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New possibility for characterization of dissociation behaviour of supramolecular electrolytes: results obtained for the International Humic Substances Society standard and reference samples by coulometry

Martina Klučáková*

Abstract

Background: Humic substances can be considered as polyelectrolytes with supramolecular character and complicated behaviour in water environment. The fractions of humic substances dissolved in water are the most active ones and determinative for their functioning in nature, where the proton-binding and dissociation ability play a crucial role. The dissociation behaviour of humic and fulvic acids can be affected by different circumstances including their concentration which is directly connected with the molecular organization of humic particles in solution and the accessibility of their ionizable functional groups. This study is focused just on these active fractions and their dissociation behaviour in the dependence on their content in studied system.

Results: Standards and reference samples of International Humic Substances Society were used. Flow-through coulometry was used to determine the total content of acidic functional groups in fulvic solutions and humic leachates. The amount of dissociated acidic groups was determined on the basis of potentiometry. Several differences between the behaviour of humic and fulvic acids were found. While whole samples of fulvic acids including the weakest functional groups were analysed, only the active dissolved humic fractions containing stronger acidic functional groups were characterized. The fractions containing higher amounts of the weakest functional groups remained insoluble. The dissociation degree of fulvic acids decreased with their increasing content, but a maximum on its concentration dependence obtained for humic acids was observed.

Conclusions: Two different values of dissociation constants were determined for each sample. The first was determined on the basis of the extrapolation of infinite dilution, second was determined as their average value in the region of high concentrations in which it was constant. Obtained values characterize the functioning of dissolved humic substances from point of view of their dissociation ability. The results obtained by this approach can help to predict the acid–base behaviour of dissolved organic carbon in soil and generally in nature. The acid–base behaviour of dissolved organic carbon depends on its ratio to water. It behaves differently in soil which is dry and in soil which is wet or saturated by water.

Keywords: Dissolved organic matter, Supramolecular polyelectrolyte, Humic acids, Fulvic acids, Solubility, Dissociable acidic functional groups, Coulometry

*Correspondence: klucakova@fch.vutbr.cz
Faculty of Chemistry, Brno University of Technology, Purkyňova 464/118, 612 00 Brno, Czech Republic

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Background

Humic substances are heterogeneous mixtures of organic materials formed by the humification of animals, plants and microbes [1, 2]. They are the most widespread natural complexing agents occurring in soils, sediments and water. Due to their colloidal and poly-functional character, these substances play important roles in the mobility and bioavailability of nutrients and contaminants in the environment [3, 4]. The major role in the interactions of humic substances with other components is played by their dissociable acidic functional groups [2–7], which include mainly carboxylic acids, phenols and alcohols [2, 3, 8–10]. While carboxylic functional groups are generally considered as strong ones with pK_a around 3.8–4.4, phenolic and enolic ones belong to weak groups with pK_a around 8.7–10 [2, 3, 6, 8, 10, 11]. One of the most important properties of humic substances is their large buffer capacity over a wide pH range, which arises essentially from the dissociation of acidic functional groups. The exact contribution to the total soil buffer capacity is not well known. In general, soils rich in humic substances are well buffered [8]. A study of the interactions of humic substances cannot disregard a knowledge of their proton exchange capacity and the strength of their functional groups which is not easy to acquire—particularly in the case of natural polyelectrolytes, whose heterogeneous structure makes the modelling of protonation equilibria very complicated [11–13]. As a consequence of such pronounced heterogeneity with respect to humic structure and composition, a strong influence on the acid–base properties and on binding capacities of humic substances and their functioning in nature must be expected [14, 15].

Humic substances are principal constituents of natural organic matter and play a crucial role in its functioning in natural systems (waters, soils, sediments). They can be characterized by complex supramolecular structure and considered as associations of small molecules self-assembled by weak forces and hydrogen bonds. Such associations in solution are formed by the self-organization of hydrophobic and amphiphilic compounds: highly polar subunits of humic substances are in contact with solution, that less polar subunits are in subsequent layers, and non-polar subunits are inaccessible in the core [16–21]. Therefore, the dissociation behaviour of humic substances can be affected by different circumstances including their concentration which is directly connected with the molecular organization of humic particles in solution and the accessibility of their ionizable functional groups. It is assumed that the fractions of humic substances dissolved in water are the most active humic fractions which are determinative for their functioning in nature. This study is focused just on these active fractions and their dissociation behaviour in the dependence on their content in studied system.

The most widely used methods to characterize the dissociation behaviour of humic substances are acid–base titrations [2, 22, 23]. Fukushima et al. [24] characterized the acid–base behaviour of humic acids by means of the continuous pK_a distribution model, in which the acidic functional groups had two centres of distribution in the humic molecule. They suggested that there are strong and weak acidic groups in the humic molecules and that they deprotonate continuously according to their pK_a distribution. In their latter work [25], humic acids fractionated by molecular weight were analysed. The largest amounts of acidic groups were found in the fraction with the lowest molecular weight. The pK_a value was the lowest for the unfractionated humic sample; therefore, the authors deduced that intermolecular interactions and conformational changes influence acidic sites in the humic structure. The method of non-aqueous conductivity titration was used in ref. [2] for the determination of the contents of carboxylic and phenolic functional groups in the collection of 29 humic samples. Garcia-Mina [26] described the proton-binding behaviour of humic acids using the polyelectrolyte model. A characterization can be made on the basis of both the electrostatic factors affecting the binding site and the site heterogeneity. Ritchie and Perdue [11] used the Henderson–Hasselbalch model modified for two types of proton-binding sites in order to obtain the "best fit parameters" which describe the proton-binding curves for humic samples. The computed parameters were only empirical ones and it was necessary to know the ratio between carboxylic and phenolic groups in the sample. Crea et al. [13] determined the dissociation constants of several humic and fulvic acids by means of four various models. A statistical analysis of the dissociation constants of all investigated polyelectrolytes was made, confirming that very similar results can be obtained using any considered model. Campitelli et al. [8] determined seven different dissociation constants of the humic acids extracted from compost, soil and amended soil by means of differential scanning potentiometry. Each one of these values corresponded to known acidic groups that could be present in the humic molecule.

