

Interaction of humic acids with soil minerals: adsorption and surface aggregation induced by Ca^{2+}

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Environmental context. Humic acids, important components of natural organic matter in soils, sediments and aquatic media, can interact with the surface of minerals affecting key environmental processes. In the presence of calcium, humic acids can also interact among themselves leading to molecular aggregates. We demonstrate that a solid mineral surface facilitates the formation of humic acid aggregates, and thus surface aggregation occurs under conditions where normal aggregation in solution does not occur.

Abstract. Humic acids (HAs) interact with the surface of mineral particles leading to the formation of clay–humic complexes that affect the transport of nutrients and contaminants in the environment, soil structure, soil erosion and carbon sequestration by soils. The interaction is influenced by the presence of multivalent ions, such as Ca^{2+} , which enhances the uptake of HAs by the particles. This article reports the effects of Ca^{2+} on the interaction between a HA and a soil clay fraction, both obtained from the same soil sample. The study was performed by using zeta potential measurements, HA adsorption isotherms, Ca^{2+} adsorption isotherms and microscopy. The results show that at low HA concentrations and low Ca^{2+} concentrations HA adsorption takes place, but that at high concentrations surface aggregation and precipitation also takes place, a process that is seldom reported or analysed in the literature. HA adsorption isotherms only give the overall HA uptake by the solid but they do not allow differentiation of HA adsorption from surface aggregation. However, HA adsorption v. Ca^{2+} concentration plots and Ca^{2+} adsorption isotherms at different HA concentrations can distinguish these two processes quite clearly. In addition, surface aggregation could be undoubtedly observed with optical microscopy. Surface aggregation starts to take place at a 0.7 mM Ca^{2+} concentration, which is lower than the Ca^{2+} concentration needed to start HA aggregation in solution. This indicates that the surface of soil minerals acts as a nucleation centre for HA aggregation.

Additional keywords: carbon sequestration; humic acid precipitation; soil aggregates.

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Introduction

Humic acids (HAs) are important components of the natural organic matter in soils, sediments and aquatic media. HA molecules can interact with the surface of mineral particles modifying the wettability, surface charge and aggregation of the particles,^[1] therefore affecting soil erosion, soil fertility and transport of nutrients and contaminants in the environment. The formation of clay–humic complexes by the association of HA to the mineral fraction of a soil is, in addition, recognised as a process that has a significant effect on conservation of soil organic matter and carbon sequestration.^[2,3] These clay–humic complexes have the potential of making an important contribution towards reducing emissions of greenhouse gases to the atmosphere through the accumulation of organic matter in the solid phase.^[3,4]

The adsorption of HAs to mineral particles depends on the properties of the organic matter and the mineral surface.

On iron oxides and aluminium oxides, HA adsorption occurs mainly by ligand exchange, where carboxylate and other groups of HAs act as ligands that substitute hydroxyls or aquo groups initially bonded to Fe or Al cations at the surface.^[2,5] In neutral or acidic media this adsorption is usually favoured by electrostatic attraction between the negatively charged HA molecules and the positively charged Fe or Al oxide surfaces. In contrast, HA molecules are electrostatically repelled by clay mineral particles, such as montmorillonite, kaolinite and illite, which usually carry negative structural charges attributable to isomorphic substitutions. Although some ligand exchange can take place at clay mineral edges, the interaction with basal surfaces is usually hindered unless charges are screened by electrolytes like NaCl at high concentrations.^[6] In the presence of di- or trivalent cations, however, there is a very significant adsorption of HA on clay minerals based mainly on cation bridging.^[7–10] The

presence of Ca^{2+} , for instance, largely enhances HA sorption on clay mineral particles in comparison to Na^+ .^[8]

HA molecules can also interact among themselves leading to the formation of molecular aggregates. HA aggregation may be very important for carbon sequestration in soil because the accumulation of organic matter is not limited by the availability of 'free' mineral surfaces, as it occurs with adsorption. In fact, much more organic matter could be accumulated if aggregation of HA molecules takes place in addition to adsorption. Although the more traditional viewpoint regarding the structure of HAs proposes that HA is formed by fairly large polymeric molecules, there is a growing consensus that HA is a supramolecular assembly of small to large molecules,^[11,12] forming dynamic associations stabilised by hydrophobic interactions, hydrogen bonds and the presence of metal ions, in particular divalent or trivalent ions. The aggregation of HA is the result of molecular interactions that depend on environmental conditions such as pH, ionic strength, presence of multivalent metal ions, organic compounds and solid particles. Aggregation in the absence of multivalent cations is usually favoured by decreasing the solution pH because protonation of functional groups leads to a decreased electrostatic repulsion among the molecules,^[13,14] favouring hydrogen bond formation. In the presence of multivalent cations, aggregation is promoted by charge neutralisation and cation bridge formation between different HA molecules.^[15] Calcium binding to HA, for example, seems to play two key roles in the aggregation process: it decreases the repulsive forces between the molecules by decreasing the negative zeta potential (ζ), and induces the formation of calcium bridges between two approaching molecules.^[16] This facilitated approach of HA molecules also allows interactions by hydrogen bonds that help hold the molecules to each other.^[17]

Although HA adsorption on mineral surfaces and HA aggregation are processes that are often studied and reported in the literature, there is very scarce information about surface aggregation of HAs, which is the process where HA aggregation takes place at a solid surface such as a mineral surface. As it usually occurs in natural media, the surface of a mineral can act as a nucleation centre for the formation of a new phase,^[18] and it is then possible that HA aggregation is favoured by the presence of mineral surfaces. Surface aggregation and precipitation is sometimes mentioned in the literature as a possible process, but methodologies to detect it have not been explored in detail. Therefore, there is a need for studies that allow insights into the mechanism and environmental consequences of this process.

