

Environmental Research Advances

Humic Substances and Natural Organic Matter

Laurence Bates
Editor

NOVA

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HUMIC SUBSTANCES AND NATURAL ORGANIC MATTER

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LAURENCE BATES
EDITOR



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PREFACE

Humic acids (HA) make up an important component of soil humus related to the maintenance of soil water-holding capacity, stabilization of soil structure and fertility and vital activity of soil micro-organisms, plants and animals. Additionally, obtaining humic substances (HS) from vermicompost has shown to be a promising alternative for large scale use in agriculture. Humic substances (HS) are also major components of natural organic matter (NOM) in soil and water as well as in geological organic deposits. This book includes chapters on vermicompost-derived liquid humus in low-input and small-scale farming; the production of liquid organic fertilizers enriched with humic substances from olive mill wastes; mechanisms of protective action of the HUMI preparation on wheat plant response to toxic ions; the application of surface-enhanced Raman scattering and fluorescence spectroscopy on silver plasmonic nanoparticles as innovative techniques to study humic substances; the complexation of metal ions with humus substances in soils and water and the final chapter examines the natural organic matter in drinking water.

Chapter 1 – Obtaining humic substances (HS) from vermicompost has shown to be a promising alternative for large scale use in agriculture. Part of the experience accumulated during several years of research on the use of liquid humus as an agricultural alternative is discussed in this chapter. Liquid humus obtained from vermicompost have in their composition, significant amounts of fulvic and humic acids; as well as amino acids, plant hormones, minerals, bacteria and fungi. Foliar application of liquid humus on crops has been shown to increase yields considerably, being a viable alternative for low-input small-scale producers. The accumulated experiences show that foliar applications of liquid humus promote increases in productivity of several crops such as corn, cucumber, cabbage, radish, tomato and improves preservation and conservation of flowers with ornamental purposes. This implies increases in farmers' income, primarily due to reduced use of industrial chemical fertilizers. The experiments shown in this chapter are intended to inform potential uses of these humified liquids products and aims to contribute to the small scale agriculture, organic and urban agriculture as well as to reduce the consumption of chemical fertilizers.

Chapter 2 – The introduction and extended use of chemical fertilizers has been a determinant factor in the development of modern intensive agriculture. However, in recent years, interest has grown in agriculture in the possibility of recycling organic residues and wastes derived from a variety of human activities. Amendment with non-toxic organic waste is absolutely necessary to improve soil fertility and crop production. The use of organic waste and residues of any nature for soil amending requires appropriate treatment to eliminate or, at least, reduce any compounds that may have harmful effects on soil properties and to optimize

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the efficiency and the fertility of the final product once in the soil. The process of composting is generally considered as the most efficient treatment for producing environmentally safe and agronomically advantageous organic waste amendments for soil. Composting is an accelerated version of the decomposition processes naturally occurring in the soil. The humification process typically occurring under natural soil conditions transforms the organic material into a stable and mature humic like end-product. The close relationship between the humified organic matter content in soil and its fertility explains the persistent interest in studying humic substances. Much work has been carried out concerning the use of organic waste in soils either as solid fertilizers or amendments. But, little information is available on the production of liquid organic fertilizers. In this objective, in order to release commercial liquid organic fertilisers from Alperujo (olive mill wastes) composts, several extraction conditions (extraction time, extraction temperature, heat, time applied, extraction ratio, extractant agent and alkali concentration) were tested. The obtained results showed that extracted humic-like substances presented characteristics close to those of standard humic materials, confirming that the hot alkaline extraction dissolved more polymerized compounds, probably including lignin degradation by-products and/or newly formed aromatic materials.

As a consequence, the alkaline extraction of Alperujo or olive mill wastes compost with 1M KOH at 70°C during 24H can be used to produce liquid organic fertilizers, thus diversifying the marketable products obtained from Alperujo, the most abundant by-product of the Spanish olive-oil extraction industry.

Chapter 3 – Humic acids (HA) make up an important component of soil humus related to maintenance of soil water-holding capacity, stabilization of soil structure and fertility and vital activity of soil micro-organisms, plants and animals. At present a wealth of information has become available on the effectiveness of HA and HA-based preparations for growth stimulation and improvement of resistance and productivity of different plant species. The authors have conducted a comprehensive analysis of growth-stimulating and protective effects of HUMI preparation (NVP "BashInkom, Ufa) on wheat plants. In these experiments the authors have used presowing treatment of wheat (*Triticum aestivum* L., cv. Bashkirskaya 24) with HUMI (0.3 mg/g of seeds). The treatment was shown to promote seedling growth. This effect is likely to be due to the HUMI-induced alterations in the plant hormonal system associated with a 1.5-fold persistent accumulation of cytokinins without any changes in the contents of IAA and ABA. The protective effect of HUMI on wheat seedlings exposed to the toxic effects of sodium chloride and cadmium acetate manifested in preventing stress-induced decline in cytokinin content and in reduction of both ABA accumulation and decline in the IAA content. These shifts in the hormonal balance led to decreased damaging stress effects on plant growth and to acceleration of the growth processes recovery during the post-stress period. Lignin deposition in root cell walls is known to promote strengthening of their barrier properties thereby reducing penetration of toxic ions into the plants. HUMI increased lignin deposition in root cell walls of basal regions of seedlings exposed to toxic ions, indicating protective effect of this regulator. Since cytokinins have been shown to increase lignification, this allows us to suggest that accelerated lignification in stressed plants pretreated with HUMI is due to higher cytokinin content in these seedlings than in untreated plants. Therefore, the ability of HUMI to induce accumulation of cytokinins and maintain their high level in plants exposed to toxic ions is important for establishment of growth-stimulating and protective action of this natural growth regulator on wheat plants.

Chapter 4 – The application of normal Raman spectroscopy is not able to characterize the soil humic substances (HS) structure due to the intense fluorescence emission, which overlaps the Raman bands. Nevertheless, surface-enhanced Raman scattering (SERS) spectroscopy, based on localized surface plasmon (LSP) in nanostructured metals, leads to a remarkable local electromagnetic field enhancement and to the fluorescence quenching via a charge-transfer mechanism.

The SERS technique was thus applied to investigate the structure and conformational changing of HS. However, the fluorescence is not quenched if the distance between fluorophores and LPS is relatively far. An intensification of the fluorescence emission through the surface-enhanced fluorescence (SEF) at optimal distance above 10–100 Å can be detected.

At distances further than this value, SERS and SEF signals are simultaneously emitted and recorded for molecular species placed in the vicinity of metal nanoparticles. Since the average size of HS is larger than this value, the fluorophores included in the HS structure are valid candidates causing intense SEF *plus* SERS joint emission spectra, without the intrinsic quenching observed on a surface.

In the present chapter, surface-enhanced Raman scattering and fluorescence spectroscopy on silver plasmonic nanoparticles are applied to investigate the HS structure in aqueous medium at different pH.

Chapter 5 – The chapter describe the complexation of metal ions with humus substances in soils and water. Humus substances as the major biochemical components of the soil and water have a significant impact on the forms and migration of metal and the toxicity of natural objects. In this chapter presents the results of large-scale chemical experiments: the study of the structural features (zonal aspects) of humus substances extracted from soil and water natural climatic zones (more than 300 objects) of Russia (European Russia and West Siberia); the influence of structural features on the physic-chemical parameters of humus acids and, in particular, on their complexing ability. The functional specifics of humus matter extracted from soils are estimated using spectrometric techniques. Conditional stability constants for Fe(III), Cu(II), Pb(II), Cd(II), Zn(II), Ni(II), Co(II), Mn(II), Cr(III), Ca(II), Mg(II), Sr(II), and Al(III) are experimentally determined with used electrochemical, spectroscopic analysis methods. The activities of metals are classified according to their affinity to humus compounds in soils and water. The determined conditional stability constants of the complexes are tested in model experiments, and it is demonstrated that Fe and Al ions have higher conditional stability constants than ions of alkali earth metals, Pb, Cu, and Zn. Furthermore, the influence of aluminum ions and iron on complexation of copper and lead as influence of lead and copper on complexation of cobalt and nickel have been identified. The metal forms a large number of lakes are calculated based on the results of the experiments. The forms of finding metals in more than 900 lakes in Russia were analyzed with used of zonal features of humus substances. This chapter describes the main chemical mechanisms of distribution of metals in forms in water lakes of European Russia and West Siberia.

Chapter 6 – In this work, vermicompost, which is one the most important humified substrates, was employed for decontaminating aqueous media enriched with Cd(II), Cu(II), Pb(II) and Zn(II), as well as three synthetic dyes (Congo red - CR, malachite green – MG and methanile yellow - MY). Besides evaluating the efficiency of metallic ion removal, this investigation also considered the influence of different sources of production on the

adsorptive properties of vermicompost. In this regard, samples were collected from four Brazilian States (Bahia, Espírito Santo, Minas Gerais and São Paulo) and characterized by X-ray diffraction, infrared molecular absorption spectrophotometry, thermogravimetry and electron microscopy in order to obtain information about mineralogical composition and presence of organic functional groups as well as morphological and structural aspects. Subsequently, the different vermicomposts were used to withdraw Cd(II), Cu(II), Pb(II) and Zn(II) from mixed solutions or CR, MG and MY from individual solutions. Parameters such as vermicompost mass, the pH of the mixed solutions, and the time of contact of these solutions with vermicompost were varied in order to obtain satisfactory adsorption responses. For each experimental design, the remaining quantities of metallic cations and dyes were determined, respectively, by flame atomic absorption spectrometry and visible molecular absorption spectrophotometry. Analyses of the characterization pointed to discrete differences among the different samples of vermicompost, including aspects related to their mineral and organic composition. Independent of the source, vermicomposts contributed large numbers of carboxyl groups ($-\text{COOH}$). Besides the carboxyl, all samples of vermicompost also exhibited large analytical signals associated with Al-O bonds. In turn, thermogravimetric analyses revealed accentuated percentages of volatile components in accordance with the expected composition of composted materials, while electron microscopy and X-ray diffraction highlighted, respectively, the complex disposition of many porous and mineral components. The results pointed to very efficient retentions ($> 70\%$) of all metallic analytes after 1 minute of dynamic contact among the solutions and all samples of vermicompost, thus evidencing predominantly reversible adsorptions. Specifically for Pb(II), adsorptions greater than 90% were observed. Nevertheless, depending on the source of the vermicompost, it was possible to identify a preferential order concerning the retention of Cd(II), Cu(II), Pb(II) and Zn(II). Based on criteria associated with the efficiency of retention, vermicompost samples from Bahia and Minas Gerais States were selected to build adsorption isotherms (individual solutions) with subsequent mathematical treatment of the data according to the Langmuir and Freundlich models. The following maximum adsorptive capacities (mg g^{-1}) were obtained: 0.4 (Cu) to 3.4 (Zn) for vermicompost from Bahia, and 0.5 (Pb) to 22.1 (Cu), for vermicompost from Minas Gerais. In turn, adsorptive tests with synthetic dyes were performed only with the vermicompost from Bahia and always employing individual solutions. The results indicated effective retentions ($> 90\%$) after 1 minute of mechanical agitation for all dyes. The best pH for adsorption of CR and MY was 2, while pH 6 yielded the greatest adsorption of MG. With vermicompost from Bahia State, adsorption isotherms indicated maximum adsorptive capacities up to 45.9 mg g^{-1} .

Chapter 7 – In order to improve project and operation of drinking water facilities is important to be aware about the kind of organic matter present. Various physical and chemical fractionation techniques have been developed, mainly focused in the identification of the most reactive components in dissolved organic matter that originate harmful disinfection by-products. These fractionation techniques include resin fractionation, membrane filtration (ultrafiltration, nanofiltration and reverse osmosis), size exclusion chromatography/high performance size exclusion chromatography and reverse phase high performance liquid chromatography. Several methods have been used for characterization of natural organic matter (NOM) fractions, namely total and dissolved organic carbon, ultraviolet-visible spectrometry, fluorescence, Fourier transform infrared spectroscopy, liquid chromatography coupled with different detectors, nuclear magnetic resonance spectroscopy,

gas chromatography–mass spectrometry (after pyrolysis or derivatization with Tetramethylammonium hydroxide) and biological methods for quantification of biodegradable organic matter.

In this chapter the more relevant contributions of the last ten years to the current knowledge regarding fractionation and characterization of NOM are discussed together with their advantages and limitations. Due to the complexity and variability of NOM there must be a special concern in planning water sampling due to seasonal and local variation of water characteristics, in order to get representative samples and take consistent conclusions about NOM nature. The best way to overcome the limitations identified in the methods of fractionation and characterization of NOM is their combination. Knowledge of molecular properties of NOM is decisive to understand and improve drinking water treatment processes.

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Chapter 1

VERMICOMPOST-DERIVED LIQUID HUMUS IN LOW- INPUT AND SMALL-SCALE FARMING

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ABSTRACT

Obtaining humic substances (HS) from vermicompost has shown to be a promising alternative for large scale use in agriculture. Part of the experience accumulated during several years of research on the use of liquid humus as an agricultural alternative is discussed in this chapter. Liquid humus obtained from vermicompost have in their composition, significant amounts of fulvic and humic acids; as well as amino acids, plant hormones, minerals, bacteria and fungi. Foliar application of liquid humus on crops has been shown to increase yields considerably, being a viable alternative for low-input small-scale producers. The accumulated experiences show that foliar applications of liquid humus promote increases in productivity of several crops such as corn, cucumber, cabbage, radish, tomato and improves preservation and conservation of flowers with ornamental purposes. This implies increases in farmers' income, primarily due to reduced use of industrial chemical fertilizers. The experiments shown in this chapter are intended to inform potential uses of these humified liquids products and aims to contribute to the small scale agriculture, organic and urban agriculture as well as to reduce the consumption of chemical fertilizers.

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1. INTRODUCTION

The use of humic materials and humic extracts in agriculture has increased in recent years (IFOAM). Although the production yields may be lower (20-25%) in organic than in conventional agriculture (Seufert et al. 2012, De Ponti et al. 2012), organic, urban, family and low-cost agriculture are still important to ensure the production and accessibility of healthy food for the populations of less developed regions (Badgley et al. 2007).

Vermicomposts (VCs) are natural products widely used in organic agriculture, both in the form of crude compost and as liquid humic extracts (liquid humus) (García et al. 2014). These materials have effects on production yields and plant growth and development and improve the chemical and physical soil conditions (Solis et al. 2012; Oo et al. 2013; Najar and Khan, 2013). In addition, humic substances (HSs) in VCs have positive effects on plant metabolism (Berbara and García, 2014). These substances are known to improve root metabolism by increasing the emission of lateral roots (Canellas and Olivares 2014) and to increase resistance to water stress (Garcia et al, 2012).

Important studies have been performed regarding the use of HSs as complexing agents. A study by García-Mina et al. (2004) showed the presence in alfalfa plants (*Medicago sativa* L.) of Zn, Cu and Fe complexes and HSs, which significantly increase the absorption of nutrients. Guardado et al. (2007) and Urrutia et al. (2014) demonstrated the potential of phosphorus-humus complexes for the protection of this element in the soil as well as for increasing its availability for plant nutrition.

Much progress has been made regarding the modes of action of HSs in plants, and this progress helps to explain the different effects that these substances exert on plants. Recent studies recognize that these effects of HSs in plants are exercised through hormone actions (auxin-AIA, ascorbic acid-ABA, cytokinins-CK and ethylene), along with the simultaneous involvement of additional metabolic pathways, such as those involving nitrogen (N), sulfur (S), carbon (C) and antioxidant metabolism, as well as H⁺-ATPase activity and nutritional processes (Schmidt et al. 2007; Cordeiro et al. 2011; Nardi et al. 2002; Mora et al. 2012; Jannin et al. 2012; Trevisan et al. 2012; Canellas et al. 2012; García et al. 2012; Muscolo et al. 2013; Aguiar et al. 2013).

In this chapter, we discuss results obtained during the application and use of liquid humus from VC in low-input and small-scale farming systems and selected chemical and physical characteristics of this humus.

The aim of this study is to show the potential of these easily accessible materials for use by family farmers, organic farms, urban agriculture and farmers in developing countries through the collection of data under actual field conditions and the assessment of the ability of these materials to increase basic income. Additionally, this study aims to encourage research on the subject, with the goal of producing a sufficient supply of plant-originated foods using healthy, sustainable and environmentally friendly practices.

2. LIQUID HUMUS DERIVED FROM CATTLE MANURE VERMICOMPOSTS

2.1. Characteristics of Liquid Humus Derived from Cattle Manure Vermicompost under Cuban Conditions

The HSs isolated as liquids from composted materials are of interest primarily as a sustainable alternative for the small-scale production of plant-originated foods. Because HSs have different structures associated with their bioactivity depending on their origin, knowledge concerning the structural characteristics of HSs should be acquired before their use under field conditions can be justified.

One relatively easily accessible technique that provides basic and important information for the structural characterization of HSs is ultraviolet-visible (UV-Vis) spectroscopy. Figure 1 shows the UV-Vis spectra of humic acid (HA) and fulvic acid (FA) fractions isolated from liquid humus derived from cattle manure VC and stored for three-month and one-year periods.

These spectra show the typical behavior of humic fractions reported in other studies (Vergnoux et al. 2011; Valencia et al. 2013). The maximum recorded absorption of these substances is between 222 and 230 nm because of the presence of aromatic structures that are derived from lignin and hemicellulose fragments and form the starting material in the humification process. Small peaks can also be observed in these spectra, which are generally associated with the Pg fraction that has been related to substances originating from the activity of actinomycetes and fungi (Caro 2004).

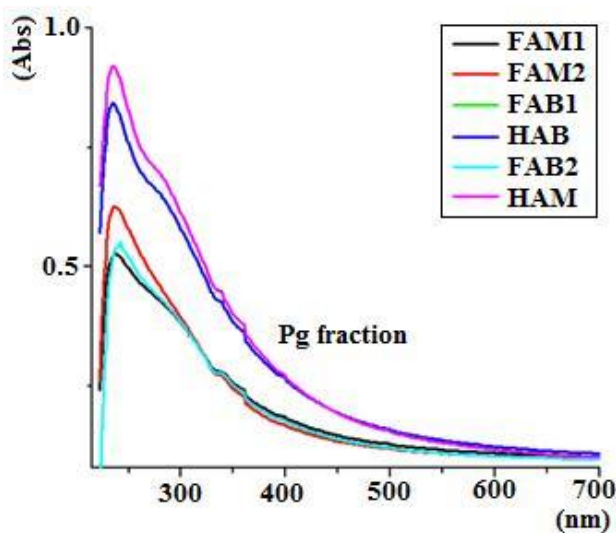


Figure 1. UV-Vis spectra of HA and FA liquid humic fractions obtained from cattle manure VC. HAM and HAB represent HA fractions stored for one year and for three months, respectively. FAM1 and FAM2 represent FAs stored for one year, whereas FAB1 and FAB2 represent FAs stored for three months (modified from Caro 2004).

Table 1. Relationship between the E₄₆₅ and E₆₆₅ coefficients for the different HA and FA fractions analyzed in liquid humus (modified from Caro 2004)

Sample	E ₄₆₅	E ₆₆₅	E ₄₆₅ /E ₆₆₅
HAM	1.010	0.281	3.59
HAB	0.587	0.126	4.65
FAM1	0.293	0.037	7.9
FAB1	0.274	0.034	8.05
FAM2	0.292	0.043	6.79
FAB2	0.202	0.033	6.12

The E₄/E₆ ratio is another important datum and provides information on the degree of carbon humification in HSs (Kononova 1966). Table 1 shows these ratios for the different fractions analyzed and indicates that lower values are observed for the HA fractions than for the FA fractions. Therefore, the HA fractions present in the liquid humus have a higher degree of aromaticity than the FA fractions.

In addition to the structural characteristics of the HSs in the liquid humus obtained from cattle manure VC, certain substances present in this pool can be of great importance, such as amino acids. Using HPLC, eight amino acids were identified and quantified in this humus (Figure 2).

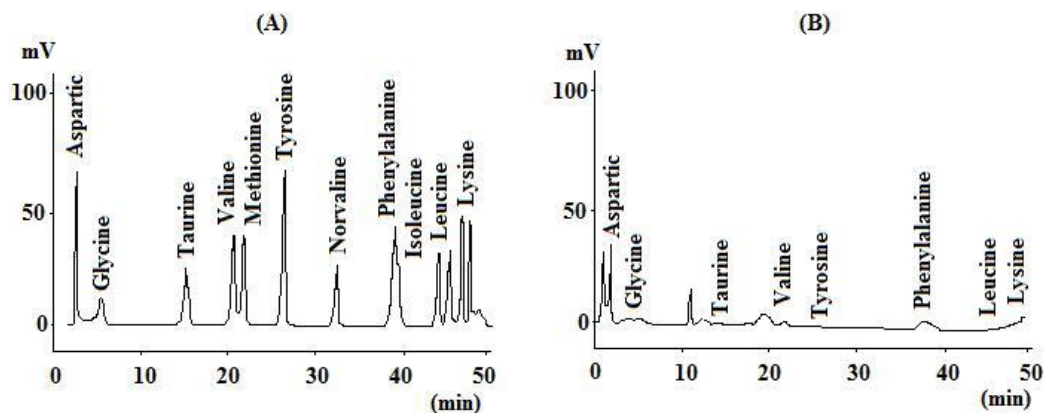


Figure 2. Chromatogram from HPLC analyses of amino acid standards (A) and of the amino acids identified in the liquid humus of cattle manure (B) (modified from Caro 2004).

The amino acids in the liquid humus pool can serve as a source of N for plants and soil when this pool of HSs is applied to crops or soil. For the soil applications, the presence of soluble amino acids in an aqueous medium indicates greater N availability, given that plants can incorporate free amino acids released into the soil by soil organic matter (SOM) and do so independently of the inorganic N supplied by microorganisms (Näsholm et al. 2000; Jones et al. 2005).

Other important components in the liquid humus pool from VC are the mineral elements, of which 12 were observed. K, Na and P were the most abundant elements, and the pattern observed is directly related to the methods for obtaining and using the extraction solution. P is

one of the most important nutrients for plants because this element is essential for energy and carbohydrate metabolism (Wrage et al. 2010). K is another important element for plants, and the intracellular regulation of this element mediates the response to water, salt and oxidative stresses (Anschütz et al. 2014).

Table 2. Inorganic composition of liquid humus from cattle manure VC determined by inductively coupled plasma (ICP) mass spectrometry (Caro 2004)

Element	Concentration (mg.L ⁻¹)
Ca	20.2
Cr	0.225
Cu	0.164
Mg	6.52
Mn	0.492
K	1,830
Fe	11.4
Na	570
Ni	0.032
Sr	0.087
Si	8.9
P	1,189

Table 3. The quantity of microorganisms in liquid humus obtained from modifications to the extraction process (Hernandez 2010)

Extraction solutions	KOH-Na ₄ P ₂ O ₇	(NH ₂) ₂ CO, KOH, KH ₂ PO ₄
Microbial groups	cfu.mL ⁻¹	
Bacteria	6.55 x 10 ⁷	3.54 x 10 ⁶
Fungi	2.21 x 10 ⁵	1.40 x 10 ⁴
Actinomycetes	5.4 x 10 ³	not available

The presence of microorganisms in VC is also an important factor to be considered. The existence of a microbial community in VC can add value to this material for agronomic use. There are reports in the literature of large quantities of bacteria in VC. Pathma and Sakthivel (2013) identified 193 types of bacteria from a straw- and goat-manure-based VC. The presence of actinomycetes isolated from a sawdust- and husk-containing VC with activity against phytopathogenic and human pathogenic fungi has also been reported (Baniya and Vaidya, 2011). In this sense, a liquid extract (liquid humus) that contains a microbial community beneficial to plants and harmless to humans could provide a valuable alternative for plant growth and the recovery of soils as well as a defense against disease and increased root growth. Table 3 shows the quantification of three microbial groups present in liquid humus that was produced from the same VC using two different extraction solutions. According to Hernandez (2010), this reduction of microorganisms in liquid humus is an advantage for the structural preservation of HSs and thus a longer storage life.

Moreover, the stability of liquid humus, in terms of the preservation of the humic structures present, ensures proper bioactivity in plants. This stability can be monitored by the

measurement of pH over time. Figure 3 shows the dynamic stabilization of two liquid humus products prepared from the same VC source. Oplant[®] liquid humus exhibits faster stabilization dynamics than Liplant[®] liquid humus. Oplant[®] stabilizes by the fourth week after preparation, with little decrease in the initial pH. In comparison, Liplant[®] displays a more drastic decrease in pH and stabilizes by the fifth week after preparation.

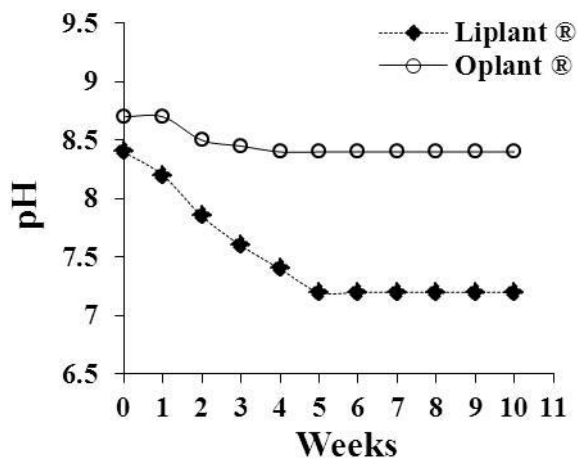


Figure 3. Stabilization dynamics over time of liquid humus from VC as a function of pH (Caro 2004, Hernandez 2010).

2.2. Experimental Application of Liquid Humus in Small-Scale Cucumber and Corn Crops

The direct, foliar application of liquid humus to crops under controlled conditions can reduce chemical inputs in small-scale systems. Figure 4 shows an example of results obtained for a cucumber crop (*Cucumis sativus* L.) when using liquid humus from cattle manure. In protected cultivation conditions (greenhouse), the applications of the Liplant[®] liquid humus at a dilution of 1:30 (v:v) increased the fruit number, size, length and weight by 26, 23.5, 6 and 2%, respectively, compared with the control treatment.

Another example of the foliar application of Liplant[®] liquid humus is shown in Figure 5 for a corn crop (*Zea mays* L.). In this case, two liquid humus dilutions (1:20 v:v and 1:30 v:v) showed the most promising effects on the measured crop production indicators. The stimulation of the number of ears per plant, ear weight with leaves, ear weight without leaves, ear length, ear diameter, mass of 100 grains, number of ears per plant and yield of dry grain for the 1:20 (v:v) dilution was 44, 29, 30, 13, 7, 14, 24 and 91%, respectively, compared with the control treatment.

The literature reports these positive effects on crops when liquid humus is applied to different types of plants. The foliar applications of liquid humus obtained from cattle manure VC and crop residues improved the yield and quality of strawberry (Singh et al. 2010) and tomato crops, increasing the yield in terms of the fruit number and quality; the N, P and K uptake; and the chlorophyll concentration (Tejada et al. 2008).

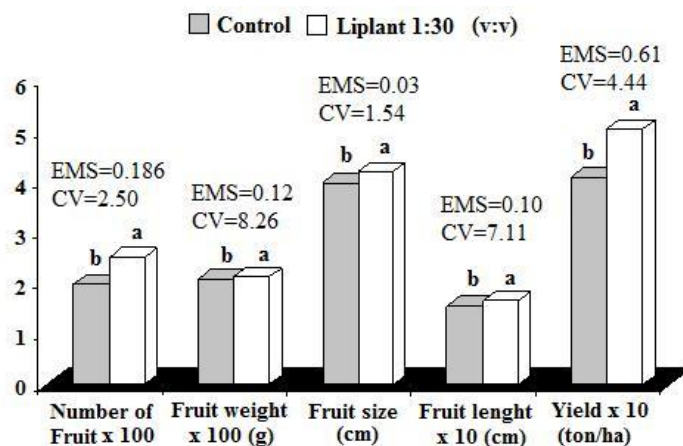


Figure 4. Effect of liquid humus from VC on certain production indicators for a cucumber crop (*Cucumis sativus* L.) of hybrid variety HA 736 (Caro 2004).

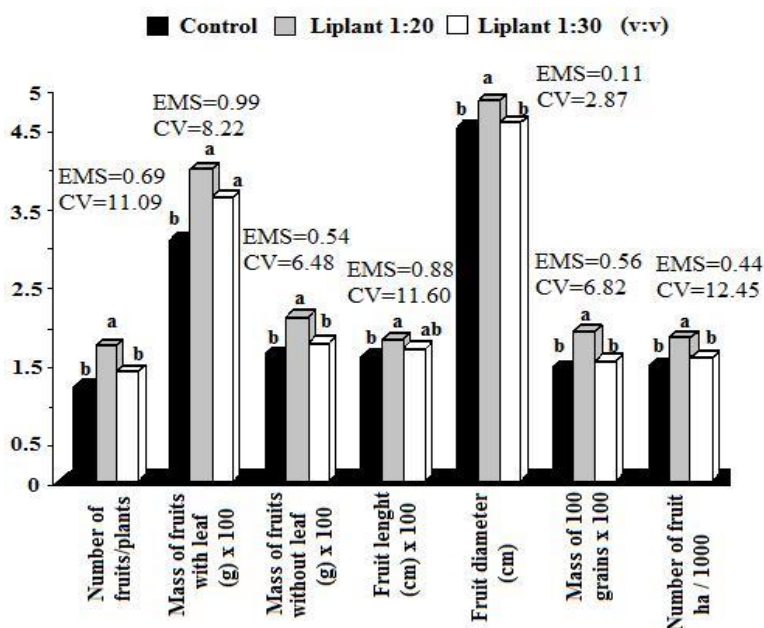


Figure 5. Effect of liquid humus from VC on certain crop production indicators in corn (*Zea mays* L.) (Caro 2004).

2.3. Effects of the Application of Liquid Humus on Ornamental Plants and Brassicas

The ability of liquid humus obtained from VC to regulate many metabolic processes in plants has increased the use of this substance, including its use in the postharvest stage, particularly in ornamental plants. Moreover, the literature contains reports regarding the use of HAs isolated from leonardite to improve ornamental plants (*Gerbera jamesonii* L.); for example, these HAs have been shown to increase the longevity of potted flowers from 2 to

3.66 days most likely because of an increase in calcium accumulation and the regulation of hormonal activity (Nikbakht et al. 2008). Certain authors, including Haghghi et al. (2014), also reported that when the HAs isolated from leonardite (500 mg.L^{-1}) were added to a nutrient solution supplied to *Gerbera jamesonii* L., there was an increase in the number of flowers per plant and in the longevity in pots of up to 9.33 days.

Table 4 presents results obtained from the application to rose plants of humified compost and liquid humus extracted from cattle manure VC. These results show an increase in the emission of roots by the plants under controlled experimental conditions for both products applied. These products, consisting of Liplant[®] and Biostan[®], influenced the emission of roots, with an increase of more than 100% under both greenhouse and natural shade conditions. The number of rosebuds, the final product of higher agricultural value in these plants, also increased under field experimental conditions by over 100% in each case compared with the control treatment. These results show an alternative use for VC-derived compounds that provides high commercial and environmental value. This use of humified compounds, which have a low production cost and are harmless to the environment, could serve as an alternative to increase yields in small-scale systems for the production of flowers and ornamental plants as well as for postharvest preservation.

Table 4. Effects of two liquid humus products derived from cattle manure VC on selected parameters of flowering plants (*Rosa* var. *multiflora*). The results of rooting under greenhouse and natural shade conditions were obtained by embedding cuttings for 30 minutes in the liquid humus. The remaining results were obtained by spraying the products on leaves

Liquid humus	Number of roots		Root dry mass	Number of leaves	Number of shoots	Number of buds
	GH ^a	NS ^b				
Biostan ^{®c}	4.7b	4.5b	1.12a	30.89a	10.69a	6a
Liplant ^{®d}	7.0a	5.5a	0.73ab	29.76b	10.85a	9a
Control ^e	2.1c	2.0c	0.47b	20.38c	8.38b	4b
CV	20.3%	18.2%	8.9%	12.3%	7.8%	10.2%
EMS	12.1	9.1	0.62	0.92	0.82	0.79

Duncan's test at 5% error probability. ^aGreenhouse conditions.

^bNatural shade conditions.

^cConcentration of 8 mg.L^{-1}

^dDilution of 1:10 (v:v).

^eH₂O.

Brassicaceae belong to a genus of plants that have value as food, as remediation agents for soils contaminated with heavy metals and as a source of green energy. In the literature, there are reports of the use of *Brassica oleracea* for extracting soil heavy metals, such as Cu, Cd, Ni, Pb and Zn (Aggarwal and Goyal 2007). In addition, these plants have potential for use as a bioenergy source, although this application requires improvements in agronomic processes and land use (Milazzo et al. 2013). Nutritionally, brassicas contain large quantities of antioxidant components (vitamins C and E and carotenoid and phenolic compounds) that act as defenses against oxidative stress (Podsędek 2007).

The nutritional and commercial value of using liquid humus to improve the yield of brassicas requires testing. Table 5 shows certain growth and production parameters that were evaluated in two cabbage varieties (*Brassica oleracea*).

Table 5. Effects of liquid humus obtained from cattle manure VC on growth and production parameters of two cabbage varieties (*Brassica oleracea*)

	Variety —CalabreseI			Variety —PatriotI		
	Plant height	Total branch length	Flower diameter	Plant height	Total branch length	Flower diameter
Control ^a	22b	15b	9b	22a	15b	11b
Liplant ^{®b}	38a	20a	15a	32b	20a	15a
Liplant ^{®c}	25b	18b	11ab	25b	18b	14a
CV	20.11%	13.21%	14.9%	23.016%	14.19%	15%
EMS	0.99	0.15	0.28	1.130	0.45	0.33

Duncan's test at 5% error probability.

All indicators are expressed in (cm). ^aH₂O.

^bDilution of 1:40 (v:v).

^cDilution of 1:20 (v:v).

In this case, two dilutions of Liplant[®] liquid humus were used under controlled experimental conditions. The use of this liquid humus at both dilutions increased plant height by between 25 and 72% and the total diameter by more than 7%.

2.4. Proving Bioactivity and Reusing Residues from the Production of Liquid Humus

One of the greatest challenges facing mankind is the avoidance of environmental contamination through the reuse of residues from existing production processes. Thus, for an agronomic alternative such as the production of liquid humus, it is important that the residues generated do not have a defined purpose. There are few studies in the literature concerning the residue remaining after the extraction of HSs from VC. However, studies conducted by García et al. (2007, 2013a) provide an alternative for reusing this solid residue to retain heavy metals because this residue exhibits a Ni and Pb retention capacity in excess of 90% in industrial wastewater (García et al. 2013b). Another alternative is the incorporation of this residue into soil as a soil improver (García et al. 2012).

To identify further reuse alternatives for this residue, this section explores results obtained from the application of a method for extracting HSs from the solid residue remaining after obtaining liquid humus from VC. Specifically, we discuss the biological activity of these liquid humus extracts. Table 6 shows the results of experiments performed with three extracts of the solid residue. Extract i was recovered from the solid generated by the production of Liplant[®] liquid humus, extract ii was derived from the solid generated by Oplant[®] liquid humus, and extract iii was obtained from a 1:1 (w:w) mixture of both solid residues.

Table 6. Morphological and production indicators at harvest in radish (*Raphanus sativus* L.) plants treated with a foliar application of three residual humic extracts obtained from cattle manure VC (modified from Rebato 2012)

Extract	Dilution (v:v)	EDTR (cm)	TRFM (g)	SFM (g)	TRDM (g)
Control		1.22d	2.05d	1.05e	1.01d
i	1/60	1.90bc	2.85b	1.3c	1.75b
	1/40	1.79c	2.89b	1.19cd	1.80b
	1/20	2.00c	2.98a	1.15de	1.90a
ii	1/60	2.59ab	2.90ab	2.0a	1.89ab
	1/40	2.45b	2.85b	1.95b	1.81b
	1/20	2.40b	2.89ab	1.94b	1.87ab
iii	1/60	3.34a	3.05a	2.04ab	2.01a
	1/40	3.30a	3.00a	2.00ab	1.90a
	1/20	3.00a	2.55c	2.00ab	1.53c
CV		11.81%	8.28%	18.65%	9.28%
EMS		0.33	0.052	0.077	0.055

Different letters indicate significant differences by Tukey's test at $p < 0.05$.

EDTR: Equatorial diameter of thickened root.

TRFM: Thickened root fresh mass.

SFM: Shoot fresh mass.

TRDM: Thickened root dry mass.

In general, when applied to radish leaves (*Raphanus sativus* L.), these extracts still showed effects on certain growth and production parameters. For extract i, a dilution of 1:20 (v:v) increased parameters such as EDTR and TRDM by between 63% and 88% compared with the control treatment. For extract ii at a dilution of 1:60 (v:v), there was a greater than 54% increase in the parameters shown Table 6, whereas for extract iii at the same dilution, there was an increase in the parameters that reached 100%.

The bioactivity of the liquid extracted from the HSs derived from cattle manure VC was also field tested under small-scale, farming conditions. In corn plants treated at three different dilutions, there was a slight increase in yield measured by the number of ears with and without leaves, with the yield of ears with leaves being 12% higher than that of the control (Table 7).

Table 7. Corn (*Zea mays* L.) crop yield after treatment at different dilutions under field conditions with a residual liquid extract (liq. ext.) obtained from the production of Liplant[®] liquid humus (Pimentel 2007)

Treatment	Yield for ears with leaves (ton.ha ⁻¹)	Yield for ears without leaves (ton.ha ⁻¹)
Control	2.33	1.37
Liq. ext. 1/10	2.17	1.42
Liq. ext. 1/20	2.27	1.47
Liq. ext. 1/30	2.62	1.48
Yield (%)	12.44	8.02

These results identify alternatives for the reuse of the solid residues generated by the production of liquid humus from VC. These alternatives could be exploited to close the use cycle of organic humified material while simultaneously benefiting agriculture and solving environmental pollution problems. However, the inclusion of these liquid extracts as a product to be applied at certain time points in the physiological crop cycle, together with the use of liquid humus obtained in the initial procedure, is an aspect that requires further analysis because it would lead to reduced costs for the use of liquid-humus-based organic fertilizers.