It is well known that the utilization of titration data for the determination of dissociation constants is connected with several problems [10, 27, 28]. Fernandes et al. [27] compared the fitting of titration data by two different models and stated that the results were strongly dependent on both the choice of model as well as the experimental conditions (e.g. titration rate). Khil´ko et al. [28] showed that the results of potentiometric titrations and the calculated dissociation constants depend on the titration method, the humic concentration, and the time taken to establish the titration rate. There are other ways to determine the dissociation constants of humic acids,

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but these methods are rarely utilized. Salma and his co-workers [29, 30] used conductometry to determine the dissociation constants of humic-like substances. Palladino et al. [15] combined potentiometric titration and constant-current coulometry as an alternative method for the determination of the dissociation constants of humic acids. UV/VIS spectroscopy [10], flow-through coulometry [31], and conductometry [32] were used in our previous works as alternative methods for the characterizing the dissociation behaviour of humic acids. Orlović-Leko et al. [33] showed that electroanalytical techniques are suitable for the investigation of physical–chemical properties of dissolved organic matter including humic and fulvic acids in water. These techniques are sensitive, rapid, and quantitative, and their cost is dramatically lower compared to other more sophisticated techniques.

In this study, flow-through coulometry [31, 34] was used to characterize the dissociation behaviour of humic and fulvic acids purchased from the International Humic Substances Society (IHSS). The method was optimized and validated for humic acids in previous work [31]. The application of the method on samples of IHSS, regarded as standards and reference materials of humic substances, is subsequent logical step in this field of humic research. Fulvic acids are the most mobile and reactive components of humic substances, and are completely soluble in water and aqueous solutions [2–6, 35]. They are characterized by a lower degree of aromaticity, as compared with that of humic acids, and a considerable predominance of aliphatic substituents. The qualitative composition of functional groups is the same as that in humic molecules [35]. Similarly, the fraction of humic acids which is dissolved in water, or rather in the aqueous phase in humic-containing systems is the most active fraction of humic acids. It is the most important medium of organic carbon transport from terrestrial to aquatic ecosystems. This fraction can be rich in acidic functional groups and its size can be the result of equilibrium between dissolved and undissolved humic acids [31, 32, 36].

Results

Figure 1 shows examples of chronopotentiograms measured by means of flow-through coulometry. As can be seen obtained results are strongly influenced by the concentration of measured samples. More fractions were observed for diluted fulvic solutions and humic leachates. The PPFA sample with concentration of 0.01 g dm^{-3} (Fig. 1a) contained minimally three main fractions of different amounts and strengths. Similar results were observed for diluted humic leachates. In contrast, only mono-modal distributions were observed in more concentrated samples (e.g. LEHA with concentration of 10 g dm^{-3} in Fig. 1b). The chronopotentiogram obtained
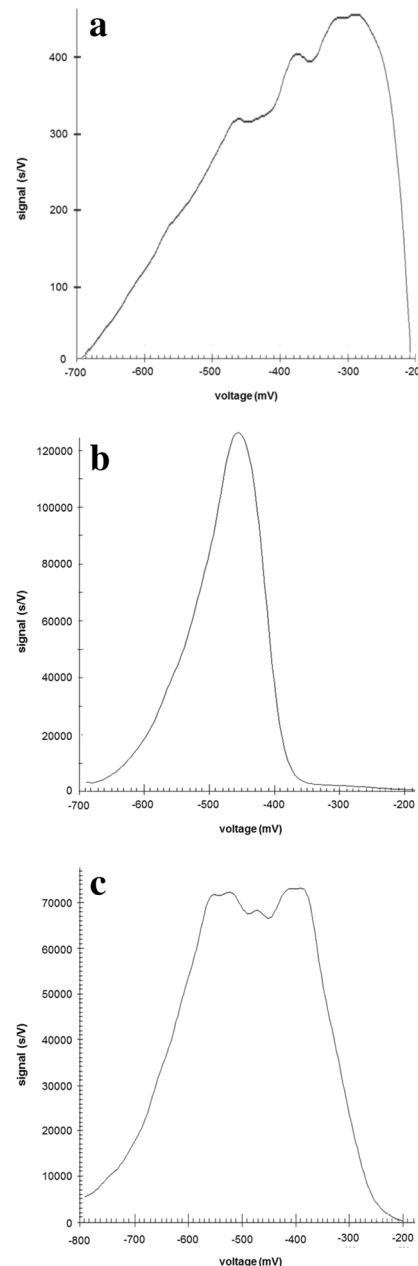
Fig. 1 The examples of chronopotentiograms measured by means of flow-through coulometry: **a** PPFA—0.01 g dm^{-3}; **b** LEHA—10 g dm^{-3} (initial concentration of suspension before centrifugation); **c** phthalic acid—5 mmol dm^{-3}

for phthalic acids with concentration of 5 mmol dm^{-3} is shown in Fig. 1c (for details of measurement see ref. [31]). In this record, two main fractions corresponding with two carboxylic acidic groups in its molecule were detected. The dibasic molecule of phthalic acids has two functional groups, which are not equal, but have two different values of pK_a: 2.943 and 5.432 (see ref.