The aim of this work is to study the interaction between a HA and a clay fraction obtained from an agricultural soil. The effects of calcium on the adsorption and aggregation behaviour of the HA are evaluated by zeta potential measurements, microscopy and HA and calcium adsorption isotherms. The possibility of surface aggregation is addressed and investigated.

Materials and methods

Both the clay fraction and the HA used in this study were taken from the same agricultural soil, which is classified as Entic Haplustoll, located in the province of La Pampa, Argentina (37° 22'S–64° 19'W). The clay fraction was obtained from a 50-g soil sample. Carbonate was removed with 50 mL of 5% CH_3COOH , and organic matter was removed with 50% H_2O_2 and heating until no frothing occurred.^[19] After that, a suspension was prepared and the clay fraction (<2- μm particles) was obtained.

These particles were then treated with 1 M NaCl, stirred for 1 h, centrifuged and the supernatant solution replaced with a fresh 1 M NaCl solution. The treatment with NaCl was repeated three times to obtain a Na^+ -exchanged clay fraction. Finally, the ionic strength of the suspension was progressively lowered by washing three times with 0.01 M NaCl,^[20] dried at 105 °C over 24 h and mortared. According to Hepper et al.,^[21] the mineralogy of this clay fraction is dominated by amorphous minerals, illites and poorly crystallised smectites, which are mostly illite-smectites intergrades. The cation exchange capacity (CEC) of the studied sample, measured using ammonium acetate at pH 7,^[22] was 0.57 meq g^{-1} .

The HA sample was obtained from the same soil sample, fractionated, purified and freeze-dried according to the procedure recommended by the International Humic Substances Society (IHSS).^[23] The C, H and N contents of the HA were 53.2, 3.8 and 3.9%, as determined with an Exeter CE 440 elemental analyser (Exeter Analytical, Coventry, UK).

In order to gain information on the electrostatic interaction between HA molecules and particles of the clay fraction, their zeta potentials under different conditions were measured with a Zetasizer ZS90 instrument (Malvern Instruments, Malvern, UK). Two types of experiments were performed: (a) ζ v. pH measurements at constant ionic strength, with either NaCl or CaCl_2 as the supporting electrolyte and (b) ζ v. CaCl_2 concentration measurements at pH 7. These two types of experiments were performed for the clay fraction, the HA and the clay fraction plus HA. For ζ v. pH measurements, either 50 mL of a 1 g L^{-1} suspension of the clay fraction, 50 mL of a 50 mg L^{-1} HA solution or 50 mL of a suspension containing 1 g L^{-1} of the clay fraction plus 50 mg L^{-1} of HA were placed in a reaction vessel with the corresponding supporting electrolyte (ionic strength, IS = 0.01). The pH was adjusted to 10 with NaOH and equilibrated under continuous stirring (450 rpm). After ζ was measured, the pH was lowered by adding a small volume of HCl, and ζ was again measured after 10 min of equilibration. This procedure was repeated until the pH was ~3. For ζ v. CaCl_2 concentration measurements, 10-mL aliquots of the three systems mentioned above were first placed in capped test tubes at pH 7. Different volumes of a 0.6 M CaCl_2 solution were then added to the tubes in order to cover a Ca^{2+} concentration range from 0 to 3 mM. After 12 h of equilibration, the pH was checked to ensure that it was 7 for all tubes, and ζ measurements were then performed. The typical standard deviation of ζ was ± 1.5 mV, as obtained from 10 measurements of the same sample.

Adsorption isotherms of HA on the clay fraction were carried out at pH 7 in 0.01 M NaCl at different Ca^{2+} concentrations. A series of centrifuge tubes were prepared containing 15 mL of a 2 g L^{-1} suspension of the clay fraction in 0.01 M NaCl, and different volumes of a 500 mg L^{-1} HA solution (also in 0.01 M NaCl), so that the initial concentration of HA varied between 0 and 160 mg L^{-1} . The Ca^{2+} concentration was adjusted by adding a known volume of a 0.05 M CaCl_2 stock solution, and the pH was adjusted with NaOH and HCl solutions. In total, 9 isotherms were determined at Ca^{2+} concentrations of 0, 0.5, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.4 mM. The tubes were shaken for 24 h, centrifuged for 5 min at 5000 rpm and the supernatants were filtered with a 0.45- μm pore size membrane to ensure complete separation of the solid phase from the solution phase. Finally the HA and Ca^{2+} concentration that remained in the supernatants were quantified.