2.5. Effects on the Soil of the Application of Liquid Humus in Agriculture

In recent years, organic agriculture has grown as an agricultural production alternative with a low cost and input use. The impacts on soils caused by products for agricultural use, including those applied to crops, should be the object of study and monitoring. Products for organic agriculture, for example, must not cause harmful changes that compromise the proper balance of the soil-plant relationship. Table 8 shows certain attributes of a soil in which tomatoes were grown with the application of Liplant[®] liquid humus. These results demonstrate that the use of this product not only leads to positive effects on production performance but also affects the amount of organic material (OM), C, Cation-Exchange Capacity (CEC), and other soil properties. A 27% increase in soil OM was observed with the use of liquid humus. This result may indicate that the contribution of OM to the soil from the use of liquid humus in this crop could occur directly or indirectly. Direct contribution occurs via the incorporation of organic compounds from the product itself, whereas indirect incorporation originates from an increase in the amount of plant biomass and the return of organic compounds to the soil by the natural deposition of plant material. Other soil attributes also improved with the use of liquid humus. The amount of C increased significantly in the soil in which tomatoes treated with Liplant[®] were grown. Additionally, CEC and the $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio, as well as the P_2O_5 and K_2O levels, also improved.

Table 8. Physical and chemical soil properties at the start and end of a tomato crop cycle (*Lycopersicon esculentum* L.) during which Liplant[®] liquid humus was applied (Barrueta 2014)

Depth 0–20 cm	% SOM	% C	pH (H ₂ O)	CEC	Ca ²⁺ / Mg ²⁺	P ₂ O ₅	K ₂ O	Ds	Moisture (%)
Start ^a	3.03c	1.76c	7.20a	19.8c	4.18b	0.63b	0.54b	1.10a	25.35a
End ^b	3.45b	2.00b	7.17a	22.10b	4.45a	0.70b	0.61b	1.10a	25.85a
End ^c	3.87a	2.25a	7.18a	23.65a	4.97a	0.75a	0.72a	1.09a	26.55a
ESx	0.09	0.15	0.02	0.13	1.12	0.18	0.02	0.09	0.11

Different letters indicate significant differences according to Tukey's test, $p \leq 0.05$.

^aBefore applying Liplant[®] to the crop.

^bWithout applying Liplant[®] to the crop (control). ^cAfter applying Liplant[®] to the crop.

CEC in cmol (+) kg^{-1}

¹ P₂O₅ in mg.kg^{-1}

K₂O in mg.kg^{-1}

Ds in mg.cm^{-1} .

Table 9 shows these same analyses on a soil in which corn was grown using a liquid extract from VC. A slight increase was observed in the amounts of Na, Ca and Mg as well as in the OM content. Although it is not possible to identify the source of the OM or C in the soil used to grow crops treated with liquid humus, the end result indicates an improvement in several soil properties.

Table 9. Soil property analyses at the start and end of a corn (*Zea mays L.*) crop cycle in which residual liquid humic extract obtained from the production of liquid humus from VC was applied to the crop (Pimentel 2007)

Depth	Cations present (cmol.Kg ⁻¹)				P (ppm)	OM (%)	pH
	Na ⁺	K ⁺	Ca ²⁺	Mg ²⁺			
0–20 cm							
Start ^a	0.13a	0.35a	12.7a	2.7a	141a	2.17a	6.8a
End ^b	0.17b	0.35a	13.4a	3.1a	140a	2.76a	6.9a
EMS	0.01	0.11	1.22	1.41	21.5	1.01	2.1

Different letters indicate significant differences according to Tukey's test, $p \leq 0.05$.

P (ppm) by the Oniani method.

^aBefore applying the residual humified extract to the crop.

^bAfter applying the residual humified extract to the crop.

There are few reports in the literature regarding the effects on soil resulting from the use of liquid humic extracts in crops. This type of study is more abundant when humified materials (VCs) are used as fertilizer. VCs are recognized as materials that can be rich in nutrients and improve plant growth (Valdez-Pérez et al. 2011). Accordingly, VCs from different sources may also be used as substrates for the inoculation of nitrogen-fixing bacteria (Balmori et al. 2013). Thus, the studies conducted with both liquid humus and composted materials show the advantages of their use; however, it is necessary to improve the conditions of their use. As we have already reported in other studies devoted to the usefulness of HSs and humified materials in agriculture (Berbara and García 2014; García et al. 2014), the most important consideration in using these resources is the preservation of the ecological balance and the maintenance of an adequate soil-plant-environment relationship.

2.6. Economic Impact of the Use of Liquid Humus in Small-Scale Agriculture

Although there are reports in the literature that indicate a decline in agricultural yields using organic compared with conventional agricultural practices (Seufert et al. 2012), the IFOAM (International Federation of Organic Agriculture Movements) reports that in 2013, 1.8 million farms grew organically in 162 countries. Therefore, there were over 37 million hectares cultivated using an organic agricultural practice.

The experience accumulated using humified materials and liquid humus under agricultural conditions of low input and fertilizer consumptions shows that this agricultural practice can be a viable and sustainable alternative for the production of plant-derived foods (Badgley et al. 2007).

In economic terms, the foliar application of liquid humus to crops produces a net increase in cash inflow for small-scale farmers. Artega et al. (2006) showed increased income when

using liquid humus obtained from VC for the cultivation of tomato crops (*Lycopersicon esculentum* Mill.) grown in two consecutive harvests. The gains recorded at the first and second harvest were 54.20 and 39.36%, respectively (Caro, 2004).

CONCLUSION AND FUTURE PROSPECTS

In this chapter, we present certain features regarding liquid humus obtained from cattle manure VCs. According to the experience accumulated, these products include both humic (HAs and FAs) and other organic substances (phytohormones and amino acids) as well as various inorganic nutrients. When applied to plants under both laboratory and field conditions, increases in physiological parameters and consequently agricultural parameters were observed. These results indicate and encourage the use of HSs as an alternative method in small-scale, family and urban agricultural production systems. In addition, this method represents a viable alternative for obtaining organic foods, which are pesticide-free and therefore healthier foods. The results presented here could serve as an incentive for this type of farming and as a knowledge base for the expansion and continuation of studies on this topic, always adopting a multidisciplinary approach that considers both sustainability and environmental stability.

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Chapter 2

**THE PRODUCTION OF LIQUID ORGANIC
FERTILIZERS ENRICHED WITH HUMIC SUBSTANCES
FROM MATURE ALPERUJO (OLIVE MILL WASTES)
COMPOST**

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ABSTRACT

The introduction and extended use of chemical fertilizers has been a determinant factor in the development of modern intensive agriculture. However, in recent years, interest has grown in agriculture in the possibility of recycling organic residues and wastes derived from a variety of human activities. Amendment with non-toxic organic waste is absolutely necessary to improve soil fertility and crop production. The use of organic waste and residues of any nature for soil amending requires appropriate treatment to eliminate or, at least, reduce any compounds that may have harmful effects on soil properties and to optimize the efficiency and the fertility of the final product once in the soil. The process of composting is generally considered as the most efficient treatment for producing environmentally safe and agronomically advantageous organic waste amendments for soil. Composting is an accelerated version of the decomposition processes naturally occurring in the soil. The humification process typically occurring under natural soil conditions transforms the organic material into a stable and mature humic like end-product. The close relationship between the humified organic matter

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content in soil and its fertility explains the persistent interest in studying humic substances. Much work has been carried out concerning the use of organic waste in soils either as solid fertilizers or amendments. But, little information is available on the production of liquid organic fertilizers. In this objective, in order to release commercial liquid organic fertilisers from Alperujo (olive mill wastes) composts, several extraction conditions (extraction time, extraction temperature, heat, time applied, extraction ratio, extractant agent and alkali concentration) were tested. The obtained results showed that extracted humic-like substances presented characteristics close to those of standard humic materials, confirming that the hot alkaline extraction dissolved more polymerized compounds, probably including lignin degradation by-products and/or newly formed aromatic materials.

As a consequence, the alkaline extraction of Alperujo or olive mill wastes compost with 1M KOH at 70°C during 24H can be used to produce liquid organic fertilizers, thus diversifying the marketable products obtained from Alperujo, the most abundant by-product of the Spanish olive-oil extraction industry.

Keywords: Composting, Alperujo, humification, humic-like substances, liquid organic fertilizers

I. INTRODUCTION

Composting is a cheap technology which speeds up the natural biodegradation of organic matter by providing a controlled environment for a great diversity of microorganisms that lead to the mineralization of a large part of the raw organic matter and the synthesis of new organic compounds via humification processes.

In this respect, Tortosa et al. (2014) reported that this process permits to transform organic wastes and by-products into quality materials that can be used as soil amendments and/or fertilisers.

As a consequence, it can be considered as a humification technology (Saber, 2001). Also, composting has been shown as an effective treatment for adding value to many wastes such olive mill wastes called in Spanish Alperujo, through the production of commercial solid organic amendments and fertilisers (Tortosa et al. 2012; Muktadirul Bari Chowdhury et al. 2013; Tortosa et al. 2014).

Indeed, it has been shown that Alperujo valorization, as amendments and/or fertilisers, has a good performance in agricultural experiments (Albuquerque et al. 2006, 2007; Fernandez-Hernandez et al. 2013; Altieri et al. 2014, Nair et al. 2014). Therefore, the compost obtained has the advantage of containing a large proportion of humic-like substances which play a major role in soil fertilization.

These substances are ubiquitous in the environment, occurring in composts, soils, sediments and waters and may be isolated in abundance from coals like lignite and leonardite. Previous studies (Garcia et al. 1993; 1994) highlighted their use in fertigation as liquid organic fertilizers in modern intensive agriculture.

Nowadays, there is considerable evidence about the pollutant impact of mineral fertilizers on the environment, due to their massive application and the fact that crops use them inefficiently; thus increasing attention has been paid to organic fertilizers that might provide plant nutrients more properly and help growers maximize crop yields. Furthermore,

fertigation satisfies the requirements of many plant species through their different stages of development and avoids nutrient losses, thus enhancing crop yield and reducing water evaporation and irrigation frequency (Parrado et al. 2008). On the basis of the above background and from previous studies by our team about pomace or alperujo composting (Ait Baddi et al. 2004, Alburquerque et al. 2004, 2006, 2007, 2009) and in order to diversify the range of commercial products obtained from alperujo in either solid (compost) and liquid (organo-mineral Fertiliser) forms; a new objective of our research was developed to produce liquid organic fertilizers containing humic substances from mature alperujo composts, by using alkali extraction (Ait Baddi et al. 2012, Tortosa et al. 2012, Tortosa et al. 2014).

2. HUMIC SUBSTANCES (HS)

2.1. Introduction to HS

The HS are described as naturally occurring biogenic, heterogenous organic substances (Parsons 1988). Nardi et al. (2007) reported that HS are heterogeneous organic molecules that form in the soil as byproducts of microbial metabolism of dead organic matter. They are the resulting products of humification process, which is typically occurring under natural soil conditions, transforming the organic material into a stable and mature humic like end-product (Senesi and Brunetti 1996). The close relationship between the humified organic matter content in soil and its fertility (Senesi and Brunetti 1996) explains the persistent interest in determining the chemical and physico-chemical properties of humic substances. They are an extremely important soil component because they constitute a stable fraction of carbon, thus regulating the carbon cycle and the release of nutrients, including nitrogen, phosphorous and sulphur. Additionally, the presence of humic substances improves water-holding capacity, pH buffering and thermal insulation (Stevenson 1994).

These substances are considered to be a necessary component of the ecosystem and the most stable part of decomposing organic matter (Ait Baddi and Hafidi 2008). Humic substances can be separated into fulvic, humic acids and humin (McDonnell et al. 2001). Humic acids represent a significant proportion of soil organic matter and are considered to be an important factor in soil fertility. This was confirmed by Ulu et al. (2014), who explained that humic substances do not have certain or general structure, hence the phrase “_humic substances” is used as a general term to express colored material or its fraction is attained on the basis of solubility characteristics depending on the pH functions as follows: Fulvic acid is soluble at all pH conditions. Humic acid fraction is alkali-dissolving (soluble at higher pH values) and insoluble in water under acidic conditions ($\text{pH} < 2$). Humin is the fraction of humic substances that is insoluble in any pH and cannot be done extracted by acid or base. Humic and fulvic acids differ from each other by the variations in molecular weight, the number of functional groups (carboxyl and phenolic OH) and the extent of polymerization. Generally, fulvic acids have lower molecular weights than humic acids, and the structures of fulvic acid are more aliphatic and less aromatic than humic acids (Ulu et al. 2014).

Much work has been carried out concerning the use of organic waste in soils either as fertilizers or amendments by using different process. In this respect, as mentioned before composting is one of the technologies aimed at producing a fertilizer or amendment from

wastes. This process permits the return to croplands of the nutrients contained in wastes. Moreover, it avoids some of the drawbacks (phytotoxicity, microbiota inhibition) of direct application of wastes to the soil. The compost properties depend on the degree of transformation reached during the bioremediation. Obviously, the transformation process is not completely achieved in the composting plant, but it continues in the soil after applying the compost, thereby increasing long-term fertility, which can be also reached owing to the high content of humic matter in the compost (80%) (Ait Baddi and Hafidi 2008).

2.2. Extraction of Liquid Organic Fertilizers (LOF)

The AL composts were obtained in a previous experiment where trapezoidal piles of 1.5 m high and 4×6 m base, each weighing 10 tons, were composted using the mechanical turning strategy. The piles were made by mixing a wet lignocellulosic solid byproduct of the olive oil industry (alperujo, AL) with the bulking agents (horse, poultry and sheep manure) (Ait Baddi et al. 2012, Tortosa et al. 2012).

The LOF (liquid organic fertilizers) were obtained by constant shaking of sealed bottles containing the mature composts and 1M KOH (1:3 w/v) for 24h in a water bath at 70°C. After centrifugation at 23,000g for 30 min, the alkaline extracts were carefully removed, dialyzed and freeze dried (Ait Baddi et al. 2012). From an agricultural point of view, Potassium hydroxide (KOH) was used instead of sodium hydroxide (NaOH) as alkali extractant due to the important role played by K in the plant metabolic processes and several enzymatic reactions, and in order to avoid the known detrimental effects of sodium on soil (Ordóñez et al. 2006).

3. CHARACTERIZATION OF AL COMPOSTING

Tortosa et al. (2012) have reported that composting is an effective technology for adding value to AL through its transformation into commercial organic amendments and fertilisers. The manures composted with AL acted as appropriate co-substrates for AL composting and the mechanical turnings applied were enough to aerate the piles, homogenizing the substrate simultaneously. The addition of the Fe and P-enriched acidic mineral amendments reduced the pH values and also the water-soluble organic carbon, watersoluble phenols and hemicellulose contents.

The composts obtained lacked phytotoxicity and had valuable contents of nutrients and high contents of OM with humic properties and predominance of the lignocellulosic matrix.

4. CHARACTERIZATION OF LIQUID ORGANIC FERTILIZER (LOF)

Tortosa et al. (2014) reported that Alkali-extractant agent (KOH) concentration significantly affects the extraction performance. The concentration of the alkali-extracted organic carbon obtained with 1 M KOH was around twice compared to 0.1 M KOH.

- The extraction time increased the amount of organic carbon extracted and the extraction efficiency, especially using 0.1 M KOH. With 1M KOH, a saturation condition was reached after 24 h.
- The application of heat (70°C) significantly affected the nature of humic alkali-soluble organic matter, increasing efficiency and concentration and showing a chemical degradation of the humic-like acid fraction.
- The extraction ratio affected the concentration of both organic, including polyphenols and carbohydrates, and inorganic fractions.

Based on these conclusions, the new process developed to produce LOF from two mature Al composts using 1M KOH at 70°C for 24h (compost with fatty proteinaceous animal Waste (CO-AWK) and without adding fatty proteinaceous animal Waste CO-K acting as control treatment) (Ait Baddi et al. 2012).

A comparison of the obtained results with the humic substances characteristics obtained by the IHSS protocol from the same materials by using NaOH and with those of Suwannee River humic and fulvic acids were performed.

The generated black liquids (LOF) are characterized by high total organic carbon and nitrogen contents (more than 40 and 5g/L, respectively) and rich in humic substances (80% humic acids and a polymerization degree of 4) (Ait Baddi et al. 2012). The characterization of these compounds by elemental analysis showed lower contents of carbon, hydrogen and nitrogen in LOF extracted from CO-AWK than in LOF extracted by NaOH composted Alperujo-fatty proteinaceous animal Waste (COAWN_a) and higher oxygen contents which suggest an increase in O-containing functional groups in LOF CO-AWK and LOF COK samples, probably due to the higher alkalinity of the extractant and the high temperature employed. Therefore, the use of FTIR and fluorescence spectroscopy confirmed the predominance of more polymerised compounds, including probably lignin degradation by-products or newly formed materials generated by 1M KOH and high temperature (70°C). These results were confirmed by statistical analysis by Principal Components Analysis, which underlined the difference between alkali humic-like substances and humic like substances extracted according to the IHSS protocol and Suwannee River fulvic and humic acids. Consequently, the use of 1M KOH at 70°C permitted the extraction of LOF from Al composts. The LOF obtained is expected to have high fertilizing potential and a positive impact on soil quality and fertility, owing to the enrichment in humic-like substances.

Despite the significant advances made in humic substances studies, such kinds of works producing liquid organic fertilizers are very scarce in bibliography. In this respect, Li et al. (2009) confirmed that few studies have been conducted to evaluate the extraction of sludge humic acid for use as a fertilizer. Thus, these authors have developed a method of reducing sludge while simultaneously obtaining humic acid fertilizer with the use of alkaline treatment. The obtained results from this study showed that alkaline extraction application do not affect the quality of obtained humic acids. Indeed, these authors reported that humic acid is particularly difficult to degrade during subsequent treatment of the wastewater. Conversely, humic acid can be extracted as a kind of highly-efficient fertilizer. Li et al. (2009) compared the obtained humic acids after alkaline extraction with commercial humic acid and they found that sludge humic acid contains a wider variety of organic substances, more lipids, more nitrogen and a lesser degree of oxidation. However, in spite of these structural differences,

there are no significant differences in the effects of the two types of humic acid on plant growth, according to Li et al. (2009) This was confirmed also by other study (Ait Baddi et al. 2013) which the objective was to develop indicators that can provide information about the maturity reached by municipal solid waste anaerobically treated in alkaline conditions. The obtained results in later study highlighted the decrease of the protein-like structures and the increase of fulvic acids-like during anaerobic degradation, and an increase of humification of leachate from municipal solid wastes in bioreactor inoculated with a residual fraction of centrifuged leachate in anaerobic conditions. So, the alkali-extracted humic-like substances presented characteristics close to those of standard humic materials.

In the objective to verify the impact of liquid organic fertilizer on plant growth, Canfora et al. (2015) studied the short-term effect of two very different organic fertilizers (stillage obtained from the production of bakery yeasts, and vermicompost extract), with regards to the production process and the composition, on Eubacteria and Archaea associated with *Solanum lycopersicum* L., on plant growth and root morphology, and on some rhizospheric soil chemical parameters. Later authors reported that the application of the two liquid organic fertilizers derived from alternative sources of organic matter differentially affected the size and biodiversity of rhizospheric Archaea and Eubacteria populations as well as the root architecture, even after a short period of the plant growth, divergently to the common mineral fertilizer. According to these authors, it appears that such outcome can sustain plant growth even with a very limited application of mineral nutrients, underlining the importance of biological fertility in the overall management of soil fertilization (Canfora et al. 2015). These results were confirmed before by Gutiérrez-Miceli et al. (2008), which investigated the effect of different combinations of vermicompost leachate, NPK fertilizer, and different concentrations of an adherent and dispersant on sorghum growth. They confirmed that vermicompost leachate can be used as liquid fertilizer for the cultivation of sorghum without dilution because they are a source of micro-elements but also of humic and fulvic acids that promote growth of sorghum plants. Furthermore, Romero et al. (2013), studied the effect of leachate from composted municipal solid wastes as a potential liquid fertilizer. After anaerobic treatment of these compost leachates, the results have been compared with those for a commercial liquid fertilizer. Romero et al. (2013) find that experimental comparison of raw and anaerobic digested leachates have better fertilizing qualities than the commercial liquid fertilizer. In addition, raw leachate could be used as potential fertilizer because it meets the requirements for commercial fertilizers: low concentrations of heavy metals, absence of pathogens, nutrients such as nitrogen, phosphorus and potassium, C/N ratio under 20 and a high germination index. Anaerobic digestion of raw leachate yields to a digested leachate also with good qualities as fertilizer, lower content in solids but higher content in salts. As a consequence, according to the same authors (Romero et al. 2013), the treatment of raw leachate before use as fertilizer may be not recommended but it should be noted that biological treatment reduces odors and stabilizes residues. So, the possible drawbacks of raw leachate as soil fertilizer (high content in solids and salts) could be overcome by a simple dilution of 1/10. However, the presence of a high salt content in these leachates could be considered as an inconvenient, which can be resolved by only an adequate dosage of these products (Romero et al. 2013).

CONCLUSION

As it can be concluded that alkali liquid organic fertilizers extracted from many sources (compost, vermicompost and leachate) can be used in fertigation in modern intensive agriculture in order to avoid the pollutant impact of mineral fertilizers on the environment and to limit their massive application and the fact that crops use them inefficiently. Thus increasing attention must be paid to alkali liquid organic fertilizers that might provide plant nutrients more properly and help growers maximize crop yields. Furthermore, fertigation satisfies the requirements of many plant species through their different stages of development and avoids nutrient losses, thus enhancing crop yield and reducing water evaporation and irrigation frequency.

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Chapter 3

**MECHANISMS OF PROTECTIVE ACTION OF THE
HUMI PREPARATION ON WHEAT PLANT RESPONSE
TO TOXIC IONS**

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ABSTRACT

Humic acids (HA) make up an important component of soil humus related to maintenance of soil water-holding capacity, stabilization of soil structure and fertility and vital activity of soil micro-organisms, plants and animals. At present a wealth of information has become available on the effectiveness of HA and HA-based preparations for growth stimulation and improvement of resistance and productivity of different plant species. We have conducted a comprehensive analysis of growth-stimulating and protective effects of HUMI preparation (NVP "BashInkom," Ufa) on wheat plants. In these experiments we have used presowing treatment of wheat (*Triticum aestivum* L., cv. Bashkirskaya 24) with HUMI (0.3 mg/g of seeds). The treatment was shown to promote seedling growth. This effect is likely to be due to the HUMI-induced alterations in the plant hormonal system associated with a 1.5-fold persistent accumulation of cytokinins without any changes in the contents of IAA and ABA. The protective effect of HUMI on wheat seedlings exposed to the toxic effects of sodium chloride and cadmium acetate manifested in preventing stress-induced decline in cytokinin content and in reduction of both ABA accumulation and decline in the IAA content. These shifts in the hormonal balance led to decreased damaging stress effects on plant growth and to acceleration of the growth processes recovery during the post-stress period. Lignin deposition in root cell walls is known to promote strengthening of their barrier properties thereby reducing penetration of toxic ions into the plants. HUMI increased lignin deposition in root cell

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walls of basal regions of seedlings exposed to toxic ions, indicating protective effect of this regulator. Since cytokinins have been shown to increase lignification, this allows us to suggest that accelerated lignification in stressed plants pretreated with HUMI is due to higher cytokinin content in these seedlings than in untreated plants. Therefore, the ability of HUMI to induce accumulation of cytokinins and maintain their high level in plants exposed to toxic ions is important for establishment of growth-stimulating and protective action of this natural growth regulator on wheat plants.

Keywords: Humic acids, HUMI, abscisic acid, indoleacetic acid, cytokinins, salinity, cadmium stress, *triticum aestivum* L

INTRODUCTION

Stress-resistance is most important for maintaining plant growth and productivity under unfavourable conditions thereby attracting universal attention to the search of effective means of its increase. Development of natural protective mechanisms taking place in plants under stress conditions is well known to be accompanied by a decline in their productivity (Wu et al. 2007, Farooq et al. 2009, Atkinson and Urwin 2012). Yield losses in agriculture due to stress-prone environments are known to reach 50-80% of genetically determined potential productivity. In accordance with this, information concerning the chain of events initiated in response to extreme conditions may really contribute to an increase in plant resistance and productivity. These goals are achieved not only by selecting genotypes with increased stress tolerance, but also through the purposeful manipulation of adaptation with the help of plant growth regulators (PGR) (Shakirova et al. 2010, 2012, Ha et al. 2012; Santino et al. 2013; Fariduddin et al. 2014, Calvo et al. 2014, Asgher et al. 2015). Due to this, the interest of researchers to growth regulators is not casual. These substances combine the properties of growth activators and inductors of unspecific resistance that determines the perspective for their practical application in agricultural production. The survival of plants is due to their ability to cope with and to adapt to challenging environment. And since crop production demands an intensification of not only plant resistance, but of productivity, in the first place, purposeful application of regulators of plant growth and development capable of increasing both of these characteristics is of high priority. It is obvious that not every PGR may be used, but those, which are characterized by a wide spectrum of their protective action (Shakirova et al. 2010, 2012, Asgher et al. 2015).

In relation to this, preparations based on humic substances (HS) are of great practical interest. HS constitute the end product of decomposition of organic residues and represent a major proportion of organic matter in water, soil and sediments. HS participate in the majority of reactions that take place in the environment, controlling transport and transformation of chemical compounds, formation of complexes by chelation of metals and other mineral elements, thus influencing the availability of nutrients and maintenance of soil structure and fertility (Nardi et al. 2002, Quaggiotti et al. 2004, Muscolo et al. 2007a, Canellas et al. 2008, Canellas and Olivares 2014). HS and humic acids (HA, being alkaline fraction of HS) should be considered as supra-molecular complexes containing several heterogeneous compounds, which have a relatively low molecular mass but are dynamically associated by means of

hydrophobic interactions and hydrogen bonds (Sutton and Sposito 2005). HA is most widely used in different branches of agriculture (Pena-Mendez et al. 2005).

Thus, stimulating effect on plant growth has been demonstrated for low molecular weight fractions of HS and HA, in particular (Nardi et al. 2002; Dobbss et al. 2007, Canellas et al. 2008, Muscolo and Sidari 2009), although it should be mentioned that biological activity of these substances may be better related to their chemical structure than to their molecular weight (Russell et al. 2006, Muscolo et al. 2007a, Canellas et al. 2008, Canellas and Olivares, 2014). Effects of HS on acquisition of mineral macro- and micronutrients by plants may significantly contribute to HS growth-stimulating action. This may be due to ability of HS to accumulate in cell walls and (at least partially) reach cell membranes and influence the state of membrane ATPase, thereby increasing permeability of cell membranes for phosphates, nitrates, potassium and other components of mineral nutrition necessary for plant growth and development (Nardi et al. 2000, 2002; Quaggiotti et al. 2004). Thus, it was shown for maize that HS-induced stimulation of nitrate uptake by roots and anion translocation to leaves is linked to increased expression of genes coding for H⁺-ATPase and its activity in root cell membranes, as well as to activity of nitrate transporters in shoots (Quaggiotti et al. 2004). Induction of lateral root formation is related to activation of ATPase that served as an evidence of hormonal (auxin-like) action of HA on lateral root emergence (Canellas et al. 2002, 2008, Jindo et al. 2012, Canellas and Olivares 2014). It is of interest that stimulation of root cell extension by IAA and HA is due to activation of vacuolar H⁺-ATPase as well as of H(+)-pyrophosphatase (Zandonadi et al. 2007). Comparative analysis of the HS and auxins effects on morpho-physiological and biochemical characteristics of plant tissues revealed great similarity in their action (Nardi et al. 2002). Furthermore, the data on interaction of either HS or auxins with carrot cell membranes in cell culture indicate possibility of HS binding to IAA receptors (Muscolo et al. 2007b). The use of different methods, immunoassay being one of them, enabled identification of not only IAA (Muscolo et al., 1998), but also cytokinins (CKs) as constituents of HS (Zhang and Ervin, 2004). Furthermore, root application of HA was found to promote cucumber shoot growth associated with an enhancement of root H⁺-ATPase activity and an increase in nitrate shoot concentration. These effects were related to significant increases in the shoot concentration of several active CKs (Mora et al. 2010), indicating the important role of endogenous cytokinins in the manifestation of the diversity of the physiological effects of HA. Thus, HS action appears to resemble action of hormones that are capable of activating plant growth (auxins and cytokinins). HS resemble hormones in that both contribute to activation of cell metabolism, being the basis of growth processes, and to the total increase in productivity of different crops (Canellas et al. 2002, 2008; Carletti et al. 2008, El-Ghamry et al. 2009, Jindo et al. 2012; Muscolo et al. 2013). It is necessary to emphasize the fact that different variants of HS-treatment protected plants against stress factors thereby promoting a significant increase in crop yield (Nardi et al., 2002; Zhang and Ervin, 2004, Muscolo et al. 2007a, Asik et al. 2009, El-Ghamry et al., 2009; Shakirova et al. 2014).

Thus, preparations of HS combine the characteristics of growth stimulators and inductors of plant stress resistance and are natural plant growth regulators widely used all over the world and contributing to yield stability and quality of the products. However, application of these preparations with the aim of increasing the yield of agricultural crops and plant resistance demands understanding the diversity of their effects on plants that are still not entirely clear.

The present work addresses a complex analysis of the mechanisms of action of HUMI preparation (NVP —BashInkom, Ufa, Russia) consisting of sodium salts of humic acids recovered from brown coal on wheat plants under normal conditions and under the influence of toxic sodium chloride, and cadmium ions.

INFLUENCE OF HUMI ON GROWTH AND HORMONAL STATUS OF WHEAT PLANTS UNDER NORMAL GROWTH CONDITIONS

In experiments we applied pre-sowing semidry treatment of wheat *Triticum aestivum* L. cv. Bashkirskaya-24 seeds with HUMI, the dose of application being 0.3 mg per g of seeds.

Initially the analysis of the effect of HUMI on intensity of seedling growth processes was carried out. The data presented in Table 1 demonstrate that treatment of the seeds with HUMI activates plant growth. HUMI-treated wheat seedlings were distinguished by significantly greater mitotic index (MI) of root apical meristem cells.

Table 1. The effect of presowing HUMI treatment on mitotic index of apical meristem cells in wheat seedling roots during germination*

MI, %	Variant	Age, d		
		2	3	4
	Control	5.4±0.22	5.3±0.23	5.2±0.23
	HUMI	7.8±0.34	7.5±0.32	7.3±0.29

* Data are mean ± SE of three independent measurements.

Hormonal system is well-known to play a decisive role in regulation of growth processes. During germination, it responds sensitively to the changes in environment as well as to the treatment with growth regulators (Shakirova et al., 2010; 2012). It is necessary to emphasize that a study of different hormones in the same plants allows to follow a complex pattern of changes in hormonal status and to evaluate if the treatment with a growth regulator is favourable for plants. Therefore it was of interest to carry out a comparative analysis of hormonal status during germination of seedlings either treated or untreated with HUMI.

Figure 1 shows that HUMI-treatment resulted in 1.5 fold increase in the concentration of free cytokinins (CKs) immunoreactive to antibodies raised against zeatin riboside. This effect persisted in the seedlings throughout the whole experiment, while there were no significant changes in the content of IAA and ABA as compared to control.

The obtained data indicate that, first of all, growth-stimulating effect of HUMI on the wheat plants is due to persistent accumulation of cytokinins known to play the key role in the activation of cell metabolism, which is the basis of plant growth and development during their whole ontogenesis (Argueso et al. 2009, Shakirova et al. 2010, Ha et al. 2012, Reguera et al. 2013, Kohli et al. 2013). Recent studies have reported the growth promoting effect of HA in cucumber associated with the increase in the contents of various forms of cytokinins during incubation of plants on medium with HA (Mora et al. 2010). However, it is worth mentioning that these changes in CKs concentration were transient and moderate (Mora et al. 2010),

whereas our studies have demonstrated 1.5 fold stable accumulation (throughout the whole experiment) of cytokinins in the wheat plants, after presowing treatment with HUMI.

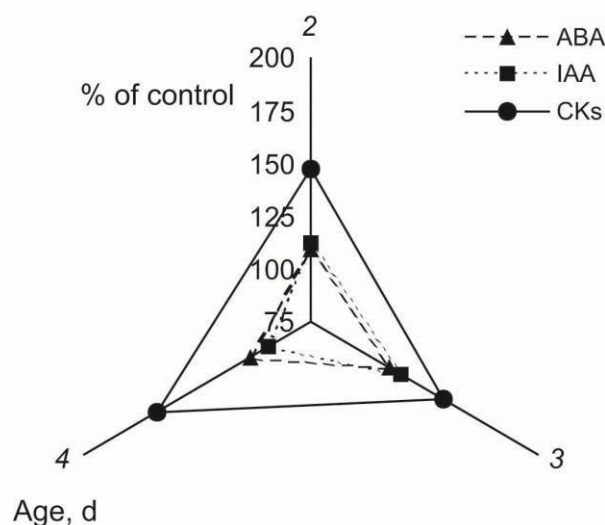


Figure 1. Dynamics of ABA, IAA and cytokinins contents (% of control) in wheat seedlings pretreated with HUMI on the 2nd, 3rd and 4th days of germination.

PROTECTIVE ACTION OF HUMI ON STRESSED WHEAT PLANTS

Salt Stress

NaCl salt stress is one of the most widely spread detrimental ecological factors all over the world leading to disturbance of water balance and toxic effects, manifested by morpho-physiological, biochemical, molecular-genetical changes and reflected in inhibition of growth processes and yield losses (Wu et al. 2007, Munns and Tester 2008, Plaut et al. 2013). Efficiency of application of HS with the aim of increasing plant resistance to sodium chloride salinity was demonstrated for maize and bean plants (Khaled and Fawy, 2011, Aydin et al. 2012).

In order to assess the ability of a HUMI preparation to exert a protective action on wheat plants, experiments have been carried out on 4-5-d-old HUMI-pretreated and untreated wheat seedlings exposed to 2% NaCl. Figure 2 shows that salinity led to significant inhibition of mitotic activity of root cells of both HUMI-treated and untreated plants 3 h after the start of the salt stress, although it is worth mentioning that MI was maintained at the control level (unstressed plants untreated with HUMI) in the variant subjected to pre-sowing HUMI-treatment. 24 h after removal of stressor from the medium, there was observed gradual recovery of MI in both variants, but it is important to note that MI of HUMI-pretreated plant slightly exceeded the level of control.

Comparative analysis of the effect of salinity on the growth of plants untreated and pretreated with HUMI have revealed that, according to the changes in the dimensions of the plants, salinity led to significant inhibition of the seedlings growth within 7 h (Figure 3). Even

after withdrawal of sodium chloride from the nutrient medium the growth of seedlings exposed to short-term salinity remained lower than in control plants during subsequent 24 h of post-stress period.

Stressed plants pretreated with HUMI experienced strain suggested by some inhibition of seedling growth as compared to HUMI-treated unstressed plants. However, since HUMI-treatment itself exerted growth-stimulating effect on plants, the size of stressed plants was significantly greater than in control plants (Figure 3). Furthermore, one day after withdrawal of the stressor from the nutrient medium, plants treated with the preparation completely restored their growth intensity up to its level in HUMI-treated plants not subjected to salinity.

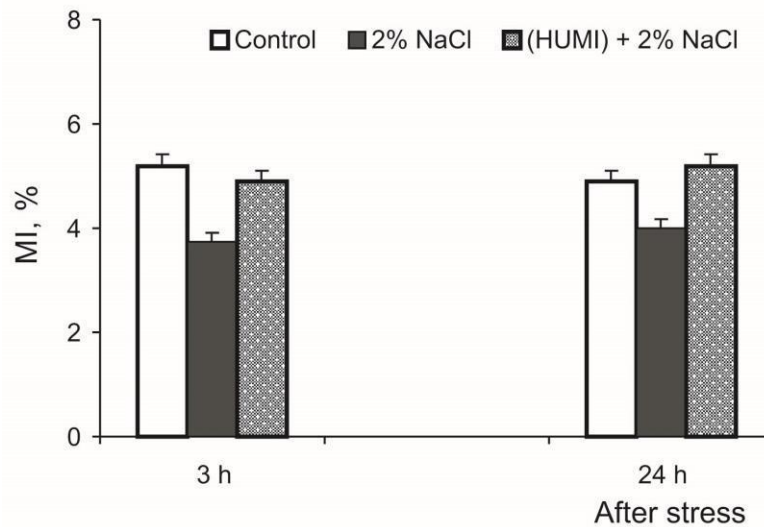


Figure 2. Effect of pre-sowing HUMI-treatment on MI of cells of apical root meristem in 4-d-old wheat seedling in the course of salinity action (3 h) and 24 h after stress withdrawal. Data are mean + SE of three independent measurements.

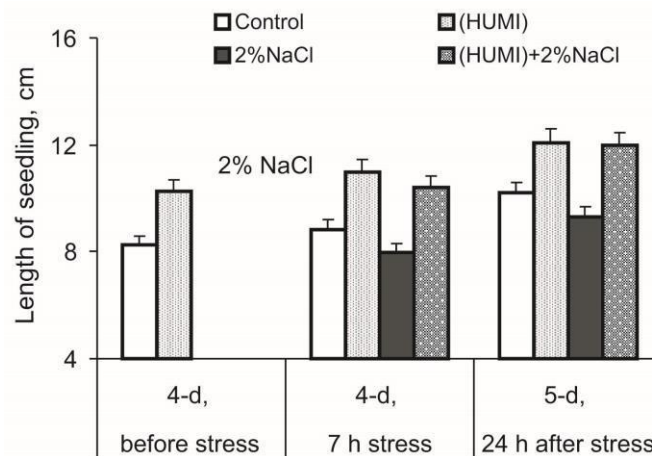


Figure 3. Effect of pre-sowing treatment with HUMI on the changes in the length of wheat seedling in the course of salinity action (7 h) and 24 h after stress withdrawal. Data are mean \pm SE of three independent measurements.

Thus, although the treatment with HUMI did not prevent inhibition of growth processes in wheat plants under conditions of short-term salinity, it did diminish significantly the level of damage exerted by the stressor contributing to maintenance of growth at higher level even in comparison with the control. This allowed us to suggest that protective effect of HUMI on growth is due to its effect on the status of hormonal system of wheat seedlings under stress. In relation to this, we conducted a comparative analysis of the changes in concentration of ABA, IAA and cytokinins in wheat seedlings treated and untreated with HUMI and subjected to 2% NaCl, as well as one day after withdrawal of stress.

