N_d^a | R^2^b | RMSE ^c |
| Elliot HA | 0.55 | 9.8 | 0.80 | 0.49 | 0.70 | 0.40 | | 228 | 0.9990 | 0.058 |
| Fluka HA | 0.50 | 5.8 | 0.90 | 0.52 | 0.49 | 0.89 | | 209 | 0.9993 | 0.039 |

156 ^a Total data points. ^b $R^2 = 1 - \frac{\sum (Q_i - Q_{i,calc})^2}{\left[\sum Q_i^2 - (\sum Q_i)^2 / N_d \right]}$. ^c $RMSE = \sqrt{\sum (Q_i - Q_{i,calc})^2 / N_d}$.

157

158 Figure 1 shows the results for the HA-Pb titrations with good agreement; the generic NICA-Donnan
 159 (ND)¹⁹ predicted curves being also shown for comparison. The pH dependence is similar to that reported
 160 by Milne et al.³ and references therein. The influence of I has been much less studied; here it is observed
 161 that the curves at different ionic strengths merge as Pb(II) activity increases; some literature studies
 162 show similar behavior: Cu binding to Suwannee River fulvic acid,³³ Am complexation with Gohy-573
 163 humic acid,³⁴ and Cu complexation with Sable silt loam HA³⁵. The NICA-EPN model predicts well this
 164 dependence, whereas the generic ND curves are nearly parallel. Table 1 collects the Pb intrinsic binding
 165 parameters and the fitting quality results for the full data set fitting (proton and metal curves).

166 Figure 2 shows the speciation of bound Pb(II) at pH = 5.5 and $I = 0.1$ M (other cases are similar),
 167 writing eq 1 in the form:

168
$$Q_{Pb} = Q_{Pb,1}^{int}(a_H^{int}, a_{Pb}^{int}) + Q_{Pb,2}^{int}(a_H^{int}, a_{Pb}^{int}) + Q_{Pb,1}^{ext}(a_H, a_{Pb}) + Q_{Pb,2}^{ext}(a_H, a_{Pb}) + Q_{Pb,el} \quad (10)$$

169 where $Q_{\text{Pb},k}^{\text{int}} = g_f (n_{\text{Pb},k} / n_{\text{H},k}) Q_{\text{max},k} g_{\text{Pb},k} (a_{\text{Pb}}^{\text{int}}, a_{\text{H}}^{\text{int}})$ and so on. As it is observed, most Pb is bound to high
170 affinity sites in the activity range studied, specially for EHA. From $a_{\text{Pb}} \approx 10^{-4}$ up the binding to low
171 affinity sites shows an increase. The electrostatic binding in the gel fraction (eq 6) is negligible in all
172 cases; note also that for FHA this contribution changes sign; this is due to inversion of the gel fraction
173 charge caused by the Pb loading.

174 There are several published studies of Pb binding to HS;^{3,36-42} however, direct comparisons are
175 difficult because the different HS and conditions employed. The values of $\bar{K}_{\text{Pb},k}$ found here (with the
176 exception of high affinity sites for EHA) are within the ranges reported by Milne et al.,³ Gustafsson,^{8,40}
177 and Tipping.⁶ Puy et al.³⁷ reported somewhat higher values of $\log \bar{K}_{\text{Pb},1}$ (4.37) and $\log \bar{K}_{\text{Pb},2}$ (7.57). The
178 high affinity constant for EHA found here is higher than the values reported by all these authors; in
179 some studies, Pb(II) binding to soil organic matter was found to be considerably higher than predicted
180 by current models, free Pb being about one order of magnitude lower than predicted (see Gustafsson et
181 al.⁴⁰ and references therein). Thus, being EHA a soil HA, whereas FHA is originated from coal, the
182 difference in their $\bar{K}_{\text{Pb},2}$ values is consistent with the findings reported by Gustafsson et al.⁴⁰

183

184 **Fluorometric titrations.** During titration of HA with Pb(II), fluorescent emission is quenched as
185 Pb(II) concentration increases, until a limiting emission is reached, which does not change as Pb(II)
186 concentration is further increased (Figure S2, Supporting Information). In all cases, the lowest emission
187 intensity observed was taken as I_F^∞ in eq 9 to compute θ_F . This quenching has been observed by a
188 number of researchers and for several cations.^{11,43} Along the titrations, the absorbance spectra of the
189 HA-Pb solutions were checked. It was found to remain unchanged until the appearance of flocculation
190 and/or precipitation, were the absorbance started to decrease (Figure S3, Supporting Information); only
191 fluorescence measurements before this point were considered in the analysis, thus in all the results
192 presented here the UV spectra remained constant. Figure 3 compares the results for fluorometric
193 titrations with Pb binding curves calculated with the NICA-EPN model for EHA and FHA. The general

194 features are coincident with previous studies.^{3,44,45} It is observed that the results are qualitatively similar
195 but not quantitatively coincident, showing the fluorometric response a tendency to saturation at $pPb \sim 5$,
196 which is not observed for the potentiometric curves. This behavior was also observed in preliminary
197 experiments conducted with other cations such as Cu(II) and Mn(II) (not shown). Similar comparisons
198 were done previously by Saar and Weber⁴³ who measured binding of several cations to fulvic acids,
199 finding good correlation for Cu(II) but only poor agreement for Pb(II), and even poorer for Co(II) and
200 Ni(II). Other studies^{11,46} were instead focused on obtaining binding equilibrium constants directly from
201 quenching data.

202

203 **Excitation-Emission matrices.** The EEM plots for Pb(II) binding to HA show typical shapes
204 observed for HS^{14,16,47} (Figure S4, Supporting Information). The shape changes as Pb(II) concentration
205 increases; the emission and excitation maxima shift towards lower wavelengths in both cases, but its
206 intensity always decreases with increasing metal concentration. The changes in shape indicate that the
207 EEMs are linearly independent, and consequently are analyzable using PARAFAC.