HA was quantified by UV-visible spectroscopy, at 500 nm with a Metrolab 1600 spectrophotometer (Metrolab, Buenos Aires,

Argentina) or an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Calibration curves at pH 7 and 500 nm were constructed with several HA solutions with concentrations ranging from 0 to 80 mg L⁻¹. In order to achieve a reliable spectrophotometric quantification, it is important to check that the shape of the HA spectra does not change by varying the concentration or after adsorption and aggregation. The spectrophotometric behaviour of HA in the experiments was tested by comparing (i) the spectra of the dissolved HA sample at different concentrations, (ii) the spectra of the HA left in solution after aggregation induced by calcium (absence of the clay fraction) and (iii) the spectra of the HA left in solution after adsorption and aggregation on the clay fraction in the presence of calcium. The results (Supplementary material) show that in all cases normalised spectra were the same, indicating that the spectrophotometric quantification was feasible and reliable. In some cases, in order to check for errors in quantification as a result of possible changes in the shape of the spectra, quantifications at 350 and 500 nm were also compared.

Ca²⁺ was quantified by titration with 2 mM ethylenediaminetetraacetic acid (EDTA) using mordant black 17 (calcon, CAS number: 2538-85-4) as indicator. The method quantifies total Ca²⁺ concentration in the supernatant, i.e. free Ca²⁺ ions in solution plus Ca²⁺ bound to HA that remained in solution. This was checked by titrating a 1.40 mM Ca²⁺ solution with varying HA concentrations (from 5 to 220 mg L⁻¹). The measured Ca²⁺ concentration with the EDTA titration method was always 1.40 mM (standard deviation ±0.03 mM, 12 points), irrespective of the HA content.

Adsorbed HA or adsorbed Ca²⁺ were calculated as

$$\Gamma = \frac{(c_i - c)V}{m} \quad (1)$$

where Γ is either Γ_{HA} (adsorbed HA) or Γ_{Ca} (adsorbed Ca²⁺), c_i is the initial concentration of HA or Ca²⁺, c is either the concentration of HA (c_{HA}) or calcium (c_{Ca}) that remained in the supernatant after equilibration, m is the mass of the clay fraction and V the volume.

The mentioned adsorption experiments allowed not only the plotting of HA adsorption isotherms (Γ_{HA} v. c_{HA} curves) at different calcium concentrations but also the construction of Γ_{HA} v. c_{Ca} and Γ_{Ca} v. c_{Ca} curves for different HA concentrations.

Imaging was conducted to provide insights into the nature of interactions between HA molecules, clay fraction particles and calcium. A HOKENN optical microscope equipped with *HOKENN Micro Image Analysis Software* was used to examine samples at different experimental conditions. The systems were prepared under the same conditions as those employed in the adsorption experiments: three centrifuge tubes were prepared containing 15 mL of a 2 g L⁻¹ suspension of the clay fraction and 100 mg L⁻¹ HA in 0.01 M NaCl. The Ca²⁺ concentration was adjusted at 0, 0.5 and 1.6 mM, by adding a known volume of a 0.05 M CaCl₂ stock solution, and the pH was adjusted to 7 with NaOH and HCl solutions. After shaking the tubes for 24 h, a drop of each suspension was placed on a glass slide and observed with the microscope.

Results and discussion

Fig. 1 shows the effect of pH on the zeta potential of the clay fraction, the HA and the clay fraction plus HA. ζ is negative in all cases. For the clay fraction in NaCl, ζ is ~-20 mV at pH 3 and becomes monotonously more negative up to a value of