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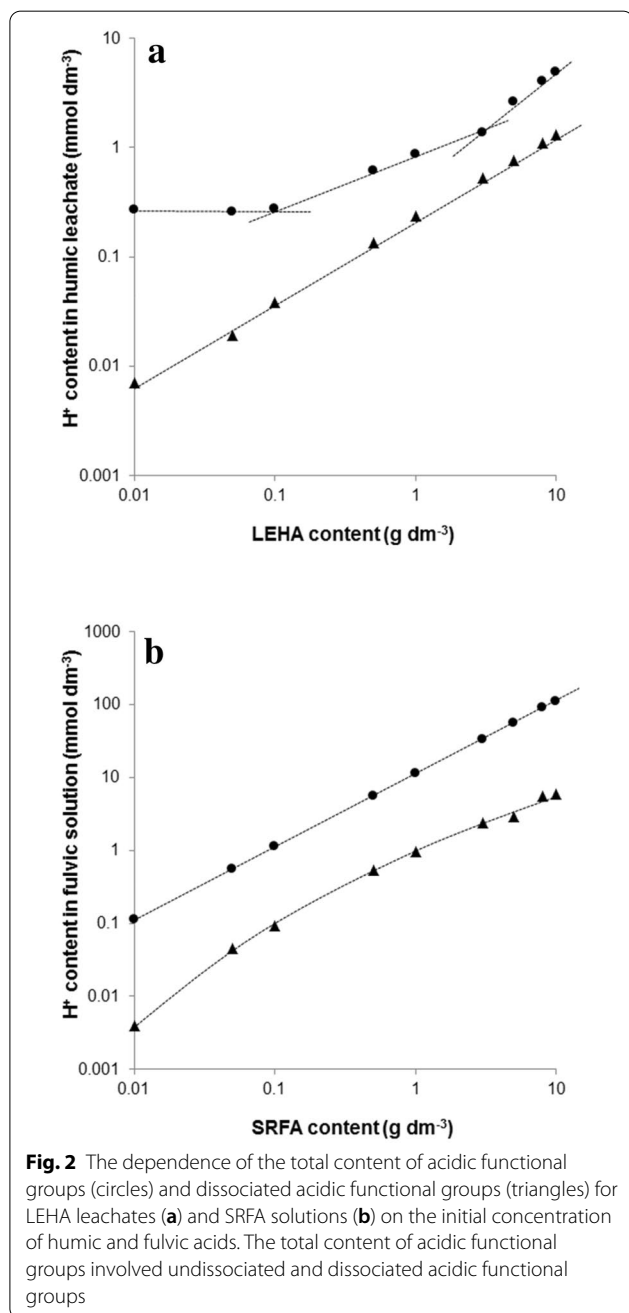
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[37]). Amounts of acidic functional groups determined on the basis of flow-through coulometry involved both protonated (undissociated) and dissociated groups in studied systems. It is very useful mainly in the case of humic acids which are only partially soluble in water and the determination of their concentration in leachates is accompanied with several problems [29–31, 36]. Amounts of dissociated acidic groups were determined on the basis of pH-measurements. In Fig. 2, the comparison o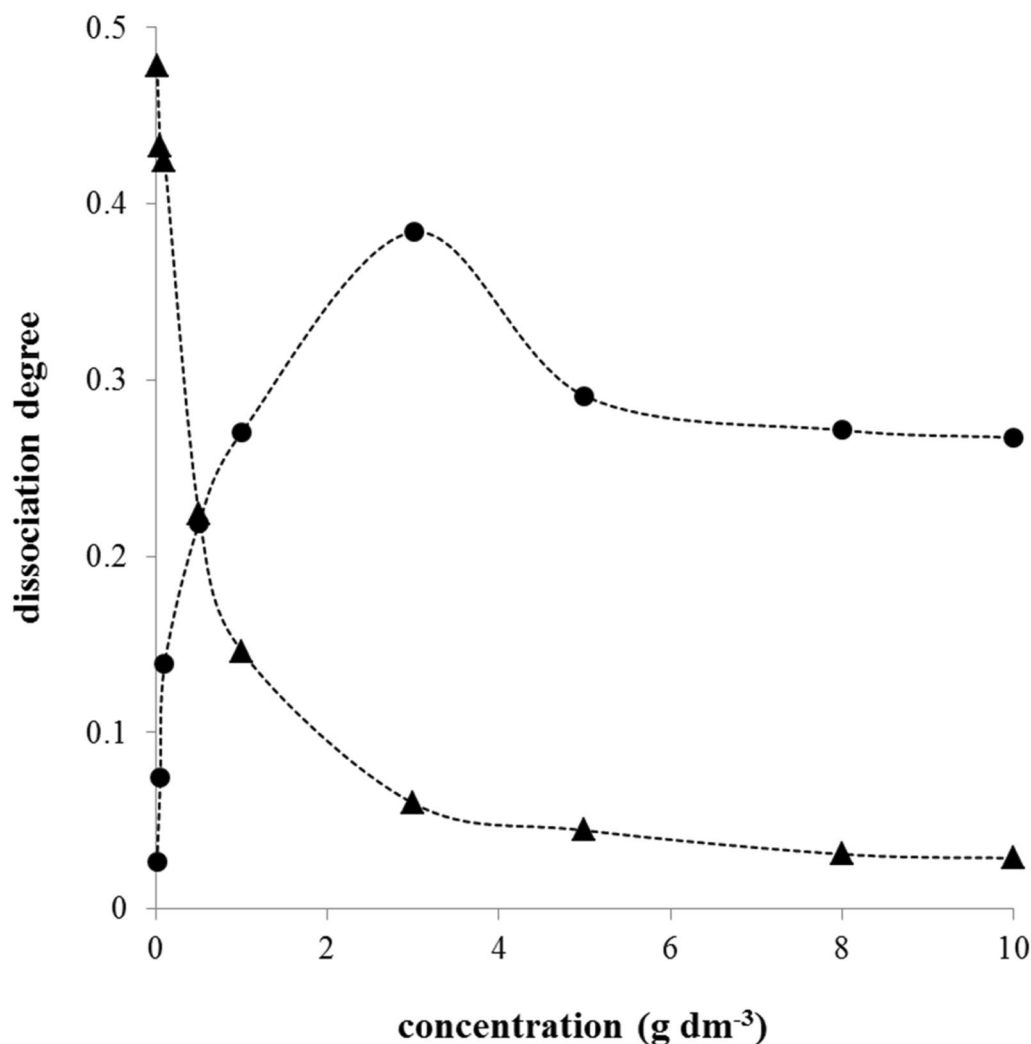f total amounts of acidic groups and dissociated functional groups is shown. While the concentration dependence of dissociated acidic functional groups in humic leachates had a linear character in logarithmical coordinates, that of total amounts of acidic functional groups could be divided into two (NLHA, ESHA, SRHA and PPHA) or three (LEHA) parts. However, for fulvic solutions, the concentration dependence of the total amount of acidic functional groups also had a linear character in logarithmical coordinates. The concentration of dissociated groups deviated slightly from a linear character and its growth slowed down for more concentrated systems as a result of a strong decrease in dissociation degree. These differences in dissociation behaviour were connected with the different solubilities of humic and fulvic acids, which were, in the case of humic acids, strongly influenced by the ratio between the total amount of humic particles and the amount of humic particles in the aqueous phase. The humic content in the leachates was thus given by the equilibrium between solid undissolved and dissolved humic molecules. The humic concentration in the leachate also affected the dissociation of dissolved humic fractions and their active coefficients.