208 After PARAFAC processing changing the number of fluorophores, N_f , between 1 and 5 it was found,
209 in all cases, that $N_f = 3$ gave an optimal fit of the model, revealed by a low value for residual standard
210 deviation, σ_R , and a high value for core consistency, CC, for that number (Figure S5, Supporting
211 Information). This result is consistent with that reported in literature for IHSS reference humic
212 substances⁴⁸ and is also consistent with measurements of fluorescence lifetime of HS, showing three
213 distinct values.⁴⁹ Figure 4 shows contour maps of the different fluorophores for EHA and FHA, which
214 are similar to others HA.^{13,48,50} The components have been named starting from the emission maximum
215 with the higher wavelength, the spectra showing only a small dependence with pH and ionic strength;
216 Table S2 (Supporting Information) collects the average peak emission and excitation wavelengths for
217 FHA and EHA. The maxima location is related to the fluorophore structure:^{18,51} the higher the degree of
218 conjugation, the higher the excitation wavelength; this suggests that the A component is the more

219 aromatic, with aromaticity decreasing in the B and C components. The component C is also observed in
220 fulvic acids.⁵² The three have spectral characteristics consistent with the operationally defined humic
221 acids by Chen et al.⁵³ based on pyrolysis results.⁵⁴ The quenching efficiency for these components lies in
222 the same order: the more aromatic is more efficiently quenched. In similar studies with dissolved
223 organic matter and aquatic HS, 13 fluorophores were reported,⁵⁵ including aminoacid-like components
224 with emission $\lambda_{em,max} \leq 350$ nm; one of such fluorophores (tyrosine-like) was found⁵⁶ to give high
225 deviation from linearity in inner filter effects for A_{254} (the absorbance at 254 nm) > 0.3 , all the rest
226 showed low deviations up to $A_{254} \cong 1.0$. In this study, no such fluorophore is found; moreover, the
227 fluorescence titrations are treated as relative values (eq 9) and the HA concentration and the full
228 absorbance spectra are constant in the measurements, thus the correction should be the same for all data,
229 even being nonlinear. Consequently, the PARAFAC resulting spectra were corrected for inner filter
230 effects. In view of the above considerations, the results shown in Figure 5 should be considered as
231 semiquantitative.

232 Figure 5 present typical results for the scores (concentrations) of each fluorophore as a function of
233 total Pb(II) concentration, c_{pb}^0 . Other results are presented in Figures S6 and S7 (Supporting
234 Information). It is seen that component A has the higher scores and shows greater quenching, followed
235 in both aspects by B and then C. Usually, the behavior is similar for all three components; in some
236 cases, the component C shows no observable quenching. No essential differences are found between
237 components; similar results were found by Yamashita and Jaffé.⁴⁷

238

239 **IR-ATR spectra.** In Figure 6 the spectra of EHA in the absence and presence of Pb(II) are shown; as
240 detailed in Appendix B (Supporting Information) the 4000-3000 cm^{-1} was recorded with lower
241 sensitivity due to water band saturation, thus presenting higher noise. The bare EHA spectrum is typical
242 of HS,² having relatively broad bands resulting from the multiple chemical environments of the
243 absorbing groups. Here, we will discuss mainly the carboxylic C=O stretching at 1720 cm^{-1} and the
244 carboxylate bands at 1585 (ν_a , asymmetric stretching) and 1380 cm^{-1} (ν_s , symmetric),^{57,58} these bands

245 are marked in the figure. Also, a sharp band is observed at *ca.* 1105 cm⁻¹, attributed to C-O stretching of
246 polysaccharide-like substances⁵⁹ and a weak band at *ca.* 1260 cm⁻¹ attributable to phenol groups.⁶⁰
247 Finally, the OH stretching region shows a negative band due to differences in OH absorption between
248 the HA solution and the electrolyte reference.

249 Two spectra with Pb in different concentrations are shown; using NICA-EPN parameters, the metal
250 loadings are estimated as 0.36 and 2.4 mol kg⁻¹, thus these can be considered as representative of low
251 and high loadings, respectively. In the low loading case, the carboxylic band decreases, whereas the
252 carboxylate bands increase and shift to 1564 and 1386 cm⁻¹, respectively, indicating that carboxylate
253 groups are involved in binding; the other bands mentioned do not change, indicating that OH groups are
254 not involved. Under high loading, those changes appear enhanced, except for ν_s , where a sharp increase
255 is found and new band arises at 1350 cm⁻¹; the sharp increase has been observed in other cases,^{28,59}
256 whereas the new band indicates a different carboxylate binding mode. According to Nakamoto,⁵⁸ the
257 difference $\Delta = \nu_a - \nu_s$ is indicative of the binding mode, a decrease being attributable to a chelate (metal
258 bound to both O atoms) or a bridging complex (one metal bound to each O atom); here Δ decreases from
259 205 to 178 cm⁻¹ at low loading, which is likely due to chelate formation, whereas the new band at high
260 loading shows $\Delta = 208$ cm⁻¹, a small increase which could be indicative of monodentate binding. The C-
261 O bands again do not show changes, whereas in the OH region a small increase is observed, which could
262 be attributable to OH⁻ ions coordinating the Pb ions; involvement of HA OH groups in binding would
263 result in a decrease due to deprotonation. Thus, we conclude that Pb binding to the HA proceeds via the
264 carboxylate groups in different modes depending on metal loading, without appreciable involvement of
265 OH groups.