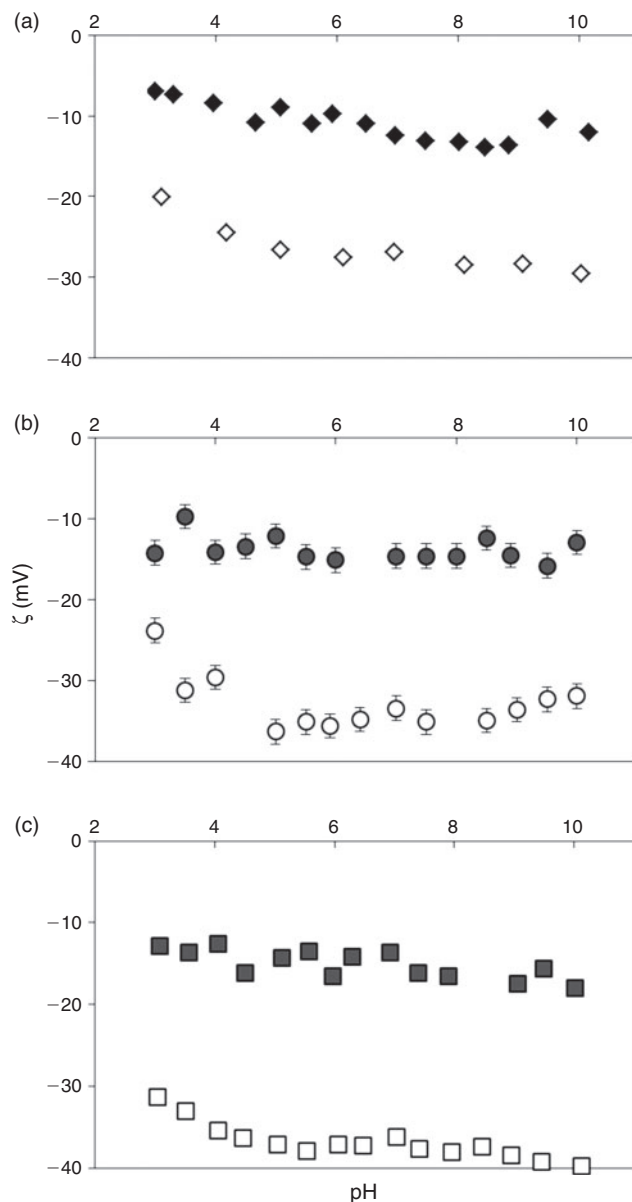


Fig. 1. Zeta potential (ζ) of the clay fraction (a), humic acid (HA) (b) and clay fraction plus HA (c) in the presence of NaCl (open series) and CaCl₂ (closed series). Part (b) shows typical error bars corresponding to standard deviations of ±1.5 mV, as an example. In all cases the pH was 7 and the ionic strength was 0.01.

~ -30 mV at pH 10. This is the expected behaviour for an inorganic soil fraction where silicoaluminates and phyllosilicates dominate the composition,^[24,25] and results from the presence of negative structural charges on clay minerals and dissociation of surface groups such as Al-OH and Si-OH groups.^[26] In CaCl₂ the zeta potential of the clay fraction is always less negative than in NaCl. It is -7 mV at pH 3 and becomes ~-12 at high pH. The adsorption of the divalent cations at the mineral surface is surely causing the decrease in the negative zeta potential when Ca²⁺ is present.^[27] For the case of HA, in NaCl ζ is ~-22 mV at pH 3, changes until it becomes ~-37 mV at pH 5 and then remains nearly constant up to pH 10. This is a known behaviour for HA and results mainly from deprotonation of carboxylic groups as pH increases,^[11,28] which are the most abundant ionisable groups in humic acids. Although some extra deprotonation at pH > 9 could occur because of the

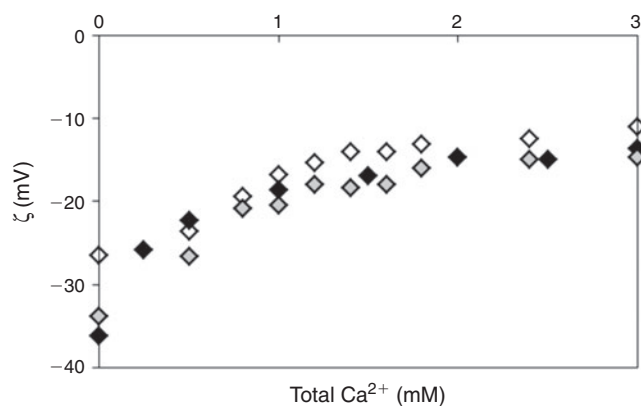


Fig. 2. Influence of Ca^{2+} concentration on the zeta potential (ζ) of clay fraction (white symbols), humic acid (HA) (black symbols) and clay fraction plus HA (grey symbols). pH 7, ionic strength 0.01.

presence of the less abundant phenolic groups, this is not evidenced in the curves. In the presence of Ca^{2+} ζ is always less negative than in NaCl alone, remaining at ~ -15 mV at all studied pH as a consequence of Ca^{2+} binding to HA molecules, binding that has been demonstrated in many publications.^[29] The system clay fraction plus HA behaves as the two other systems, having a less negative ζ at all pH in the presence of Ca^{2+} , which can be attributed to calcium binding to both mineral particles and HA molecules.^[9,23]

Fig. 2 shows the effects of calcium concentration at pH 7 on ζ for the clay fraction, the HA and the clay fraction plus HA. The behaviour is rather similar in all cases, ζ starts with a high negative value and becomes less negative as Ca^{2+} concentration increases. Ca^{2+} effects are rather significant up to a concentration of 1.5 mM, where ζ becomes ~ -18 mV. Above this concentration the effect is less important and ζ reaches values of ~ -15 mV at 3 mM Ca^{2+} . Although Ca^{2+} binding decreases the negative charge of mineral particles and HA molecules, making ζ less negative in the three systems, it is clear that the binding is not able to produce charge reversal. Results are similar to those shown by Majzik and Tombácz^[6] for the zeta potential of a HA at varying Ca^{2+} concentration and pH 6.5, and similar to our earlier results for HA in the presence of Ca^{2+} at three different pH.^[14]