The different behaviours of humic and fulvic acids could also be detected in virtue of the comparison of their dissociation degrees (see Fig. 3). The dissociation degree of fulvic acids decreased gradually with their increasing concentration similarly as in the case of simple organic acids. The concentration dependence of the dissociation degree for humic leachates was not the same as that for fulvic solutions. It was observed that the dissociation degree strongly increased with the increasing concentration of the dissolved humic fraction up to the initial humic content in suspension of around $1-3$ g dm^{-3} and then gradually decreased. A maximum of dissociation degree in this concentration region was observed for all studied humic samples.

Discussion

The amount of dissociable acidic groups in humic leachates was determined by means of the method of flow-through coulometry. It is well known that humic acids are only partially soluble in water and neutral aqueous solutions and therefore it is necessary to specify the amount of acidic functional groups which are contained in the dissolved humic fraction. The solubility of humic acids is affected by many factors such as pH, amount of humic acids per unit volume, ionic strength, and the content of acidic functional groups in humic acids and their strength and dissociation ability, etc. [30, 33]. The dissolved humic fraction is then the most active one, which can play a transport role in, for example,

Fig. 2 The dependence of the total content of acidic functional groups (circles) and dissociated acidic functional groups (triangles) for LEHA leachates (**a**) and SRFA solutions (**b**) on the initial concentration of humic and fulvic acids. The total content of acidic functional groups involved undissociated and dissociated acidic functional groups

Fig. 3 The dependence of dissociation degree on the concentration of NLFA (triangles) and LEHA (circles)–the initial concentration of suspension before centrifugation is used in the case of LEHA

soil solutions. In contrast, fulvic acids are fully soluble in water and aqueous solutions and the determined amounts of dissolved acidic groups should agree with their total amounts.

In this work, we used a mathematical model derived for monobasic acids [38, 39] and applied for the first time to humic acids in our previous works [31, 32]. In the case of highly diluted humic leachates or fulvic solutions, the activity of water can be considered as unity and the equilibrium dissociation constant K_a can be defined as

$$K_a = \frac{a_{H_3O^+} a_{A^-}}{a_{HA}} = \frac{c_{H_3O^+} c_{A^-}}{c_{HA}} \gamma_{\pm}^2, \quad (1)$$

where $a_{H_3O^+}, a_{A^-}$ and a_{HA} are the activities of H_3O^+ and A^- ions, and HA or FA molecules in equilibrium,

respectively. Similarly, $c_{H_3O^+}, c_{A^-}$ and c_{HA} are equilibrium concentrations of H_3O^+ and A^- ions, and HA or FA molecules. Activity coefficients of individual reaction components were replaced by square of mean activity coefficient $\gamma_{\pm}$ (activity coefficient of undissociated molecules was considered as unit) [38, 39]. The mathematical model derived in refs. [31, 32] resulted in Eq. (2):

$$\log K_c = \log K_a + 2 \log \gamma_{\pm} = \log K_a + 2A_c \sqrt{\alpha c_0}, \quad (2)$$

where K_c is the concentration dissociation constant, A_c is a constant characterizing used solvent at given temperature, α is the dissociation degree of acidic functional groups of humic and fulvic acids and c_0 is the total concentration of acidic functional groups in the humic leachate or fulvic solution. The concentration dissociation

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constant K_c is defined by means of equilibrium concentrations of reaction components. The product of K_c and square of mean activity coefficient $\gamma_\pm$ is equal to the equilibrium dissociation constant K_a [38, 39].

The dissociation degree α is the ratio between the amount of dissociated acidic functional groups (c_{A^-}) and the total amount of acidic functional groups contained in dissolved humic and fulvic acids (c_0):

$$\alpha = \frac{c_{A^-}}{c_0}. \tag{3}$$

Figure 4 shows an example of experimental data fitting for the NLFA sample. The dependence of log K_c on $\sqrt{\alpha}\, c_0$ obtained for fulvic solutions is linear only for higher concentrations (compare with Eq. 2). The dilution of fulvic solutions resulted in a significant decrease in values of log K_c, which achieved their minimum for