Figure 4 shows that salinity led to significant changes in the hormonal status of the seedlings manifested in accumulation of ABA and decline in the content of IAA and cytokinins that may serve as an illustration of a negative effect of salinity on plants and as an evidence of the stress-induced inhibition of cell metabolism (by the decline in the level of IAA and cytokinins) resulting in inhibition of growth processes as well as development of ABA-controlled protective reactions (Ghanem et al. 2008, Shakirova et al. 2012, Kohli et al. 2013).

Thus, quantitative assay of phytohormones in wheat plants under salinity revealed their characteristic stress response manifested in hormonal imbalance. One day after withdrawal of sodium chloride from the medium, the seedlings were almost similar to the control ones, which may serve as an indicator of recovery of the cell metabolic activity and gradual recovery of the growth processes during the post-stress period. Pre-treatment of plants with HUMI did not diminish the salinity-induced decline in auxin level, but reduced significantly the stress-induced accumulation of ABA and completely prevented the drop of CKs level in the seedlings, being seemingly due to the higher initial level of cytokinins in plants pre-treated with HUMI (Figure 4).

This fact is of special interest, due to the well-known ability of cytokinins to increase stress-resistance of plants (Srivastava et al. 2007, Havlova et al. 2008, Peleg et al. 2011, Reguera et al. 2013, Shakirova et al. 2014). Consequently, the effect of HUMI on persistent accumulation of cytokinins and normalization of their content in stressed plants made an important contribution to the development of its protective action on wheat seedlings reflected in characteristics of their growth. And it is the ability of HS to increase cytokinins concentration in plants (Mora et al. 2010) that is related to their ability to increase their drought-resistance (Zhang and Ervin 2004).

One of the main consequences of NaCl stress is the loss of intracellular water, and accumulation of compatible solutes is often regarded as a basic strategy for plant protection against dehydration. Thus survival of plants under salinity depends on ability to protect water relations from disturbance, proline being one of key osmoprotectants (Szabados and Savoure 2010, Slama et al. 2015). Proline accumulation is believed to improve adaptation to salt and drought stresses by acting as a protein-compatible hydrotrope and radical scavenger (Hayat et al. 2012). In connection with this, it was interesting to carry out a comparative analysis of salinity-induced changes in the content of proline in untreated and HUMI-pre-treated wheat seedlings.

Figure 5 shows that salinity led to significant increase in the concentration of proline in wheat seedlings in comparison to the control, while the level of proline accumulation was significantly lower in plants pre-treated with HUMI. These results may serve as an argument in favour of protective action of HUMI on wheat plants, reflected in lower level of damaging effect of salinity on seedlings pre-treated with HUMI.

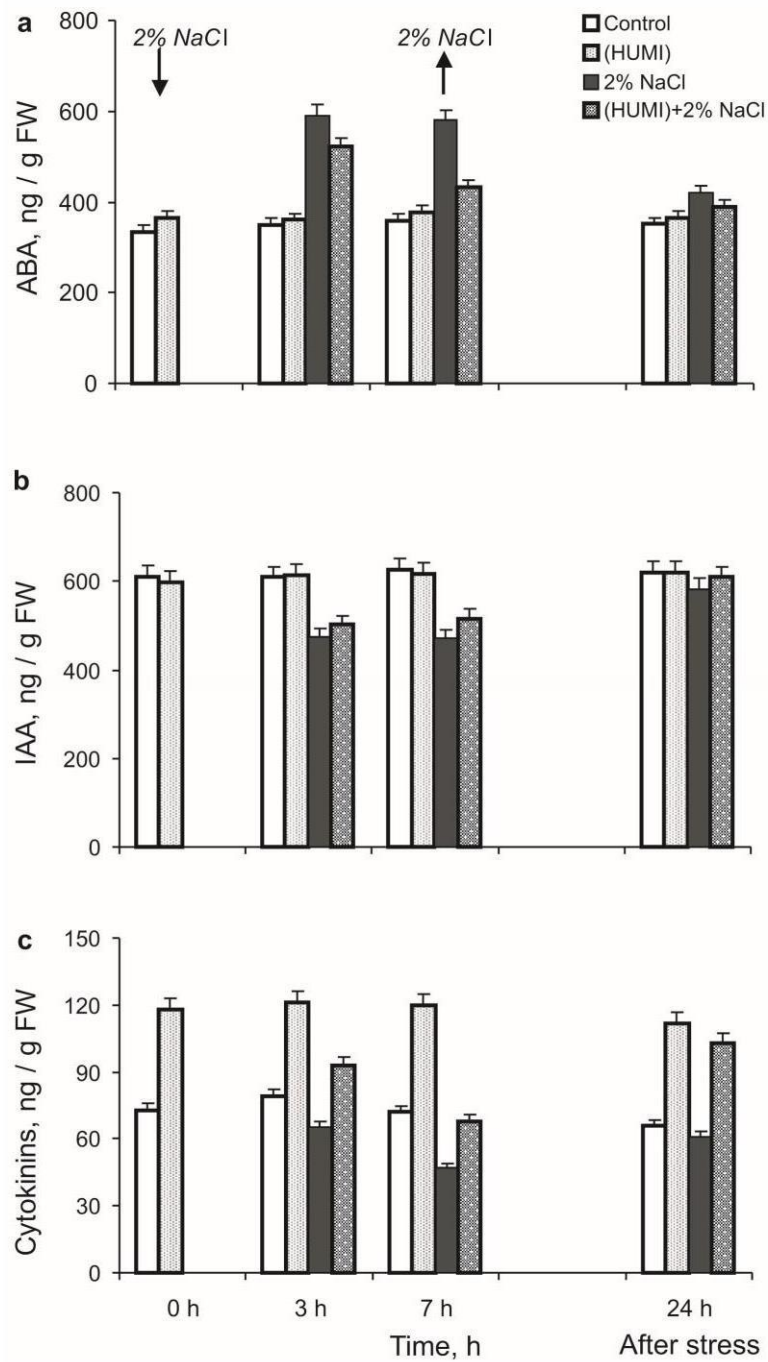


Figure 4. Changes in the levels of ABA (a), IAA (b) and CKs (c) in HUMI-pretreated and untreated 4-d-old wheat seedlings in the course of salinity action (7 h) and one day after withdrawal of 2% NaCl from the medium. Data are mean \pm SE of three independent measurements.

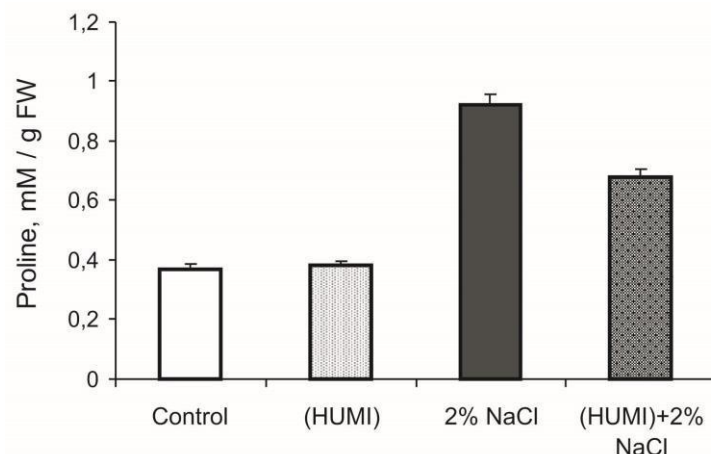


Figure 5. Effect of pre-sowing treatment of wheat seeds with HUMI on the proline content in 5-d-old wheat seedlings 18 h after exposure of 4-d-old wheat seedlings on 2% NaCl. Data are mean + SE of three independent measurements.

Salinity, as well as other stress factors, leads to oxidative burst due to activated production of reactive oxygen species (ROS), accompanied by peroxide oxidation of lipids, as estimated by the level of malondialdehyde (MDA), which, in turn, results in disturbance of integrity of membrane structures and activation of electrolyte leakage from the plant tissues (Bezrukova et al. 2008, Choudhury et al. 2013). Thus, it is possible to estimate the level of injury exerted by stressors on plants as well as the extent of protective effect of preparations according to these characteristics. Analysis of the MDA content in plants untreated with HUMI and exposed to 2% NaCl showed an increase in these characteristics (Figure 6), as well as an increase in electrolyte leakage from the tissues (Figure 7). Meanwhile, HUMI-pre-treated wheat seedlings were distinguished by a significantly lower level of MDA and leakage of electrolytes under salinity (Figures 6 and 7). It is necessary to emphasize that HUMI-treatment itself had no effect on these characteristics.

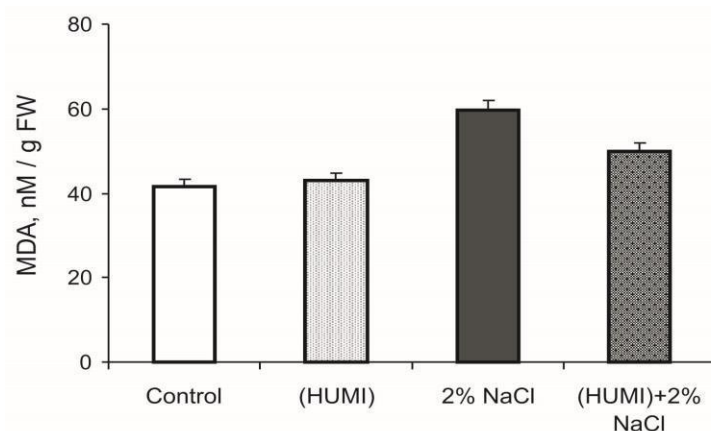


Figure 6. Effect of pre-sowing treatment of seeds with HUMI on the concentration of MDA in 5-d-old wheat seedlings 18 h after exposure of 4-d-old wheat seedlings on 2% NaCl. Data are mean + SE of three independent measurements.

Thus, the obtained data illustrate a clear effect of the preparation as a protecting agent against salinity in case of a pre-sowing treatment of wheat seeds that became apparent due to a decline in the level of stress-induced accumulation of MDA and the extent of disturbance of the membrane integrity generally reflected in physiological status of the plants.

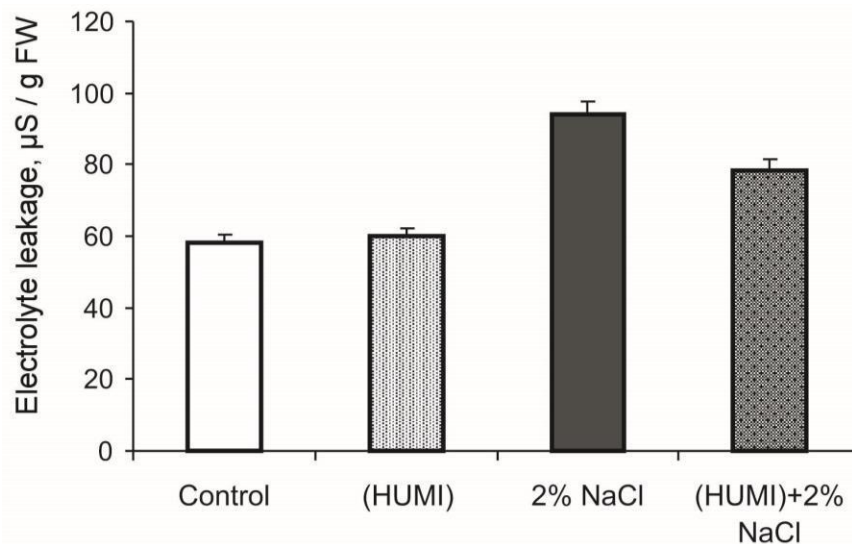


Figure 7. Effect of pre-sowing treatment of seeds with HUMI on electrolyte leakage 3 h after exposure of 4-d-old wheat seedlings on 2% NaCl. Data are mean \pm SE of three independent measurements.

Lignin and suberin are known to contribute significantly to strengthening the barrier properties of plant cell walls enabling inhibition of the uptake of toxic ions, such as sodium chloride and cadmium (De Simone et al. 2003; Moura et al. 2010, Parrotta et al. 2015). In relation to the revealed effect of HUMI on wheat plants as a protecting agent against salinity, it was important to carry out a comparative analysis of dynamics of lignin deposition in cell walls of the basal root parts under normal conditions of growing as well as under exposition of 4-d-old seedlings pre-treated and untreated with HUMI for 24 h on 2% NaCl. Specific staining of lignin with phloroglucinol allowed us to estimate lignin deposition in cell walls of roots.

Data presented in Table 2 demonstrate acceleration of lignin formation in roots of seedling pre-treated with HUMI as compared to the control. Thus, in the roots of experimental plants lignin was detectable by the fifth day and the process was intensified by the sixth day, while in the control, lignin started to appear in the walls of xylem vessels only on the sixth day. Basing on the information about involvement of exogenous and endogenous CKs in the synthesis and accumulation of lignin (Guo et al. 2005), it may be assumed that intensification of lignin deposition in root cell walls of seedlings treated with HUMI is due to 1.5 fold accumulation of endogenous cytokinins in them (Figure 1).

Table 2. Qualitative assay of specific lignin staining with phloroglucinol in the basal part of roots 24 h after exposure of seedlings pre-treated with HUMI on 2% NaCl*

Variant	Days after sowing		
	5	6	7
Control	-	+	++
HUMI	+	++	++
2% NaCl	+	+	++
HUMI+2% NaCl	++	+++	+++

*Notes: (-) no staining; (+/-) very weak staining; (+) weak staining; (++) strong; (+++) very strong staining.

NaCl-treatment also accelerated the lignification of root cell walls – lignin was already registered in 5-d-old seedlings and lignin staining increased with the age of the seedlings. Seedlings pre-treated with HUMI were characterised by additional intensification of NaCl-induced lignification of cell walls in the basal part of roots: one day after withdrawal of salt from the medium, the roots of the seedlings showed maximum staining registered not only in the cells of stele but also in the cortex epidermis (Table 2). Consequently, HUMI-pre-treatment strengthened root cell walls and increased their barrier characteristic. This was likely to due to prevention of salinity-induced gradual decline in endogenous cytokinin content in HUMI-treated plants (Figure 4).

Thus, presented results demonstrate the ability of HUMI applied as pre-sowing seed treatment to increase wheat resistance to salinity manifested by the strengthening of cell wall barrier properties as a result of increased lignification, the decline in MDA level and electrolyte leakage, the decreased amplitude of stress-induced shifts in the balance of IAA and ABA and by preventing the drop of cytokinin content in seedlings, which, in total, is reflected in reduced growth inhibition exerted by salinity and in accelerated recovery of plant growth during the post-stress period.

Impact of Toxic Cadmium Ions

Under natural environment, plants experience the action of adverse conditions, including presence of heavy metals (HM) (DalCorso et al. 2008; Chmielowska-Bąk et al. 2014). Due to the progressive contamination of soils with salts of toxic HM inducing oxidative stress and dehydration in plants and causing not only a decrease in yield, but also a deterioration of its quality, investigation of the molecular mechanisms of plant resistance to HM acquires special significance (Azevedo et al. 2012, Chmielowska-Bąk et al. 2014). Cadmium can be characterized as the most toxic of HM, being a non-essential chemical element, which does not perform any physiological functions in plants (Azevedo et al. 2012, Chmielowska-Bąk et al. 2014). It is known that plant hormones such as abscisic, salicylic and jasmonic acids, ethylene, cytokinins, brassinosteroids, nitric oxide play crucial roles in the adaptation and survival of plants exposed to different HM, including Cd (Chmielowska-Bąk et al. 2014, Asgher et al. 2015). It is known from the literature that HS-treatment contributes to a decrease in the level of toxic action of heavy metals on plants (Kharytonov et al. 2005, Zhang et al.

2013), although there is scanty information concerning mechanisms of their protective action on wheat plants exposed to heavy metals.

Addition of 1 mM cadmium acetate to the seedling nutrient medium resulted in a significant decline in MI of roots cells already within 3 h (Figure 8). HUMI pre-treatment turned out to be less effective in protecting against toxic effects of cadmium than it was in case of sodium chloride (see previous section). Nevertheless, MI of HUMI-treated stressed plants was somewhat higher than in untreated plants but lower than in control.

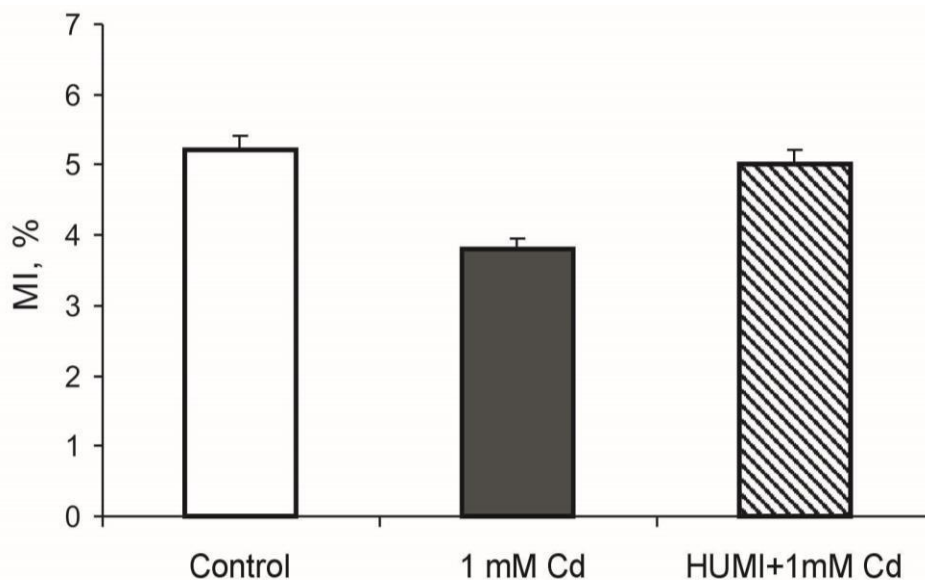


Figure 8. Effect of pre-sowing HUMI-treatment on MI of cells of apical root meristem of 4-d-old wheat seedling exposed to 1 mM cadmium acetate during 3 h. Data are mean \pm SE of three independent measurements.

We analysed long-term effects of cadmium on growth characteristics of 3-d-old wheat seedlings pre-treated and untreated with HUMI (Figure 9). As with MI analysis, seedlings pre-treated with HUMI experienced great stress due to exposure on cadmium made apparent by significant decline in their line length as compared to the control: toxic effect of cadmium increased with time. However, when compared to the plants untreated with HUMI, these plants had significantly higher growth characteristics throughout the experiment and were less inhibited under prolonged cadmium treatment (Figure 9). Pre-treatment with HUMI failed to prevent stress-induced inhibition of growth processes, however both MI of roots and their line length in seedlings treated with the preparation remained at higher level than in the variant without the treatment although lower than in control (Figures 8 and 9).

The obtained data provide evidence in favour of protective action of HUMI on wheat seedlings under conditions of cadmium stress, which is most clearly apparent during short (up to 24 h) exposition. The revealed effect of HUMI on plant response to cadmium may be due to its impact on the status of plant hormonal system.

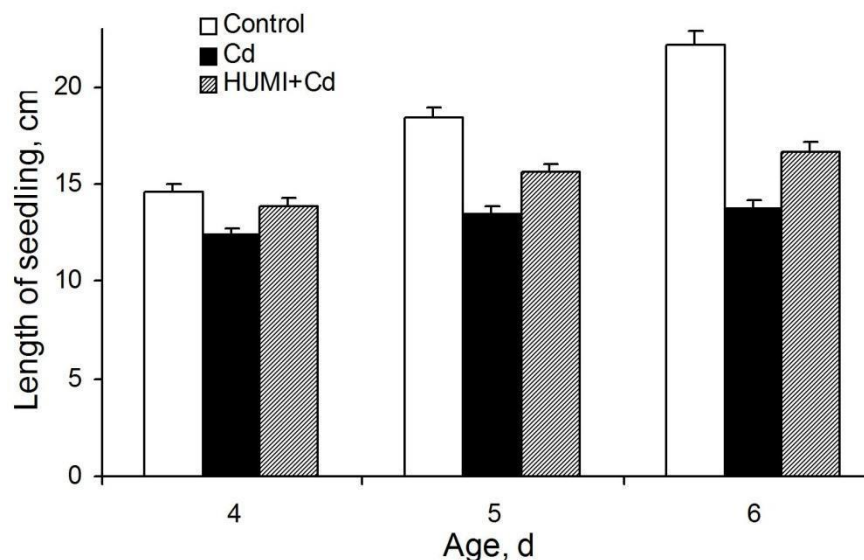


Figure 9. Effect of HUMI on the dynamics of growth of wheat plants in the course of cadmium stress. 3-d-old seedlings were transferred to the solution containing 1 mM cadmium acetate. Data are mean \pm SE of three independent measurements.

Actually, analysis of dynamics of hormone content in seedling exposed to cadmium treatment (Figure 10), revealed sharp shifts in the hormonal balance, manifested in simultaneous accumulation of ABA and drop of cytokinins level and, especially, of IAA, which is consistent with other data (Xu et al., 2010; Stroiński et al. 2013, Hashem 2014). These results are likely to serve as an illustration of the fact that wheat plants experienced severe stress as a result of toxic cadmium effect. Plants pre-treated with HUMI were characterised by lower level of Cd-stress-induced accumulation of ABA and by smaller decline in auxin concentration, while CKs remained on the control level (Figure 10).

In total, comparison of results of quantitative analysis of phytohormones in seedlings treated with sodium chloride and cadmium acetate shows that pretreatment with HUMI led to similar beneficial effect on hormonal system of both NaCl- and Cd-stressed seedlings related to reduced stress-induced shifts in ABA and IAA and maintenance of concentration of CKs on the level of the control plants. The latter is likely to be important for the manifestation of protective effect of HUMI on plants in regard to different stress factors.

Deposition of lignin in cell walls is one of protective reactions to the toxic effect of cadmium (Kováčik, Klejdus 2008, Bezrukova et al. 2011). Analysis of lignin accumulation in cell walls of the basal part of roots showed that Cd-treatment of 4-d-old seedlings led to acceleration of lignin formation within 24 h (Table 3). However, in roots of seedlings pre-treated with HUMI, reaction with phloroglucinol was significantly greater than in untreated plants. One and two days after withdrawal of cadmium, lignification of root cell walls of HUMI-treated seedlings continued to increase unlike untreated plants.

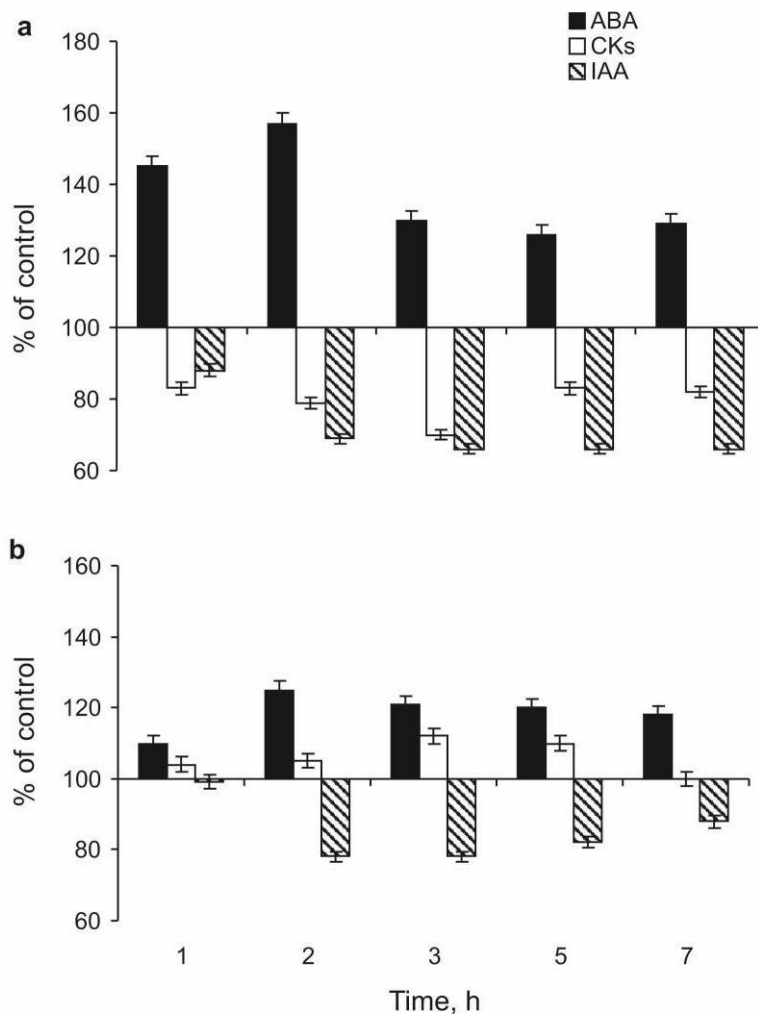


Figure 10. Effect of 1 mM cadmium acetate on concentration of ABA, CKs and IAA in wheat seedlings untreated (a) and pretreated with HUMI (b). Data are mean \pm SE of three independent measurements.

Table 3. Accumulation of lignin in the basal part of roots of wheat seedlings pre-treated and untreated with HUMI 24 h after exposure to 1 mM cadmium acetate*

Variants	Days after germination		
	5	6	7
Control	-	+	++
Cd	+	++	++
HUMI+ Cd	++	+++	+++

* Symbols are the same as in Table 2.

It is known that while entering a plant heavy metals are localized in different tissues and organs, where they exert their toxic action on cell metabolism (DalCorso et al., 2008). Pre-treatment with HUMI was shown to contribute to additional activation of lignin deposition in

cell walls of Cd-stressed plants (as well as in plant exposed to 2% NaCl), that strengthens their barrier properties against penetration of toxic ions into plant tissues. Thus, it was of interest to compare the pattern of cadmium distribution in tissues of wheat seedling roots untreated and treated with HUMI. With this aim, we applied histochemical method of cadmium registration in different root tissues with the help of ditizone, forming water insoluble salts with cadmium (ditizonate), having red colour (Bezrukova et al. 2011). This approach enables discovery of cadmium localization in living tissues.

Table 4. Distribution of cadmium in the cells of roots of 5-d-old wheat seedlings pre-treated and untreated with HUMI*

Variants	Root zone					
	Epidermis	Primary cortex	Endodermis	Pericycle	Meta-xylem	Central cylinder
Control	-	-	-	-	-	-
Cd	+	+	+	+	+	+
HUMI+Cd	++	-	-	-	-	-

* 4-d-old seedlings were treated with 1 mM cadmium acetate during 24 h.

Data in Table 4 show that cadmium ditizonate was revealed in practically all tissues of transverse section of roots untreated with HUMI, while in sections of HUMI-pre-treated plants it was present only in epidermis tissue. Thus, pre-treatment of plants with HUMI prevents massive penetration of cadmium into roots and, consequently, inhibits its distribution throughout the plant. Nevertheless, it is important to emphasize that HUMI did not prevent toxic effect of cadmium on a whole plant level, and data on hormonal status of the seedlings and their growth process serve as an evidence of this.

CONCLUSION

Due to their sessile life style plant organisms have to adjust to frequent changes in environment although they are limited in the choice of strategy for survival. Understanding various mechanisms forming the basis of plant adaptation has a pivotal fundamental and applied importance. It may really not only help the advance of plant breeding for resistance, but enable purposeful management of the mechanisms of adaptation to extreme external conditions with the help of growth regulators. Growth stimulating and adaptogenic effects of humic acid to unfavourable environmental factors have been known for a long time and, due to this, they found a broad application on different crops all over the world. In this manuscript the main attention was paid to effects of pre-sowing semidry treatment of seeds with HUMI preparation used as a natural growth regulator on the status of hormonal system in wheat plants in relation to induced resistance to toxic ions.

Accomplishment of the research allowed revealing a long-term growth-stimulating and protective action of HUMI on wheat plants against toxic effects of sodium chloride salinity and cadmium manifested in decreased negative effect of these stressors on the intensity of growth processes. Since HS are acknowledged stimulators of growth processes, it may be expected that growth stimulating effect of HUMI on wheat plants is due to its impact on the

endogenous system regulating cell metabolism and forming the basis of growth and development, i.e., due to its effect on hormonal system. Actually, we have shown, for the first time that HUMI applied as a pre-sowing treatment rendered a prolonged effect on phytohormonal balance manifested in persistent 1.5 fold accumulation of cytokinins against the background of the absence of significant changes in the IAA and ABA content in plants. This is evidence in favour of assumption that growth promotive action of HS may be attributed to CKs playing a key role in regulation of cell metabolism and forming the basis of plant growth. Moreover, another property of HS, important for their application in crop production has been revealed. It was shown that HUMI increases unspecific resistance of plants to stress factors, made apparent by growth characteristics of wheat seedlings. This protective effect of HUMI is also due to its impact on the status of hormonal system manifested by the reduced stress-induced shifts in the balance of IAA and ABA and prevention of the CKs drop. CKs are known to be characterised by a broad spectrum of regulatory action, including activation of growth processes and implementation of resistance against stress factors of different nature.

This allows us to conclude that combination of growth promoting and protective action against stress factors in HUMI preparation is due to induction of accumulation of endogenous cytokinins in HUMI-treated wheat plants.

ACKNOWLEDGMENTS

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Chapter 4

**SURFACE-ENHANCED RAMAN SCATTERING AND
FLUORESCENCE SPECTROSCOPY
ON SILVER PLASMONIC NANOPARTICLES
AS INNOVATIVE TECHNIQUES
TO STUDY HUMIC SUBSTANCES**

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ABSTRACT

The application of normal Raman spectroscopy is not able to characterize the soil humic substances (HS) structure due to the intense fluorescence emission, which overlaps the Raman bands. Nevertheless, surface-enhanced Raman scattering (SERS) spectroscopy, based on localized surface plasmon (LSP) in nanostructured metals, leads to a remarkable local electromagnetic field enhancement and to the fluorescence quenching via a charge-transfer mechanism.

The SERS technique was thus applied to investigate the structure and conformational changing of HS. However, the fluorescence is not quenched if the distance between fluorophores and LPS is relatively far. An intensification of the fluorescence emission through the surface-enhanced fluorescence (SEF) at optimal distance above 10–100 Å can be detected.

At distances further than this value, SERS and SEF signals are simultaneously emitted and recorded for molecular species placed in the vicinity of metal nanoparticles. Since the average size of HS is larger than this value, the fluorophores included in the HS structure are valid candidates causing intense SEF *plus* SERS joint emission spectra, without the intrinsic quenching observed on a surface.

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In the present chapter, surface-enhanced Raman scattering and fluorescence spectroscopy on silver plasmonic nanoparticles are applied to investigate the HS structure in aqueous medium at different pH.

Keywords: Humic substances, surface-enhanced raman scattering, surface-enhanced fluorescence, silver nanoparticles, functionalization, methyl viologen

INTRODUCTION

Humic substances (HS) are natural organic components, which are widely distributed in soils, river sediments, and brown coals [1]. Based on their solubility in aqueous solution, HS are operationally divided into three different fractions: (i) humic acid (HA), the fraction soluble at alkaline pH values; (ii) fulvic acid (FA), the fraction soluble at all pH values; and (iii) humin, the fraction insoluble at all pH values [2].

The variety of chemical and biological reactions involved in their genesis make HS very complex. Thus, the characterization of the shape, conformation, structure, and composition of HS is a focal point in understanding their physico-chemical reactions and in predicting the fate of environmental pollutants, and the biogeochemistry of soil organic carbon [3].

Models such as polymer and polymer distribution have been developed to characterize the HS molecular structure [4-5]. However, evidence emphasizes the relevance of non covalent intermolecular interactions (e.g., $\pi = \pi$, CH- π , van der Waals, charge-transfer, and hydrogen-bonding) between small organic moieties of HS [6]. A micellar structure of HS formed through a spontaneous aggregation has been proposed [7]. The hydrophilic domain consists of polar groups (e.g., carboxylic acids), whereas the hydrophobic domain is located in the inner sides and is composed of vegetal macromolecules. Because the intermolecular interactions are very weak, the micelle is disaggregated easily into several smaller aggregates in the presence of organic acids (mono-, di- and tricarboxylic acids) and pH variation [8]. This implicates that the broken segments reform micelles, and micellization and disaggregation may then continue on and on.

As in the micellar theory, the supramolecular association of heterogeneous molecules held together by hydrophobic interactions (van der Waals, π - π , ion-dipole) and hydrogen bonds was proposed by Piccolo (2002) [9]. A major aspect of the supramolecular association is that hydrophobic and hydrogen bonds stabilize the structure of molecular aggregates while the former become more important with the changing environmental conditions [10-12].

Most of the conclusions have deduced that HS are able to rearrange and restructure in response to pH: i) at the alkaline pH, HS are in disperse form because intramolecular hydrogen bondings are completely disrupted [13]; ii) acidification of HS with weak acids solutions produces a significant decrease of apparent molecular sizes or disaggregation through the disruption of weak non covalent interactions, such as van der Waals, π - π , and CH- π [13]; iii) at the lowest pH values (<3), the HS collapse. This phenomenon is mainly due to the protonation of carboxylate groups that favor an increase in the number of intra- and intermolecular hydrogen bonds inside the humic macromolecules [14].

The nature and distribution of the carboxyl groups are of especial interest for determining the HS solubility and aggregation but also the ability to complex metals and organic molecules. In previous papers [15-17] it was shown that manure amendment led to an

increase of the organic carbon and carboxylate groups content in comparison to other treatments. These structural changes may be of great importance in determining the mechanisms involved in interactions of HS with xenobiotic molecules and, consequently, their fate in the environment.

Despite the indisputable contribution of several spectroscopic analytical methods (e.g., NMR, FT-IR) in evaluating the HS structure, there are some limitations which reduce their potential, such as sensitivity, sample concentration and purity, etc. Thus, there is a clear need to find advanced new techniques in order to better understand the complex structure of HS.

In recent years, several optical spectroscopy techniques (Raman, IR and fluorescence) have undergone a renaissance due to the notable characteristics of metal nanostructures where localized surface plasmon resonance (LSPR) can occur (Figure 1). LSPR is generated by the plasma electron oscillations occurring in nanostructured active metals (Au, Ag and Cu), leading to a remarkable local enhancement of the electromagnetic field and, thus, of the spectroscopic signal (from 6 to 14 orders of magnitude) mainly for those molecules close to the surface [18], allowing the spectral recognition of even single molecules [19].

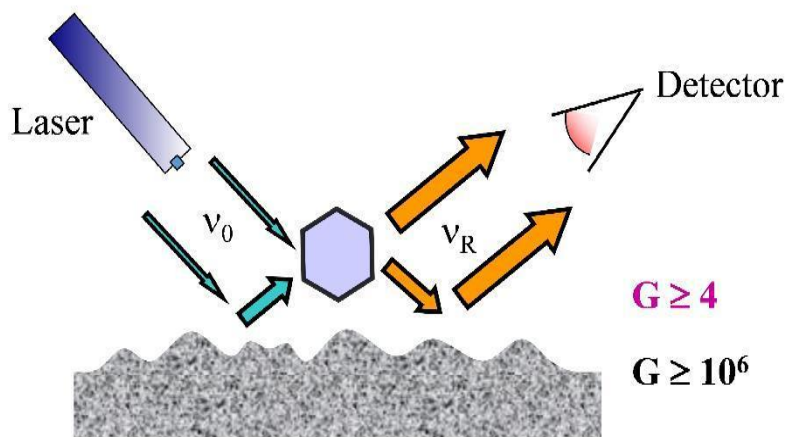


Figure 1. SERS effect of molecules adsorbed onto metal nanoparticle (Ag or Au). The molecules in proximity of the surface are perturbed and this strongly enhances the spectral signal by two different processes: a) intensification of the incident radiation (left) and the scattered radiation (right). The intensification (G) is *ca* 4 in the case of planar surfaces and can reach 10^6 in the case of nanostructured plasmonic surfaces.

SERS thus becomes a powerful tool in characterizing molecules close to plasmonic metallic surfaces: the mechanism of interaction and the tridimensional structure can also be deduced from the SERS spectra. By virtue of this interaction, molecules may undergo a structural change that makes the SERS spectra different from the normal Raman spectrum. Moreover, the metal nanoparticles are charged and may influence the molecules' behavior, interacting with the metal surface [20-21]. This phenomenon is particularly important in the case of humic substances, as they carry a strong negative charge. In addition, another advantage of the SERS technique is the possibility to modify the metal surface either by modifying the surface density of charge or by introducing a specific functionalization that makes the surface more accessible for those molecules exhibiting a poor affinity for the surface [22]. These modifications allow more detailed analysis of molecules.

Surface-enhanced Raman scattering (SERS) technique has provided important information on structural and chemical composition of humic substances [23-24] in relation to extraction and purification procedures [25] and soil fertilization [14]. In these studies the SERS intensity of HS depends on parameters such as pH and the aromaticity degree [23-27]. In particular, the SERS intensity of HS decreases in a pH range from 5.0 to 7.0, and the metal/HS interaction takes place through carboxylate moieties and highly substituted phenols [23-26].

Recent works have demonstrated that the joint application of SERS and surface-enhanced fluorescence (SEF) is a useful approach in characterizing humic substances [14, 27-28]. The SEF technique has not been employed as much as SERS because of the charge transfer effect, which takes place on the surface and which, in turn, leads to an actual quenching of the fluorescence. The intensification of the fluorescence occurring in SEF is also induced by the LSP resonance of nanostructured plasmon metals [29].

However, the net effect seems to vary depending on the distance between the fluorophore and the surface, as well as on the intrinsic quantum yield of the fluorophore [30]. In general, the fluorescence is not quenched if the molecule is relatively far from the surface. The estimated optimal distance for a good SEF enhancement is achieved above 100\AA [31]. At distances further than this value, SERS and SEF signals can be simultaneously emitted and recorded for molecular species placed in the vicinity of nanoparticles [32].

The humic substances can ensure an intense emission of both Raman and fluorescence signals because they are characterized by aromatic or polyaromatic moieties, which can be adsorbed or placed in the vicinity of plasmonic nanoparticles. Thus, HS are suitable candidates for application of SERS *plus* SEF combined emission.