266

267 **Affinity spectra.** Figure 7 shows the affinity spectrum for Pb(II) binding to EHA corresponding to the
268 NICA fitting results shown in Table 1, compared with the conditional spectra for fluorometric
269 measurements obtained solving numerically eq 8; similar results are obtained for FHA (Figure S8,
270 Supporting Information); Figure S9 (Supporting Information) shows the NICA affinity distributions for

271 protons. These are bimodal distributions with peaks at $\log K_{Pb}$ values ~ 0.5 for the low affinity sites,
272 being for high affinity 5.8 for FHA and 9.8 for EHA. On the other hand, fluorometric results show
273 monomodal distributions peaking at ~ 5 for FHA and $\sim 6-7$ for EHA, roughly coincident with the higher
274 affinity peak from the potentiometric experiments. This suggests (considering also IR results) that
275 fluorescent quenching is sensing only high affinity sites for Pb(II), whereas potentiometric analysis finds
276 two types. Comparison of potentiometric and fluorometric measurements has been conducted by several
277 researchers,^{4,43,61,62} usually finding that metal binding quenches the HS fluorescence; however, it was
278 noted that both techniques do not sense the same complexation phenomena:⁴ for Cu binding to fulvic
279 acid, it was observed that the two measurements agreed only at low levels of copper loading,⁶¹ which is
280 consistent with our findings. Also, Saar and Weber found poor agreement between potentiometric and
281 fluorometric measurements for Pb.⁴³ These binding sites are expected to be, given the IR results,
282 carboxylates; this is also consistent with the fact that carboxylate groups are known to form stable
283 complexes with Pb(II)⁶³ whereas phenol groups are not expected to bind Pb(II) at the present pH
284 values.⁶⁴ There are a number of studies which seek to obtain binding constants between metal cations
285 and humic substances from fluorescence measurements, based on the assumptions of 1:1 binding and
286 that fluorescence was detecting all types of binding sites.⁴⁶ However, as already observed by Senesi,⁴ the
287 two techniques are not equivalent and, as found here, fluorometry senses only the strongly binding sites.
288 Therefore, the binding constants obtained exclusively through fluorometry should be considered valid
289 only in the context of this technique.

290

291 **Fluorescence quenching.** Quenching of fluorescence can be caused by several mechanisms, mainly
292 dynamic (collisional) and static (complex formation).⁵¹ For single fluorophores, the Stern-Volmer plot, a
293 graphic of I_F^0/I_F as a function of the total quencher concentration, is useful to reveal the type of
294 quenching taking place.⁵¹ For HS, their complex nature introduces difficulties because the observed
295 emission is the result of the combination of several fluorescence phenomena. Here, we treat the
296 PARAFAC components as independent fluorophores to analyze the quenching behavior. In most cases,

297 the Stern-Volmer plots show an upward curvature (Figure S10, Supporting Information); for simple
298 cases, this is indicative of mixed static-dynamic quenching,⁵¹ however dynamic (collisional) quenching
299 is usually important at concentrations several orders of magnitude higher.⁶⁵ Furthermore, attempts to
300 deduce quenching parameters by usual methods⁵¹ from these plots lead to unreasonably high values for
301 the dynamic quenching constant. Thus we conclude that static quenching is present in this case, as found
302 before for other metal-HS systems.⁴ The effect could be due to different causes,⁵¹ for HS it has been
303 attributed to intersystem crossing;⁴ that is, enhanced transference from the singlet excited state to the
304 triplet ground state. Aromatic carboxylic acids show usually this type of quenching.^{4,46,61,66} However, in
305 the case of HS this is not necessarily true: some studies⁶⁶ show that complexes of model compounds
306 with heavy metal ions (such as Tb(III)) show quenching whereas binding of the same cation to HS does
307 not. Furthermore, a molecular quenching mechanism such as intersystem crossing would require the
308 carboxylic groups to be directly bound to the fluorescent aromatic groups, but this is not necessarily the
309 case: on one hand not all the fluorescent aromatic groups would have a carboxylic substituent, and on
310 the other not every carboxylate would be attached to a fluorophore group.^{1,2,4} Thus, we propose as an
311 alternative quenching mechanism the aggregation of humic molecules induced by Pb(II) binding.
312 Quenching by dimerization of aromatic and highly conjugated substances is well known^{67,68} and shows
313 increasing efficiency at higher wavelengths, as it is observed here; on the other hand, aggregation of HS
314 by cation binding is well known.⁶⁹ Starting from this assumption, the quenching can be rationalized in
315 the following way, considering the process schematically depicted in Figure 8. When Pb(II) is added, it
316 first binds in bidentate form promoting aggregation by bridging two humic molecules; one or several
317 metal ions are expected to participate in the bridging. This process corresponds to the high affinity part
318 of the spectrum, which is thus assumed to correspond to bidentate complexation, but not necessarily all
319 Pb(II) bound in this way participate in bridging. Further increase in Pb(II) concentration results in singly
320 coordinated binding to carboxylate groups, corresponding to the low affinity part.