$c_0 \rightarrow 0$. The reason for this was the increase in dissociation degree (see Fig. 3) with dilution being connected with an increase in the activity coefficients. Surprisingly, the data fitting according to Eq. 2 seemed to be the same for humic leachates (not shown, please see ref. [31]). However, the reason for such data fitting was different when compared with that for fulvic solutions. The total contents of dissolved humic fractions in leachates changed according to the initial contents of humic acids in suspensions. The increase in humic concentrations in leachates was necessarily connected with changes in the amount and composition of the dissolved particles and the content and strength of acidic functional groups. When the equilibrium between dissolved particles and undissolved residue was attained different humic fraction were divided between dissolved and undissolved particles according to their
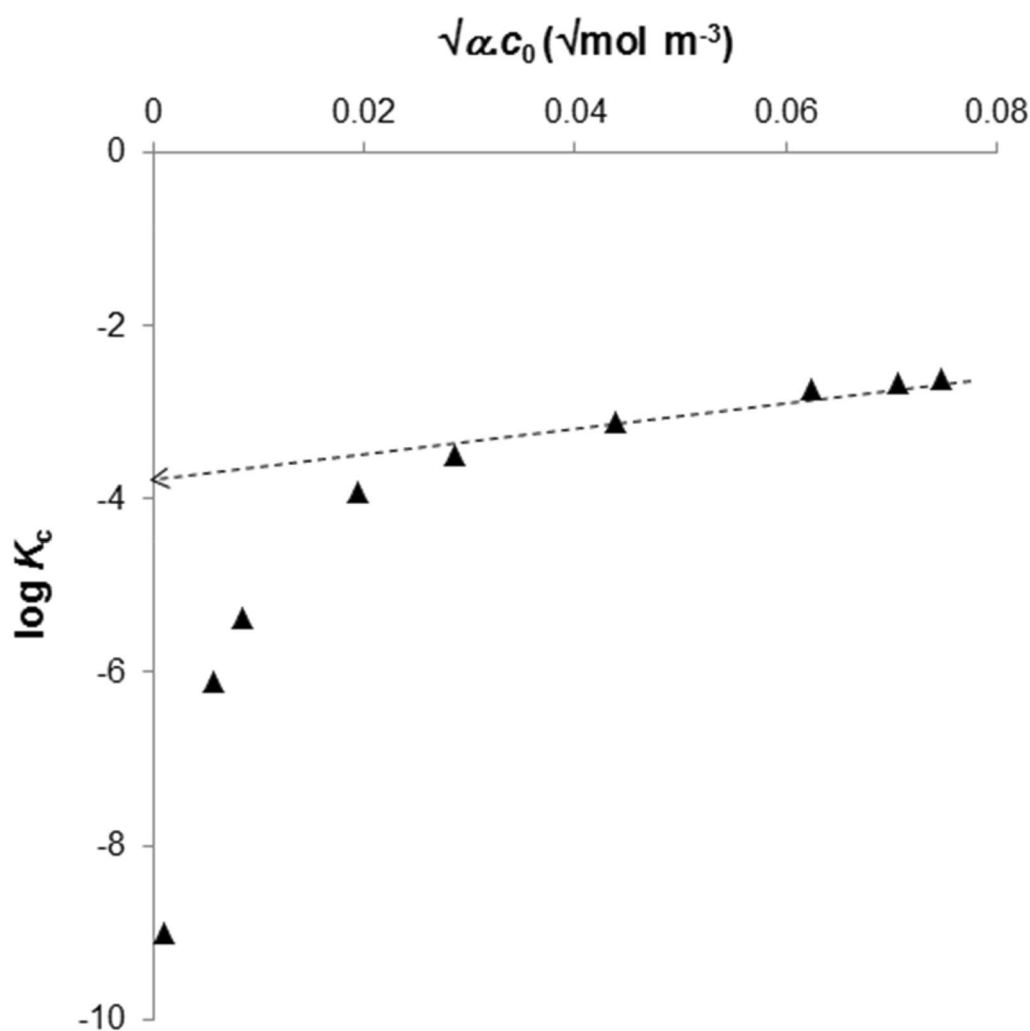
Fig. 4 An example of data fitting by Eq. (2) for the NLFA sample

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acidic functional groups. In general, humic fractions containing more functional groups with higher dissociation ability can be easily dissolved. In contrast, humic fractions low in acidic functional groups can be difficult to dissolve [4, 10, 24, 25, 31, 31, 32, 34, 35]. The dissociation ability of acidic functional groups of humic acids is closely associated with their solubility. If the ratio between solid humic acids and water is low, humic fractions containing weaker functional groups can be dissolved. In contrast, higher ratios can suppress the solubility of fractions less rich in dissociable functional groups. Therefore, the chemical structure of dissolved humic particles is dependent on the ratio between solid humic particles and water which are equilibrated [10, 31].

As described above, the concentration dependence of the dissociation degree for humic leachates was not the same as that for fulvic solutions. In contrast to fulvic acids a maximum was observed in the concentration dependence of dissociation degree of humic acids. An explanation of the observed maximum can be based on the following hypothesis. The solubility and dissociation of humic acids is strongly affected by the strength of their acidic functional groups. Therefore humic molecules containing weaker functional groups can dissolve more easily in systems with low ratio between humic acids and water (aqueous solution). In contrast, highly concentrated systems contain mainly humic molecules rich in strong acidic functional groups. We suppose that the observed maximum is therefore caused by two opposite effects. If the concentration of humic leachate is low, less soluble humic particles can be dissolved due to the fact that the leachate is little saturated. It means that the concentration of humic acids in leachate is low and the fractions with lover contents of acidic functional groups and that the groups have lower dissociation ability. Therefore, the functional groups can remain as non-dissociated (macro)molecules. In more concentrated systems, humic acids containing worse dissociable functional groups can remain as undissolved which results in the increase in dissociation degree up to the maximum, because corresponding humic leachate contains mainly humic (macro)molecules rich in strong functional groups. In highly concentrated humic leachates, their dissolved particles can interact which can cause the decrease in their activity coefficients and also in their dissociation degree. The differences observed between dissociation of humic and fulvic acids can be connected by other factors. One of them can be their difference in molecular organization of humic and fulvic acids [12, 25, 26, 36, 40]. In general, supramolecular associations and macromolecules can co-exist in the structure of humic acids [41–43] and a supramolecular arrangement is usually proposed for fulvic acids [44]. Nuzzo and Piccolo [40] indicated the possibility of increasing the molecular size of supramolecular structures via oxidative polymerization and formation of new covalent bonds, chemically stabilizing humic substances. The differences in molecular organization of humic and fulvic acids can co-affect their dissociation behaviour and support the dissociation ability of humic acids in the concentration range where maximum of dissociation degree was observed.

Figure 5 shows the dependence of pK_c on the centration of LEHA. As can be seen, the dependence of pK_c on concentration decreases with the increase in humic content and the dissociation constants of highly concentrated systems are practically constant. These constant values of $pK_{a,2}$ were much lower in the case of fulvic acids (see also Table 1) as a result of higher contents of stronger acidic functional groups. On the other hand, the values of pK_{a1} obtained by extrapolation to $c_0 \rightarrow 0$ were lower for humic leachates. It resulted in the conclusion that highly diluted humic leachates contained in average stronger acidic groups in comparison with fulvic acids. It seems that this finding is contrary to the character and properties of humic and fulvic acids; however, it is, in fact, logical. The reason lies in the different solubilities of these two groups of humic substances. While fulvic acids are totally soluble and all their acidic functional groups including the weakest ones are dissolved in fulvic solutions, humic particles containing the weakest functional groups can remain in solid undissolved form, which can apparently improve the dissociation ability of humic acids when compared with fulvic ones. It means that humic leachates based on humic suspensions with different contents of humic acids contained not only different amounts of dissolved humic fractions, but also dissolved humic acids with different structure and composition of functional groups, and also with different molecular arrangement affecting their dissociation behaviour. Therefore, values determined for the dissociation constants are valid for the active dissolved fractions (at given circumstances) in the case of humic acids and for the whole sample in the case of fulvic acids.