MATERIALS AND METHODS

Humic Substance Extraction

The humic substances were isolated from soil samples taken from the experimental farm of the University of Bologna (Cadriano, Italy). This soil is classified (Soil Survey Staff - USDA SCS 1989) as a *Typic Udochrept*. The main physical-chemical characteristics of the soil studied were the following: pH (in water) 6.9; texture: sand 56%; silt 16%; clay 28%; total calcium carbonate (CaCO_3) < 1%; total organic C (TOC) 7.5 g kg^{-1} ; total N 1.1 g kg^{-1} ; cation exchange capacity $16.5\text{ cmol}_c\text{ kg}^{-1}$ [33].

Silver Nanoparticles Preparation

Silver nanoparticles were prepared by using hydroxylamine hydrochloride as a reducing agent as previously reported [34]. Samples for SERS measurements were prepared by adding 10 L of 10^{-3}M solution of PQ to 100 μL of an aqueous solution of HA, which was obtained by dissolving 1 mg of the corresponding humic fraction in 1 mL of tri-distilled water. This solution was then left to interact for 24 h. Ten μL of this solution were then added to 900 μL .

of the silver colloid. The colloidal suspension was activated for SERS by the addition of 40 μL of 0.5 M KNO_3 .

Raman Spectroscopy

The SERS spectra were recorded on a Renishaw Raman Microscope System RM2000, using the macro configuration equipped with a notch filter and an electrically refrigerated CCD camera. The Raman and Fluorescence emission spectra were recorded by the CCD camera. The 514.5 nm line of an Ar^+ laser was used. The laser power at the sample was set to 2.0 mW. Spectral resolution was 2 cm^{-1} , by selecting an appropriate slit. The SERS spectra were baselined to eliminate the contribution of the fluorescence by using the algorithm provided by the Origin 8.0 program.

FT-Raman spectra excited at 1064 nm were obtained with a Bruker RFS 100/S instrument by using the line at 1064 nm equipped with a Nd:YAG laser and a Ge detector cooled by liquid nitrogen. The resolution was set to 4 cm^{-1} , and 180° geometry was employed.

RESULTS AND DISCUSSION

Surface-Enhanced Emission (Raman and Fluorescence) Spectra of Humic Substances

The normal Raman spectrum of soil HS ($1\text{ }\mu\text{g mL}^{-1}$) was compared with that of the surface-enhanced emission on Ag NPs presence (Figure 2).

The emission spectrum of the solution showed the water Raman bands at 1640 cm^{-1} (very weak) and 3420 cm^{-1} (strong) and a broad fluorescence band produced at 630 nm by HS. No Raman band of HS was seen. Instead, the SEE spectrum of HS showed strong Raman bands between $1200\text{-}1650\text{ cm}^{-1}$, and a fluorescence emission with a maximum at 650 nm. The latter was threefold intensified in relation to that seen in aqueous solution. The intensification of Raman bands corresponding to the HS and the shift observed for the fluorescence emission maximum suggest structural changes in the HS upon adsorption on the metal. Thus, the SEE spectrum of the HS provided a combined enhanced Raman and fluorescence information because the cross-sections corresponding to both emissions are of the same order of magnitude.

Although the total emission measured from adsorbates adsorbed on metal NPs is also a combined luminescence signal with contributions from both the molecule and the metal background luminescence [35], we can consider that the latter is negligible in relation to the luminescence of HS in the SEE spectra.

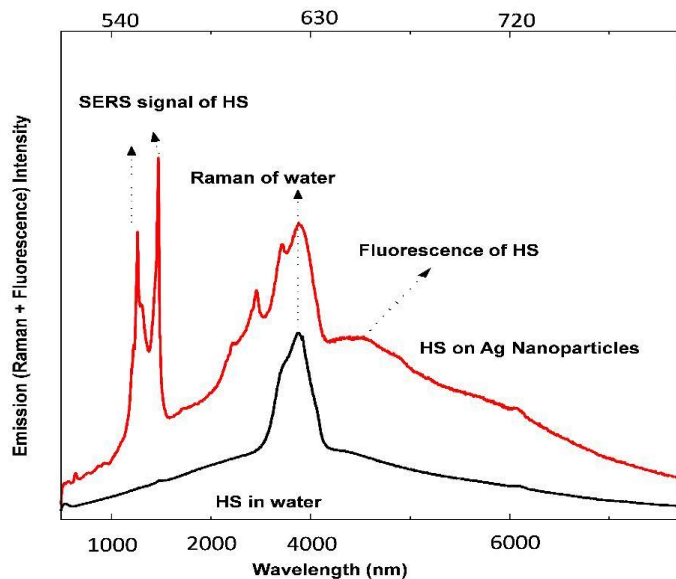


Figure 2. Normal Raman spectra of soil humic substance (HS) (bottom) and in the presence of Ag nanoparticles (top).

Effect of pH on the SERS and SEF

The SERS effect is strongly dependent on the distance of the scattering group from the metal surface, in such a way that only the Raman bands of those chemical groups close to the surface are actually enhanced in the SERS spectrum.

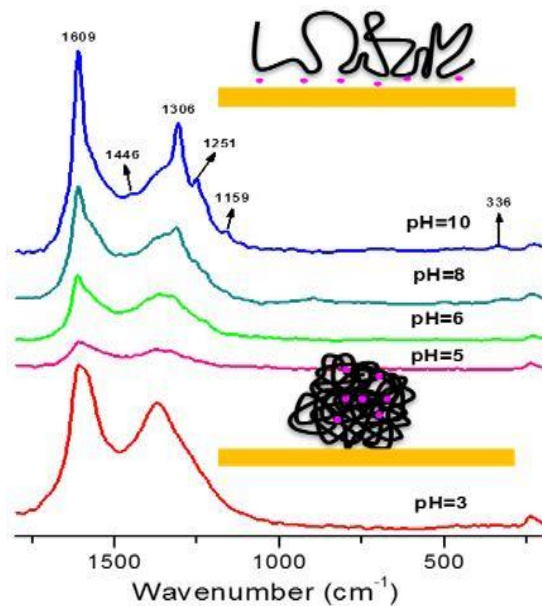


Figure 3. SERS spectra of HS in water solution at different pH values.

The distance from the surface can change by macromolecules when the tridimensional structure changes as a result of some environmental modification. In this context, the pH is one of the main factors that can influence the tridimensional structure of HS due to the modification of the protonation state that affects carboxylate and phenolate groups involved in H-bonds. Consequently, the SERS spectra of soil HS are good candidates for undergoing strong changes on pH variation. The SERS spectra of HS at different pH values are shown in Figure 3.

At alkaline pH the SERS spectrum of HS is dominated by two strong and narrow bands at around 1300 and 1610 cm^{-1} . They are attributed to the aromatic ring vibrations in polycyclic aromatic hydrocarbon (PAH). Under this condition, an expansion or dispersion of the HS macromolecular structure, due to the ionization of carboxylic and phenolic groups, is expected. The Raman intensity changes with the pH, reaching a minimum at pH 5.5 (Figure 4), while the fluorescence emission undergoes the opposite behavior, i.e., a maximum emission is seen at precisely pH 5.5 (Figure 4).

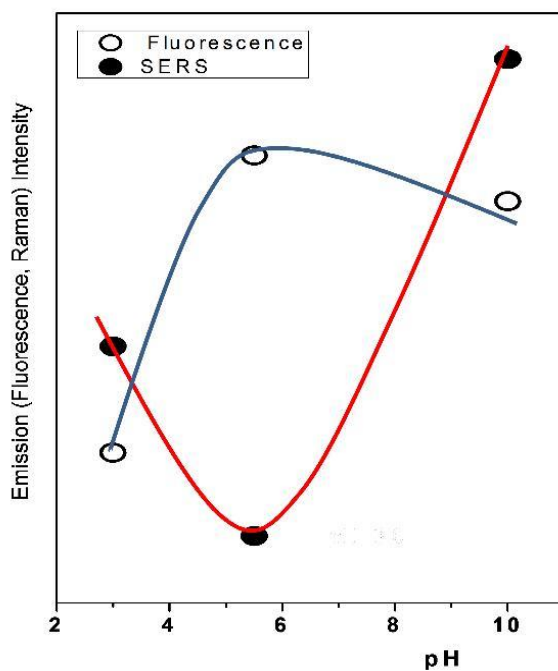


Figure 4. Effect of pH on the Raman and fluorescence emission of HS spectra.

The opposite behavior of fluorescence and SERS emissions in soil HS can be accounted for on the basis of the structural modifications induced on HS upon acidic groups ionization (carboxylic and phenolic groups) on increasing the pH. Other authors have also reported similar variations with pH of the HS fluorescence emission [36]. Soil HS undergo a structural expansion in the pH range between 8-10, resulting in an opposite response on the fluorescence and Raman intensities.

On one hand, this favors the approach of the inner polycyclic aromatic hydrocarbon (PAH) moiety residues localized in the inner part of HS to the metal leading to an enhancement of the SERS signal. However, this expansion induces the fluorescence

quenching due to two main reasons: a) the higher exposure of fluorophores to the aqueous polar medium, and b) a charge-transfer induced by the closer metal surface. Even if at alkaline pH the carboxylic groups (hydrophilic domain) are mostly favored to approach the metal nanoparticle surface, interactions between polyaromatic groups (hydrophobic domain) and metal surface are not excluded. We may infer that PAH residues may be mainly localized inside the HS macromolecular structure.

At pH 5, a considerable decrease in the band intensity at 1308 and 1609 cm^{-1} was observed (Figure 3). These changes are a consequence of: i) an increase of intermolecular interactions, due to both a decrease of electric repulsions and an increase of H-bonds, and ii) coagulation of smaller HS micelles into larger ones. These facts lead to a macromolecular shrinkage. Consequently, HS have a more compacted form, and thus PAHs residues remain inside the HA structure, i.e., far from the metal surface, yielding a weaker SERS intensity. At these conditions, the fluorescence signal is enhanced due to the fact that fluorophore groups are protected from the aqueous medium and the metal surface remains far from them, thus leading to lower quenching effects.

On the contrary, at pH 3 a significant enhancement of the SERS intensity was observed (Figure 3). This effect is caused by the protonation of acidic groups that strongly reduce the negative charge of HS. The higher hydrophobicity of the macromolecules induces a massive adsorption on the metal surface that places the HS in proximity to the metal nanoparticle surface. The increase of HS molecules adsorbed on the metal is furthermore intensified by the area reduction per molecule induced by the molecular shrinkage at low pH [37].

However, the analysis of these bands is very complex because of the sum of different components and, in particular, carbonaceous material [38] might be produced due to photodegradation [39]. The strong pH dependence of HS, as revealed by the variation of the SERS spectra, excludes a massive photodegradation of the adsorbed HS. We attribute these changes to rearrangements of functional groups such as benzoic, polyphenolic and aliphatic ones. The fluorescence quenching observed at lower pH can now be attributed to the protonation of its acidic groups and the lower quantum efficiency of protonated groups [14].

The dependency of SERS intensity on pH is better displayed in Figure 4, where a minimum at ca. 5.5 was observed. Terashima et al. [37] demonstrated that the apparent pK (pK_{app}) of HS was approximately 5.5. As a consequence, at pH 5.5 great changes in physical properties, such as surface tension, surface area occupied by HS molecules adsorbed onto a surface and hydrophobicity, occurred. A similar pH value was described by other authors when studying HS aggregation [40].

Ag Nanoparticles Functionalized with Methyl Viologen

The functionalization of metal nanoparticle surfaces (NPs) has been applied in the field of Raman spectroscopy for compounds which are SERS inactive due to their inability to approach the metal surface. The nanoparticle surface coated with appropriate host molecules (e.g., calixarenes or nanotubes [41] may promote the adsorption or be bound specifically to the target molecule. Such coated surfaces have been used to detect molecules with no affinity for NP surfaces, such as polycyclic aromatic hydrocarbons [42].

Bipyridinium dications are also known as viologen compounds. In particular, dimethyl viologen is widely used as a herbicide and it forms an interesting interaction with soil HS.

These interactions have been studied by fluorescence spectroscopy [43]. In the present work we show how the dimethyl viologen/ HS complex leads to a great enhancement of the emission (Raman and Fluorescence) signal.

SERS and SEF emissions of HS are shown in Figure 5. These spectra are dominated by the Raman bands corresponding to HS and the broader fluorescence emission with a maximum at 3700 cm^{-1} (corresponding to 635.5 nm). The Raman intensity depends on the pH and dimethyl viologen presence. As discussed above, at alkaline pH the SERS intensity of the bands at 1610 and 1309 cm^{-1} is assigned to polyaromatic compound vibrations closer to the Ag surface. On the contrary, the SEF emission corresponds to the excitation of aromatic groups at 514.5 nm , which undergoes a decrease (relative to the water band at 3400 cm^{-1}) due to the closer position of these groups with respect to the surface because of a quenching mechanism based on the energy transfer from the polyaromatic compounds and the metal surface.

Functionalization of Ag NPs with dimethyl viologen influences the SERS and SEF intensities of HS at pH 6.0 and 10 (Figure 5). The intensities corresponding to the SERS band at 1610 cm^{-1} and the SEF band at 3700 cm^{-1} (635.5 nm) are normalized to the Raman band of water at 3400 cm^{-1} .

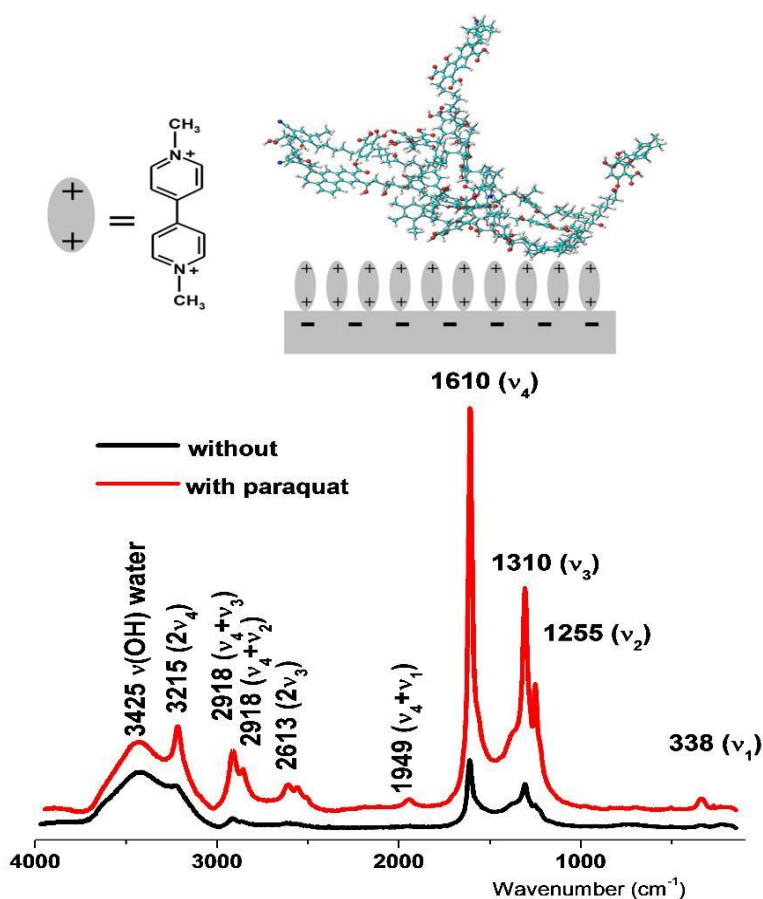


Figure 5. Functionalization of Surfaces with methyl viologen: Effect on the Surface enhanced Emission (SERS+SEF) spectrum of soil humic substances.

At pH 6.0, the dimethyl viologen leads to a weakness of SERS and SEF intensities of HS. At this pH value, the carboxylic groups are deprotonated and, thus, the presence of dimethyl viologen would balance the HS negative charges and favor the aggregation forms (Figure 5). As a consequence, most of the humic molecules remain far from the metal surface to undergo the electromagnetic field enhancement, leading to a decrease of both SERS and SEF intensities.

At pH 10, the SERS intensity is remarkably intensified while a reduction of SEF intensities is observed (Figure 4). This opposite behavior between the SERS and SEF intensities displayed at high pH values was described above. These results can be explained bearing in mind the conformational changes that can be experimented by HS as a function of the pH and the possible interactions for which methyl viologen can be adsorbed onto HS.

At alkaline pH, HS are under a relatively extended conformation due to the uncoiling of the tridimensional structure (Figure 3). Their aromatic residues are easily exposed to the exterior due to the electrostatic repulsion from the high negative site density, becoming more accessible to interact with dimethyl viologen. In addition, inner carboxylic groups are completely deprotonated and, as a consequence, the overall electric charge of the HS increases and also the electrostatic interaction with dimethyl viologen is higher. This explains the marked intensification of the SERS signal in the spectrum. On the other hand, the fluorescence decreases due to the quenching induced by aromatic groups approaching the metal surface. In addition, charge-transfer interactions between HS π -electron rich groups and dimethyl viologen π -electron deficient aromatic rings are also possible.

Two possible mechanisms influence the SERS and SEF emission in dimethyl viologen/HS complexes (ionic and stacking) by affecting the aggregation state of HS and their adsorption onto the metallic surface. In general, a large aggregation implies a lower adsorption onto the surface, and induces an increase in the SEF emission, while the SERS undergoes a reduction. In contrast, a large adsorption, involving a lower aggregation, leads to an increase of SERS and a concomitant decrease of the SEF. Regarding the SERS emission, the predominant effect is the adsorption on the metal surface, because a large adsorption makes it possible to approach the surface and benefit from the large intensification of the electromagnetic field on plasmonic nanoparticles due to large localization of the field.

CONCLUSION

Surface-enhanced Raman scattering (SERS) and fluorescence (SEF) spectroscopy on silver plasmonic nanoparticles are promising techniques to investigate the structure and dynamics changes of HS under different environmental conditions. The SERS and SEF emissions are greatly affected by several phenomena occurring in the soil HS, such as aggregation, coiling/uncoiling and the adsorption onto the metallic surfaces.

These processes mostly depend on the HS chemical composition and pH values. SERS and SEF emissions follow opposite trends due to the effect of the metal surface on the groups responsible for these emissions.

The functionalization of Ag NPs with dimethyl viologen greatly influenced the SERS and SEF intensities of HS. Two different mechanisms are possible in dimethyl viologen /HS complex formation: ionic and stacking of aromatic rings, and their relative importance also

depends on the chemical composition of the HS. These interactions are more intense at high pH values, since at these conditions the anionic groups of HS are more accessible to the dimethyl viologen.

It is expected that future improvements in this technique will be directed towards the development of new functionalized Ag NPs to provide structural information on the target molecule.

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Chapter 5

**INFLUENCE OF HUMUS SUBSTANCES FUNCTIONAL
CHARACTERISTICS ON THE SPECIATION OF METALS
IN NATURAL WATERS (MODEL EXPERIMENTS
AND CALCULATIONS)**

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ABSTRACT

The chapter describe the complexation of metal ions with humus substances in soils and water. Humus substances as the major biochemical components of the soil and water have a significant impact on the forms and migration of metal and the toxicity of natural objects. In this chapter presents the results of large-scale chemical experiments: the study of the structural features (zonal aspects) of humus substances extracted from soil and water natural climatic zones (more than 300 objects) of Russia (European Russia and West Siberia); the influence of structural features on the physic-chemical parameters of humus acids and, in particular, on their complexing ability. The functional specifics of humus matter extracted from soils are estimated using spectrometric techniques. Conditional stability constants for Fe(III), Cu(II), Pb(II), Cd(II), Zn(II), Ni(II), Co(II), Mn(II), Cr(III), Ca(II), Mg(II), Sr(II), and Al(III) are experimentally determined with used electrochemical, spectroscopic analysis methods. The activities of metals are classified according to their affinity to humus compounds in soils and water. The determined conditional stability constants of the complexes are tested in model experiments, and it is demonstrated that Fe and Al ions have higher conditional stability constants than ions of alkali earth metals, Pb, Cu, and Zn. Furthermore, the influence of aluminum ions and iron on complexation of copper and lead as influence of lead and copper on complexation of cobalt and nickel have been identified. The metal forms a large number of lakes are calculated based on the results of the experiments. The forms of finding metals in more than 900 lakes in Russia were analyzed with used of zonal features of humus substances. This chapter describes the main chemical mechanisms of distribution of metals in forms in water lakes of European Russia and West Siberia.

ROLE OF WATER ORGANIC MATTER IN FORMATION OF CHEMICAL COMPOSITION

Anthropogenic intake of soluble compounds metals in the soil and water environment - a widespread phenomenon. Forms of finding metals in natural objects have varying degrees of toxicity. Especially dangerous are the ionic forms of occurrence for most metals.

A unique feature of metals distinguishing them from other pollutants is that their potential toxicity and bioavailability in the environment depend to a large extent on their speciation [1-4]. In natural waters, metals exist as free hydrated ions, complexes with organic and inorganic ligands, and compounds sorbed on the surface of solid mineral and organic particles.

The speciation of a metal is controlled by the conditions of the aqueous medium: pH, concentration of various ligands, size and amount of suspended particles, etc. [3]. The prediction of biological activity (accumulation and toxicity) is based to some extent on the knowledge of equilibrium conditions in solutions and physicochemical properties of metal ions.

The biological activity of a metal is defined as its ability to penetrate and accumulate in living organisms, as well as associated toxic effects [1-4]. For instance, the investigation of metal complexation showed that biological uptake and toxicity are in essence chemical processes: metal ions react with functional groups (sulphy

dryls, amines, carboxyls, hydroxides, and oxides) on the surface of cells, in membranes, enzymes, etc. [5]. The physicochemical activity of metals depends on a number of factors, the most important of which are the identity of the metal ion and its complexation capacity and conditions in the system affecting metal speciation.

The metal ions in natural waters are capable of complexing with the anions of organic and inorganic nature. The most important organic anions, which make the largest contribution to the inactivation of most metals ions - humus substances (HS), extracted from the soil. Their concentration in the surface water is in the range of 1-100 mg / l and due to the specific structure of these compounds to possess excellent makroligands properties.

In the territory of Russia are widespread colored water with high concentrations of humus substances due to the nature of formation waters [6]. Increased concentrations of Fe(III), Al(III), Mn(II) due to humification watersheds are characteristic of feature of the natural objects chemical composition.

The rules of water quality standards in Russia (about concentrations of metals and ecotoxic properties) do not include regional and local characteristics of the chemical composition of water and do not use information about complexing forms of metals with HS. However, direct determination of the forms of metals in natural waters are extremely complex and time-consuming task [6-7]. The study of complexation in each natural object - a problem that requires a generalization of contradictory data in the literature, extensive experimental studies, mathematical calculations and accounting for many natural factors. An urgent task of our time is to develop methods that allow based on water chemistry to predict the speciation of metals and toxicity level of water. In order to a correct and comprehensive study of the migration of metals in the system "soil-water" is very important to investigate the processes of complexation of metal ions with humus substances extracted from soil (various natural areas) and use the data to predict of the forms of metals in natural waters of these areas with a

high degree of reliability. Particular attention is paid to the research of such qualitative features of humus substances as dominant functional groups by NMR and IR spectrometry and to identify the changing in the IR spectra after interaction with metal ions. Also, the study of complexation with the help of various physical and chemical methods of analysis, such as voltammetry, potentiometry, ionometry. The experimental constants make it possible to calculate the form of finding metals in natural water bodies using equation material balance, equilibrium constants of mathematical expressions, equations proton equilibria and electrical neutrality.

HUMUS SUBSTANCE: STRUCTURE AND FORMATION

Humus acids refer to organic objects of stochastic character along with oil and lignin [8]. Due to their chemical nature is randomized polymers oksipolikarbonic aromatic acids [3]. Due to the specific structure (Figure 1) - hydrophobic aromatic skeleton and functional richness - humus substances exhibit makroligands properties. They complexing with metal ions [1-4] that determines, as noted [1-4] their protective properties in the biosphere.

The formation of HS of soils - a very complex process of biological and biochemical reactions of plant residues (and animal) origin in the humus horizon - the third and final layer of the leaf and grass litter [3]. At the heart of this process is, as noted I Perminova [6], selection of the most biothermodynamic stable structures and macromolecules in their connections randomly. This could mean a sufficient variety structure of humus compounds depending on the conditions of formation [3].

The biochemical synthesis of natural humus substances (the views of the scientists [5-6]) based on melanoidin mechanism of interaction of amino acids and carbohydrates. It mince enzymic oxidation of aromatic compounds (polyphenols and lignin) formed quinones and semiquinone radicals, which react with amino groups of polypeptides and amino acids melanoidins. The reaction products enter into the polycondensation to form high molecular weight natural substances of variable composition [3]. HS, according to many scientists [5], composed of fractions (acid groups), which are characterized by different physic-chemical characteristics.

From the point of view of generally accepted gradation [1-7] HS consist of fulvic and humic acids, each of which, in turn, include a plurality of low molecular weight fractions (acid groups), also differ in physical and chemical characteristics [7-9]. According to [7], contain more oxygen FA (wt.% for the JMA-spetrometry), HA - carbon, especially the aromatic structure. Increasing the degree of aromaticity in the transition from FA to the HA is accompanied by the deepening of color, it is believed that the color of surface water by 80% due to the presence of HA [2].

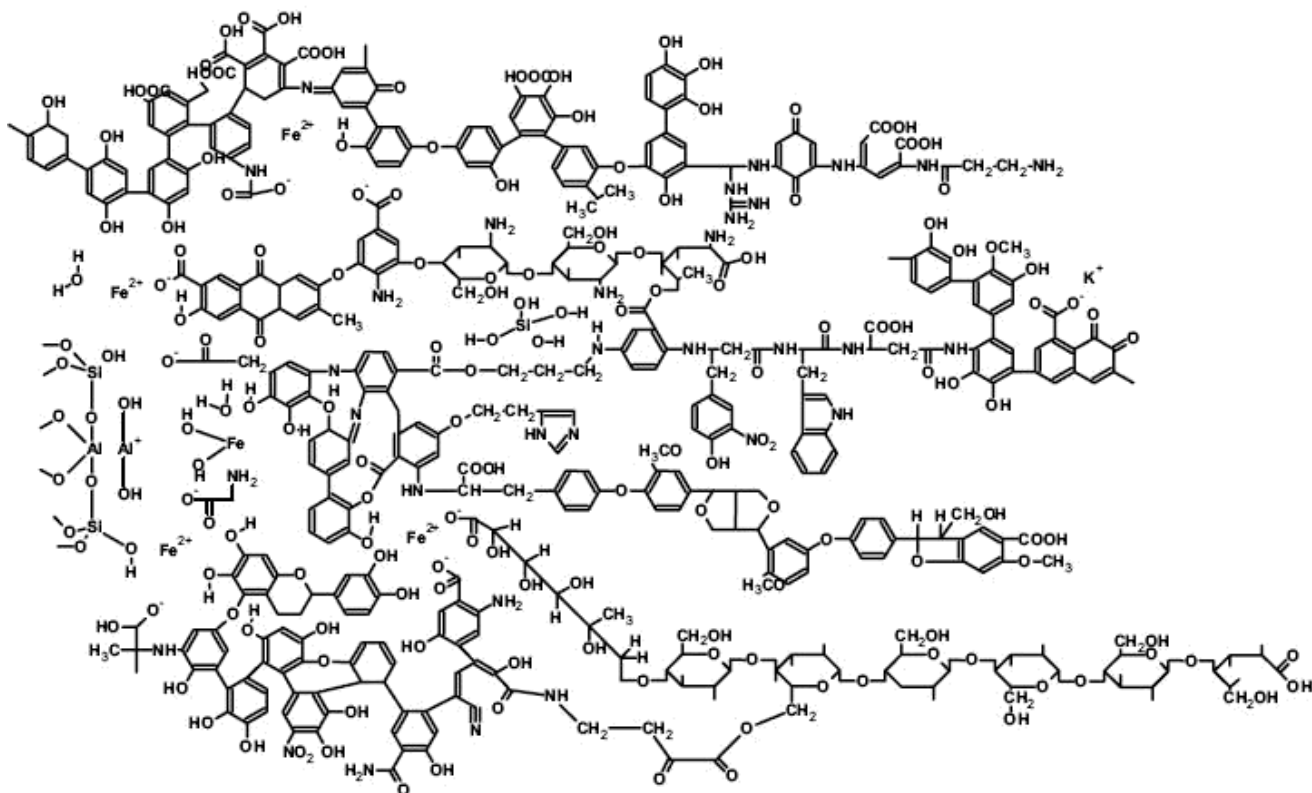


Figure 1. Fragment of humus acids.

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RESEARCH PARAMETERS OF HUMUS SUBSTANCES

In order to investigate structure of humus-fulvic-acid (HFA) used and applied various analytical and physicochemical methods. For example, by NMR spectrometric analysis identified contents of elements in the FA (Table 1) of different soils. Reduction of acid groups and phenolic groups from the organic horizons to mineral may be explained by molecular microbiological degradation of FA and cleavage of the carboxyl groups. Phenolic fragments are more resistant to degradation and consequently accumulate in the structure of the photonic crystal. In contrast, less oxidized black soil, the main group is phenol fragment.

Table 1. The elemental composition of HA preparations (wt.%) [9]

HA sod-podzolic and gray forest soils	C	H	N	O	H/C	O/C
sod-podzolic soils (forest)	51.1	4.2	5.2	39.4	1.0	0.6
gray forest soils (forest)	49.2	4.3	5.0	41.5	1.0	0.6
gray forest soil (arable)	48.3	4.2	4.8	42.7	1.0	0.6
<i>HA of black soil</i>						
black soil pratense	54.3	3.9	4.6	37.2	0.9	0.5
the typical black soil	54.8	3.5	4.0	37.8	0.7	0.5
pratense-gley black soil	54.9	4.0	2.6	38.5	0.9	0.5
<i>HFA of natural waters</i>						
r. Moscow	51.2	4.9	1.6	42.3	1.2	0.6
r. Istra	52.7	5	1.6	40.4	1.14	0.58

The study of acid-base properties of potentiometric method allowed to determine [9] constant of acid-based acidity (3-5 steps) for FA and HFA. The values of acid-based constant are ranged from 10^{-2} to 10^{-7} degrees and indicating sufficient reactivity acids. In [9] to identified seasonal changes in pH values of the constants for the FA and is explained by intramolecular conversion of compounds and changes in their molecular weights. In recent years, actively used adapted to soil science method of calculation of acidity constants of HS. This method of studying complex polyelectrolyte to allow calculating the distribution function of ionic group's concentration; the distribution function is called the pK spectrum.

As result, we use not discrete dissociation constants but a function of the distribution of ionic groups concentrations [4-5]. However, in this process ignores the change of the electric double layer, so the results may differ from the true. However the differences in pH depending on the volume of titrant (by graphs) for various soils HS are traced.

Many articles are devoted to determine the molecular weight of HS and their fractions, such information is very important for the study of their chemical properties [1-9]. Almost each of published articles are described the unique results that can be associated with different conditions of allocation of the trial and the study of certain subtypes of FA and HA. Much attention is paid to the conditions under which the formation of HS or FA and HA - their mass concentration and pH. In addition, these parameters depend on the degree of association and hence the magnitude of the molecular weight substances.

HS are play an important ecological role in natural waters due to the makroligand properties [1]. Currently, there are two main source of the HS migration in surface water [3-6].

- 1) leaching, and soil humus clastic oxidation of other organic substances;
- 2) synthesis of HA and FA in the reservoir due to enzymatic degradation of aquatic organisms

This may mean similar qualitative composition of organic matter treatment with soil organic matter (especially watershed). Analysis of published data [9] to allow revealing a number of features of humus acids that determine their behavior when entering the aquatic environment:

1. Structural features of HS. The presence in the structures of the HS certain functional groups to affect their physical and chemical properties.
2. Acid-base properties of HS. HFA is sufficiently strong acids and influence on pH, acid balance water, and biochemical reactions.
3. The intramolecular reaction. The variety of functional groups and the ability mezameric and inductivic effects within the structure of HS to facilitate changing their physic-chemical properties.
4. HS Association. Fulvic and humic acids to form associates and polidispersing system. The monomeric FA and polymeric structure of FHA associates are differently reacted due to of physic-chemical properties in natural waters

Based on literature data can be concluded the humus substances is sufficient natural acid of high strength and their physicochemical properties depend on molecular weight and functional features. The molecular weight, in turn, determined by the values of pH, concentration of substances in the system. The study of physical and chemical characteristics of HS a complex process that requires taking into account a variety of factors and the application of a variety of physical and chemical methods to obtain reliable results. In addition, various types of soils HS have specific features that effect on processes of their interaction with metal ions. HS podzolic soils in natural waters are present mainly in the form of oxidized aggressive FA. Black soil is rich in humus, which is dominated by humic acid.

METALS AFFINITY TO HS

The metal ions in natural waters are capable of forming compounds of varying degrees of strength depending on their physicochemical characteristics and physic-chemical characteristics of the anions present in the system.

There are many theories to describe of possible prediction of the metals forms. Metal ions are able to participate in many processes in the environment. The impact of various external factors can significantly change the direction of complexation reactions and, therefore, finding the form of the metal. Reflected in the literature of the "ranks of active metals" (RAM) in the reactions of complexation with HS are very different, and generally do not reflect the trend of any of the forms of metals.

There is a "ranks of metal activity" in complexation reactions are based on the study of specific water bodies or model experiments and mathematical calculations. For example, in

[9] is specified the following to the ranks of active metals (metals affinity to HS) in water based on the in-site experiments (ion exchange resins):

Mn (II) <Co(II) <Ni(II) <Zn(II) <Cd(II) <Cu(II) =Cr(III) = Al(III) = Pb(II) = Fe(II) = Hg(II). Cr(III) extent of binding is equivalent to the Cu (II), Al (III), Fe (III) and Hg (II). Co (II) and Ni (II) occupy the position after Zn (II).

In [8] reviewed the extent of binding of metal ions with humus substances identified waters and other RAM: Sr (II) <Cr (III) <Mn (II) <Zn (II) <Ni (II) <Al (III) <Cu (II) <Fe (III). Cr (III), in the present range, less able to bind to complexes than Fe (III) and Al (III), Cu (II). Sr (II), as the representative of the group of alkaline earth metals, it has a low ability to form complexes with humus, which coincides with the findings of other authors [1-4].

These differences in results may explain the zonal features of humic substances and the influence of the material balance system. Such issues according to the literature, is paid to very little attention. However, we found same similar trend: ions of metals such as Fe (II), Cu (II) complex formation with HS is more typical than the ions Ca (II) and Mg (II).

Speaking about the research methods of metal complexes with humic substances, should be singled out electrochemical methods. The using of electrochemical methods of analysis allows to study the processes of complexing metal ions or their fractions of HS at concentrations of metal ions 10^{-6} mmol / L, which is much closer to the experimental conditions of natural waters. In combination with spectroscopic methods for studying the structure of humic substances, information on the extent of the strength of their connections with metals gives a multidimensional picture of the biochemical transformations of organic matter in natural waters and soils.

MODELING EXPERIMENTAL RESEARCH

The experiment consisted of the following steps (Figure 2):

1. Extraction of humus acids and fractions (acid groups) from the typical soils of different climatic zones;
2. The study of functional characteristics of HS and factions, and their acid-base properties;
3. Study of the interaction of HS metal ions, determination of appropriate stability constants of complexes;
4. Theoretical calculation of some of the forms of metals in natural water bodies.

In this paper we were investigated common soil humus substances (European Russia and West Siberia): the zone of northern taiga - gleypodzolistye soil, the zone of mixed forests - sod-podzolic soils, the steppe zone - black soil.

In the course of detailed experiments were studied (Figure 2). Humus substances - alkaline soil extractor; alkali-soluble and acid-soluble fraction of humus substances – fulvic acid; and humic acids + gimatomelanic (gimetomelanic) acids (HA-HmA) - alkoly-non soluble. Electrochemical methods of analysis have been investigated processes of complexation of metal ions from the HS and their factions.

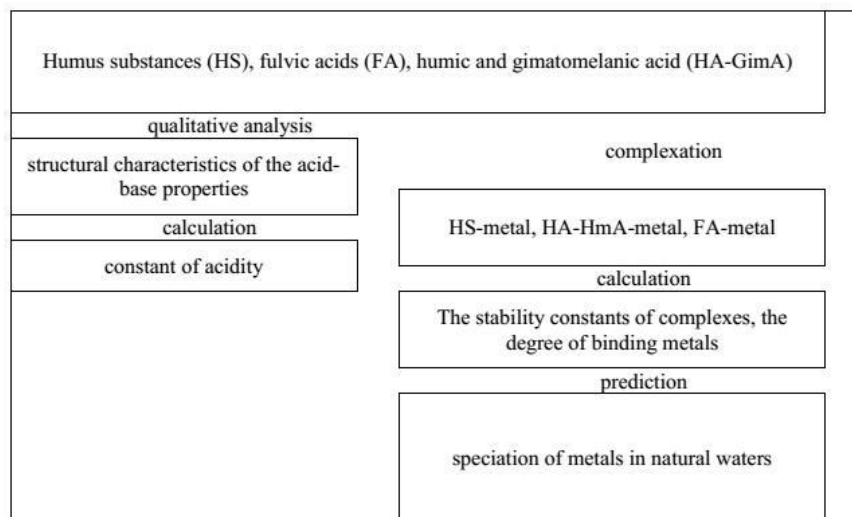


Figure 2. Scheme of investigation of humus substances and fractions.

We have chosen the following metal ions: Fe (III), Cu (II), Pb (II), Cd (II), Zn (II), Ni (II), Co (II), Mn (II), Cr (III), Ca (II), Mg (II), Sr (II), Al (III).

Samples extraction was performed because the procedure of HS leaching from soils into water is the main source of organic matter. And, the structural features of organic matter of water similar to the composition of the soil humus substances. Also, HS of water contain in composition as the proportion of fulvic acids, which are readily soluble in water, and mixtures gimatomelanic and humic acids, which are soluble in alkali.

Investigation of fractions HS such as fulvic and humic and gimatomelanic mixture of acids were carried out for more in-depth examination of complex formation taking into account functional characteristics of each faction. Research complexation FA as the dominant component of HS revealed the metal ions that have an affinity for oxygen-containing functional groups, as well as to compare the obtained stability constants of metal complexes with FA with numerous data in the literature, most of which is devoted to the study of this fraction HS.

Study of complex formation of metal ions with a mixture of humic acids and gimatomelanic acid that are necessarily present in natural waters, but in lower concentrations compared to FA, revealed differences in the mechanisms of complex formation for these fractions.