321 The process for the high affinity sites, which causes quenching, can be written as



323 where n is the average number of Pb^{2+} ions bridging humic molecules, considering the HA
324 heterogeneity. The reaction will have a conditional equilibrium constant given by

$$325 \quad K = \frac{[(\text{HA})_2\text{Pb}_n]}{[\text{HA}]^2 c_{\text{pb}}^n} \quad (12)$$

326 where c_{pb} is the free Pb(II) concentration. Now, if it is assumed that free HA molecules show emission
327 whereas those bound do not, it can be written for the total fluorophore concentration F_0 :⁵¹

$$328 \quad F_0 = F + 2C \quad (13)$$

329 where F is the concentration of free fluorophores and C is the complexed fluorophores (not emitting)
330 concentration. Identifying F with $[\text{HA}]$ and C with $[(\text{HA})_2\text{Pb}_n]$, it can be found from eqs 12 and 13, and
331 taking into account that fluorescent intensity is proportional to fluorophore concentration, that

$$332 \quad \frac{I_F^0}{I_F} = \frac{F_0}{F} = \frac{1 + \sqrt{1 + 8KF_0 c_{\text{pb}}^n}}{2} \quad (14)$$

333 Figure 9 shows the fit of FHA fluorophore scores (in Stern-Volmer form) to eq 14 using a common n
334 value for all curves, and different values for the product KF_0 . The free Pb(II) concentration are obtained
335 from the potentiometric results with the Davies equation for activity coefficients. A good fitting is found
336 in general, the C component in some cases show a less satisfactory fitting, but it can be due to its lower
337 intensity, resulting in higher errors. EHA data shows similar fitting (Figure S11, Supporting
338 Information). All FHA curves are fit with $n = 1.6$, whereas EHA fits with $n = 1.3$. Unfortunately, K and
339 F_0 cannot be separated, because the many unknown factors; the resulting values for their product are
340 presented in Table S3, Supporting Information.

341 Thus, we conclude that fluorescence quenching is consistent with metal-induced aggregation of HS;
342 this aggregation proceeds at a molecular scale, to be distinguished of precipitation, however, more
343 experimental information is needed to confirm this mechanism. Other spectroscopic studies are
344 consistent with the present findings. Xia et al.⁷⁰ studied by X-ray absorption the interaction of Pb(II)
345 with a humic extract from a silt loam soil suggesting the presence of two C atoms in the second

346 coordination shell, which would indicate bidentate binding. A study by EPR measurements combined
347 with DFT analysis is consistent with a structure of Pb (II) bound to two carboxylates.⁷¹

348 Concluding, the NICA-EPN model here introduced describes adequately the binding of proton and
349 Pb(II) to humic acids; fluorescence quenching has a different dependence with Pb concentration
350 compared with potentiometric titrations: it is proposed that the addition of Pb(II) causes dimerization (or
351 aggregation) of humic molecules. Thus, fluorescent quenching senses multiligand Pb(II) binding (which,
352 from IR results, should be a chelate type complex), and the second site type (with lower affinity)
353 observed in potentiometric measurements is proposed be due to 1:1 binding.

354 **Acknowledgments.** The authors wish to thank Dr. A. Olivieri for his assistance with PARAFAC
355 processing. The authors gratefully acknowledge financial support from the Universidad de Buenos Aires
356 (UBACYT 2008-2010 X148), the Consejo Nacional de Investigaciones Científicas y Técnicas and the
357 Agencia Nacional de Promoción Científica y Tecnológica (grant N° 06-12467). F. V. M. is a member of
358 the Carrera del Investigador Científico of CONICET.

359 **Supporting Information Available.** Additional figures and tables. Appendix A details the
360 experimental procedures, and Appendix B does the same for data analysis. This material is available free
361 of charge via the Internet at <http://pubs.acs.org>.

362

363 **FIGURE CAPTIONS**

364 Figure 1. Pb(II) binding curves at different pH and ionic strengths for Elliot soil humic acid (a) and
365 purified Fluka humic acid (b). The dotted lines are the NICA-Donnan predicted curves with the generic
366 recommended parameters of Milne et al.³ 1 g L⁻¹ HA in NaClO₄ at the ionic strengths shown.

367

368 Figure 2. Speciation of Pb(II) binding at pH = 5.5 and $I = 0.1$ M in NaClO₄ (1 g L⁻¹ HA) for Elliot soil
369 humic acid (a) and purified Fluka humic acid (b). The closed symbols (total and high affinity binding)
370 correspond to the left side scale, and open symbols (low affinity and electrostatic binding) to the right
371 side scale.

372

373 Figure 3. Amount of metal bound (closed symbols) and relative fluorescence quenching (open symbols)
374 for simultaneous potentiometric and fluorescence titration of EHA (a) and FHA (b) with Pb(II). 20 mg
375 L⁻¹ HA in NaClO₄ at the ionic strengths shown.

376

377 Figure 4. Fluorescence components as obtained from PARAFAC. (Top) Elliot soil humic acid, pH =
378 4.0; $I = 0.02$ M; (bottom) Purified Fluka humic acid, pH = 5.5, $I = 0.1$ M. Blue is zero intensity, red is
379 maximum (arbitrary scale). 20 mg L⁻¹ HA in NaClO₄.

380

381 Figure 5. Scores of the fluorescence components (shown in Figure 3) deduced from PARAFAC as a
382 function of total Pb(II) concentration, for 20 mg L⁻¹ FHA at pH = 5.5, $I = 0.02$ M (NaClO₄).

383

384 Figure 6. ATR-IR spectra of Elliot soil HA (0.28 g L⁻¹) in the absence and in the presence of Pb(II) at
385 different concentrations. pH = 5.5, $I = 0.1$ M (NaClO₄). See text for details.

386

387 Figure 7. Affinity spectrum found for EHA by NICA fitting (solid lines) and conditional spectra found
388 from analysis based on eq 8 of fluorometric quenching experiments (dashed lines).

389

390 Figure 8. Simplified scheme of the humic-Pb(II) interaction. In the first stage (high affinity sites) the
391 Pb^{2+} ions promote aggregation of humic molecules through bridging; the Pb ions bind to carboxylate
392 groups in chelate form as indicated by IR results. In the second stage, 1:1 binding takes place yielding
393 the low affinity sites; mainly single bond Pb binding is assumed, as suggested by IR results.