Conclusions

In this work, flow-through coulometry was used to characterize the dissociation behaviour of dissolved organic carbon—fulvic solutions and humic leachates. While whole samples of fulvic acids including the weakest functional groups were analysed, only the active dissolved humic fractions containing stronger acidic functional groups were characterized. The fractions containing

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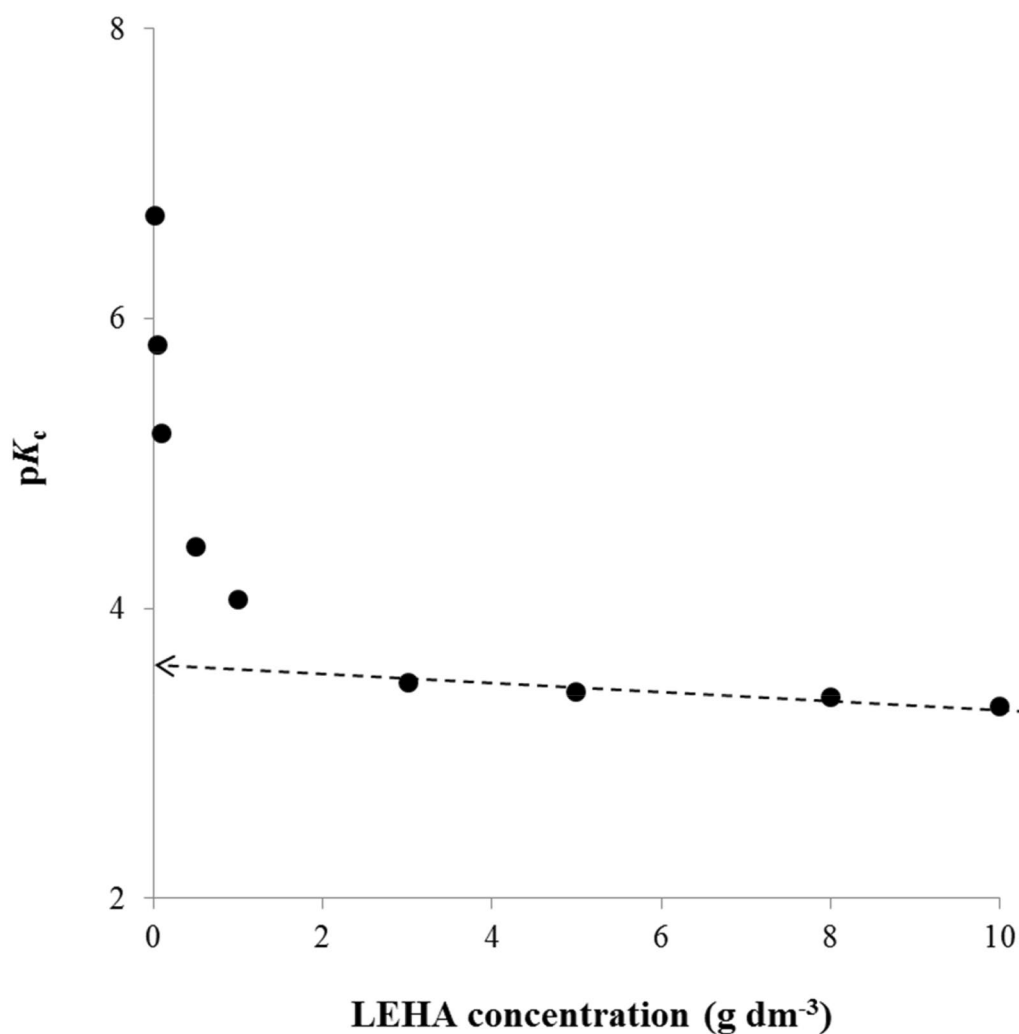
Fig. 5 The dependence of pK_c values on the concentration of LEHA (the initial concentration of suspension before centrifugation)

higher amounts of the weakest functional groups remained insoluble. Obtained data resulted in two different values of dissociation constant which can characterize acid–base behaviour of dissolved organic carbon in dependence on its concentration. This work thus characterizes the functioning of dissolved organic carbon from point of view of the dissociation ability. The results obtained by this approach can help to predict the acid–base behaviour of dissolved organic carbon in soil and generally in nature. The acid–base behaviour of dissolved organic carbon depends on its ratio to water. It behaves differently in soil which is dry and in soil which is wet or saturated by water.

Table 1 Values of p$K_{a,1}$ and p$K_{a,2}$

Sample	p$K_{a,1}$	p$K_{a,2}$
NLHA	6.18 ± 0.07	3.35 ± 0.08
ESHA	6.46 ± 0.04	3.48 ± 0.09
SRHA	6.32 ± 0.12	3.78 ± 0.15
PPHA	6.52 ± 0.06	3.41 ± 0.07
LEHA	7.13 ± 0.10	3.89 ± 0.05
NLFA	9.98 ± 0.12	3.82 ± 0.10
ESFA	9.86 ± 0.08	3.03 ± 0.08
SRFA	9.21 ± 0.07	3.08 ± 0.04
PPFA	8.92 ± 0.05	3.05 ± 0.02

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Experimental

Materials

Humic substances

Samples of humic and fulvic acids were purchased from the International Humic Substances Society. Samples of Nordic lake humic acids (NLHA), Elliot soil humic acids (ESHA), Suwannee river humic acids (SRHA), Pahokee peat humic acids (PPHA), Leonardite humic acids (LEHA), Nordic lake fulvic acids (NLFA), Elliot soil fulvic acids (ESFA), Suwannee river fulvic acids (SRFA), and Pahokee peat fulvic acids (PPFA) were used in this study. The main characteristics such as elemental composition or the contents and properties of acidic functional groups can be found on the website of the International Humic Substances Society (http://humic-substances.org/acidic-functional-groups-of-ihss-samples/).