Physical and chemical methods used to study the complexation of metal ions with the HS, are shown in Figure 3. As the figure shows, the qualitative features of the soil acids were studied by IR and NMR spectrometric methods [1-9] (information on the functional groups, causing the connection to a specific metal ions). According to Moiseenko [6-8] observed a significant correlation (99%) between the amount of organic matter in waters humic nature and values of permanganate oxidation, therefore the method of permanganatometric concentrations were used for analyzez HS, FA and HA-HimA and in following, for calculated concentrations [7].

The average molecular weight of humus substances were found by sedimentation equilibrium [1-8]. Using the obtained molecular weights were calculated molar

concentrations of acid and fractions, which are used in further for asses conditional stability constants of the complexes.

The stability constants of metal complexes with HS have been determined by various physical and chemical methods. The main physical and chemical methods used in the work - a method of voltammetry (VA) [3-6]. Potentiometric titration method was used to study the interaction of the ions Ca (II), Mg (II), Sr (II), Al (III) with FHS.

Application of ionometry to determine the stability constants of metal complexes with HS was implemented in the form as direct ionometry and potentiometric titration [8]. Using these methods have been studied species complexation processes ions Ca (II), Mg (II), Pb (II), Zn (II), Cu (II), Cd (II) with GA and fractions.

In the final stage of the experiment, as shown in Figure 3, was conducted of the testing stability constants of the complexes by preparing systems containing multiple metal cations and HS. They also discussed the processes of complexation of metal ions with HS in a competitive binding, ie, in the presence of several cations or anions, close in composition to the waters of the natural systems. The concentrations of cations and anions were chosen according to the chemical composition of natural waters, reflected in [6-8].

The amount of unbound metal:

The basis of the methodology used in isolation of humic substances fractionation is the process that was reflected in the D.I.Orlov [1-4]. Known extraction method was supplemented by a few steps, namely: HS (Figure 3.3.) Undergoes a process of desalting cation-exchange resin and purification of low molecular weight organic substances with activated carbon; mixture during the isolation of HA and HA-HmA not divided into lower molecular weight fraction by adding an alcohol, and has been considered as a set of acids falling into water in the leaching process.

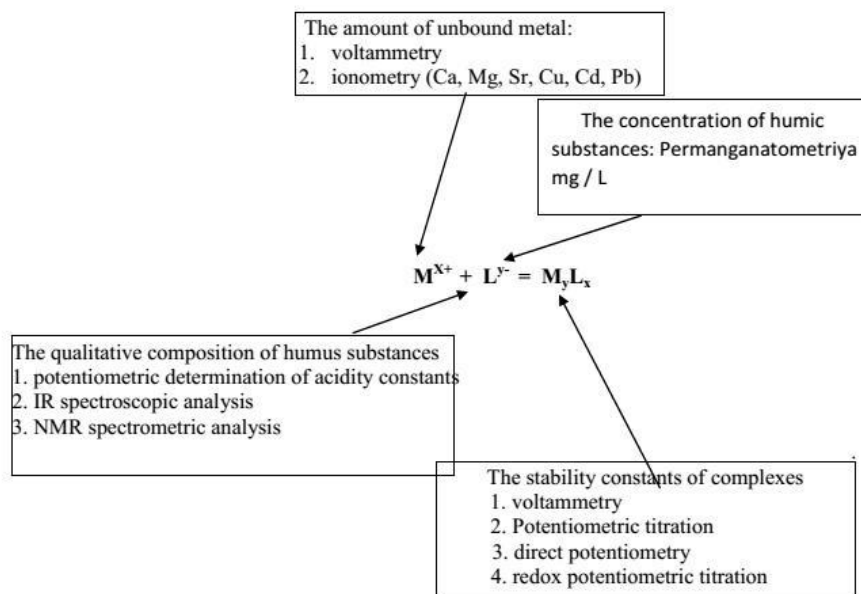


Figure 3. Scheme of investigation of metal complexation with humus substances and factions.

According to these amendments, a detailed soil sample preparation system and the allocation of HS and fractions consisted of the following steps (Figure 3.3.). We used infrared spectrometric analysis and NMR spectrometry analysis for evaluating qualitative features of humus substances and their fractions - IR-spectrometr NicoletTM380, NMR-spectrometr JEOL.

To evaluate the properties of the organic components of potentiometric method using potentiometric titration. The calculation of acidity constants was carried in three ways: graphic method, method Berumma and buffer capacity ("buffer"). The system of calculation of acidity constants for FA steppe zone is shown below, the values obtained logarithms acidity constants are shown in Table 2.

The definitions of stability constants of metal complexes with HS and fractions were conducted by electrochemical methods of analysis (Tables 3-4).

Table 2. Constant acid-base equilibrium (lgK)

the degree of dissociation	I			II			III		
	HS	FA	HA-HimA	HS	FA	HA-HimA	HS	FA	HA-HimA
1	3,0	3,0	5,0	2,0	2,4	3,0	1,7	1,5	5,0
2	6,0	7,3	10,0	5,0	6,7	6,0	2,2	2,0	15,0
3	10,8	12,0		4,3	12,0		4,3	5,0	
4	13,4	15,0			15,0		5,1	7,0	

The main results of determination of the stability constants of complexes are presented in Tables 3-4.

Table 3. Experimentally obtained by voltammetry logarithms of the stability constants of complexes of metal ions ($1 \cdot 10^{-3} - 1 \cdot 10^{-2}$ mmol / l) 1 - humic substances ($1 \cdot 10^{-3} - 3 \cdot 10^{-2}$ mmol / l) 2 - with the factions, lgK1,2 - logarithms of the constants stability in the first and second dissociation stage (n = 12; P = 0,95)

Ions	lgK _{1,2}	lgK		
		Natorel zone		
		Northern Taiga	Mixed forests	Steppe
	lgK 1	8.0±0,3	6.5±0,3	5.7±0,3
Cu(II)	lgK 2	-	12.9±0,3	10.3±0,5
	lgK 1	4.5±0,4	5.2±0,5	6.7±0,3
Pb(II)	lgK 2	7.2±0,5	-	-
Zn(II)	lgK 1	4.7±0,5	5.4±0,5	3.7±0,5
Cd(II)	lgK 1	3.7±0,4	3.7±0,3	4.1±0,3
	lgK 1	8.2±0,4	7.7±0,3	10.2±0,4
Fe(III)	lgK 2	16.0±0,5	-	-
Ni(II)	lgK 1	5.0±0,5	5.7±0,3	4.5±0,3
Co(II)	lgK 1	3.9±0,5	4.8±0,2	5.8±0,3
Cr(III)	lgK 1	2.5±0,3	3.2±0,5	3.5±0,4
Mn(II)*	lgK 1	2.4±0,3	3.2±0,3	2.8±0,2

Mn(II)* - n = 15, P = 0,9.

Ions	Naturel zone	LgK	
		Fraction	
		FA	HA
Fe(III)	northern taiga	10,3±0,5	6,3±0,3
	mixed forests	8,4±0,5	7,1±0,3
	steppe	7,4±0,5	10,2±0,5
		-	12,0±0,5
Cu(II)	northern taiga	5,5±0,5	5,3±0,5
	mixed forests	6,3±0,5	6,3±0,5
	steppe	7,0±0,5	7,4±1,0
		-	9,9±0,5
Pb(II)	northern taiga	7±0,3	6,5±0,5
	mixed forests	6,0±0,5	5,0±0,5
	steppe	-	6,0±0,5
		5,0±0,5	4,3±1,0
Cd(II)	northern taiga	4,0±1,0	3,6±0,5
	mixed forests	6,0±0,5	5,0±0,5
	steppe	3,5±0,4	2,5±0,5
Zn(II)	northern taiga	3,0±0,3	2,7±1,0
	mixed forests	5,0±0,4	4,5±0,5
	steppe	5,6±0,5	3,0±1,0

The concentrations ranged of HS $1 \cdot 10^{-3}$ mmol / L to $3 \cdot 10^{-2}$ mmol / L for all applicable methods. The concentrations of FA and HFA ranged from $1 \cdot 10^{-3}$ to $0,3 \cdot 10^{-2}$ mmol / L. The concentrations of metal ions used for voltammetry methods constituted $1 \cdot 10^{-3}$ mmol / l to $5 \cdot 10^{-2}$ mmol / L. The concentration equal from $1 \cdot 10^{-3}$ to $5 \cdot 10^{-3}$ mmol / l used for ionometri methods (as in voltammetry method) and $1 \cdot 10^{-1}$ mmol / L and 5 mmol / l of ions Ca (II), Mg (II). The method of potentiometric titration – $1 \cdot 10^{-2}$ mmol / l to $3 \cdot 10^{-2}$ mmol / l.

Table 4. The experimentally obtained values of the logarithms method ionometrii stability constants of metal complexes with HS, FA and HA
($P = 0,95$; $n = 7$), the concentration of metal ions 1 - $5 \cdot 10^{-3}$ mmol / l; 2 - $5 \cdot 10^{-2}$ mmol / l; 3. - 0,5 mmol / l

Ions	LgK								
	Naturel zone								
	steppe			mixed forests			northern taiga		
	HS	FA	HFA	HS	FA	HFA	HS	FA	HFA
Cu(II)	8	5	5,5	6,5	6	6,5	5,7	7	7,5
Pb(II)	4,5	7	6,5	5,1	6,5	5	6,7	5	4
Zn(II)	4,6	3	2,7	5,3	5	4,5	3,7	5,6	3
Cd(II)	3,7	4	3,6	3,7	6	5	4,1	3,5	2,3
Fe(III)	8,2	7	6,7	7,6	8	7,5	10,2	10	10,2
Ca(II)	4,1	-	-	2,2	-	-	3,1	-	-
Mg(II)	2,7	-	-	2,2	-	-	3,5	-	-

Table 4. (Continued)

Ions	LgK								
	Naturel zone								
	steppe			mixed forests			northern taiga		
	HS	FA	HFA	HS	FA	HFA	HS	FA	HFA
Cu(II)	8,2	5,5	6	6	6,5	7,5	6	7,5	6,5
Pb(II)	4,5	7,5	6	4,9	5,6	6	7	5,5	4
Zn(II)	4,5	2,5	2	5	5	5,5	4	6	3
Cd(II)	4	4,5	4	4	4,5	4	5,5	4	2,5
Fe(III)	8	7,5	7	7,9	7	6,5	10	10,5	10
Ca(II)	4	-	-	2,5	-	-	4,1	-	-
Mg(II)	2,9	-	-	2,5	-	-	4,5	-	-
Ions	LgK								
	Naturel zone								
	steppe			mixed forests			northern taiga		
	HS	FA	HFA	HS	FA	HFA	HS	FA	HFA
Cu(II)	6	4	5	6	5	6	5	7	7,5
Pb(II)	4,5	6,5	6	5	5	5,5	6	5	3,4
Zn(II)	4,5	4,3	2,2	5	3	4	3,5	4,5	2,5
Cd(II)	3,5	3,5	3	2,7	5	4,5	4	3,5	2,3
Fe(III)	8	6,7	6,5	7,6	8	7,5	10	10,3	10
Ca(II)	4	-	-	2,2	-	-	3,1	-	-
Mg(II)	2,6	-	-	2,2	-	-	3,5	-	-

FEATURES OF HUMUS SUBSTANCES OF VARIOUS NATURAL-CLIMATIC ZONE

1) Heavy Metals in Humus Substances

According to the experimental data (tab. 5), the soil humus substance contained metal ions: Cd (II), Zn (II), Pb (II), Cu (II), Fe (III). For the northern boreal forest zone HS content Fe (III) and Cd (II) (typical hard Pearson acids) sufficiently predominant over the content of other metal ions, the least likely to humus substances zones are ions Cu (II) and Zn (II). The HS steppe zone revealed large amounts of ions Cu (II) and Pb (II) - softer acid Pearson. The concentrations of ions Fe (III) less than the previous zone. The HS zone of mixed forests showed a high content of ions Cd (II) ions predominate over the Fe (III) and Pb (II). Dominance of certain metal ions in a given sample HS indicates the structural features of HFS from each soil, which are discussed below.

Table 5. The average concentration of metal ions (mg / l), located in areas of natural humic substances (n = 20, P = 0,95)

Naturel zone	Cd(II)	Pb(II)	Cu(II)	Zn(II)	Fe(III)
northern taiga	0,95	0,30	0,15	0,17	0,34
mixed forests	0,54	0,21	0,11	0,20	0,27
steppe	0,06	0,12	0,10	0,30	0,07

2) Spectrometry Research

According to NMR spectrometry analysis HS steppe zone characterized by a predominance of substituted aromatic and aliphatic hydrocarbon fragments of the oxygen-containing fragments, HS of northern taiga soil – oxygen and nitrate group.

Analysis of IR-spectrograms revealed:

1. Humus substances isolated from soil of typical northern taiga zones are characterized by a high content of oxygen-containing groups (fig 4-5). Humus substances derived from soil zone typical mixed forests contain about the same amounts as oxygen and aromatic aliphatic hydrocarbon fragments. Variability oxygenated fragments large compared to the rest of submitted samples. Humus substances isolated from soil of typical steppe zone, characterized by a predominance of aromatic and aliphatic hydrocarbon fragments.
2. Fulvic acids isolated from soils of the taiga, represented the most extensive part of the spectrum corresponding to the presence of large amounts of oxygen-containing groups. For fulvic chernozems is characterized by narrow spectra. Part of the spectrum of humic acids of chernozem, reflecting the widest aromatic moieties, unlike of humic acids of soils of northern taiga zone.
3. The acid-base properties of fulvic and humic acids differ due to the influence of oxygen, nitrogen and hydrocarbon-containing functional groups in the fractions. These differences lead to different processes of dissociation of acids. The biggest acidity constants have acid soil humus, acid soil lowest gleepodzolic soil.
4. The metal ions have different affinities to specific functional groups. As shown in Figure 6-7. For the ions Fe (III) is most typical bond formation through an oxygen bridge. Alkaline earth metal ions - Hard Pearson acids form strong bonds through oxygen and nitrogen atoms. Cu (II) and Pb (II) can bind to the HS via a soft center - sulfur-containing groups.

COMPLEXATION WITH METAL IONS (ZONAL FEATURES)

The results of the study revealed the peculiarities of interaction of metal ions with different soils HS and HS with fractions.

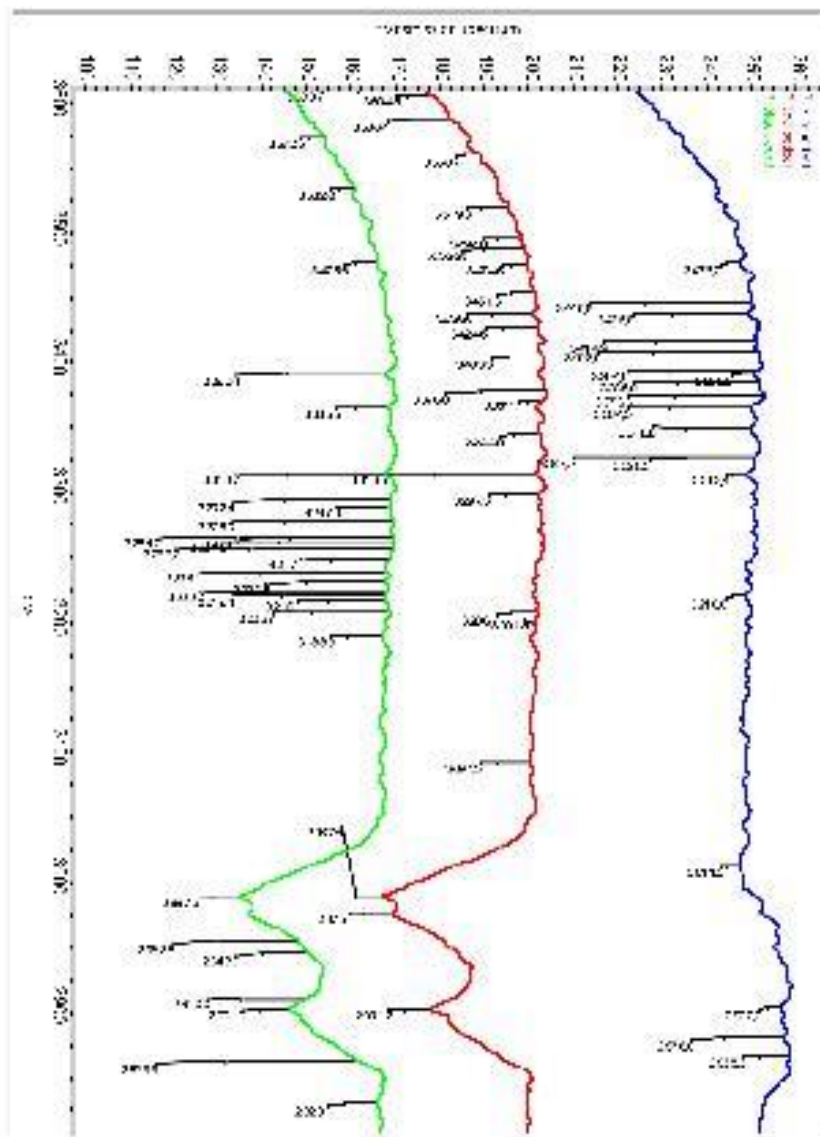


Figure 4. IR-spectrogram of HS.

As was found by the results of studies, alkaline earth metal ions - Sr (II), Ca (II) and Mg (II) to form complexes with humic substances gleepodzolistyh soil in a ratio of 1: 1. Many articles [1-4] are reflected such data that is associated low capacity alkaline earth metal ions to form complexes, in particular due to the large ionic radius as compared with other metal ions (Table 2.1.). The strength of the formed complexes is low (about 103) and the degree of formation of complexes with alkaline earth metal ions depends on the degree of humification and mineralization of the water body [1-4].

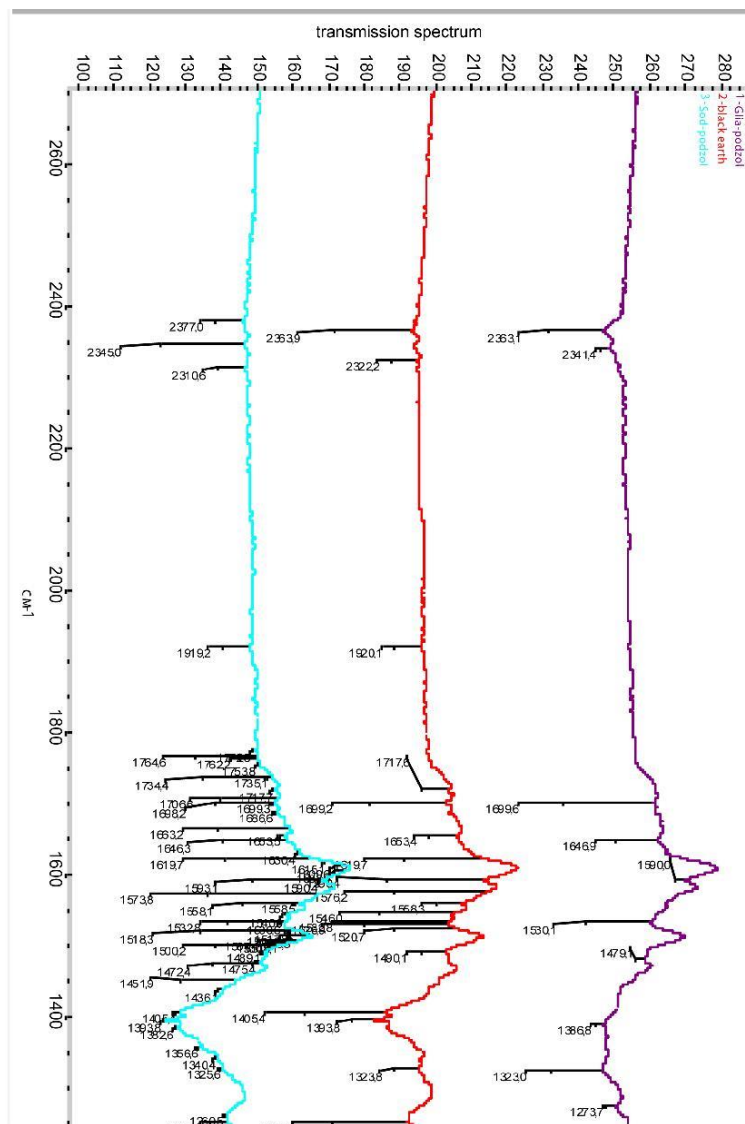


Figure 5. IR-spectrogram of HS.

Ions Fe (III) form with HS gleepodzolic monodentantnye soil complexes. Corresponding values presented in Table 5.1. Comparable to common literature data [1-4, 9], which indicate high affinity ions Fe (III) to HS, regardless of the soil type. How tough Pearson acid ions Fe (III) form complexes with HS northern taiga zone characterized by high stability constant ($K_1 = 1.010$), due to the predominance of oxygen-containing groups (Pearson hard grounds) in them.

The stability constant of the complex Al (III) is equal to 10^6 HS, the stoichiometry of the complex - 1: 1. Published data on quantitative indicators binding Al (III) with HS do not have much. From modern research - is the work [1-4] in which the methods of gel chromatography and fluorescent analysis determined stability constants of aluminum with FA and HS treatment (lgK range from 5 to 6).

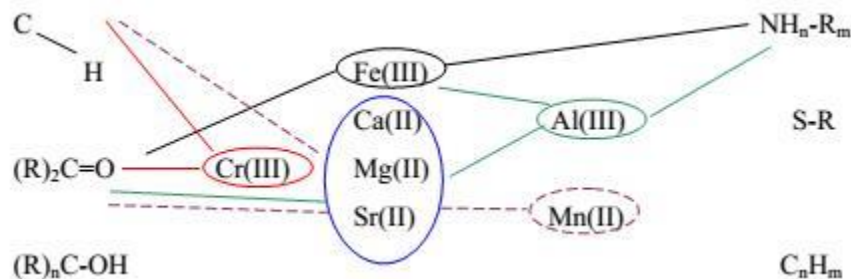


Figure 6. The affinity of hard acids Pearson to the functional groups of HS.

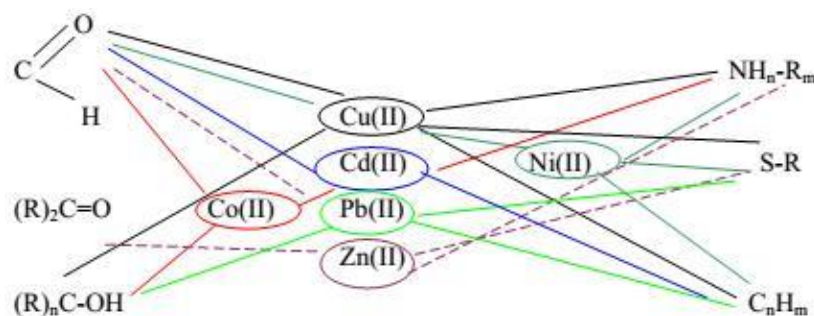


Figure 7. The affinity of soft acids Pearson to the functional groups of HS.

Ions of Ni (II) and Co (II) exhibit high ability to form complexes with HS glepodzolic soil: According to the data obtained from the stability constants of the compounds, HS (in the ratio 1: 1) are 4.4 and 5.8, respectively. Ni (II) and Co (II) - intermediate acid Pearson different from Fe less affinity for oxygen atom and Cu (II) - a lower affinity for soft grounds Pearson. Therefore, the obtained values of stability constants of the complexes is lower than complexes ions Fe (III) with HS.

Ions Zn (II) in the process of complexation form complexes in a ratio of 1:1 with a constant equal to 3.7. The data on the interaction of Zn (II) with organic substances waters are different, but the results are the same as reflected in the work of J. Schnitzer [51], in which samples HS as seen glepodzolic extract from the soil of Canada. Ions Zn (II), possess an affinity for oxygen-containing groups, but due to the size of the ionic radius having steric hindrance and stability constants of the complexes is lower than that for other metal ions.

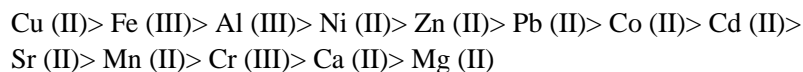
Mn (II) and Cr (III) by reaction with HS complexed in a ratio of 1: 1 with low values of stability constants, because of low complexing power for ions and a sufficiently large radius (Table. 2.1.).

Based on these results, we can conclude that such metal ions as Fe (III), Pb (II), Cu (II) capable of forming a complex with HS glepodzolic soil in a ratio of 1: 2. For the Mn (II), Cr (III), Cd (II), Ni (II) and Co (II), Ca (II) and Mg (II) - 1: 1.

According to the calculations of the stability constants of complexes was built following "a range of active metals»: Fe (III)> Cu (II)> Pb (II)> Al (III)> Co (II)> Ni (II)> Cd (II)> Zn (II)> Cr (III)> Mg (II)> Sr (II)> Ca (II)> Mn (II).

Complexes of alkaline earth metal ions with HS from zone of mixed forests is characterized by lower values of stability constants compared with complexes with HS zone

of northern taiga. For ions Ca (II) and Mg (II) values of stability constants are much lower - average $\lg K = 2.2$ instead of 3.3. The stability constants of complexes of Sr (II) c HS of sod-podzolic soils are equal. The values of the stability constants of the complexes HS zone of mixed forests with ions of calcium and magnesium, as reflected in the work [9], and also show a low ability of metals to form complexes ($\lg K < 3$). The stability constant of complexes of Fe (III) -HS is $10^{7.6}$ which is lower than the stability of the complexes of Fe (III) with HS zone of northern taiga. The reasons for this difference can serve as a smaller proportion of oxygen-containing groups, which is not advantageous from the viewpoint of the formation of complexes for hard acids Pearson. Ions Cu (II) form complexes with HS, characterized by stability constant ($\lg K$) about 6.5, which is slightly higher than for systems with HS zone of northern taiga. According to a preferred functional groups for ions Cu (II), as the bond is formed through the rigid center - oxygen and nitrogen, as well as through the soft center - sulfur. Comparison of the stability constants of metal complexes with HS describes the following series:



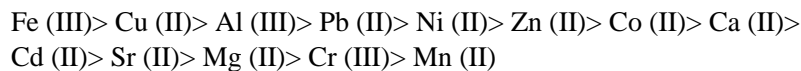
Humic substances of black soil have an increased content softer centers Pearson (sulfur-containing groups, aromatic moieties) compared with podzolic soils. These features create favorable conditions for complexation milder acids Pearson.

Ions Ca (II) and Mg (II) to form complexes with stability constants of the logarithms of 3.8 and 2.7, respectively; that is different from the values obtained for the previous bands, but as well as in the area of the northern taiga Ca (II) is more constant than the Mg (II). Fe (III) forms complexes at a ratio of 1: 1 and 1: 2, with the logarithms of the stability constants of 8.2 and 15.9, that could be due to the presence of HS in the sample zone steppe contribution complexation oxygenates and nitrogen containing groups. It can be assumed that the dominance of the substituted aromatic groups in the structure HS determines various mesomeric and inductive effects in the aromatic ring.

For ions Cu (II), obtained stability constants of complexes with black soil HS higher than for systems with HS podzolic soils. As milder acid Pearson, unlike Fe (III) and Al (III), Cu (II) complexes with better milder bases Pearson, which dominate the samples submitted.

Pb (II) complexes with HS in the ratio 1: 2, which differs from the process flow of chelation complexes with other soil HS ($C = 10^{4.5}$ and $10^{7.2}$).

Comparing the values of stability constants of metal complexes with HS can form the following series:



As samples boreal forest, Fe (III) forms complexes with better HS than other metals. Position Cu (II) and Al (III) changed compared to the complexation c HS sod-podzolic soils. Cr (III) and Mn (II) in the same manner as in the previous results have low ability to form complexes with HS.

The affinity of the metal ion to a specific fraction HS characterizes most preferred functional group for each of them.

For FC revealed the following patterns (taiga - gleepodzolistye soil zone of northern taiga, forests - sod-podzolic soils of the mixed forest, steppe - black earth steppe zone):

Pbtayga> Pbforest> Pbstep; Znstep> Znforest> Zntayga; Custep>
Cuforest> Cutayga
Cdforest> Cdtayga> Cdstep; Fetayga> Feforest> Festep

As seen from the series, ions Fe (III) have high affinity for FK gleepodzolistyh soils, characterized by high amounts of oxygen-containing fragments.

CALCULATION OF THE FORMS OF METALS IN NATURAL WATERS ON THE BASIS OF THE EXPERIMENTAL DATA ON THE HS

The calculations of the forms of metals in natural waters lay study of small lakes in the European part of Russia, from the tundra to the steppe zone, held at the Institute of Water Problems of RAS. The method of sampling and analysis is described in detail [6-8]. The concentrations of cations and anions, which were carried out according to the calculations, as reflected in [6-8] in the process of calculation takes into account the effect of these anions - humus substances, sulfate ions, chloride ions, sodium ions, hydroxide ions, as well as in the presence of other cations metals.

Analyzing the forms of occurrence of metals can be concluded about the great variability of forms of distribution of metals in natural waters, which are determined by the composition and concentration of trace elements and characteristics of humus substances. The chemical properties of GW in natural waters depends, in turn, the properties of the soils located in the catchment area and therefore have a zonal specifics.

There are complex mechanisms of binding and distribution of the forms of metals in natural waters, which are defined not only by the properties of metal ions as the overall chemistry of the waters, including the entire range of metal content, as well as individual quality features of humus substances in them.

The distinctive features of the chemical composition of the waters of the northern taiga zone are low salinity and high variability of the concentrations of HS, Fe (III) and Al (III) due to the presence of lakes are located in the mountainous landscape, as well as among the wetlands and woodlands. Natural waters boreal forest characterized by a large variety of metal ions, the simultaneous presence which, together with sufficient alkalinity, cause the following pattern in an advantageous distribution of metal ions bound in organic complexes:

Fe> Al> Pb> Co> Ni> Zn> Cd> Cu> Mg> Ca> Cr> Mn> Sr

Based on the chemistry of the waters of this natural area, structure and properties of metals HS, one can conclude that Fe (III) and Al (III) are the main competitors for binding into organic complexes and other metals inactivation probability is very low, most of them will be in ionic form.

The natural water areas of forests are characterized by a high content of mineral salts, a fairly wide range of alkalinity and pH, the presence in the waters of a large group of metals.

The general rule in the distribution:

Fe > Cu > Al > Zn > Cd > Pb > Ni > Co > Mg > Ca > Cr > Mn > Sr

In this zone, as in the taiga, Fe (III) is the main binding elements. Cu (II) is located at the second position due to the possibility to communicate with HS in the absence of Fe (III). Al (III) is in third place due to the difference between the stability constants of Cu-Al-HS and HS.

Natural water steppe zone characterized by the presence or absence of aluminum ions, Fe (III), Cu (II), a high total alkalinity. These features are manifested in changes in the extent of binding metals. The presence of all the metals in the system generally shown in the following patterns:

Al > Fe > Cu > Zn > Ni > Co > Mg > Ca > Sr > Mn > Cr

Lack of Fe (III), aluminum and Cu (II) several changes this pattern. In this case, a number of substantially unchanged, but increases the degree of binding metals in the following sequence:

Ni > Co > Zn > Sr > Ca > Mg > Mn > Cr

Every water system has a unique set of chemical components and GW. Physicochemical properties of metal ions and their content as well as the concentration of cations and anions cause specific trends metal migration. Therefore, each of the proposed series is quite arbitrary and generalized to specific conditions. It should be noted that the patterns of distribution of metals according to the calculations do not repeat the pattern of the values of stability constants of complexes - this confirms the finding of an impact on the form of metals of many factors. Therefore, the stability constants of metal complexes with humus substances - an important but not the only factors affecting the speciation of elements in natural waters.

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Chapter 6

**EVALUATION OF VERMICOMPOSTS FOR
DECONTAMINATING AQUEOUS MEDIA CONTAINING
METALLIC IONS AND SYNTHETIC DYES**

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ABSTRACT

In this work, vermicompost, which is one the most important humified substrates, was employed for decontaminating aqueous media enriched with Cd(II), Cu(II), Pb(II) and Zn(II), as well as three synthetic dyes (Congo red - CR, malachite green – MG and methanile yellow - MY). Besides evaluating the efficiency of metallic ion removal, this investigation also considered the influence of different sources of production on the adsorptive properties of vermicompost. In this regard, samples were collected from four Brazilian States (Bahia, Espírito Santo, Minas Gerais and São Paulo) and characterized by X-ray diffraction, infrared molecular absorption spectrophotometry, thermogravimetry

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and electron microscopy in order to obtain information about mineralogical composition and presence of organic functional groups as well as morphological and structural aspects. Subsequently, the different vermicomposts were used to withdraw Cd(II), Cu(II), Pb(II) and Zn(II) from mixed solutions or CR, MG and MY from individual solutions. Parameters such as vermicompost mass, the pH of the mixed solutions, and the time of contact of these solutions with vermicompost were varied in order to obtain satisfactory adsorption responses. For each experimental design, the remaining quantities of metallic cations and dyes were determined, respectively, by flame atomic absorption spectrometry and visible molecular absorption spectrophotometry. Analyses of the characterization pointed to discrete differences among the different samples of vermicompost, including aspects related to their mineral and organic composition. Independent of the source, vermicomposts contributed large numbers of carboxyl groups ($-\text{COOH}$). Besides the carboxyl, all samples of vermicompost also exhibited large analytical signals associated with Al-O bonds. In turn, thermogravimetric analyses revealed accentuated percentages of volatile components in accordance with the expected composition of composted materials, while electron microscopy and X-ray diffraction highlighted, respectively, the complex disposition of many porous and mineral components. The results pointed to very efficient retentions ($> 70\%$) of all metallic analytes after 1 minute of dynamic contact among the solutions and all samples of vermicompost, thus evidencing predominantly reversible adsorptions. Specifically for Pb(II), adsorptions greater than 90% were observed. Nevertheless, depending on the source of the vermicompost, it was possible to identify a preferential order concerning the retention of Cd(II), Cu(II), Pb(II) and Zn(II). Based on criteria associated with the efficiency of retention, vermicompost samples from Bahia and Minas Gerais States were selected to build adsorption isotherms (individual solutions) with subsequent mathematical treatment of the data according to the Langmuir and Freundlich models. The following maximum adsorptive capacities (mg g^{-1}) were obtained: 0.4 (Cu) to 3.4 (Zn) for vermicompost from Bahia, and 0.5 (Pb) to 22.1 (Cu), for vermicompost from Minas Gerais. In turn, adsorptive tests with synthetic dyes were performed only with the vermicompost from Bahia and always employing individual solutions. The results indicated effective retentions ($> 90\%$) after 1 minute of mechanical agitation for all dyes. The best pH for adsorption of CR and MY was 2, while pH 6 yielded the greatest adsorption of MG. With vermicompost from Bahia State, adsorption isotherms indicated maximum adsorptive capacities up to 45.9 mg g^{-1} .

Keywords: Humic substrate, adsorption, metallic ions and ionic synthetic dyes

INTRODUCTION

Humic substances comprise a class of highly complex compounds derived from the chemical and microbiological decomposition of organic matter, which is driven by random processes of molecular recombination [1]. During humification, biopolymers such as proteins and nucleic acids are continuously broken down into smaller fragments that are then recombined in order to generate humic precursors with variable sizes and degrees of aromaticity [2]. Under adequate physical-chemical conditions in soils, sediments, water and other environments, effective changes can be observed in the organic precursors so that compounds with large structural differences are obtained.

In this sense, a single humic molecule can contain more than 1,000 aromatic rings [3]. According to Stevenson (1982), humic substances are so complex that there are no two identical molecules in nature, but it is possible to group them into three categories based on

criteria of solubility. In this regard, humines present very small solubility over a wide range of hydrogenionic concentrations, while fulvic acids are completely soluble in acidic, neutral or alkaline aqueous media. In turn, humic acids present poor solubility in diluted acid. These differences in solubility exist because the percentages of oxygen, nitrogen and sulfur are higher for humic and fulvic acids, which present the respective minimal chemical formulas: $C_{10}H_{12}O_5N$ and $C_{12}H_{12}O_9N$ (disregarding sulfur). Based on these chemical formulas, it is possible to note that fulvic and humic acids present many hydrophilic groups that are responsible for high adsorptive capacities related to ionic species.

For this reason, it is a common practice to add humified substrates to soils in order to increase the cationic exchange capacity (CEC) and fertility of these environments. Additional to their fertile aspects, humified substrates are also responsible for increasing the temperature of soils as well as their capacity to retain water [4].

Naturally, humification occurs very slowly over many decades, but composting is capable of producing humified substances in a few months [5].

Conceptually, composting conglomerates all procedures in which organic residue sources are recycled to restore nutrients to soils [6], while an alternative definition [7] classifies composting as any procedure able to transform organic materials by means of indigenous and aerobic microorganisms. In addition to classic procedures of composting, there is vermicomposting, which is performed using specific species of earthworms. In the digestive system of these animals, a complex microbial population accelerates molecular fragmentation as well as their recombination with a consequent increase in humification rates. Vermicompost is the final product of vermicomposting and it has all of the desirable aspects previously described for humified substrates. Further, vermicomposts are excellent sources of plant nutrients such as calcium, magnesium, potassium, sodium, and phosphorus [3]. According to a recent review about the environmental uses of vermicomposts [4], these humified materials have excellent potential for removing ionic pollutants from aqueous media. This characteristic was attributed to their accentuated surface area, high content of adsorptive functional groups and reduced costs of acquisition, since one ton of vermicompost can be acquired for US\$100.

However, vermicomposts can be obtained from many different raw materials and ways of production. In this sense, these substrates are inherently heterogeneous and an evaluation of this heterogeneity upon the adsorptive efficiency of vermicompost should be performed since there are few studies concerning this subject. Thus, this work aimed to evaluate the retention of Cd(II), Cu(II), Pb(II) and Zn(II) in vermicomposts from four Brazilian States (Bahia, Espirito Santo, Minas Gerais and São Paulo). Moreover, the adsorptive capacity of one vermicompost sample (from Bahia State) was verified against the following synthetic dyes: Congo red (CR – $C_{32}H_{22}N_6Na_2O_6S_2$, Figure 1), malachite green (MG – $C_{23}H_{25}ClN_2$, Figure 2), and methanile yellow (MY, $C_{18}H_{14}N_3NaO_3S$, Figure 3). This last goal was considered in order to amplify the uses of vermicompost as a natural adsorbent for decontaminating colored aqueous media. This environmental application of vermicompost is scarcely reported in the literature. The elements cadmium and lead were chosen for this investigation because they present high toxicity to humans and other forms of life, including effects related to infertility, anemia, cancer, and psychiatric disturbances [8-12].