394

395 Figure 9. Stern-Volmer plots as a function of free Pb(II) concentrations for FHA fluorophores (symbols).

396 Lines show the fitting to eq 14.

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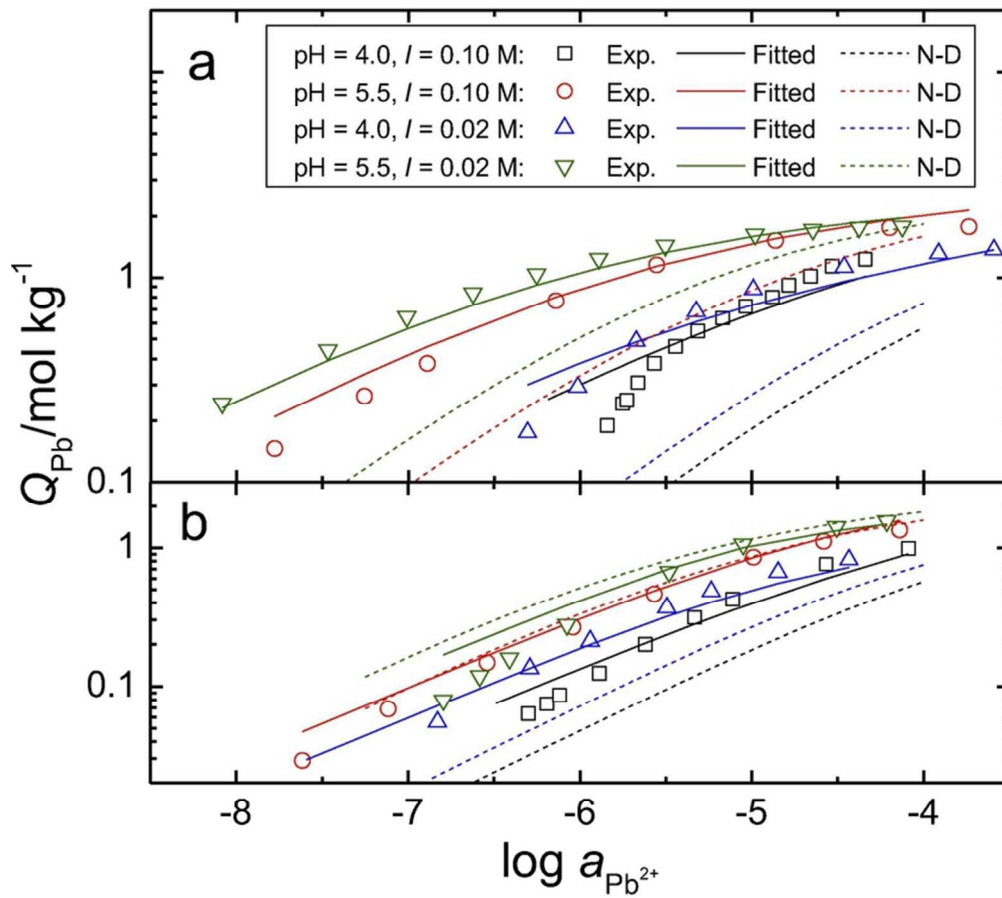


Figure 1
71x63mm (300 x 300 DPI)

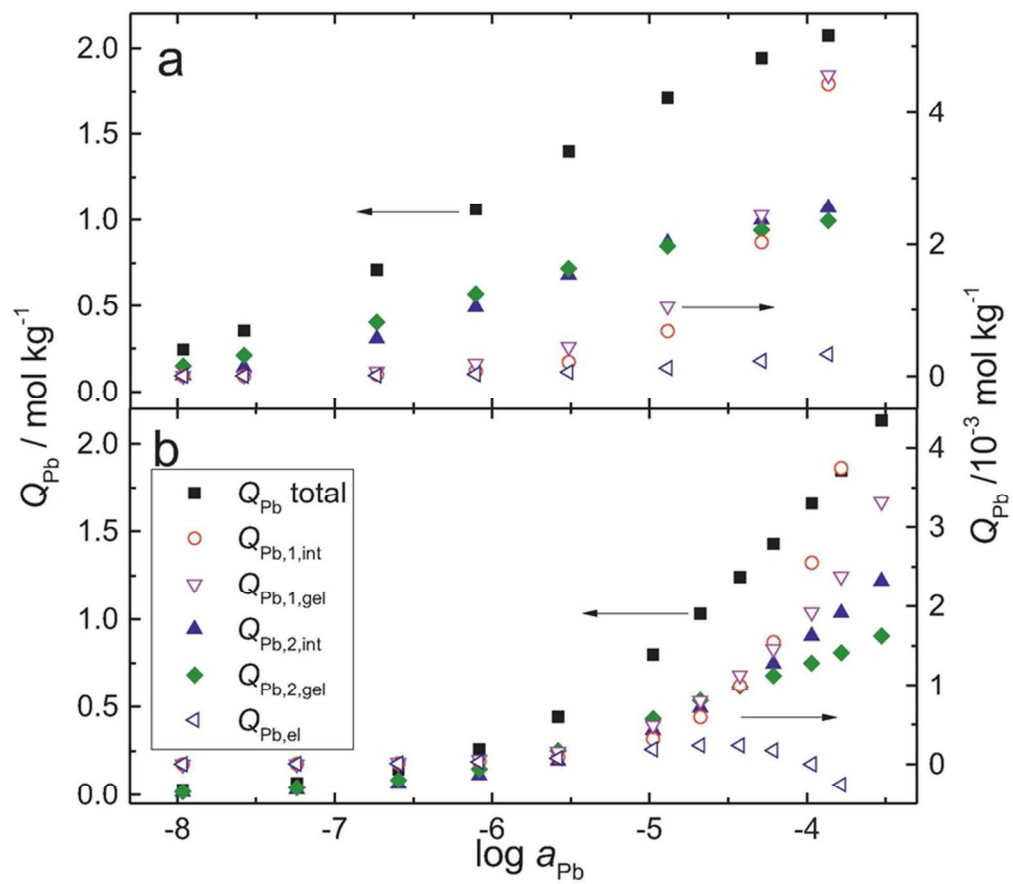


Figure 2
70x61mm (300 x 300 DPI)