Fulvic solutions and humic leachates

Fulvic acids were dissolved in a 1.5% solution of Na_2SO_4 (the background electrolyte for the flow-through coulometry) in order to achieve concentrations from 0.01 g dm^{-3} to 10 g.dm^{-3}. The solutions were stirred for 24 h. up to total dissolution. Suspensions of humic acids with humic contents from 0.01 g.dm^{-3} to 10 g.dm^{-3} were prepared by mixing powdered samples with a 1.5% solution of Na_2SO_4. The suspensions were stirred for 24 h in order to achieve equilibrium between the dissolved and undissolved humic fractions. Equilibrated suspensions were centrifuged in order to remove the solid humic residue and the obtained leachates (contained only dissolved fractions of humic acids) were used for the determination of pK_a.

Methods

Study of dissociation behaviour of humic substances

In order to investigate the dissociation behaviour of the fulvic acids and the dissolved humic fractions, the total concentrations of fulvic acids in the solutions and of humic acids in the leachates and their dissociated portions were determined by means of flow-through coulometer EcaFlow 150 GLP [30, 33]. This method is based on direct electrochemical conversion of the analyte species in the pores of the electrode or in a thin layer. At a suitable potential, the working electrode is filled with the sample solution and the H^+ ions splitting-off from acidic groups are reduced galvanostatically to hydrogen and thus neutralized. In this step, the signal (a chronopotentiogram) is recorded and the acid content in the sample is evaluated. The concentrations of dissociated acidic functional groups were calculated on the basis of pH-measurements (Mettler Toledo Seven Easy).

References
1. Kimuro S, Kirishima A, Sato N. Determination of the protonation enthalpy of humic acid by calorimetric titration technique. J Chem Thermodynamics. 2015;82:1–8. https://doi.org/10.1016/j.jct.2014.10.010.
2. Xu P, Wang Y, Li X, Chen Q, Hao L, Zhang J, Zhu X, Jia B. An acidic-groups detection method and its application to analysis of Chinese humic acid samples. PLoS ONE. 2020;15(8):e0238061. https://doi.org/10.1371/journal.pone.0238061.
3. Badr MH, El-Halafawi MH, Abd El-al Zeid ER. Comparison between the effect of ionic strength on acidity and dissociation constants of humic acids extracted from sewage sludge and Nile water hyacinth composts. Global J. Environ. Res. 2012;6(1):36—43. https://doi.org/10.5829/idosi.gjer.2012.6.1.64117
4. Klučáková M. Complexation of metal ions with solid humic acids, humic colloidal solutions, and humic hydrogel. Environ Eng Sci. 2014;31(11):612–20. https://doi.org/10.1089/ees.2013.0487.
5. Kalina M, Klučáková M, Sedláček P. Utilization of fractional extraction for characterization of the interactions between humic acids and metals. Geoderma. 2013;207–20:92–8. https://doi.org/10.1016/j.geoderma.2013.04.031.
6. Tan WF, Norde W, Koopal LK. Humic substance charge determination by titration with a flexible cationic polyelectrolyte. Geochim Cosmochim Acta. 2011;75(19):5749–61. https://doi.org/10.1016/j.gca.2011.07.015.
7. Sedláček P, Klučáková M. Simple diffusion method applied in evaluation of metal transport in model humic matrices. Geoderma. 2009;153(1–2):286–92. https://doi.org/10.1016/j.geoderma.2009.07.002.

8. Campitelli PA, Velasco MI, Ceppi SB. Chemical and physicochemical characteristics of humic acids extracted from compost, soil and amended soil. Talanta. 2006;69(5):1234–9. https://doi.org/10.1016/j.talanta.2005.12.048.

9. Enev V, Pospíšilová L, Klučáková M, Liptaj T, Doskočil L. Spectral characterization of selected natural humic substances. Soil Water Res. 2014;9(1):9–17. https://doi.org/10.17221/39/2013-SWR.

10. Klučáková M, Kolajová R. Dissociation ability of humic acids: Spectroscopic determination of pKa and comparison with multi-step mechanism. React Funct Polym. 2014;78:1–6. https://doi.org/10.1016/j.reactfunctpolym.2014.02.005.

11. Ritchie JD, Perdue M. Proton-binding study of standard and reference fulvic acids, humic acids, and natural organic matter. Geochim Cosmochim Acta. 2003;67(1):85–96. https://doi.org/10.1016/S0016-7037(02)01044-X.

12. Atalay ZB, Carbonaro RF, Di Toro DM. Distribution of proton dissociation constants for model humic and fulvic acid molecules. Environ Sci Technol. 2009;43(10):3626–31. https://doi.org/10.1021/es803057r.

13. Crea F, De Stefano C, Gianguzza A, Pettignano A, Piazzese D, Sammartano S. Acid-base properties of synthetic and natural polyelectrolytes: Experimental results and models for the dependence on different aqueous media. J Chem Eng Data. 2009;54(2):589–605. https://doi.org/10.1021/je800518j.

14. Klučáková M, Pekař M. Study of diffusion of metal cations in humic gels. In: Ghabbour E, Davies G, editors. Humic substances: Nature`s most versatile materials. New York: Taylor & Francis; 2004. p. 263–73.

15. Palladino G, Ferri D, Manfredi C, Vasca E. Potentiometric determination of the total acidity of humic acids by constant-current coulometer. Anal Chim Acta. 2007;582(1):164–73. https://doi.org/10.1016/j.aca.2006.08.060.

16. Giachin G, Nepravishta R, Mandaliti W, Melino S, Margon A, Scaini D, Mazzei P, Piccolo A, Legname G, Paci M, Leita L. The mechanisms of humic substances self-assembly with biological molecules: The case study of the prion protein. PLoS ONE. 2017;14:e0213673.

17. Cao X, Drosos M, Leenheer JA, Mao J. Secondary structures in a freeze-dried lignite humic acid fraction caused by hydrogen-bonding of acidic protons with aromatic rings. Environ Sci Technol. 2016;50:1663–9.

18. Conte P, Piccolo A. Conformational arrangement of dissolved humic substances. Influence of solution composition on association of humic molecules. Environ Sci Technol. 1999;33:1682–90.