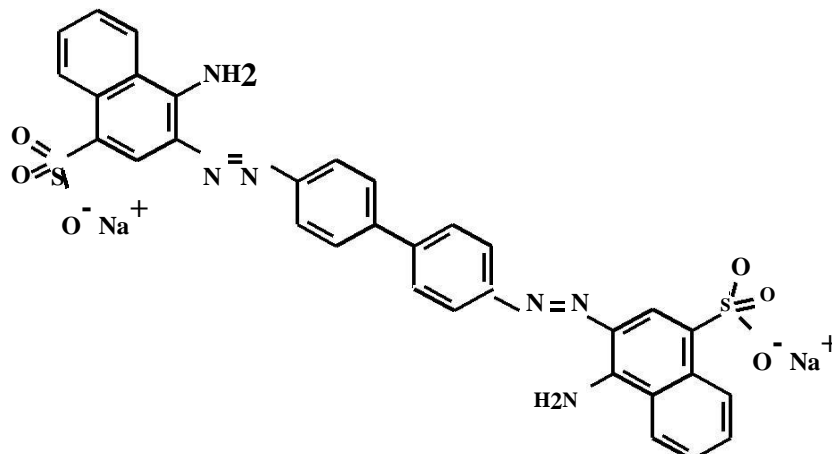


Figure 1. Structure of Congo red.

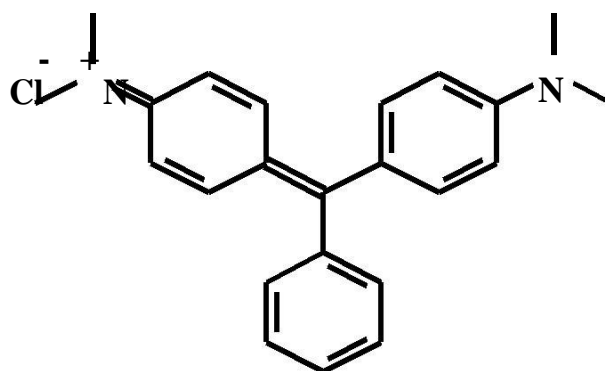


Figure 2. Structure of malachite green.

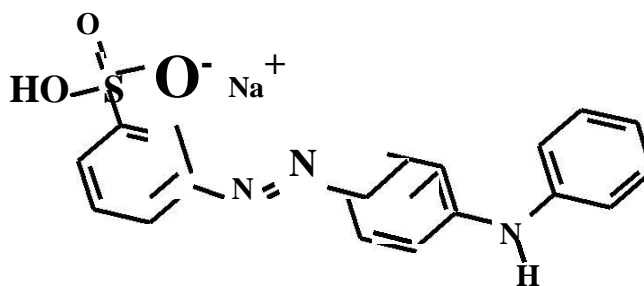


Figure 3. Structure of methanile yellow.

In turn, copper and zinc are essential elements but can also be toxic in excessive doses. In humans, an excess of zinc is responsible for dermatological infections and cellular mutation [12], while an excess of copper is able to cause blindness among other disturbances [12]. In addition to the potential biological risks of these elements, they are extensively employed in many human activities, thus explaining their large distribution throughout the environment as well as the necessity of finding efficient and viable ways to remove them from aqueous media.

In turn, it is also necessary to find procedures able to remove the cited synthetic dyes because they find large applicability in industries as well as microbiological, pharmaceutical, and chemical laboratories [13, 14]. Besides their intensive uses, all of these dyes can present notable toxicity to humans and other mammals. In this regard, neurological effects were reported in rats exposed to MY for long periods of time [15], and doses as low as 22 nmol L^{-1} of MY were enough to double the rate of mutation in human cells [16]. MG is responsible for chromosomal breakage and emergence of tumors in humans [17], while CR is classified as a powerful carcinogenic agent [18].

EXPERIMENTAL

Reagents and Instruments

All reagents (acids and dyes - analytical quality) were acquired from Merck (Germany) or Vetec (Brazil). For the preparation of all solutions, ultrapure water obtained from a purification unit (Gehaka, Brazil) was employed. The standard solutions of metals that were employed for calibrating the flame atomic absorption spectrometer were prepared daily by diluting stock solutions at $1,000 \text{ mg L}^{-1}$ (SPC Science, US).

For quantification of cadmium, copper, lead, and zinc, a GBC (Malaysia) model SensAA flame atomic absorption spectrometer equipped with a deuterium lamp for correcting background radiation was employed. In all the analyses, an air-acetylene flame was used; wavelengths for Cd, Cu, Pb, and Zn were 214.438, 324.754, 220.353, and 213.856 nm, respectively. In order to measure the absorbance of dye solutions, a Fenton (Brazil) model 600 molecular absorption spectrophotometer with a double beam was employed.

Glass cuvettes (10 mm optical length) were used in all spectrophotometric determinations of absorbance.

Acquisition of Vermicomposts and Their Initial Preparation

All samples of vermicompost were acquired in floricultures or city markets in each of the cited Brazilian states. After acquiring them, these samples were heated in a stove at 60°C until a constant mass was reached. Afterwards, they were passed through a 270-mesh stainless steel sieve, stored in polyethylene flasks (previously decontaminated in HNO_3 solutions at 10%, v/v) and kept at 4°C .

Physical-Chemical Characterization of Vermicomposts

All samples of vermicompost were submitted to analyses by infrared molecular absorption spectrophotometry, thermogravimetry, X-ray diffractometry and electron microscopy. Additionally, total contents of organic matter and ashes, humidity as well as pH of the samples were also determined. These four last analyses were performed according to protocols of the Brazilian agricultural research company (Embrapa, in Portuguese) [19].

The thermogravimetric analyses were performed by heating the samples from room temperature to 1,000°C at 10°C min⁻¹ in an oxidant atmosphere composed of synthetic air. For X-ray analyses, powdered samples were submitted to an incidence of radiation derived from a cobalt source ($\lambda = 1.78890 \text{ \AA}$). During these analyses, 2Θ angles varied from 5 to 50°, and the applied voltage and current were kept at 40 kV and 30 mA, respectively. For electron microscopy, samples of vermicompost were covered with a thin layer of gold and an electron acceleration voltage of 20 kV was applied.

In turn, infrared spectra were obtained after pressing the samples with KBr and subsequent spectral scanning between 4,000 and 400 cm⁻¹. Vermicomposts *in natura* or enriched with metals (both passed through 270-mesh sieve) were submitted to analyses by infrared spectrophotometry. Moreover, vermicomposts, also passed through 270-mesh sieve and not enriched with metals, were heated at 200, 400 and 600°C using a thermogravimetric analyzer and were subsequently analyzed by molecular absorption infrared spectrophotometry.

In all procedures involving infrared spectrophotometry, the same proportion of mass (1:100) among the samples of vermicompost and KBr was always used. This proportion was maintained in order to allow comparisons among the different sets of spectral data.

Adsorptive Profiles of Cd(II), Cu(II), Pb(II) and Zn(II) against Vermicomposts from Different Sources

After initial preparation and characterization of the vermicomposts, 500 mg of each were stirred with 25.00 mL of mixed solutions of Cd(II), Cu(II), Pb(II) and Zn(II) at 10 mg L⁻¹ and pH 2.0. During this experiment, agitation times varied from 1 to 120 minutes. In the next step, adsorption isotherms were built using vermicomposts from Bahia and Minas Gerais. In order to achieve this latter goal, the mechanical agitation time was fixed at 1 minute and all of the experimental conditions already described were kept. However, the concentration of each ion was varied from 1 to 40 mg L⁻¹. After all adsorptive experiments, supernatants were filtered in qualitative filters, acidified with a few drops of 14 mol L⁻¹ HNO₃ and stored in polyethylene flasks at 4°C for quantifications of cadmium, copper, lead and zinc by flame atomic absorption spectrometry (FAAS). For determining the remaining contents of each metallic cation in the supernatants, calibration curves were built in the following concentration ranges (mg L⁻¹): 0.2 to 2.0 (Cd and Cu), 1.0 to 10.0 (Pb) and 0.5 to 5.0 (Zn).

Adsorptive Profiles of Congo Red, Malachite Green, and Methanile Yellow in Vermicompost from Bahia State

First, it was necessary to relate the absorption spectra of dye solutions with the hydrogenionic concentration of the media. In this regard, solutions of CR and MY at 5 mg L⁻¹ and MG at 2 mg L⁻¹ were prepared at different values of pH (2 to 6) and, for each pH and dye, spectral analyses were performed from 350 to 700 nm.

After this early investigation, the adsorptive profiles of CR, MG, and MY were evaluated by varying the initial pH of the dye solutions (25.00 mL of solutions at 5 mg L⁻¹ for CR and

MY and 2 mg L^{-1} for MG) as well as the duration of mechanical agitation with 100 mg of vermicompost. Thus, solutions were stirred with vermicompost for 1 minute, while their initial values of pH varied from 2 to 6.

In the next step, the initial pH was fixed at 2 for CR and MY and 6 for MG, while agitation times were tested over an interval from 1 to 120 minutes.

Finally, 100 mg of vermicompost were stirred with 25.00 mL of solutions of CR (5 to 100 mg L^{-1}), MG (5 to 200 mg L^{-1}) and MY (5 to 150 mg L^{-1}) in order to build adsorption isotherms. To achieve this goal, the initial pH was fixed at 2 for CR and MY and 6 for MG and the mechanical agitation time was kept at 1 minute. After each agitation experiment described in this section, the pH of the supernatants was measured and the absorbance signals were measured at the corresponding maximum wavelength, as discussed later in the text. In order to quantify the levels of CR, MG and MY remaining in the supernatants, calibration curves (0.5 to 5.0 mg L^{-1}) were constructed daily for these dyes.

RESULTS AND DISCUSSION

Physical-Chemical Characterization of Vermicomposts

A portion of the results related to the physical-chemical characterization of the vermicomposts is listed in Table 1. The accentuated values of humidity can be attributed to the peripheral hydrophilic groups (phenolic and alcoholic hydroxyls, carboxyls, and amino groups, among others) of the humified substances, which are also related to their high total contents of organic matter. It is important to note that the procedure employed to quantify total contents of organic matter, which is based upon heating samples in a muffle at 600°C , can also promote the thermal decomposition of some carbonates.

Nevertheless, considering the nature of the raw material employed to produce vermicomposts (mainly vegetal residues and soils), a significant contribution of carbonates to the loss of mass observed after calcination is not expected. The pH values (Table 1) are close to neutral; this result points to an extreme degree of dissociation of acidic functional groups from the organic matter. From an environmental point of view, this condition indicates appropriate availability of chelating groups able to retain ionic species, such as cations of heavy metals.

In turn, the total contents of ashes (or thermally stable compounds, considering temperatures higher than 880°C) presented remarkable differences between two groups of samples: those from Bahia and São Paulo States and those from Espírito Santo and Minas Gerais States. Considering that vermicomposting employs variable amounts of soils, inconstant levels of ash that are largely derived from aluminosilicates of those soils are expected.

Similar amounts of different soils can present notable variations in their natural contents and geodiversity of minerals, which is an important aspect related to the adsorptive capacity of vermicomposts. The types of minerals in the different samples of vermicompost were identified by X-ray analyses, as indicated in Figures 4a-d. As can be noted, quartz - Qz (SiO_2) was the major mineral component in all samples and this prevalence of quartz is consistent with the advanced stages of weathering that is found in Brazilian soils. In addition to quartz,

valerite (Vt) - CaCO_3 , and caulinite (Ct) - $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$ were also identified. As quartz presents surfaces with few functional groups and this mineral dominated the inorganic fraction of all vermicomposts, it is possible to conclude that their adsorptive capacities are predominantly influenced by the humified organic components.

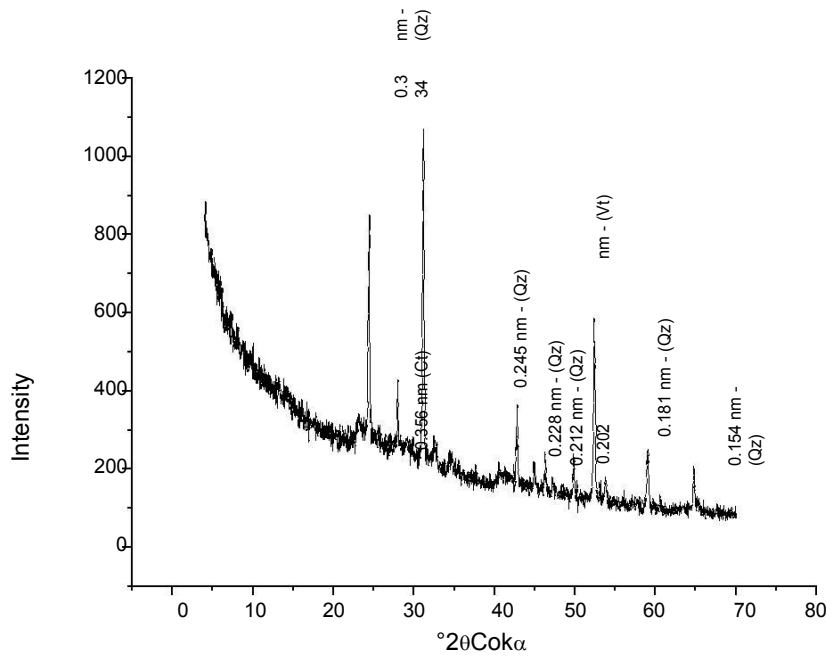
Table 1. Data from physical-chemical characterization (N = 3) of vermicomposts

Precedence of vermicompost	pH	Humidity (% m/m)	Total organic matter (% m/m)	Ashes (% m/m)
Espírito Santo	6.5 ± 0.1	77.4 ± 2.6	74.2 ± 0.5	27.0 ± 0.2
São Paulo	6.8 ± 0.4	92.1 ± 0.8	89.6 ± 0.2	10.4 ± 0.2
Bahia	7.0 ± 0.0	63.3 ± 2.3	84.5 ± 0.4	15.5 ± 0.4
Minas Gerais	7.2 ± 0.0	58.3 ± 0.5	70.0 ± 0.8	30.0 ± 0.8

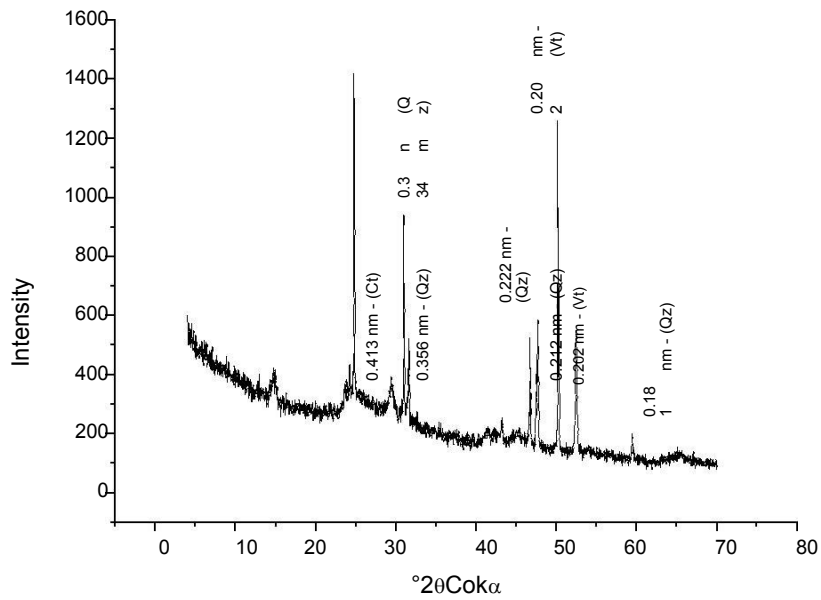
Figures 5a-d contains the thermogravimetric profiles of all evaluated samples of vermicompost. In all thermograms, the first drop in mass can be associated with volatilization of water that is weakly adsorbed on the surface of minerals or occluded into them. Independent of the vermicompost source, their water content was less than 10% (m/m): 5.87% (Espírito Santo), 6.25% (São Paulo), 7.99% (Bahia) and 8.96% (Minas Gerais). Between 200 and 400°C, two additional losses of mass (% m/m) were observed in the following ascending order: 14.78 (São Paulo), 17.13 (Espírito Santo), 17.96 (Bahia) and 23.46 (Minas Gerais). These losses are related to the combustion of carbohydrates and thermal degradation of aromatic compounds. In turn, between 400 and 700°C, a new reduction in mass was observed that was associated with the volatilization of many types of organic compounds such as proteins and nucleic acids, among others. Additionally, this range of temperatures also includes the decomposition of some carbonates [20]. Within this third range of loss of mass, the following percentages (% m/m) were observed: 12.69 (Bahia), 14.54 (Minas Gerais), 17.62 (Espírito Santo) and 21.61 (São Paulo). The infrared spectra (Figures 6a-d) gave typical evidence of stretches of phenolic and alcoholic hydroxyls as well as of carboxylic acids involved in hydrogen bonds. For these analytical signals, the corresponding wavenumbers (cm^{-1}) were: 3445 (São Paulo), 3450 (Minas Gerais), 3436 (Bahia) and 3432 (Espírito Santo) [21].

From 3000 to 2800 cm^{-1} , the signals were attributed to symmetric and asymmetric stretches of C-H bonds in aromatic groups. In this specific case, the wavenumbers (cm^{-1}) were: 2937 (São Paulo), 2910 (Minas Gerais and Bahia) and 2927 (Espírito Santo) [22].

Between 1700 and 1600 cm^{-1} , the peaks indicated stretches related to vibrations of C=C bonds in conjugated alkenes as well as COO^- and C=O in quinones: 1637 (São Paulo), 1630 (Minas Gerais), 1659 (Bahia) and 1635 (Espírito Santo). In turn, all samples of vermicompost also exhibited peaks typical of C-O bonds at the following wavenumbers: 1402 (São Paulo), 1374 (Minas Gerais), 1388 (Bahia) and 1393 (Espírito Santo) [22]. Between 1500 and 1300 cm^{-1} , the peaks were related to stretches of C-O bonds. Finally, wavenumbers less than 900 cm^{-1} corresponded to analytical signals of Si-O chemical bonds [21], which are expected due to the use of soils for producing vermicomposts.



a



b

Figure 4. (Continued).

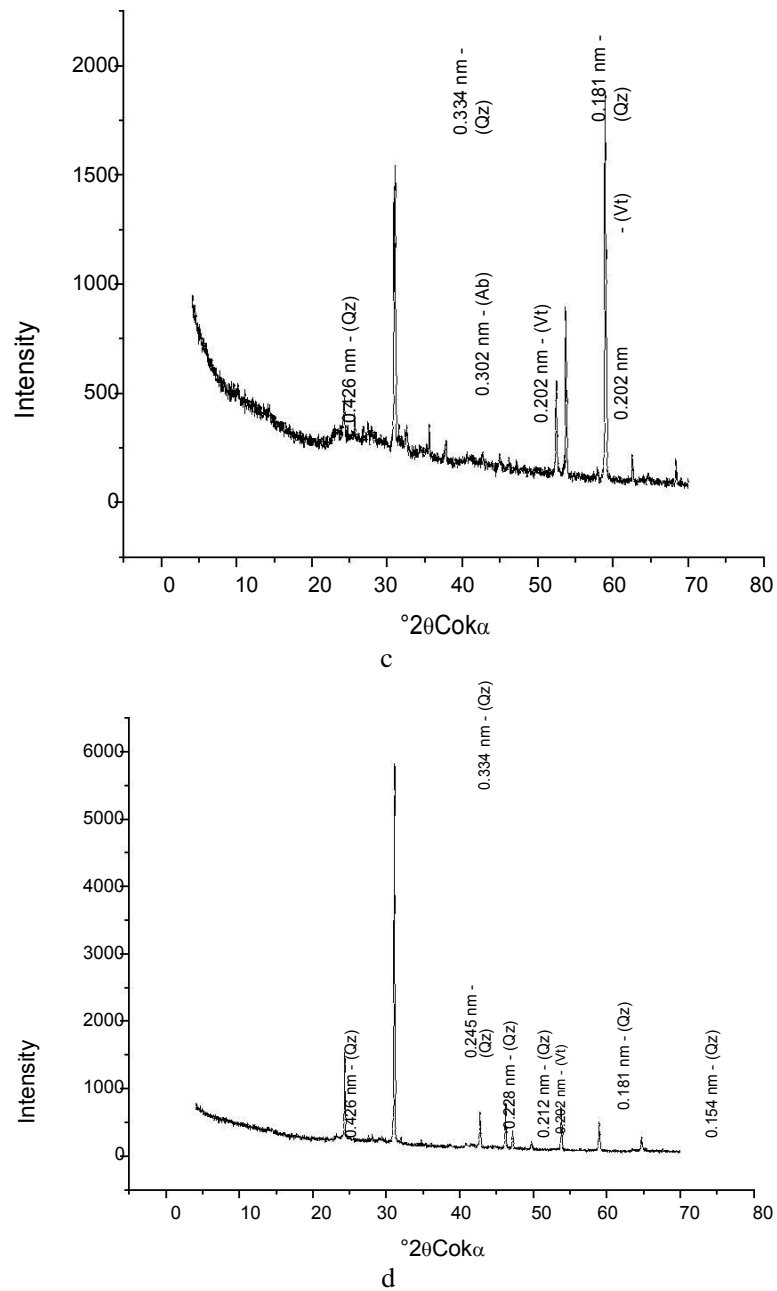
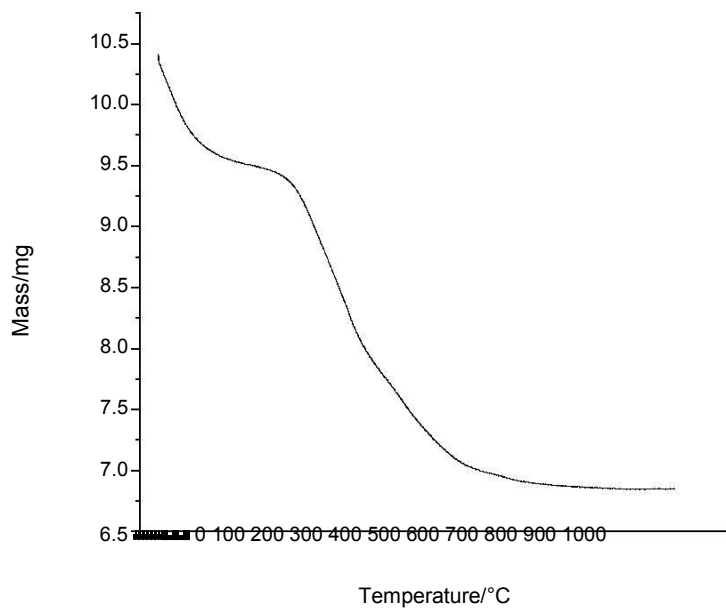
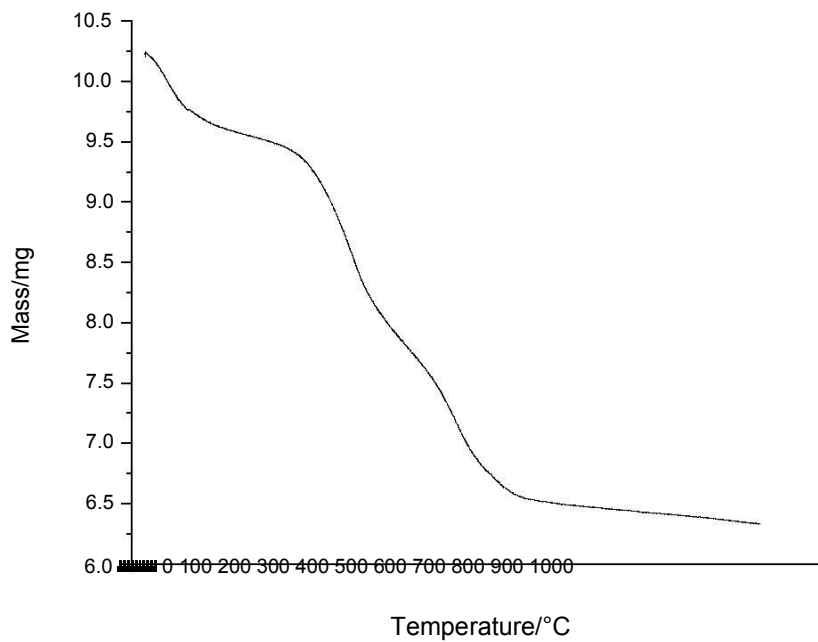


Figure 4. X-ray diffractogram of vermicomposts from (a) Bahia State, (b) Espírito Santo State, (c) Minas Gerais State and (d) São Paulo State.



a



b

Figure 5. (Continued).

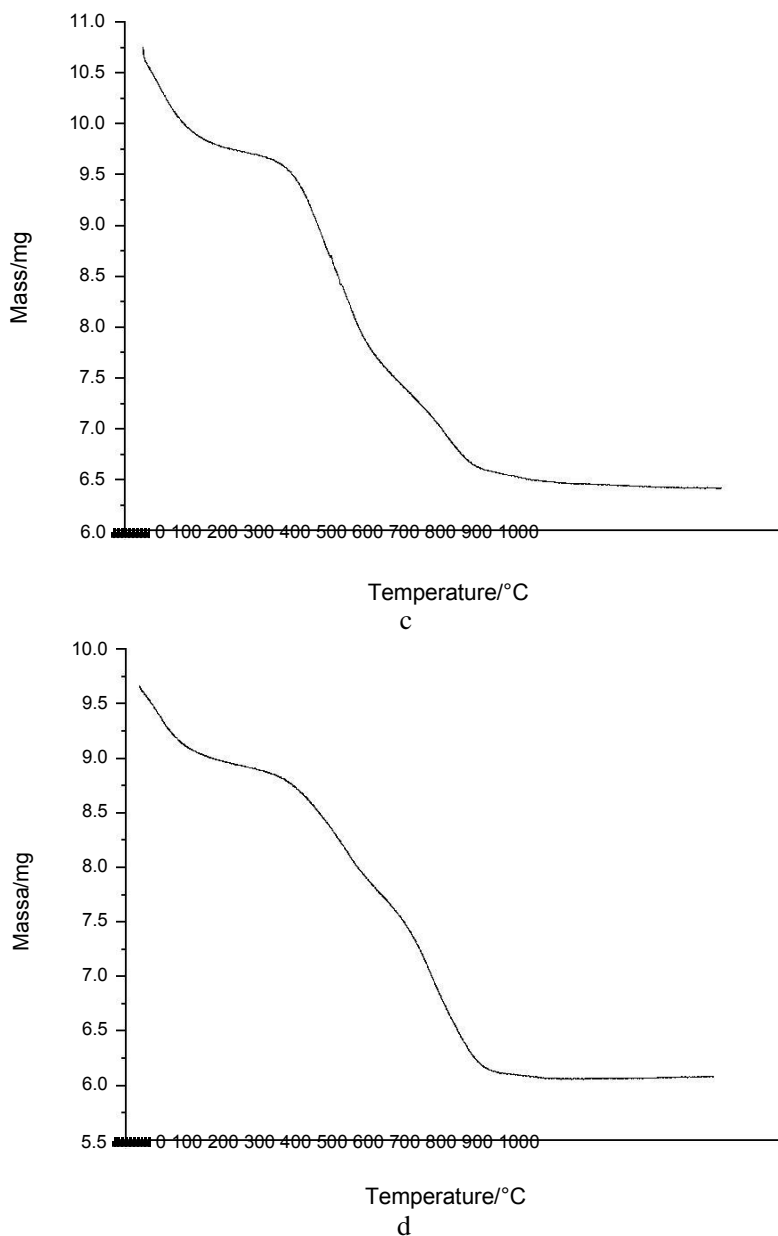


Figure 5. Thermogravimetric profiles of vermicomposts from (a) Bahia State, (b) Espírito Santo State, (c) Minas Gerais State and (d) São Paulo State.

As specified in Figures 6a-d, the red lines indicate spectra obtained after enrichment of vermicomposts with cadmium, copper, lead and zinc. As can be noted, the interactions of all vermicompost samples with Cd(II), Cu(II), Pb(II) and Zn(II) promoted modifications in the shapes of peaks as well as in signal (transmittance, %) intensity, thus evidencing strong interactions among the cited ions with the functional groups of humified organic matter. Such interactions included electrostatic attractions and the formation of chelates.

Figures 7a-d show the infrared spectra of samples of vermicompost not enriched with metallic cations. In these figures, there are spectra related to samples submitted to heating at 200, 400 and 600°C and unheated samples. From these spectra, it is possible to conclude that chemical groups with N-H (amines and amides) and C-H (aromatics) bonds presented high thermal stability (temperatures higher than 400°C were necessary for effective decomposition). In turn, temperatures less than 400°C were enough to decompose chemical groups containing C=O and C-O bonds. Figures 8a-d illustrate morphological aspects of the vermicompost particles. For all samples, it was possible to identify a great number of pores of variable size; this aspect is highly desirable for adsorptive processes.

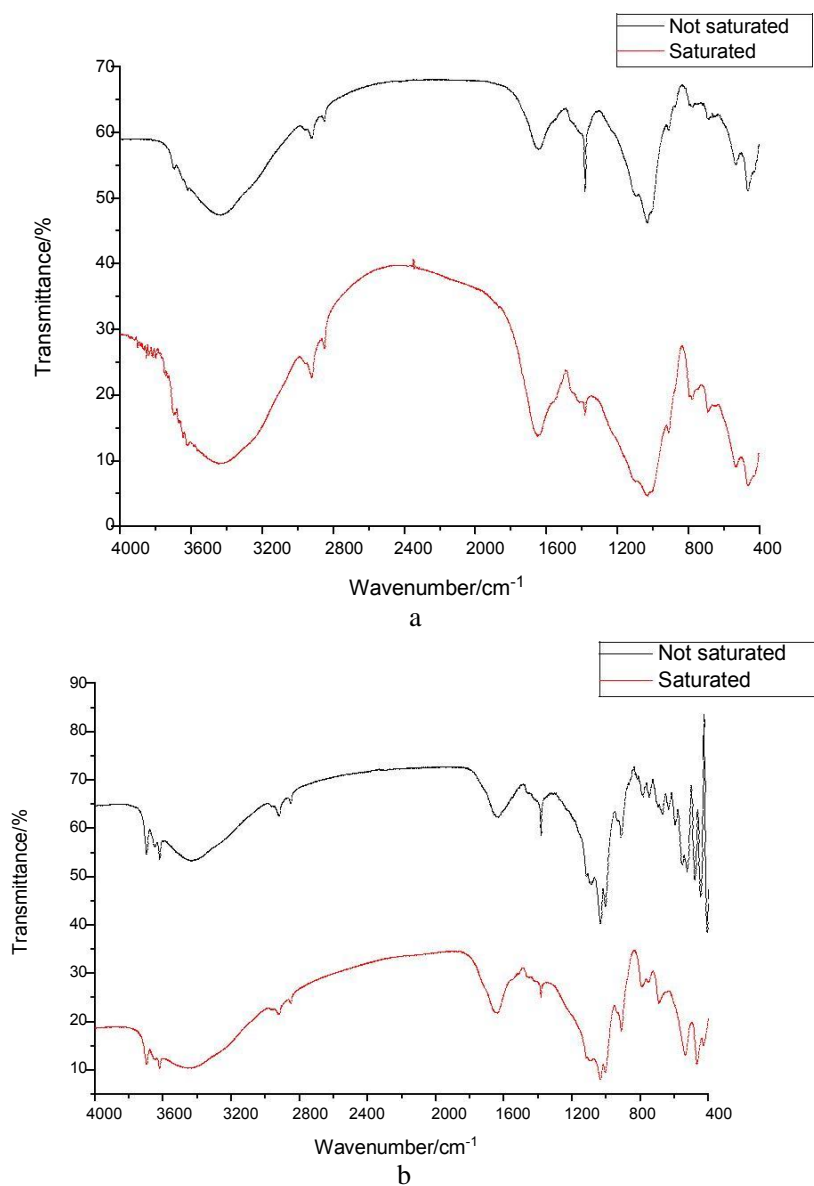


Figure 6. (Continued).

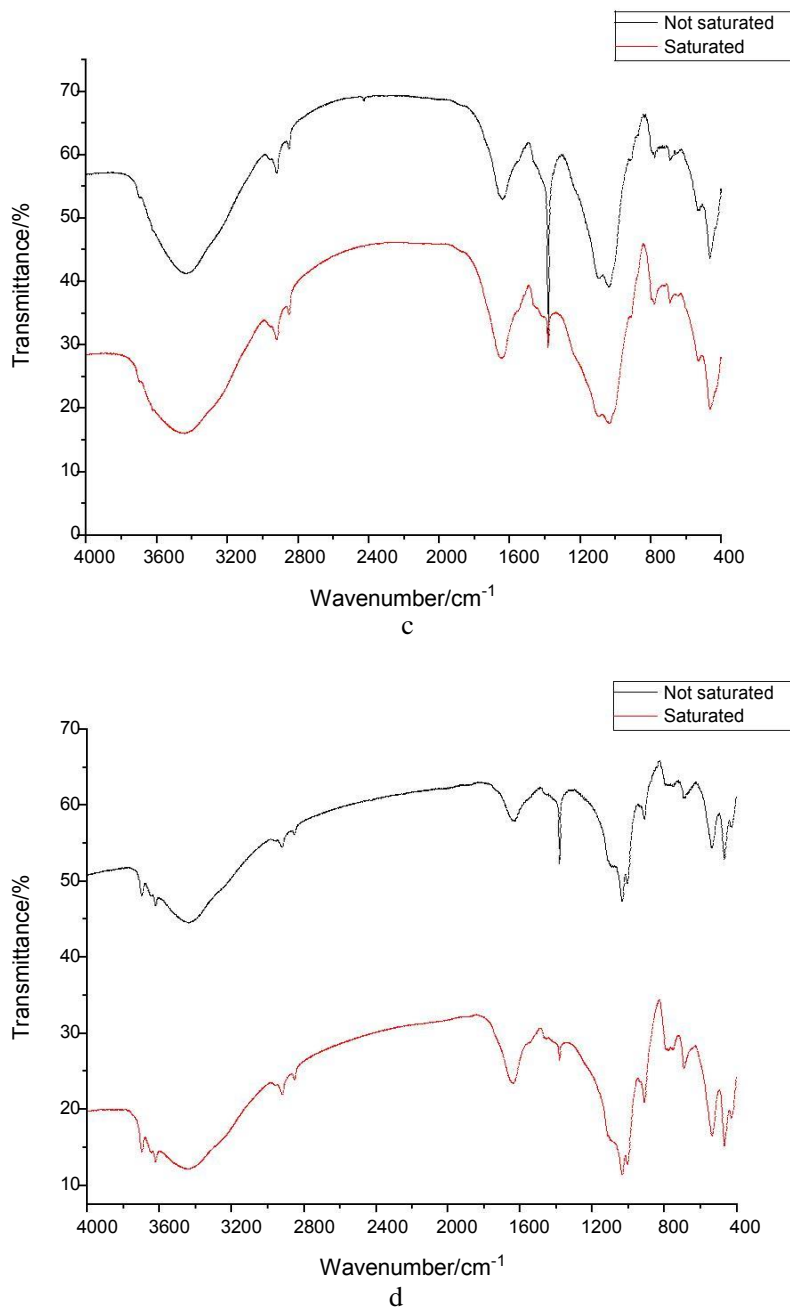


Figure 6. Fourier transformed infrared spectra (FTIR) of vermicomposts not saturated with metallic analytes and saturated with them. Samples from (a) Bahia State, (b) Espírito Santo State, (c) Minas Gerais State and (d) São Paulo State.

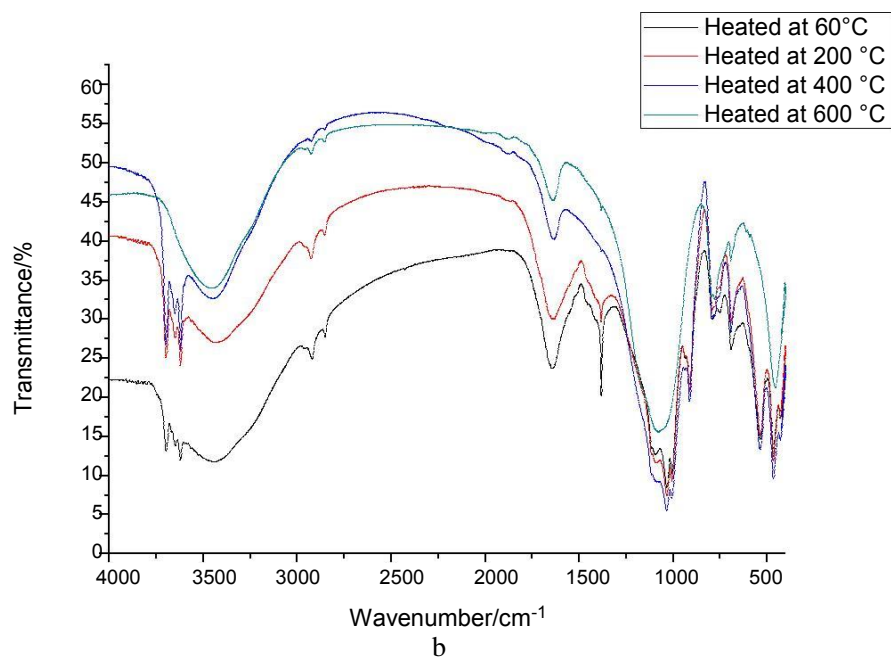
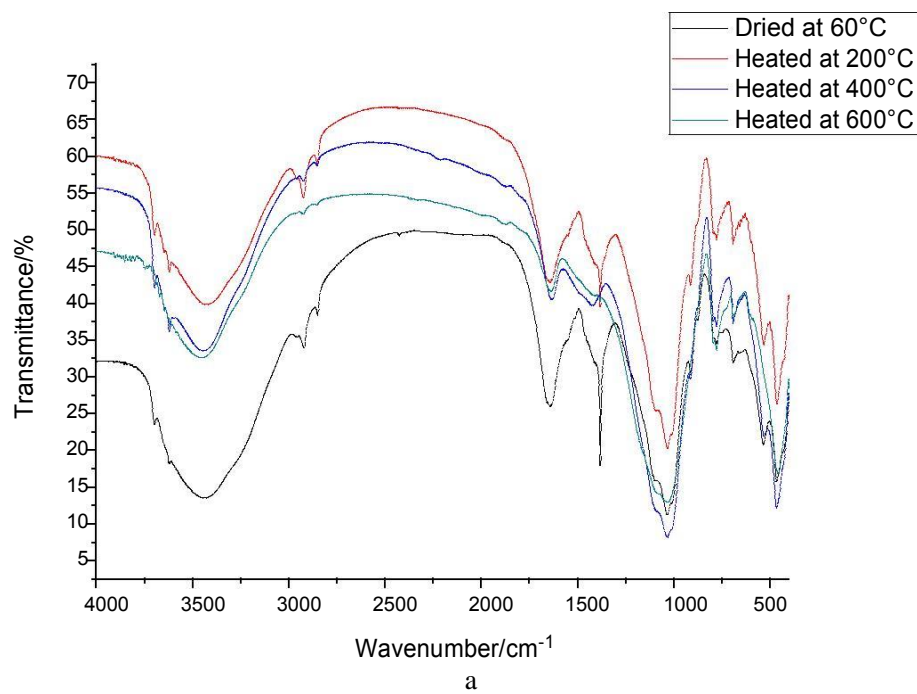


Figure 7. (Continued).