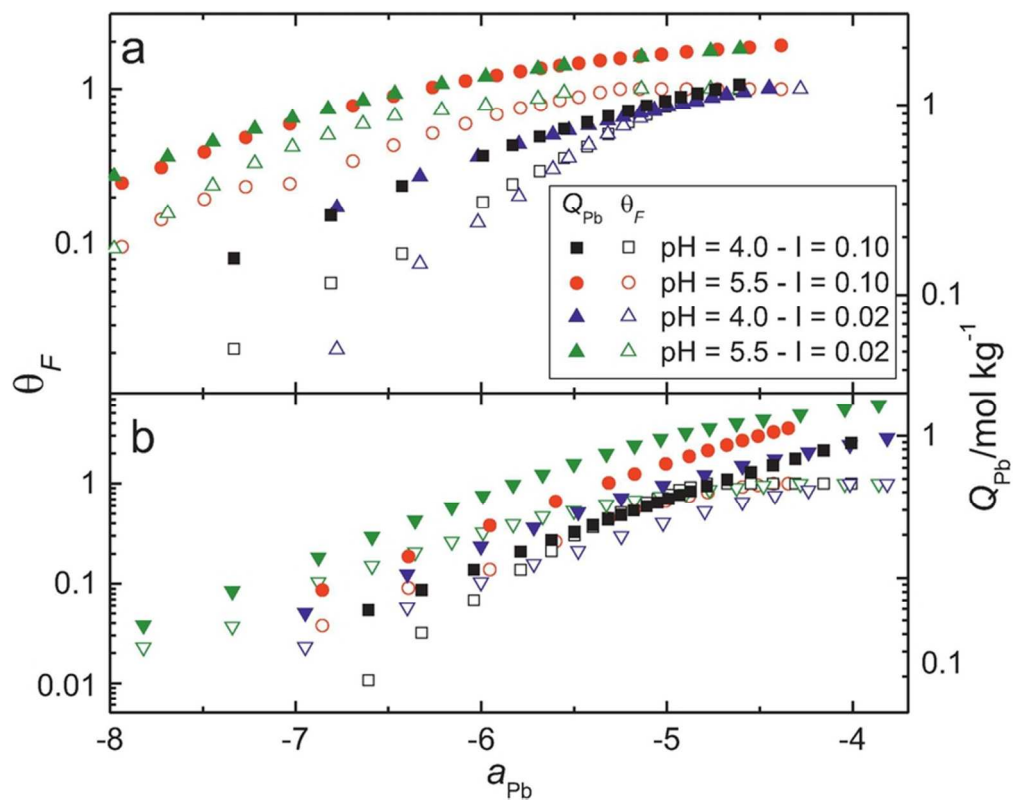


Figure 3
72x57mm (300 x 300 DPI)

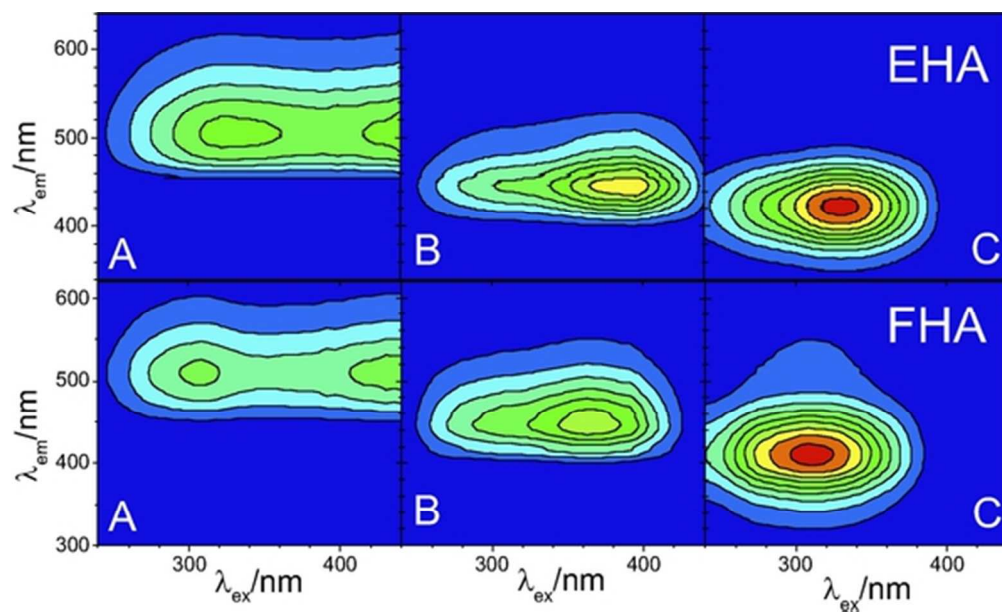


Figure 4
50x30mm (300 x 300 DPI)