Fig. 3 shows the adsorption isotherms of HA on the clay fraction at different Ca^{2+} concentrations. HA adsorption is very low in the absence of Ca^{2+} , reaching an adsorption value of 15 mg g^{-1} at high HA concentrations. Because both HA molecules and particles of the clay fraction are negatively charged as seen from ζ measurements, repulsive electrostatic interactions are impeding a significant adsorption. This low adsorption may take place at the edges of clay mineral particles, as seen by different authors for HA adsorption on montmorillonite and kaolinite,^[18,30] or at the surface of some Al^{III} and Fe^{III} oxides, which are good HA adsorbents^[31,32] but are present at low concentrations in the clay fraction. Fig. 3 also shows that Ca^{2+} significantly affects HA adsorption, which increases as Ca^{2+} concentration increases. The important enhancing effect of Ca^{2+} on HA adsorption has been observed by several authors for adsorption on pure solids.^[7,23] Majzik and Tombácz,^[6] for example, observed an increase in HA adsorption on montmorillonite by increasing Ca^{2+} concentration. They attributed this behaviour to Ca^{2+} adsorption on montmorillonite by ion exchange and formation of Ca^{2+} bridges between the montmorillonite surface and HA molecules. These authors also proposed the formation of a second HA layer on the coated surface as a

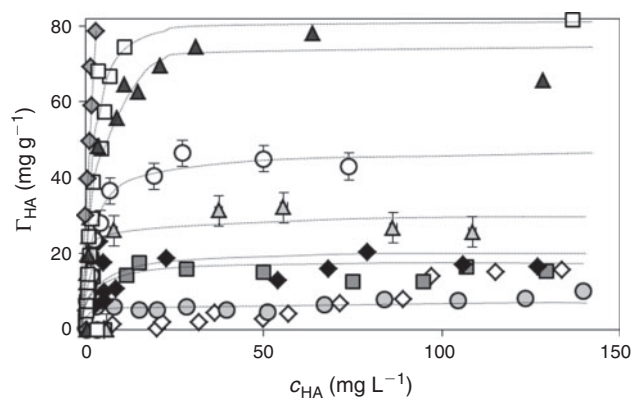


Fig. 3. Adsorption isotherms of humic acid (HA) on the clay fraction at different total calcium concentrations: 0 (white diamonds), 0.5 (grey squares), 1.0 (black diamonds), 1.2 (grey triangles), 1.4 (white circles), 1.6 (black triangles), 1.8 (white squares) and 2.4 mM (grey diamonds). pH 7, ionic strength 0.01. Some curves show typical error bars corresponding to the maximum difference in Γ_{HA} (adsorbed HA), as measured by reading absorbances at 350 and 500 nm. Lines are a guide to the eye.

result of Ca^{2+} bridges between HA molecules from the first to the second layer. Precipitation of HA as Ca^{2+} -humate was also suggested as a possible process.^[6] Martinez et al.^[23] proposed that increasing Ca^{2+} concentrations results in a more compact structure of humic molecules, leading to a higher adsorption of humic substances in minerals such as kaolinite and illite. In addition, Akbour et al.,^[7] who worked with quartz sands and kaolinite, indicated that adsorption of divalent cations reduces the negative charge of the humics and the negative surface charge of the solids, increasing consequently the adsorption. The results shown in Fig. 3 are in line with all these explanations, and thus they can be understood in terms of a combination of all the following processes: (a) the presence of Ca^{2+} reduces the electrostatic repulsion (reduction in ζ , as seen in Fig. 1 and Fig. 2) between HA and the solid surface favouring the interaction; (b) Ca^{2+} adsorption on the surface of the particles promotes the formation of Ca^{2+} bridges between particles and HA; (c) HA compaction allows a high adsorption density of HA and (d) surface precipitation or surface aggregation of HA may be possible at high Ca^{2+} concentrations. Regarding this last process, it is well known that Ca^{2+} has the ability of producing aggregation of HA molecules above a certain cation concentration. In a previous paper^[14] it was shown that the HA studied here starts to aggregate significantly at 1 ± 0.2 mM Ca^{2+} concentration in 0.01 M NaCl in the absence of solid particles, as a consequence of calcium binding to HA. This Ca^{2+} concentration is the critical aggregation concentration (CAC) of calcium for the studied HA in solution. Therefore, it is possible that HA aggregation, and not only adsorption, takes place at high Ca^{2+} concentrations. Adsorption isotherms such as those presented in Fig. 3 give the overall HA uptake by the solid, but do not allow one to distinguish the conditions where aggregation could occur. Other data need to be evaluated in order to obtain evidence of this process.

Fig. 4 shows the Γ_{HA} v. c_{Ca} curves for five different HA concentrations. A general inspection of the curves indicates that adsorption increases linearly (line 1) up to $c_{\text{Ca}} = 0.70$ mM, and that above this concentration the adsorption curves acquire a steeper slope (line 2). For the lowest HA concentrations investigated (25 and 50 mg L^{-1}), the adsorption curves never acquire