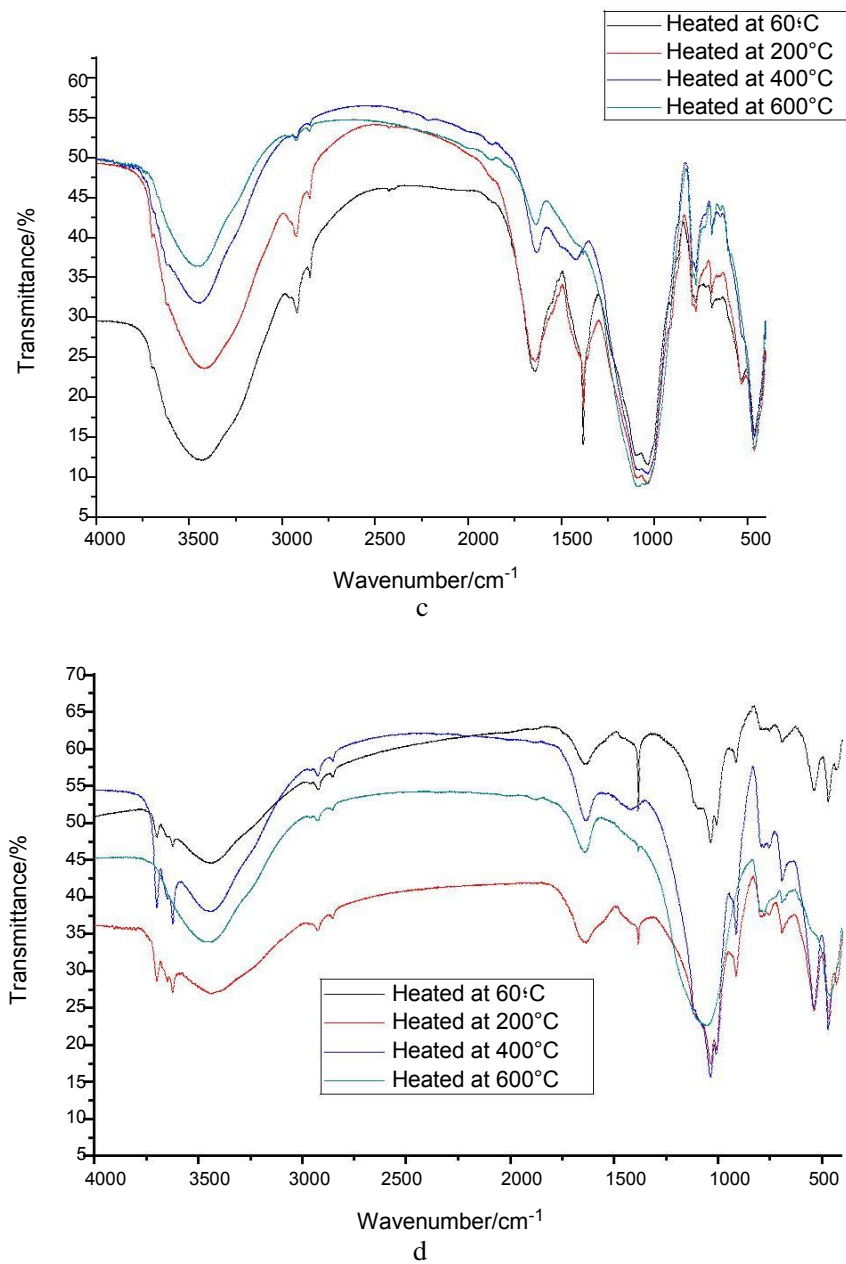
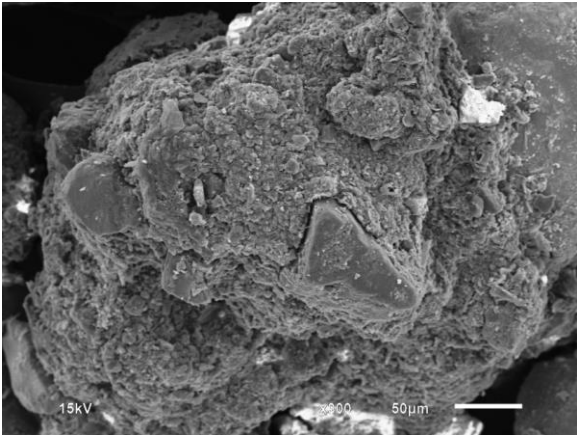
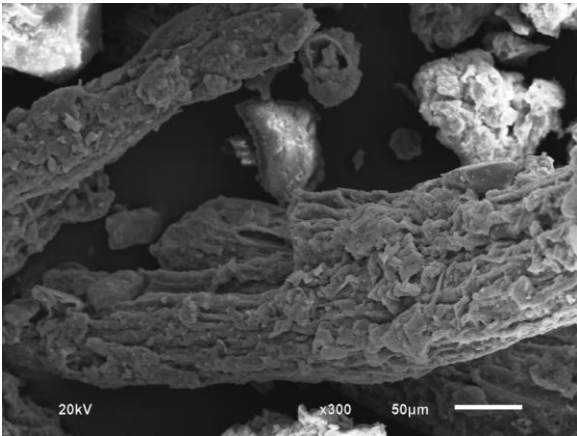


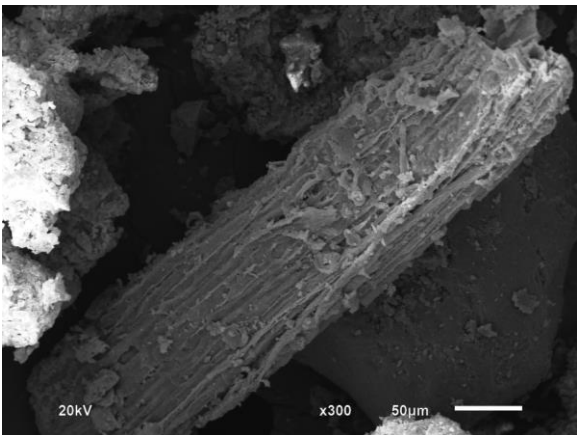
Figure 7. Fourier transformed infrared spectra (FTIR) of vermicomposts submitted to different temperatures of heating. Samples from (a) Bahia State, (b) Espírito Santo State, (c) Minas Gerais State and (d) São Paulo State.



a

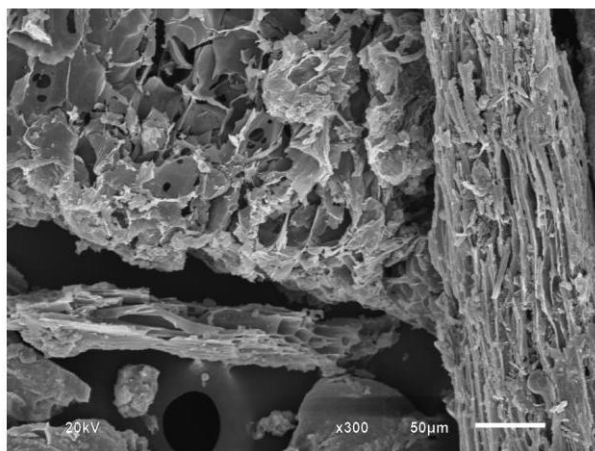


b



c

Figure 8. (Continued).



d

Figure 8. Electron microscopy of vermicomposts (magnification of 300 X) from (a) Bahia State, (b) Espírito Santo State, (c) Minas Gerais State and (d) São Paulo State.

Figure 9 shows a dendrogram that correlates the adsorptive percentages of Cd(II), Cu(II), Pb(II) and Zn(II) with data derived from the physical and chemical characterization (humidity, organic matter, ash and pH) of the vermicompost.

From these results, it is possible to evidence a strong similarity between humidity and organic matter, justified by the large amount of hydrophilic chemical groups in the humified molecules, as already discussed.

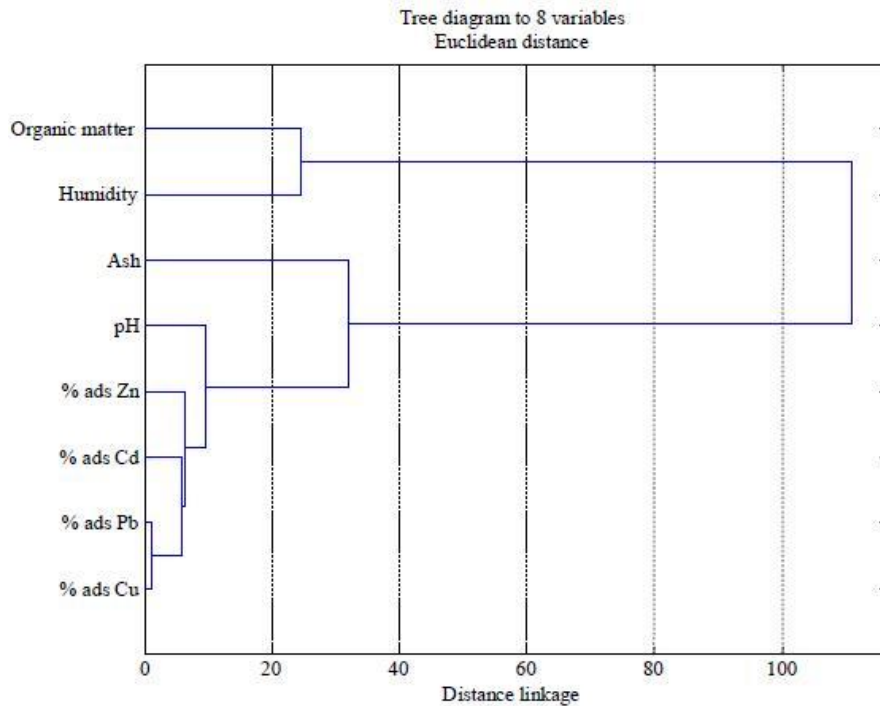


Figure 9. Dendrogram correlating analyses of the physical-chemical characterization of vermicomposts.

In spite of the small intensity, it was also possible to identify similarities between pH and contents of ash, which may be explained by the elevated buffering capacity of the humified substances that are able to compensate for changes related to the distribution of charges on mineral structures.

The adsorptive percentage of Zn(II) is highly correlated with pH of the vermicompost, and this result points to a great dependency of zinc adsorption in relation to the degree of humification in the samples of vermicompost. This statement is true, because pH near neutrality (as those listed in Table 1) indicates advanced stages of humification. As cadmium and zinc are elements with close periodic properties, the similarity between them (see dendrogram – Figure 9) is coherent.

Due to the accentuated reduction in the adsorptive percentages of Zn(II), after 60 and 120 minutes of mechanical agitation, this element presented the largest detachment from the other analytes. Despite the small variances observed between the adsorptive percentages of Cu(II) and Pb(II), they were not accentuated enough for a statistical (multivariate analysis) differentiation. Additionally, the distance of the adsorption percentages of copper and lead (as indicated in Figure 9) reveals their independence in relation to other evaluated physical-chemical parameters. This conclusion is mainly observed for Pb(II), which exhibited the highest percentages of adsorption, independently from the vermicompost source.

Adsorptive Profiles of Cd(II), Cu(II), Pb(II) and Zn(II) against Vermicomposts from Different Sources

Figures 10a-d illustrate the influence of the mechanical agitation time on adsorptive profiles of Cd(II), Cu(II), Pb(II) and Zn(II).

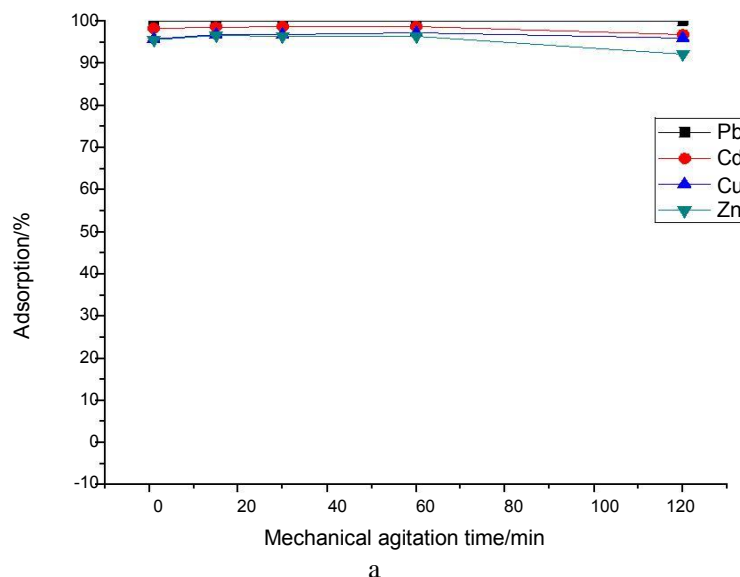


Figure 10. (Continued).

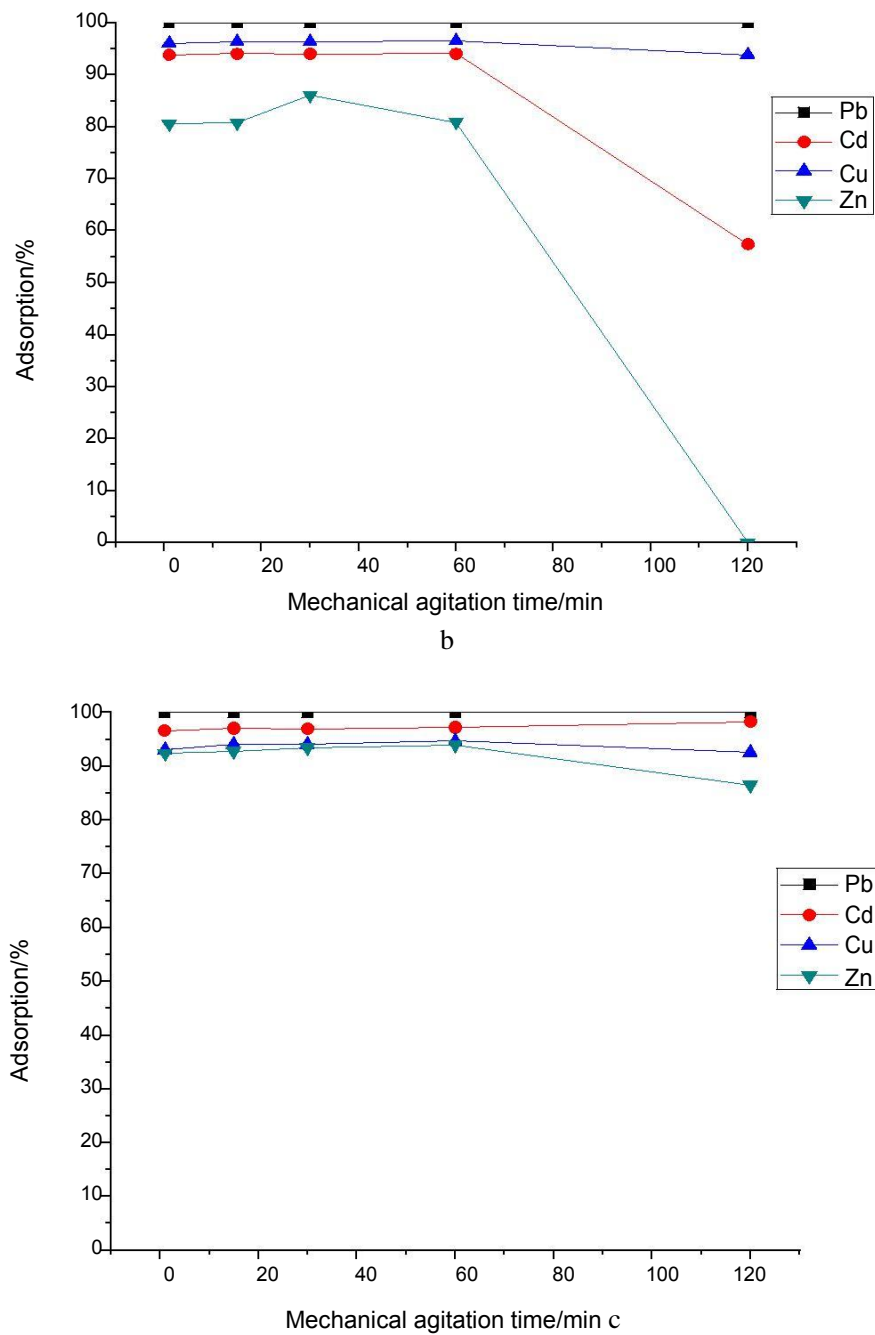


Figure 10. (Continued).

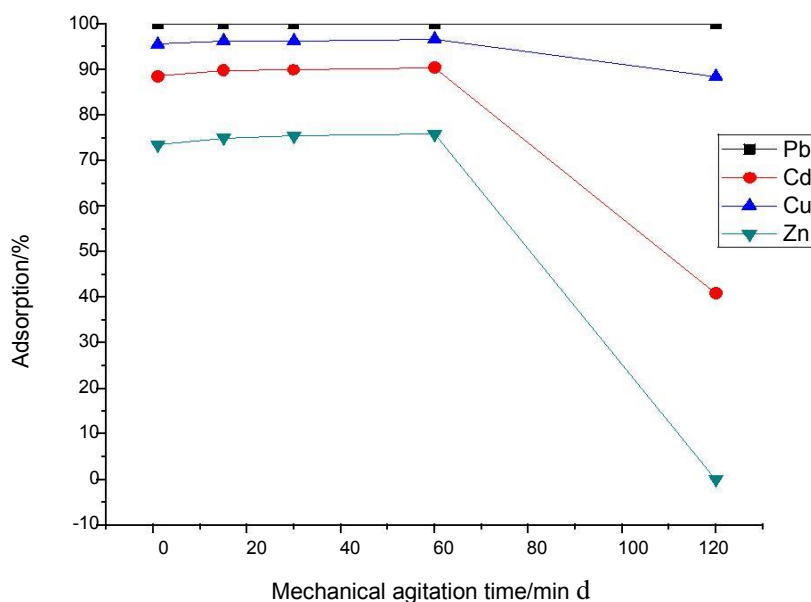


Figure 10. Influence of mechanical agitation time on the adsorptive percentages of Cd(II), Cu(II), Pb(II) and Zn(II) in vermicompost from: (a) Bahia State, (b) Espírito Santo State, (c) Minas Gerais State and (d) São Paulo State. Each point represents an average of 3 replicates. All relative standard deviations were less than 10%.

It is important to note that the concentrations of Cd(II), Cu(II), Pb(II) and Zn(II) were fixed at 10 mg L^{-1} , because this value is higher than the Brazilian environmental limits for wastewater, which are (in mg L^{-1}): 0.2 (Cd), 1.0 (Cu), 0.5 (Pb) and 5.0 (Zn) [23]. In this sense, the adsorptive tests described in this work were performed under experimental conditions that were able to simulate real situations of polluted wastewaters.

The pH of the mixed solutions was fixed at 2, because this value of pH is found in many real aqueous discharges (contaminated with metallic analytes) derived from chemical laboratories of universities and industries. In order to compensate for the high concentrations of H_3O^+ , the vermicomposts were submitted to a fine granulometry (270 mesh).

As can be noted in Figures 10a-d, no significant differences were observed among the majority of adsorptive percentages within the interval of 1 – 120 minutes, thus evidencing the predominance of physical and reversible interactions among the particles of vermicompost and selected cations. Among the possible reversible interactions, those related to electrostatic attraction should be highlighted. As discussed below, the appearance of electrical charges on the particles of vermicompost is a process submitted to changes along the contact with mixed solutions of Cd(II), Cu(II), Pb(II) and Zn(II). Initially, the high concentrations of hydrogen ions (at pH 2) indicate an excess of positive charges on the vermicompost due to the protonation of chemical groups such as, for example, hydroxyls. This excess of positive charges can retain anions from solutions (mainly NO_3^- in this specific case), and in turn this layer is responsible for attracting cations such as Cd(II), Cu(II), Pb(II) and Zn(II).

Nevertheless, the pH of the solutions was increased (from 2 to 6) during the experiments with the consequent appearance of negative charges on the vermicompost and the attraction of metallic cations. This phenomenon occurs with humified materials because the large

number of chemical groups on their surfaces promotes efficient removal of H_3O^+ from aqueous media. After this reduction of $[\text{H}_3\text{O}^+]$ and the concomitant increase in pH, acidic groups can be neutralized with a consequent drop in $[\text{OH}^-]$, thus indicating that the pH of the aqueous media was elevated to values higher than 6, and that this last value was achieved after the establishment of equilibria of dissociation. It is important to note that retention of cations by means of electrostatic force does not need specific spatial orientations and, consequently, long periods of time are not necessary for its establishment. Moreover, these types of interaction do not involve large associated energies and this characteristic confers a considerable reversibility to them. From an environmental point of view, reversible adsorptive processes comprise a desirable feature for procedures designed to recover pollutants.

As can be noted in Figures 10a-d, high adsorptive percentages were obtained even under accentuated acidity (pH 2) and the concomitant presence of Cd(II), Cu(II), Pb(II) and Zn(II). These results highlight that the use of vermicomposts, passed through a 270-mesh sieve, was able to compensate for unfavorable conditions related to competitive adsorptions.

Among the evaluated group of analytes, Pb(II) presented adsorptive percentages near 100%, which is consistent with its greater density of charge and smaller energy of hydration [24]. These features are very relevant for adsorptions predominantly based on electrostatic attractions, which were already evidenced by the short period of time (1 minute) necessary to achieve a condition of chemical equilibrium.

Tables 2 and 3 list the physical-chemical parameters derived from the mathematical treatment of the adsorption isotherms for vermicompost from Bahia and Minas Gerais, respectively. As previously commented, this work employed two mathematical models (Langmuir and Freundlich) that are commonly used for treating data derived from procedures of adsorption [4].

The graphics associated with the cited isotherms are illustrated in Figures 11a-h. The Langmuir model is based on a uniformity of the adsorptive groups and the formation of monolayers of adsorbates, while the Freundlich model considers the possibility of differences in energy among many different adsorbent chemical groups.

Since vermicomposts have very complex and heterogeneous matrices, the higher concordance among the experimental data and model of Freundlich (see Tables 2 and 3) is coherent. Here, coherency with a mathematical model means good responses to procedures of linearization of adsorption data (R^2 close to unity).

When both mathematical models are compared, the differences between them (in terms of the calculated physical-chemical parameters listed in Tables 2 and 3) can be attributed to dissimilarities in the fundamentals of both models.

As can be seen in Figures 11a-h, clear tendencies of saturation (achievement of a plateau) were verified only for cadmium and zinc in vermicompost from Bahia. For other combinations of metallic ions and vermicompost, irregular profiles of adsorption were obtained, thus indicating different degrees of removal from the theoretical assumptions associated with both mathematical models.

Table 2. Adsorptive parameters derived from linearizations of adsorption isotherms of Cd(II), Cu(II), Pb(II) and Zn(II) in vermicompost from Bahia State

Parameters	Analytes			
	Cd(II)	Cu(II)	Pb(II)	Zn(II)
Langmuir	$Y = 5.4X - 3.1$	$Y = 2.8X - 2.4$	$Y = 0.05X + 2.8$	$Y = 4.9X - 8.1$
q_{\max} (mg g^{-1})	-----	0.4	-----	-----
K_L (mg L^{-1})	-----	0.9	-----	-----
R^2	0.733	0.924	0.409	0.783
Freundlich	$Y = 1.2X - 0.4$	$Y = 1.2X - 0.1$	$Y = 0.6X + 0.1$	$Y = 1.1X + 0.5$
K_f (mg g^{-1})	2.3	1.3	1.4	3.4
$1/n$	1.2	1.2	0.6	1.1
N				
R^2	0.866	0.917	0.884	0.958

For definition of Y and X in the equations, see discussions along the text.

*Not calculated due to small values of R^2 .

Table 3. Adsorptive parameters derived from linearizations of adsorption isotherms of Cd(II), Cu(II), Pb(II) and Zn(II) in vermicompost from Minas Gerais State

Parameters	Analytes			
	Cd(II)	Cu(II)	Pb(II)	Zn(II)
Langmuir	$Y = 1.2X + 0.2$	$Y = 22.5X - 0.1$	$Y = 1.6X - 1.9$	$Y = 4.3X - 5.4$
q_{\max} (mg g^{-1})	4.8	9.9	0.5	-----
K_L (mg L^{-1})	1.0	0.004	1.2	-----
R^2	0.983	0.999	0.965	0.769
Freundlich	$Y = 0.8X - 0.2$	$Y = 1.0X - 1.3$	$Y = 1.3X + 0.2$	$Y = 1.8X + 0.1$
K_f (mg g^{-1})	1.6	22.1	1.5	1.3
$1/n$	0.8	1.0	1.3	1.8
N	1.2	1.0	0.8	0.6
R^2	0.968	0.999	0.912	0.931

For definition of Y and X in the equations, see discussions along the text.

*Not calculated due to small values of R^2 .

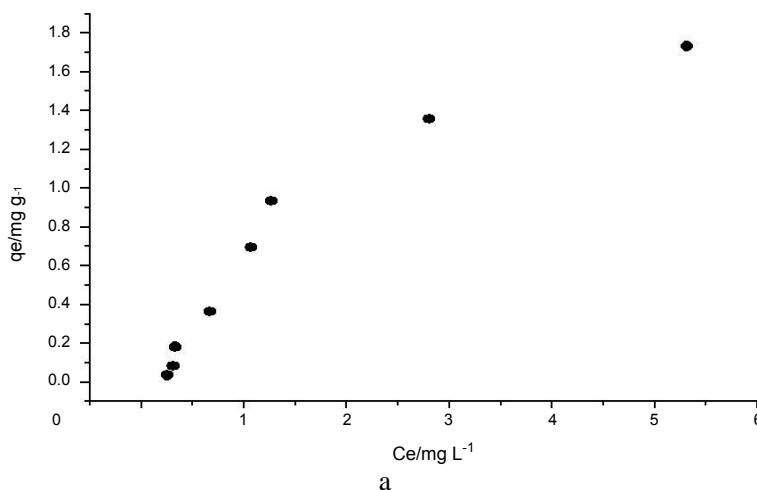


Figure 11. (Continued).

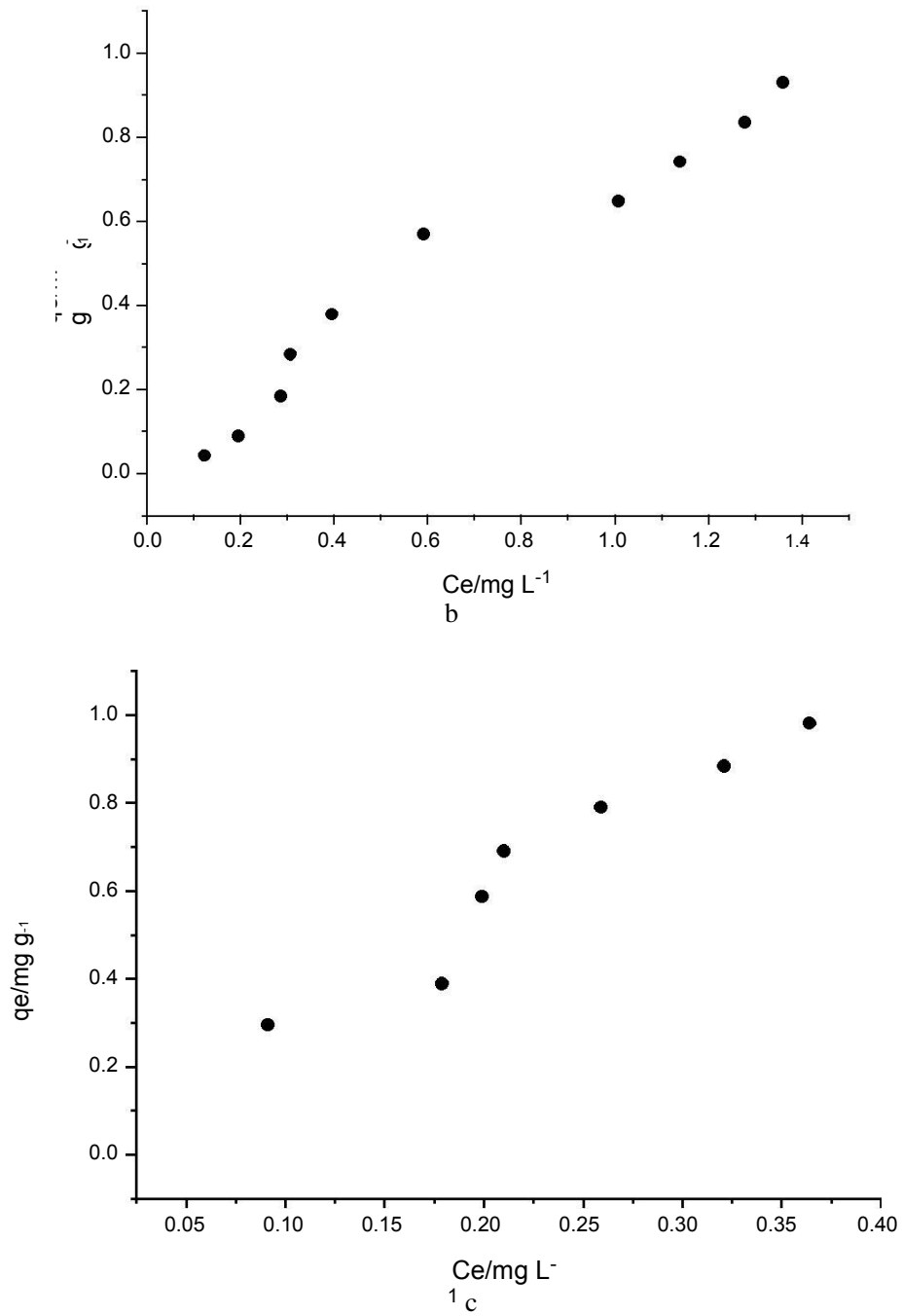


Figure 11. (Continued).

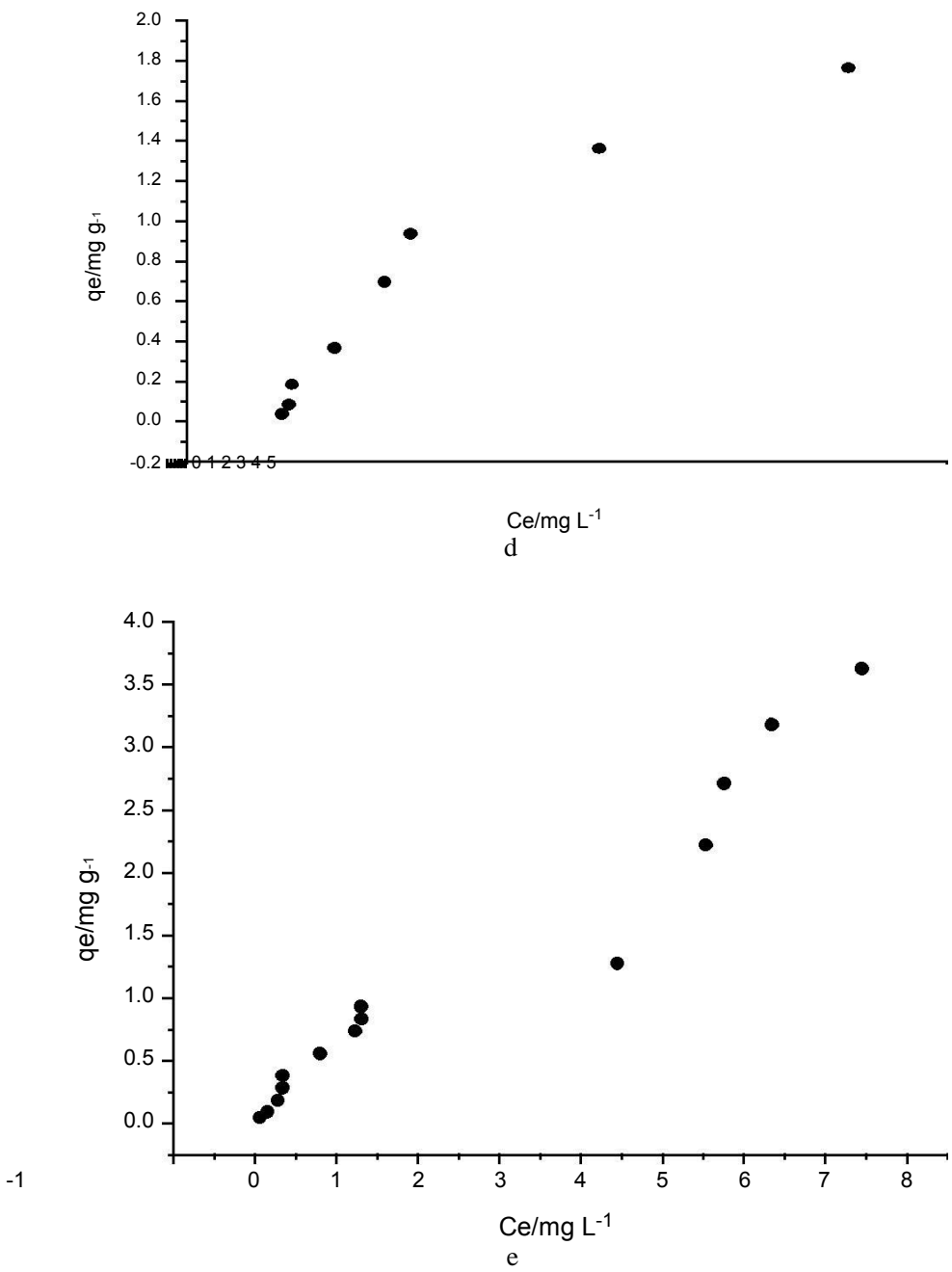


Figure 11. (Continued).

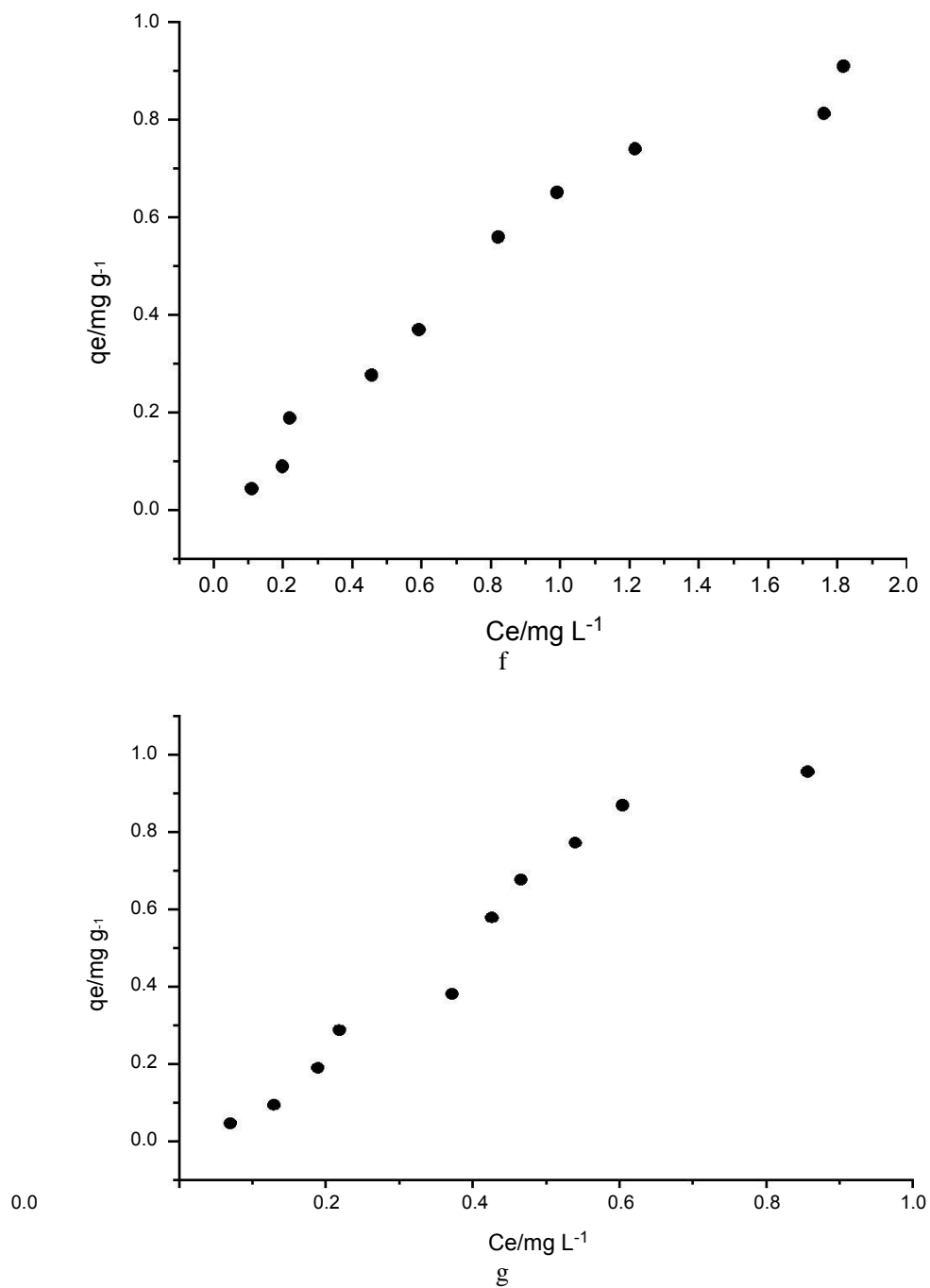


Figure 11. (Continued).