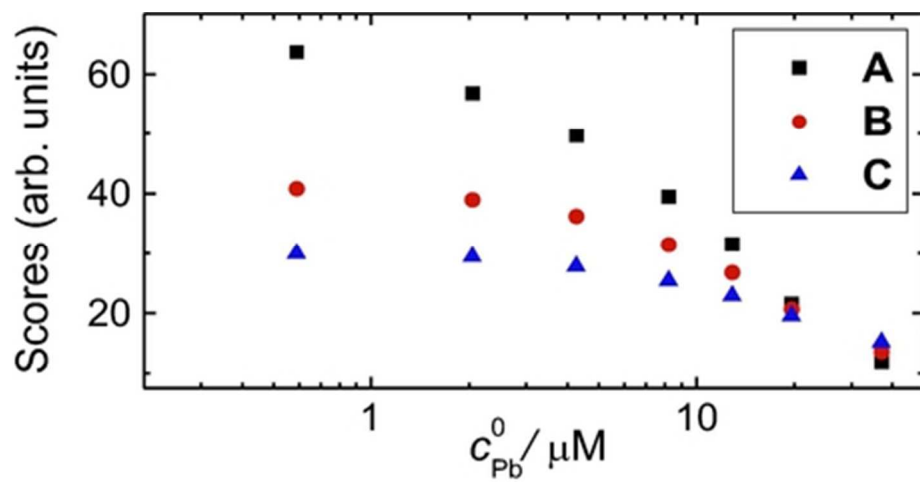


Figure 5
38x19mm (300 x 300 DPI)

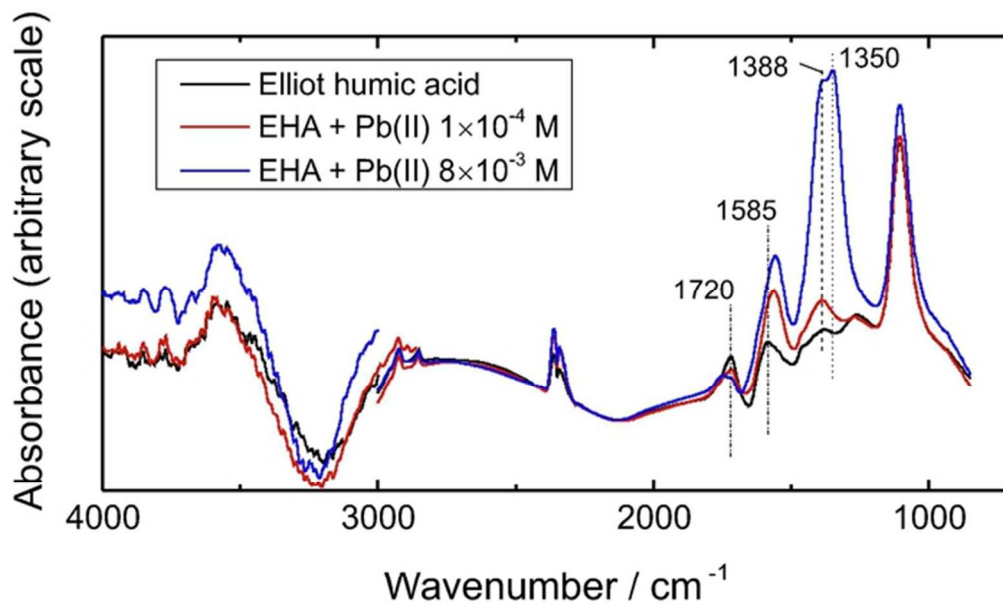


Figure 6
58x39mm (300 x 300 DPI)

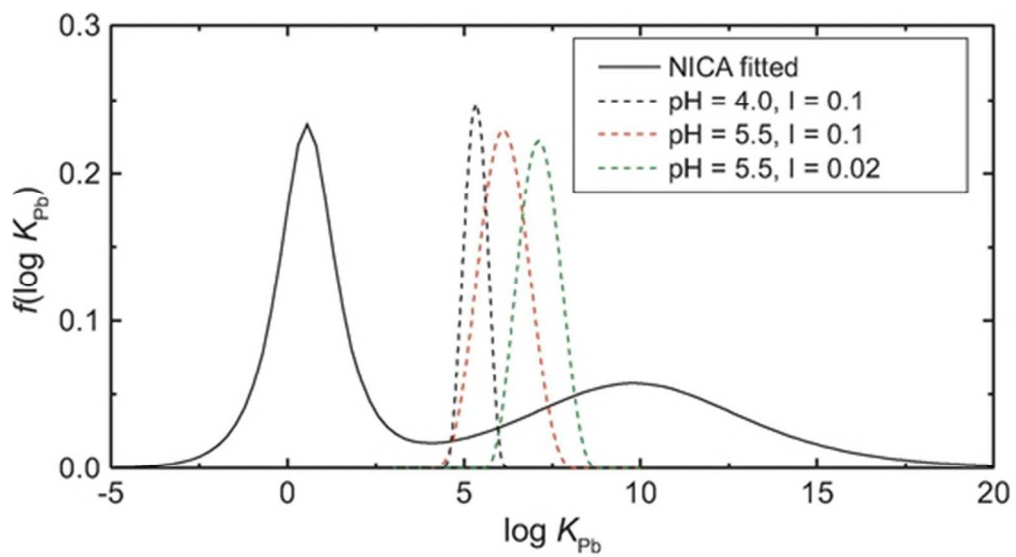


Figure 7
46x25mm (300 x 300 DPI)