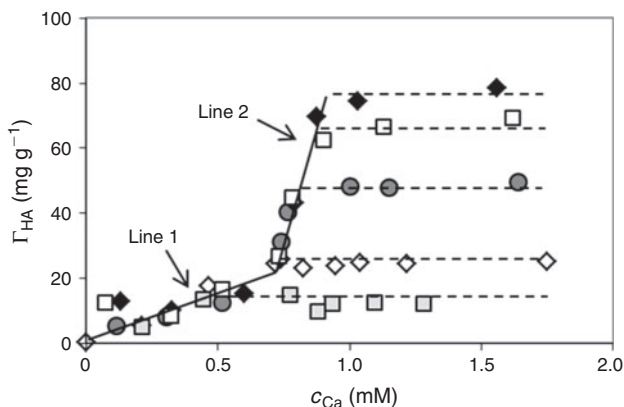


Fig. 4. Humic acid (HA) adsorption (Γ_{HA}) as a function of calcium concentration (c_{Ca}) at five different total HA concentrations: 25 (black triangles), 50 (white diamonds), 100 (grey circles), 140 (white triangles) and 160 mg L^{-1} (black diamonds). Dashed lines indicate complete removal of HA. pH 7, ionic strength 0.01. Lines are a guide to the eye.

the steeper slope because all the HA in the system is already adsorbed (marked by horizontal dashed lines in the figure) before this condition is reached. In all the other cases, the presence of a steeper slope in the curves is evident at c_{Ca} above 0.70 mM, until curves level off because of complete removal of HA from solution. The two different slopes suggest that two different processes are operating and controlling this removal. One of these processes is calcium-assisted HA adsorption, which takes place at a c_{Ca} below 0.70 mM. This HA adsorption is most probably driven by a reduction in the electrostatic repulsion between the solid surface and humic molecules, and by the formation of Ca^{2+} bridges between the surface and HA. Aggregation of HA molecules that remained in solution would not be significant under these conditions because the Ca^{2+} concentration is considerably lower than that needed (CAC) for HA aggregation, especially in the initial part of the curves. The second process might be HA aggregation, which is likely to take place together with HA adsorption at c_{Ca} above 0.70 mM, leading to the steeper slope in the curves. Although 0.70 mM is slightly below the CAC of HA in the absence of solid particles, it seems that aggregation starts to take place at this c_{Ca} value in the presence of the clay fraction. This is a rather common phenomenon; the solid surface acts as a nucleation centre favouring aggregation, precipitation or formation of a new phase at the surface.^[16] Although it is very difficult to discriminate between Ca^{2+} -induced aggregation in solution previous to the adsorption of the aggregates, and direct aggregation upon adsorption (surface aggregation), it is possible to infer that aggregation upon adsorption would be the prevailing process following the analyses by Schneider et al.^[33] According to these authors, aggregation upon adsorption is possible if there is an accumulation of calcium ions within the diffuse part of the electrical double layer. Calcium accumulation will take place because there is a net negative charge on the solid surface. Thus, even though the concentration of calcium in solution may be less than the CAC for HA aggregation, this critical concentration near the solid surface is exceeded and direct aggregation onto the surface takes place.

The formation of aggregates could be readily confirmed by optical microscopy. Fig. 5 shows images of the clay fraction with no HA in the system, with HA and Ca^{2+} under the conditions of line 1 in Fig. 4 and with HA and Ca^{2+} under the