19. Piccolo A. The supramolecular structure of humic substances. Soil Sci. 2001;166:810–32.

20. Nebbioso A, Piccolo A. Molecular characterization of dissolved organic matter (DOM): a critical review. Anal Bioanal Chem. 2013;405:109–24. https://doi.org/10.1007/s00216-012-6363-2.

21. Andjelkovic T, Perovic J, Purenovic M, Blagojevic S, Nikolic R, Andjelkovic D, Bojic A. A direct potentiometric titration study of the dissociation of humic acid with selectively blocked functional groups. Eclét Quím. 2006;31(3):39–46. https://doi.org/10.1590/S0100-46702006000300005.

22. Du J, Sato N, Tochiyama O. Potentiometric study on the proton binding of humic substances. J Nucl Radiochem Sci. 2005;6(1):25–9. https://doi.org/10.14494/jnrs2000.6.25.

23. Porasso RD, Benegas JC, van den Hoop MAGT, Paoletti S. Analysis of potentiometric titrations of heterogeneous natural polyelectrolytes in terms of counterion condensation theory: application to humic acid. Biophys Chem. 2000;86(1):59–69. https://doi.org/10.1016/S0301-4622(00)00159-9.

24. Fukushima M, Tanaka S, Hasebe K, Taga M, Nakamura H. Interpretation of the acid-base-equilibrium of humic acid by a continuous pK distribution and electrostatic model. Anal Chim Acta. 1995;302(2–3):365–73. https://doi.org/10.1016/0003-2670(94)00471-W.

25. Fukushima M, Tanaka S, Nakamura H, Ito S. Acid-base characterization of molecular weight fractionated humic acid. Talanta. 1996;43(3):383–90. https://doi.org/10.1016/0039-9140(95)01727-5.

26. Garcia-Mina JM. Advantages and limitations of the use of an extended polyelectrolyte model to describe the proton-binding process in macromolecular systems. Application to a poly(acrylic acid) and a humic acid. J Phys Chem B. 2007;111(17):4488–94. https://doi.org/10.1021/jp0689518.

27. Fernandes AM, Giacomelli C, Giovanela M, Vaz DO, Szpoganicz B, Sierra MMD. Potentiometric acidity determination in humic substances influenced by different analytical procedures. J Brazil Chem Soc. 2009;20(9):1715–23. https://doi.org/10.1590/S0103-50532009000900021.

28. Khil'ko SL, Kovtun AI, Rybachenko VI. Potentiometric titration of humic acids. Solid Fuel Chem. 2011;45(5):337–48. https://doi.org/10.3103/S0361521911050028.

29. Salma I, Ocskay R, Lang GG. Properties of atmospheric humic-like substances-water system. Atmos Chem Phys. 2008;8:2243–54. https://doi.org/10.5194/acp-8-2243-2008.

30. Salma I, Lang GG. How many carboxyl groups does an average molecule of humic-like substances contain? Atmos Chem Phys. 2008;8:5997–6002. https://doi.org/10.5194/acp-8-5997-2008.

31. Klučáková M. Dissociation properties and behavior of active humic fractions dissolved in aqueous systems. React Funct Polym. 2016;109:9–14. https://doi.org/10.1016/j.reactfunctpolym.2016.09.004.

32. Klučáková M. Conductometric study of the dissociation behavior of humic and fulvic acids. React Funct Polym. 2018;128:24–8. https://doi.org/10.1016/j.reactfunctpolym.2018.04.017.

33. Orlović-Leko P, Vidović K, Plavšić M, Ciglenečki I, Šimunić I, Minkina T. Voltammetry as a tool for rough and rapid characterization of dissolved organic matter in the drainage water of hydroameliorated agricultural areas in Croatia. J Solid State Electr. 2016;20:3097–105. https://doi.org/10.1007/s10008-016-3245-0.

34. Klučáková M. Adsorption of nitrate on humic acids studied by flow-through coulometry. Environ Chem Lett. 2010;8:145–8. https://doi.org/10.1007/s10311-009-0201-6.

35. Ivanov AA, Maltseva EV, Yudina NV. Composition of fulvic acids after the mechanical activation of peats. Solid Fuel Chem. 2016;50:7–11. https://doi.org/10.3103/S0361521916010055.

36. Klučáková M. Characterization of pH-fractionated humic acids with respect to their dissociation behaviour. Environ Sci Pollut R. 2016;23:7722–31. https://doi.org/10.1007/s11356-015-5932-2.

37. Haynes WM. CRC Handbook of chemistry and physics. 93rd ed. Boca Raton: CRC Press; 2012.

38. Monk PMS. Physical chemistry: Understanding our chemical world. 1st ed. Chichester: JohnWilley & Sons, Ltd.; 2004.

39. Moore WJ. Physical chemistry. 5th ed. New York: Longman Publishing Group; 1998.

40. Nuzzo A, Piccolo A. Oxidative and Photo-oxidative polymerization of humic supra-structures by heterogeneous biomimetic catalysis. Biomacromol. 2013;14:1645–52. https://doi.org/10.1021/bm400300mF.

41. Tarasevich Y, Dolenko SA, Trifonova MY, Alekseenko EY. Association and colloid-chemical properties of humic acids in aqueous solutions. Colloid J. 2013;75:207–13. https://doi.org/10.1134/S1061933X13020166.

42. Baigorri R, Fuentes M, Gonyaley-Gaitano G, Garcia-Mina JM. Simultaneous presence of diverse molecular pattern in humic substances in solution. J Phys Chem B. 2007;111(35):10577–82. https://doi.org/10.1021/jp0738154.

43. Trubetskoi OA, Trubetskaya OE. Reversed-phase high-performance liquid chromatography of the stable electrophoretic fractions of soil humic acids. Eurasian Soil Sci. 2015;48:148–56. https://doi.org/10.1134/S106422931502012X.

44. Fleury G, Nero MD, Barillon R. Molecular fractionation of a soil fulvic acid (FA) and competitive sorption of trace metals (Cu, Zn, Cd, Pb) in hematite–solution systems: effect of the FA-to-mineral ratio. RCS Advances. 2017;7:43090–103. https://doi.org/10.1039/C7RA06838G.

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