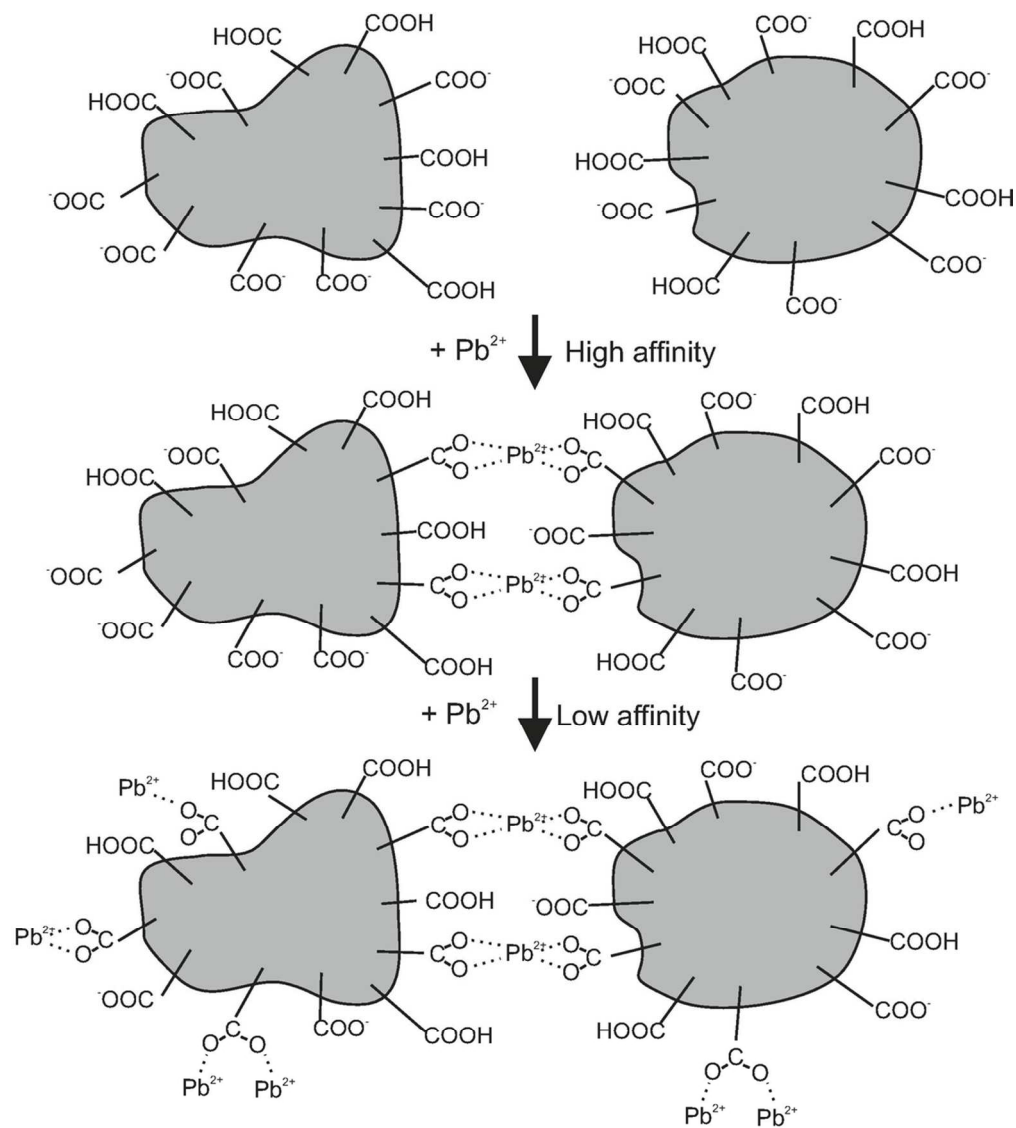


Figure 8
101x113mm (300 x 300 DPI)

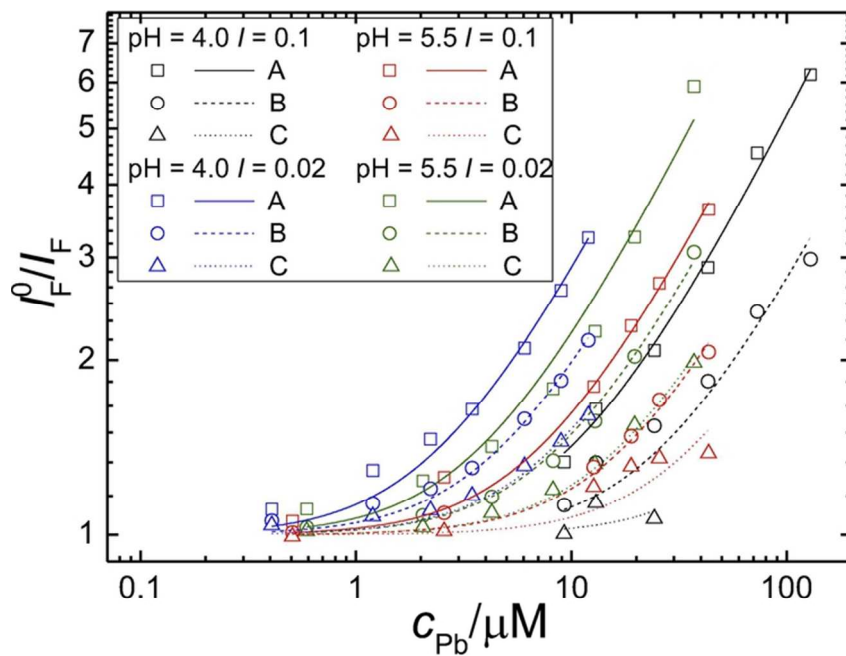
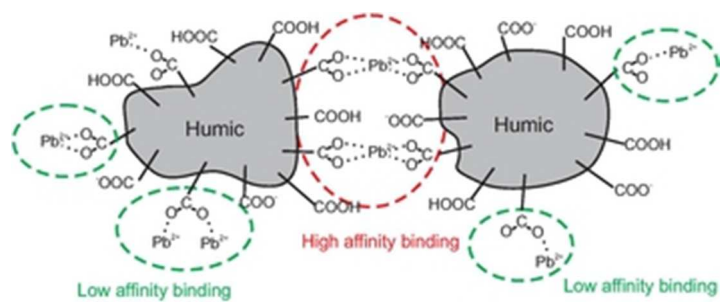


Figure 9
75x51mm (300 x 300 DPI)



TOC Art
29x12mm (300 x 300 DPI)