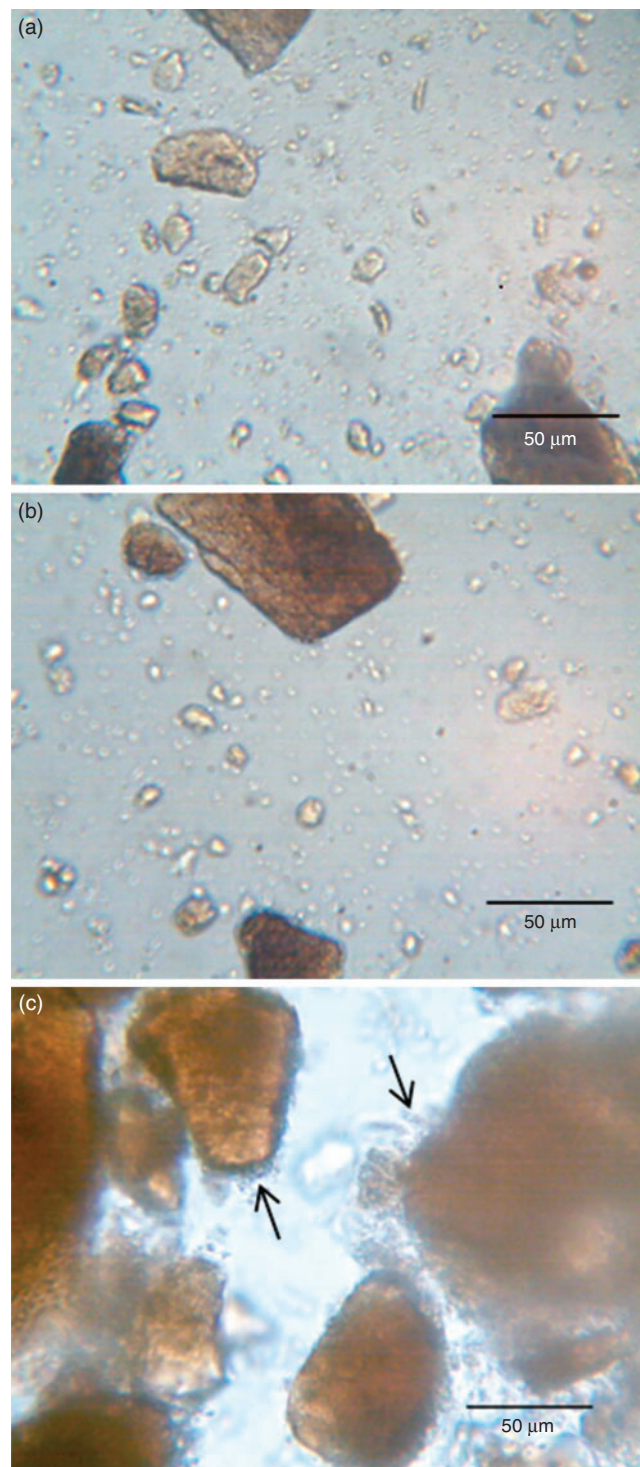


Fig. 5. Optical microphotographs of the clay fraction: (a) with no humic acid (HA) in the system, (b) with HA and calcium under the conditions of line 1 in Fig. 4 and (c) with HA and calcium under the conditions of line 2. Arrows indicate the presence of HA aggregates. pH 7, ionic strength 0.01.

conditions of line 2. With no HA in the system, bare particles corresponding to the clay fraction are observed. The same kind of image was obtained under the conditions of line 1, in agreement with the fact that the removal of HA is attributable only to adsorption, because a monolayer or sub-monolayer of adsorbed HA cannot be detected by optical microscopy or even with higher magnifications. Under the conditions of line 2,

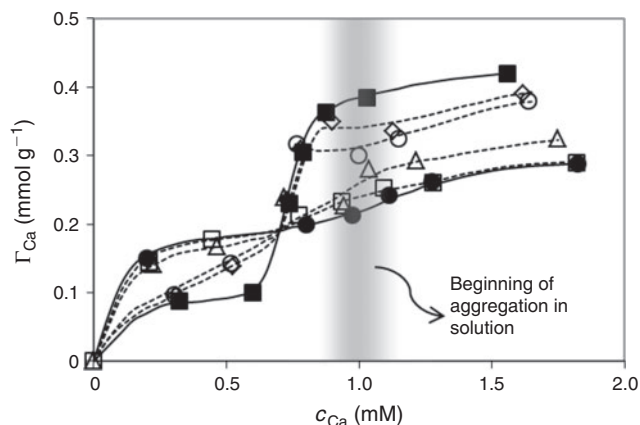


Fig. 6. Calcium adsorption isotherms (Γ_{Ca}) at different total humic acid (HA) concentrations: 0 (black circles), 25 (white squares), 50 (white triangles), 100 (white circles), 140 (white diamonds) and 160 mg L^{-1} (black squares). The grey bar indicates the calcium concentration (c_{Ca}) values where HA starts to aggregate in solution. pH 7, ionic strength 0.01. Lines are a guide to the eye.

however, the presence of HA aggregates can be clearly detected in the form of small dots (arrows in Fig. 5). Many of these aggregates are grouped together at the surface of mineral particles, and they are the product of surface aggregation. Other aggregates show up separately from the particle surface. As it is known that these aggregates are rather mobile in water and subject to Brownian motion,^[14] they may have formed as surface aggregates and then become detached by thermal movements from the surface. They may also be the product of massive HA aggregation at HA concentrations well above the CAC, without the need of a solid surface for nucleation.

Fig. 6 shows Γ_{Ca} v. c_{Ca} curves for the series of experiments shown in Fig. 4. Because Ca^{2+} binds both the mineral surface and HA molecules, Γ_{Ca} represents Ca^{2+} bound to the surface plus Ca^{2+} bound to adsorbed and aggregated HA. Two extreme cases will be first analysed: Ca^{2+} adsorption in the absence of HA and Ca^{2+} adsorption in the presence of the maximum HA concentration investigated, 160 mg L^{-1} . In the absence of HA, the Γ_{Ca} v. c_{Ca} curve is a normal Ca^{2+} adsorption isotherm on the clay fraction. Ca^{2+} adsorbs as its concentration increases reaching an adsorption value of 0.29 mmol g^{-1} , or 0.58 meq g^{-1} , which corresponds almost exactly to the CEC of the clay fraction (0.57 meq g^{-1}). In the presence of 160 mg L^{-1} HA, Γ_{Ca} starts becoming lower than in the absence of HA, but then increases rather abruptly leading to an S-shaped curve with an inflection point at $c_{Ca} \approx 0.70$ mM, and finishing with Γ_{Ca} values that are much higher than in the absence of HA. The abrupt increase in Γ_{Ca} occurs at the same Ca^{2+} concentration that triggers aggregation (Fig. 4). Thus it must be a consequence of Ca^{2+} consumption as a result of surface aggregation of HA. The decreased Γ_{Ca} at low c_{Ca} when HA is present in the system can be easily understood as a competition between the mineral surface and dissolved HA molecules for Ca^{2+} . An important fraction of HA remains dissolved in solution, and therefore adsorbed Ca^{2+} decreases because part of this Ca^{2+} becomes bonded to dissolved HA molecules. Similar competition processes take place at high c_{Ca} . Under this last condition, however, most of the HA is either adsorbed or aggregated, capturing a great deal of Ca^{2+} and resulting in an increased Γ_{Ca} . The same explanations are valid to understand all the other curves in Fig. 6. They lay between the two extreme cases analysed just

because the HA concentration is between the HA concentrations of these two cases.

It is interesting to note that all curves in Fig. 6 seem to define a crossing point at $c_{Ca} \approx 0.70$ mM, which corresponds to $\Gamma_{Ca} \approx 0.19$ mmol g^{-1} . This is an inflection point that becomes evident for those curves obtained with HA concentrations higher than 50 mg L^{-1} . The presence of a crossing point means that changing the HA concentration at this point does not produce any change in either c_{Ca} or Γ_{Ca} . It corresponds to a balance between Ca^{2+} in the solution phase, represented by c_{Ca} (dissolved Ca^{2+} in water plus Ca^{2+} bonded to dissolved HA; i.e. all Ca^{2+} forms that remained in the supernatant after centrifugation plus filtration), and Ca^{2+} in the 'solid' phase, represented by Γ_{Ca} (Ca^{2+} adsorbed to the minerals surface, plus Ca^{2+} bonded to adsorbed HA, plus Ca^{2+} bonded to aggregated HA; i.e. all Ca^{2+} forms that could be removed by centrifugation plus filtration). Therefore, for the studied system, adding HA to the suspension of the clay fraction will not change the partition of Ca^{2+} between the solution phase and the 'solid' phase if $c_{Ca} = 0.70$ mM. However, if $c_{Ca} < 0.70$ mM, adding HA will favour the solution phase; and if $c_{Ca} > 0.70$ mM adding HA will favour the 'solid' phase. This helps understand the shapes of the curves in Fig. 6, curves that also give a good evidence for surface aggregation.

As stated in the *Introduction*, there is a growing consensus in that HA is a supramolecular assembly of small to large molecules, which contradicts the most traditional, polyelectrolytic, viewpoint regarding the HA structure. It is then instructive to analyse if aggregation and adsorption experiments in the presence of Ca^{2+} as performed in this article give some useful information about this structure. The main answer comes from the analysis of the UV-visible spectra of HA, which indicates that neither aggregation with Ca^{2+} nor adsorption and aggregation at the surface of mineral particles in the presence of Ca^{2+} change the spectra. In the absence of important amounts of calcium or other multivalent cations, Andrew et al.,^[34] for example, show that oceanic dissolved organic matter adsorbs with preference the long wavelength (visible) absorbing and emitting (fluorescent) material on a C18 extracting cartridge, suggesting fractionation of the material into different components, and pointing to the supramolecular structure of the dissolved organic matter. In the light of our results, it appears that Ca^{2+} does not discriminate between large and small HA moieties, probably because of the ability to form bridges between them. Thus, aggregation, surface aggregation and Ca^{2+} -assisted adsorption occur quite homogeneously. Therefore, any attempt to fractionate HA constituents should be done in the complete absence of calcium and probably other multivalent cations.

Conclusions

The effects of calcium concentration on the interaction between the HA and the clay fraction of a soil sample were investigated. Calcium plays a key role in this interaction because it is able to bind both surface sites at mineral surfaces and functional groups in HA molecules, decreasing the negative zeta potential in both cases and favouring the interaction. In the absence of calcium, HA adsorption on the clay fraction is very low as the result of electrostatic repulsion between mineral surfaces and HA molecules. In the presence of calcium, there is a significant uptake of HA by minerals. The calcium concentration determines whether this uptake is mainly HA adsorption or HA aggregation. Low calcium concentrations favour HA adsorption, which occurs by the formation of calcium bridges between

the mineral surface and HA molecules. High calcium concentrations induce HA aggregation, which leads to a very significant removal of dissolved HA. HA aggregation could be consistently detected with $\Gamma_{\text{HA}} v. c_{\text{Ca}}$ plots from the change in the slope of the curves, and with $\Gamma_{\text{Ca}} v. c_{\text{Ca}}$ plots from the crossing point of the curves. These kinds of plots, as far as we know, have not previously been employed to detect HA aggregation. HA aggregation was readily observed by optical microscopy and is favoured by the presence of the mineral surface, which acts as a nucleation centre.

The fact that surface aggregation of HA occurs on soil mineral surfaces evidences the high potential of a soil for organic matter accumulation and carbon sequestration, which should be much higher than the accumulation produced by adsorption processes only. In addition, because aggregation is facilitated by mineral surfaces, HA aggregates are likely to be preferentially formed at the surface of soil particles, linking different particles and contributing to soil structure.

Supplementary material

UV-visible spectra of HA solutions before aggregation, after aggregation with calcium and after adsorption on the soil clay fraction are available from the journal online (see http://www.publish.csiro.au/?act=view_file&file_id=EN14157_AC.pdf). Figs S1–S4 show that all spectra of HA that remained in solution in adsorption and aggregation experiments coincide with spectra of dissolved HA used for calibration. Thus, quantification could be performed by reading absorbances at, for example, 350 or 500 nm without important variations.

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