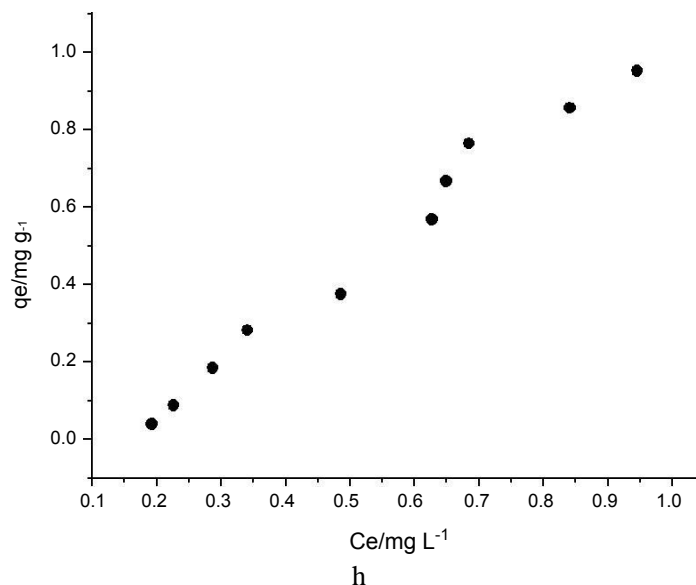


Figure 11. Adsorption isotherms of cadmium (a), copper (b), lead (c) and zinc (d) in vermicompost from Bahia State and cadmium (e), copper (f), lead (g) and zinc (h) in vermicompost from Minas Gerais State. The percentages of adsorption indicated in these figures are related to an initial concentration of 10 mg L^{-1} for each metallic cation. Each point represents an average of 3 replicates. All relative standard deviations were less than 10%.

These linearizations, according to the Langmuir and Freundlich models, were obtained from equations 1 and 2, respectively [25].

$$1/q_e = 1/(q_{\max} \times K_L) \times 1/C_e + 1/q_{\max} \quad (1)$$

$$\log q_e = \log K_f + 1/n \times \log C_e \quad (2)$$

In these equations, q_e , q_{\max} and C_e are, respectively: the quotient between the adsorbed mass of adsorbate and adsorbent mass (mg g^{-1}), the maximum adsorbed mass of the adsorbate in relation to adsorbent mass (mg g^{-1}) and the equilibrium concentration of the adsorbate in the supernatants (mg L^{-1}). In turn, K_f is a constant that describes the adsorptive capacity of vermicompost according to the model of Freundlich. The parameter n indicates the adsorptive tendency and values, between 2 and 10, indicate processes of adsorption with thermodynamic fostering.

As previously discussed, the different parameters cited above are valid if they are derived from equations with R^2 (linear correlation coefficient) values close to unity. As can be seen in Tables 2 and 3, values of R^2 for Zn(II) in vermicompost from Minas Gerais (Langmuir model) and for Cd(II), Pb(II) and Zn(II) in vermicompost from Bahia (Langmuir model) were considerably less than 1. Consequently, no adsorptive parameters were calculated for these adsorptive systems.

However, for other combinations of metallic ions and vermicompost, adequate values of R^2 ($R^2 > 0.85$ in Tables 2 and 3) were obtained and it was possible to calculate reliable values of q_{\max} and other physical-chemical parameters.

From the calculated values of q_{\max} , all the samples of vermicompost can be classified as good adsorbents for the considered analytes, even considering a reduced pH (pH = 2).

It is important to note that the adsorption isotherms were built with individual solutions in order to obtain a more detailed panorama about the influence of vermicompost composition (derived from different sources of production) on its capacity for adsorption. In this regard, the use of mixed solutions for building the isotherms of adsorption would mask specific interactions with each one of the metallic analytes.

Adsorptive Profiles of Congo Red, Malachite Green, and Methanile Yellow in Vermicompost from Bahia State

Table 4 lists the influence of pH on the maximum absorption wavelength for solutions of Congo red, malachite green, and methanile yellow. In spite of the variation related to maximum absorption of malachite green, the λ_{\max} of 617 nm was retained over a large range of pH. In turn, changes in pH caused remarkable modifications in the values of maximum absorption and λ_{\max} for Congo red and methanile yellow. For this reason, after each step of agitation, the pH of the supernatants was determined and the absorbances were measured at their respective values of λ_{\max} . Thus, decreases in absorbance (%) according to initial pH, which are listed in Table 5, were calculated by comparing the absorbance of solutions before and after agitations with vermicomposts at their respective λ_{\max} .

Table 4. Maximum absorption wavelengths (nm) of Congo red, malachite green, and methanile yellow solutions in different values of pH

pH	λ_{\max}		
	Congo red	Malachite green	Methanile yellow
2	570	617	454
3	560	617	442
4	475	617	445
5	500	617	439
6	500	617	442

Table 5. Influence of initial pH of the dye solutions on adsorption percentages of Congo red, malachite green, and methanile yellow (N = 3)

Initial pH	Reduction in absorbances (%)		
	Congo red	Malachite green	Methanile yellow
2	96.4 ± 1.9	98.4 ± 0.3	61.8 ± 0.4
3	91.8 ± 3.5	98.7 ± 0.9	39.6 ± 2.0
4	75.9 ± 5.0	98.1 ± 0.6	33.3 ± 0.7
5	67.3 ± 1.2	98.1 ± 0.3	26.0 ± 3.0
6	85.9 ± 4.5	99.9 ± 0.4	23.9 ± 3.0

As observed during the adsorption experiments with metallic cations, solutions of dyes initially at pH 2 were submitted to an elevation in pH after mechanical agitation with vermicompost. In this regard, all previous discussions about the appearance and changes in charges on particles of vermicompost can also be applied here.

For Congo red and methanile yellow, the best percentages of adsorption were observed at pH 2, while green malachite exhibited its best retention at pH 6. At pH 6, the surface of the vermicompost is negatively charged, which justifies the great capacity of this humified adsorbent to retain malachite green, which is an organic cation. However, very satisfactory retentions of malachite green were also observed at pH 2, 3, 4, and 5, thus suggesting additional mechanisms of retention. In turn, Congo red and methanile yellow are negatively charged with a consequent electrostatic attraction in relation to the positively charged particles of vermicompost at pH 2. The results related to the influence of mechanical agitation time on the retention of Congo red, malachite green, and methanile yellow are listed in Table 6. The presence of a predominantly electrostatic interaction among the three evaluated dyes and vermicompost is also reflected in the short period of time (1 minute) that was necessary to achieve a condition of chemical equilibrium (Table 6).

As can be noted in Table 7 (see values of R^2), lousy mathematical adjustments were obtained for adsorptions of Congo red (for Langmuir and Freundlich models) and methanile yellow (Langmuir model) in vermicompost and this result avoids any estimation related to the physical-chemical parameters. However, this does not mean that vermicompost from Bahia exhibits small adsorptive capacity against Congo red and methanile yellow since very satisfactory percentages of adsorption were obtained, as indicated in Tables 5 and 6. As previously discussed, extensive departures from mathematical treatments are related to a lack of concordance among the hypothetical assumptions and experimental evidence.

The discrepancies observed for adsorptions of Congo red, malachite green and methanile yellow in relation to the mathematical models of Langmuir and Freundlich can be attributed to the same principles previously discussed for metallic cations.

In this experimental section, only one sample of vermicompost (from Bahia State) was used, because it was observed that this sample exhibited satisfactory profiles of adsorption for other ionic adsorbates (Cd(II), Cu(II), Pb(II) and Zn(II)).

The maximum adsorptive capacities listed in Table 7 point to a promising field of application of vermicompost for decontaminating aqueous media containing the evaluated dyes.

Table 6. Influence of mechanical agitation time on adsorption percentages of Congo red, malachite green, and methanile yellow (N = 3)

Mechanical agitation time (min)	Reduction in absorbances (%)		
	Congo red	Malachite green	Methanile yellow
1	94.9 ± 4.2	99.3 ± 0.1	96.5 ± 1.4
5	98.0 ± 1.8	97.8 ± 1.0	99.8 ± 0.2
10	91.3 ± 3.0	93.0 ± 3.0	98.1 ± 3.2
30	97.0 ± 2.3	100.0 ± 0.1	94.9 ± 1.2
60	95.6 ± 5.3	100.0 ± 0.1	98.5 ± 0.5
120	96.1 ± 6.7	99.9 ± 0.6	97.9 ± 0.5

Table 7. Adsorptive parameters derived from linearizations of adsorption isotherms of Congo red, malachite green, and methanile yellow in vermicompost from Bahia State

Parameters	Dyes		
	Congo red	Malachite green	Methanile yellow
Langmuir	$Y = -0.04X + 0.7$	$Y = 0.02X + 0.04$	$Y = 0.02X + 0.05$
q_{\max} (mg g ⁻¹)	-----*	45.9	-----
K_L (mg L ⁻¹)	-----	0.6	-----
R^2	0.0183	0.9713	0.5987
Freundlich	$Y = 0.5X + 0.004$	$Y = 0.5X + 1.2$	$Y = 0.6X + 1.1$
K_f (mg g ⁻¹)	-----	14.3	3.0
1/n	-----	0.5	5.0
n	-----	2.1	0.2
R^2	0.4138	0.9356	0.9656

For definition of Y and X in the equations, see discussions along the text. *Not calculated due to small values of R^2 .

CONCLUSION

This work evaluated possible changes in the adsorptive profiles of Cd(II), Cu(II), Pb(II) and Zn(II) in vermicomposts derived from different sources as well as forms of production. Considering the employed mass of the vermicomposts (0.5 g) and their granulometry (270 mesh or ≤ 0.053 mm), no significant differences were observed in relation to the percentages of adsorption of all cationic analytes.

It is important to note that this conclusion was valid even considering some structural differences between the samples of vermicomposts, as highlighted after statistical treatment of the data derived from physical-chemical characterization of vermicomposts. In accordance with the data derived from isotherms of adsorption, vermicomposts (samples from Bahia and/or Minas Gerais States) presented some deviations from theoretical assumptions in relation to the models of Langmuir and Freundlich. Such deviations can be attributed to the structural complexity of vermicomposts and adsorbates (dyes, mainly).

This work was able to evidence a common feature of vermicomposts (independent of their source of production) that is related to their capacity to retain ionic pollutants from aqueous media. Moreover, the use of vermicomposts for decontaminating colored aqueous media was amplified, since this investigative field is still scarce in the literature.

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Chapter 7

NATURAL ORGANIC MATTER IN DRINKING WATER: CHARACTERIZATION METHODS

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ABSTRACT

In order to improve project and operation of drinking water facilities is important to be aware about the kind of organic matter present. Various physical and chemical fractionation techniques have been developed, mainly focused in the identification of the most reactive components in dissolved organic matter that originate harmful disinfection by-products. These fractionation techniques include resin fractionation, membrane filtration (ultrafiltration, nanofiltration and reverse osmosis), size exclusion chromatography/high performance size exclusion chromatography and reverse phase high performance liquid chromatography. Several methods have been used for characterization of natural organic matter (NOM) fractions, namely total and dissolved organic carbon, ultraviolet-visible spectrometry, fluorescence, Fourier transform infrared spectroscopy, liquid chromatography coupled with different detectors, nuclear magnetic resonance spectroscopy, gas chromatography–mass spectrometry (after pyrolysis or derivatization with Tetramethylammonium hydroxide) and biological methods for quantification of biodegradable organic matter.

In this chapter the more relevant contributions of the last ten years to the current knowledge regarding fractionation and characterization of NOM are discussed together with their advantages and limitations.

Due to the complexity and variability of NOM there must be a special concern in planning water sampling due to seasonal and local variation of water characteristics, in order to get representative samples and take consistent conclusions about NOM nature.

The best way to overcome the limitations identified in the methods of fractionation and characterization of NOM is their combination. Knowledge of molecular properties of NOM is decisive to understand and improve drinking water treatment processes.

1. INTRODUCTION

Natural organic matter (NOM) is a complex mixture of organic products from different sources, namely environmental degradation of organisms, vegetation, soil, wastewater discharge and agricultural return. The water chemistry, characterized by pH, alkalinity, salinity, nutrients, etc. and hydrological factors, such as water depth and flowrate, affect the composition of NOM (Chow et al. 2005). Their composition is different according to its origin and depends also from the climate (mainly temperature and rainfall), geology and topography (Fabris et al., 2008, Vieira et al. 2012, Wang et al. 2013).

Dissolved organic matter (DOM) is defined as the portion that passes through a 0.45 μm pore-size filter, which corresponds to the apparent molecular weight fraction lower 108 Da and represents more than 80% of NOM (Chow et al. 2005); it contains humic substances (500 – 10 kDa) that represent more than 50% of DOM, viruses and organic compounds (amino acids, carbohydrates, fatty acids and hydrocarbons). The size fraction greater than 0.45 μm , particulate organic matter, includes zooplankton, algae and organic residues (e.g., from soil); it represents a small fraction of the organic matter and can be efficiently removed by conventional treatment methods, such as coagulation and filtration processes (Chow et al. 2005). Due to its relative abundance most of the research is focused in DOM fractionation and removal, especially humic substances.

The presence of organic matter in drinking water is of major concern because (Matilainen et al. 2011); it affects directly the organoleptic properties of water (colour, taste and odour); it influences biological regrowth of microorganisms throughout the distribution systems; it can originate harmful disinfection by-products (DBP); high concentration of organic matter affects the operation of water treatment plants, namely coagulation, and contributes to the metal complexation.

The growth of bacteria in distribution networks, which occurs mainly in pipes biofilm surface, should be avoided because some of these bacteria are pathogenic for humans (e.g., *Legionella pneumophila* and *Mycobacterium avium*), therefore the biodegradable organic matter levels in drinking water should be as low as possible (Juhna and Melin, 2006).

The identification of the most reactive components in DOM that originate harmful disinfection by-products (DBP) are of major concern. All methods of disinfection (chlorine, ozone, chlorine dioxide and UV-radiation) produce DBP and bio-reactive compounds (Richardson et al. 2007). Water chlorination, the most used disinfection process, has been reported to result in the formation of a large number of chlorination by-products, such as Trihalomethanes (THM) and Haloacetic acids (HAA), which are the most abundant chlorination by-products and are considered potentially carcinogenic (Zhang et al. 2011).

The reactivity of DOM in the formation of THM content varies with different sources of organic matter and depends on the reactivity of aromatic carbons towards disinfectants, which is related on the position of functional groups, namely hydroxyl and carboxylate, this reactivity is also a function of the presence of other species, namely bromine and organic nitrogen increase the formation of THM and other DBP, not only DOM but also particulate organic matter can originate DBP, especially when its source is soil or algae (Karapinar et al. 2014, Zhao et al. 2013). DOM is also a source of N-nitrosamine precursors that are formed during disinfection, namely N-nitrosodimethylamine (NDMA) therefore it is important for water professionals to know the N-nitrosamine precursors from DOM to better control the

formation of these DPB (Wang et al. 2013), considered by USEPA (2006) as probably carcinogenic to humans (B2 group classification).

Ineffective removal of N-nitrosamines precursors during conventional water treatment and poor removal of the hydrophilic fraction (low molecular weight fraction < 1 kDa), dissolved organic nitrogen and soluble microbial by-products may lead to an increase of the N-nitrosamines concentration in the water distribution system (Wang et al. 2013).

It has been reported that the hydrophobic compounds (mainly consisting of humic acids, aromatic carbon, phenolic structures and conjugated double bonds) are the most important precursors for DBP formation in special THM and HAA, followed by the hydrophilic fraction (mainly consisting aliphatic carbon and nitrogenous compounds) (Karapinar et al. 2014; Zhao et al. 2013). Hydrophobic and charged compounds are effectively removed from drinking water by coagulation using metal salts, reducing the presence of anionic charged hydrophilic compounds that are involved in the transport of heavy metal cations (Wong et al. 2002). Treated water becomes enriched in uncharged hydrophilic compounds, which also contribute to disinfection byproducts and may have a role in bacterial regrowth in distribution systems.

High concentration of organics leads to operational problems not only by the increase itself, but also the change in NOM composition and character, which influences the treatment processes specially coagulation with traditional inorganic coagulants (Sharp et al. 2006) and may cause membrane fouling, clogging of filters and reduction of granular activated carbon adsorption capacity (Kent et al. 2014).

Although generally raw underground waters present higher quality than raw surface waters, due to soil natural filtration, the latter are available in higher quantities and more easily accessed. Therefore surface waters are commonly used as source for drinking water production. Treatments are usually focused in solids and NOM removal, and sometimes metals removal (e.g., iron and manganese), being DOM removal the most challenging task.

NOM removal in water treatment can be achieved by different processes, e.g., coagulation, membrane filtration, advanced oxidation and membrane filtration.

Drinking water regulations European Commission (1998) include parameters related with the presence of organic matter, such as colour, taste and odour, turbidity, total organic carbon (TOC) and oxidability, and also indirectly related, such as THM. Water treatment companies need to go further than the accomplishment of the parametric values. Given the negative impact of the presence of NOM in water treatment systems, the capacity to separate NOM in different fractions and to characterize of each one, is the first step to identify the compounds that must be removed and will contribute to a more efficient treatment of drinking water.

In the next sections of this chapter it will be presented a revision of the fractionation techniques and of the several methods used for characterization of NOM fractions in the last decade as well as some important findings.

2. SEPARATION METHODS

2.1. Resin Fractionation

The natural organic matter (NOM) fractionation through the use of synthetic resins has been a widely used method due to its satisfactory results and widely applicability (Lin et al.

2014; Rakruam & Wattanachira, 2014). This method was originally proposed by (Leenheer & Huffman, 1976) and has been adopted with some changes by other researchers (Chow et al. 2004; Tsuda et al. 2012). The widely used Amberlite® XAD-8 resin is no longer commercially available and was replaced by Supelite™ DAX-8 resin (Chow 2006). The tests performed with both resins indicated comparable results in general use for isolating DBP precursors from bulk aquatic humic substances (Chow 2006).

In many works the water sample is filtered through a 0.45 µm membrane in order to quantify the DOM fractions (Chow et al. 2004; Vieira et al. 2012; Wang et al. 2013). This technique allows the separation of organic matter regarding its hydrophilic and hydrophobic characteristics. For this purpose the water samples pass through the resins and the pH manipulation allows the resins to release or fix a particular fraction. After adjustment of the pH of water samples to 2 the separation of the hydrophobic and hydrophilic fractions is performed by passing through two packed columns containing respectively the resins DAX-8 and XAD-4 (Hong et al. 2013, Karapinar et al. 2014, Tubic et al. 2013, Zhao et al. 2013). The DAX-8 resin sorbs the hydrophobic fraction (HPO), which is also referred as the humic fraction (Chow et al. 2005) or very hydrophobic acids (VHA) (Chow et al. 2004). The XAD-4 resin retains the transphilic fraction (TPH) (Chow et al. 2005) also designated as slightly hydrophobic acids (SHA) (Chow et al. 2004). The hydrophilic fraction (HPI) passes through both resins (Chow et al. 2005). Additionally IRA-958 resin may be used to separate the hydrophilic charged (CHA) that are sorbed onto this resin, oppositely to the hydrophilic neutral (NEU) (Chow et al. 2004).

Another fractionation technique based on NOM polarity and XAD-8 adsorption chromatography with gradient elution has been proposed by Li et al. (2009).

Besides the XAD resins, other resins have been utilized. Hur et al. (2013) used the cationic resin AG-MP-50 to separate the hydrophilic basic (HIB) and neutral (HIN) compounds and the anionic resin AG-MP-1 to separate hydrophilic acids (HIA). More recently Kent et al. (2014) separated organic matter into six fractions concerning hydrophobicity and acidity: adjusting the pH of the water sample to 7, 10 and 2 they separated hydrophobic neutrals (HON), bases (HOB) and acids (HOA) compounds that were subsequently desorbed from DAX-8 resin, using methanol, hydrochloric acid and sodium hydroxide, respectively; the separation of the three hydrophilic fractions was done after adjustment of the samples pH to 2 and passage through the AG-MP-50 resin to separate the hydrophilic bases (HIB) fraction and Diaion WA 10 to separate hydrophilic acids (HIA) and neutrals (HIN) fractions. The main advantages of this technique is the application directly over the original water sample, avoiding pre-concentration steps, keeping fractionation consistency and comparability between samples (Chow et al. 2004), making XAD fractionation data useful for comparison of samples from similar sources.

Due to the variety of different sampling site characteristics, the results obtained are not always similar. The complexity and variability of NOM, as well as the analytical complexities and instrumentation requirements, it is difficult to draw conclusions on the nature of NOM from a single sample taken from a single site (Kent et al. 2014). Moreover they are sometimes difficult to compare because of the different classification of fractions.

Previous studies reported by Chow et al. (2005) concerning surface waters (37 sites from rivers, reservoirs and lakes) show that the hydrophobic fraction HPO was the dominant DOM in 51% of studies, when compared with TPH and HPI fractions alone, although together the non-humic fraction was dominant in 65% of cases. In table 1 it is presented a summary of the

latest reports using XAD fractionation, according to the classification made by the authors. These results are in accordance with the previous, indicating that the hydrophobic fraction is dominant in 74% of the 19 sites studied. When further fractionation of the HPO and HPI fraction in acid, basic and neutral sub-fractions, the HOA was identified as the most prevailing (Zhao et al. 2013). Though this technique has been applied very successfully, it takes several hours to process, requires large volumes of water samples, the pH variations can lead to changes in the organic matter (Chen et al. 2014) and it does not distinguish which compounds are obtained in each fraction. Since it is not possible to obtain information about the molecular weight or morphology of the compounds, for the same fraction obtained from different sampling sites, we may have different compounds (Karapinar et al. 2014, Kent et al. 2014; Wei et al. 2013). The distribution of each fraction may be affected by sample preparation, namely preservation by frizzing and filtration by hydrophobic membranes (nylon), and different operational conditions (Chow et al. 2004). Variations to level of the total amount of NOM present and the sum of all fractions have been reported (Rakruam and Wattanachira 2014).

Table 1. Resin fractionation data of DOM from different water sources

Reference	Sample	Sample Period	Fraction		
			HPO	HPI	TPH
Wong et al. (2002)	Surface water from Moorabool River	-	34% VHA	33% CHA 15% NEU	18% SHA
	Surface water from Mount Zero reservoir	-	58% VHA	11% CHA 17% NEU	14% SHA
Kennedy et al. (2005)	Ijssel Lake (Andijk, North Holland)	-	53-55%	20-22%	25%
Sharp et al. (2006)	Albert Water Treatment Works	August 2004	37% HAF 22% FAF	-	41%
Zazouli et al. (2007)	Lar, Jajrood and Karaj rivers – drinking water supply	May to July 2006	57%	43%	-
Nam et al. (2008)	Upstream of WWTP	February 2004 – August 2005	50.3%	26.1%	23.4%
Wei et al. (2008)	Intakes of drinking water treatment plants (WTP) supplied by four main source waters in China	Spring 2005	40%	25%	-
Drikas et al. (2011)	Raw water from Mount Pleasant in South Australia	November 2005	40.3% VHA VHA>SHA>NEU>CHA	-	-
Pernet-Coudrier et al. (2011)	Raw water from Marne and Seine Rivers	June 2007	62%	23%	15%
Molnar et al. (2012)	Groundwater from Central Banat (Province of Vojvodina, Serbia)	-	65% FAF 14% HAF	21%	-
Aslam et al. (2013)	River Murray, at Morgan South Australia	January–May 2011	72.52%		
Tubic et al. (2013)	Raw groundwater from the Vojvodina region (Republic of Serbia)	-	82% (68% FAF)	6% acids 12% non-acids	-
Wang et al. (2013)	Luan River in Northern China	July 2010	30%	70%	

Table 1. (Continued)

Reference	Sample	Sample Period	Fraction		
Zhao et al. (2013)	Raw water Songhua River	October 2008	47% HOA 3% HOB 17% HON	26%	7%
Kucukcongar et al. (2013)	Omerli Dam Water	-	1% HOB 33% HOA 43% HON	7.6% HIB 9% HIA 4.7% HIN	-
	Camlidere Dam Water	-	1% HOB 52% HOA 8.2% HON	6.2% HIB 14% HIA 17% HIN	-
	Surface water from sources in Eastern North America	-	22-58% HOA 1-7% HOB 1-4% HON	3-51% HIA 1-2% HIB 15-54% HIN	-
	Kent et al. (2014)				
Karapinar et al. (2014)	Terkos Lake	May 2010 to January 2011	35.2%	30.8%	43.0%
Lin et al. (2014)	Raw water from the Huangpu river in Shanghai China.	Winter, 2012	44.9%	42.6%	20.7%
	Raw water from Yangtze river in Shanghai, China		38.8%	63.4%	16.9%
Rakruam and Wattanachira (2014)	Raw surface of the Ping River (Chiang Mai Province)	July 2012	29% HOA 9% HON 4% HOB HIA>HOA>HIN>HON>HOB>HIB	38% HOA 15% HON 4% HOB	-

HPO/hydrophobics: VHA/very hydrophobic acid; HAF/hydrophobic humic fraction; FAF/hydrophobic fulvic fraction; HOA/hydrophobic acid; HOB/hydrophobic base; HON/hydrophobic neutral.

HPI/hydrophilics: CHA/charged hydrophilics; NEU/neutral hydrophilics; HIA/hydrophilic acid; HIB/hydrophilic base; HIN/hydrophilic neutral; HINA/hydrophilic non-acidic.

TPH/transfilic fraction: SHA/slightly hydrophobic acid.

The use of organic solvents (methanol and acetonitrile) for resins conditioning is expensive and high volume of water is needed for rinsing in order to avoid sample contamination (Fabris et al. 2008, Vieira et al. 2012; Wang et al. 2013). Smith and Alqabany (2009) proposed an alternative method that uses phosphoric acid and sodium hydroxide, which minimizes organic contamination. With the same purpose and aiming at optimize humic substances adsorption Tsuda et al. (2012) developed techniques for quantification of humic substances with low concentrations in clear-water samples, which involved the crush of DAX-8 resin to a fine powder and a different conditioning method using sodium hydroxide, methanol, acetonitrile, acetone, helped by a Soxhlet apparatus for extraction of the organic solvents and by a reduced pressure drying to complete their removal.

Although it is generally considered that hydrophobic NOM has higher potential for DBP formation, namely THM, there is no reproducible correlation between the fractions separated and their reactivity towards DBP formation, which might be considered the major drawback of XAD fractionation (Chow et al., 2005).

This method does not provide information on the molecular weight or morphology of compounds, which limits its utility in the selection of adequate treatment methods (Kent et al. 2014).

Nevertheless other DBP, N-nitrosamines, should also be of concern. Wang et al. (2013) used also XAD fractionation to identify the hydrophilic fraction of DOM as their major source, followed by the hydrophobic and transphilic fractions.

Moreover this method may not extract all DOM from waters, generally 50-90% (Chow et al., 2005).

Although most of the works reported that the quantification of dissolved organic carbon was used to characterize the different fractions other methods may be used, namely excitation emission matrix fluorescence spectroscopy (EEMFS). Wei et al. (2013) began by separate HOA by the traditional method, sorbing the fraction in the DAX-8 resin and subsequently instead of elute it in a continuous flow, the column was filled with the NaOH and aliquots of small volumes were taken. This method coupled with EEMFS allows the identification of different sub fractions of NOM. This technique allowed in the same way the use of XAD resins columns, which have greater flexibility and capacity in relation to a normal HPLC column. A greater versatility in the analysis of sub-fractions by various other techniques is an advantage of this new fractionation method.

2.2. Membrane Fractionation: Ultrafiltration, Nanofiltration, Reverse Osmose

Ultrafiltration (UF), nanofiltration (NF) and reverse osmose (RO) are physical processes for the separation of molecules through a semipermeable membrane by the action of a pressure differential. The difference between them lies on the different pore size of membranes (and pressures applied) and therefore the composites that each one can retain.

Regarding the resins, membranes fractionation has the advantage of not having the need of pH adjustment of the samples or chemical solvents addition (Lin et al. 2014, Ozdemir 2014).

UF usually is used to remove some organic compounds dissolved in water but it also works as a purification method in water treatment facilities. The process consists on passing the samples through membranes whose pores can vary regarding the molecular weights (MW) of the compounds: from 500 to 100,000 Da (Chow et al. 2005, Ozdemir 2014, Wyart et al. 2014). Thus it is possible to fractionate NOM taking into account the distribution of MW. UF systems for DOM characterizations are operated in dead-end mode and the flux is directed towards the membrane while in drinking water treatments they are operated in cross-flow mode and the inlet flux is directed tangentially to the membrane surface (Chow et al. 2005).

Wang et al. (2013) and Chen et al. (2014) used this fractionation methods to shown that the DOM fraction with a MW below 1 kDa tended to form nitrosamines precursors; this information is also reported by (Karapinar et al. 2014), who verified that lower molecular weight fractions are more suitable to react with chlorine and form DBPs. Fractions with low MW are often the most dominant ones (Karapinar et al. 2014, Ozdemir 2014, Wang et al. 2013).

As treatment, the biggest disadvantage of ultrafiltration lies in membrane fouling. The encrustations develop as the water flows through the membrane and affect its performance. Their formation is influenced by the type of NOM, the water flow, its physical and chemical properties and membrane properties (Chen et al. 2014, Wyart et al. 2014). To deal with this problem, periodically membrane cleaning/disinfection has to be made. Also the membrane

ageing is a disadvantage: the association of the frequent cleaning/disinfection operations with the different operating conditions lead to exhaustion of the membrane and therefore alteration of its selectivity and the loss of integrity (Regula et al., 2014).

RO and NF can be used both for NOM fractionation and for water treatment (Gorzalski and Coronell 2014), being the first method the most used for NOM fractionation (Matilainen et al. 2011).

Usually NF is used an advanced water treatment process concerning the removal of NOM and micropollutants. As in the other membrane processes, fouling is a phenomenon that reduces membrane performance, namely flux and removal efficiency, which is caused by hydrophilic high molecular weight NOM and saccharides or proteins-like substances (Park et al. 2006).

Song et al. (2009) and Green et al. (2014) reported percentages above 80% in the isolation of NOM by RO.

According to Cortes-Francisco et al. (2014), NF membranes (ESNA1LF2-4040 and NF270-4040, operating respectively at maximum pressures of 21 and 41 bar) reduce many of the amount of NOM, however, only in relation to the more hydrophilic compounds, which are the least susceptible to form disinfection by-products (Garcia-Vaquero et al. 2014) present different results, concluding that NF membranes (NF90-4040 with molecular cut off of 200 Da) are more efficient in the removal of hydrophobic compounds. These differences could depend of the type of fouling: biological, colloidal, inorganic and organic (Shen and Schafer, 2014) in the case of Garcia-Vaquero et al. (2014) the analyzed waters were rich in hydrophilic compounds and as reported by the author these compounds are more susceptible to form biofouling and may interfere with the selectivity of the membrane.

Membrane isolated fractions do not correspond to an exact MW but to a range, which depend also from the operational conditions, shape of the chemical species, presence of solutes, pH, ionic strength, membrane material and configuration. Humic substances are flexible linear colloids at low concentrations that become spherocolloids at high concentrations, very low pH or appreciable concentration of neutral electrolytes, making their removal variable upon conditions (Chow et al. 2005; Matilainen et al. 2011).

Although the different fractionation the mechanisms used for resin and membrane separation, molecular sieving in the last case, in both cases the fraction separated cannot be directly correlated with reactivity of DOM to form DBP (Chow et al. 2005). Besides THM and HAA, like other DBP, N-nitrosamines, should also be of concern.

2.3. Size Exclusion Chromatography (SEC/HPSEC)

Size exclusion chromatography (SEC) is a physical method for NOM fractionation, like membrane filtration, both based on different molecular sizes rather than in molecular weight and dependent on operating conditions. They do not provide information regarding the nature of the compounds (Kent et al. 2014).

In SEC a porous gel is used for separation. The large species are excluded from micropores and surface functional groups, due to steric interactions, and are eluted by the stationary phase presenting shorter retention times or smaller elution volumes than small species, which are delayed by their penetration in micropores; the gel pores act as a

chromatographic medium thus separating species (from 500 to 1,000 Da) based on hydrodynamic size or effective diameter (Chow et al. 2005).

The characterization of the molecular weight of NOM is based in the comparison with known molecular mass standards, mainly Poly-styrene sulphonate, assuming a hypothetical structure of NOM that may be different (branches, cross-links), therefore producing approximate results of molecular weight (Matilainen et al. 2011).

SEC has been used since the early 1960s and later the techniques developed have been applied to high performance liquid chromatography and originated high performance size exclusion chromatography (HPSEC) (Matilainen et al. 2011). Both SEC and HPSEC fractionation are dependent on operating conditions due to interactions with NOM, namely the eluent (pH and ionic strength), the gel (surface charge characteristics) and the type of NOM (charge and structure). The eluents used may be phosphate buffer solution with ionic strength adjustment using sodium chloride or sodium acetate solution; the columns are silica-based or polymer-based supports; the detectors used in HPSEC include Fourier Transform Infrared (FTIR), on-line dissolved organic carbon (DOC) analysers, excitation emission fluorescence detection, diode array, high resolution mass spectrometry (HRMS) and single and variable wavelength ultraviolet-visible (UV-vis), being the two last the most used (Matilainen et al. 2011). On-line detectors are very useful. The use of the different detectors and their combination will be discussed in section 3 of this chapter.

HPSEC uses a porous gel with a controlled pore size distribution to separate molecules. It has been compared with flow field-flow fractionation (FIFFF), which uses hydrodynamic and molecular diffusion principles to separate molecules on the basis of molecular size. HPSEC was considered a reliable molecular weight distribution characterization technique although solute-gel interactions were identified with the HPSEC system (Pelekani et al. 1999). In this method there is a higher surface area where adsorptive or electrostatic interactions can take place than in FIFFF and therefore the large molecules elute first in HPSEC, while the smaller molecules elute first in FIFFF, limiting its application to compounds with molecular weights greater than 300-400 (Pelekani et al. 1999).

Another use of HPSEC was to investigate the influence of the presence of NOM in copper release due to corrosion of household plumbing. Gao and Korshin (2013) concluded that NOM promotes copper release from corroding surfaces but NOM changes induced by chlorination or ozonation gradually reduce/eliminate these effects by reducing copper mobilization. The nature of NOM changed by chlorination and ozonation was examined using differential absorbance spectroscopy (DAS) and HPSEC. Based in strong linear correlations between specific ultraviolet absorption at 254 nm (SUVA₂₅₄) values and metal release, it was verified that both chlorination and ozonation decrease NOM apparent molecular weight, aromaticity and the abundance of carboxylic and phenolic groups, which sorb preferentially on the copper surface important for metal release, NOM complexation and colloidal dispersion.

Although SEC profiles have been used to control the quality of NOM along the different steps of the water treatment the information obtained is not enough concerning the potential formation of DBP by the remaining DOM molecules (Matilainen et al. 2011).

HPSEC is considered a simple and relatively fast technique that combined with characterization methods focused in structural information, such as ¹³C NMR, can provide useful information on the physicochemical character of NOM (Pelekani et al. 1999).

HPSEC seems to be an effective tool both for the identification of leaching of organic matter into wells, when coupled with UV detection at 254 nm, and for determination of anthropogenic influences on a well, when coupled with UV detection at 224 nm (Szabo and Tuhkanen, 2010).

2.4. Reverse Phase Liquid Chromatography

Liquid chromatography (LC) has been used for the separation and characterization of NOM in several variants.

The characterization may be made by hydrophobicity, separating hydrophobic, transphilic and hydrophilic (Kennedy et al. 2005).

High performance size exclusion chromatography (HPSEC) has been widely used to determine the molecular size/weight distribution of humic substances in aquatic environments (Bagtho et al. 2011b). If NOM is separated according to molecular size/weight, up to five fractions were achieved: (i) biopolymers comprising polysaccharides, proteins and colloids, (ii) humic substances, (iii) building blocks (hydrolysates of humics), (iv) low molecular weight humics and acids, and (v) low molecular weight neutrals (such as alcohols, aldehydes, ketones and amino acids) (Bagtho et al. 2011a).

Reverse phase high performance liquid chromatographic (RPHPLC) methods have also been trailed as a rapid method to distinguish hydrophilic and hydrophobic fractions of NOM (Matilainen et al. 2011, Xing et al. 2012).

A RPHPLC method was developed to separate NOM based on relative polarity. It is a useful tool for the optimization of water treatment process efficiencies (Duan et al. 2013, Xing et al. 2012) as well as the formation of DBP, namely haloacetic acids evaluation.

Xing et al. (2012) applied RPHPLC method as a rapid characterization technique to establish relationships between coagulation treatability (particularly DOC removal) and hydrophobic/ hydrophilic ratio. The characterization results were further compared to those of the traditional resin fractionation technique using DAX-8 and XAD-4 resins together with HPSEC (Xing et al. 2012). A chromatogram subtraction approach was conducted to determine the character (molecular weight profile – HPSEC and polarity – RHPLC) of the hydrophobic proportion and the effluent samples were used to determine the character of the hydrophilic proportion. Although RPHPLC and XAD fractionation are different methods, both confirm that the hydrophilic fraction was poorly removed by coagulation and has low molecular weight (Xing et al. 2012).

The RPHPLC results of water samples before and after coagulation showed that, the percentage peak area of each one is presented to provide quantitative characterization information of the organics. The relatively higher percentage peak area of RPHPLC-hydrophobic peaks after treatment indicates that better removal of the RPHPLC-hydrophilic fraction can be achieved by coagulation (Xing et al. 2012).

The characterisation of NOM with polarity rapid assessment method (PRAM) developed by Rosario-Ortiz et al. (2007) is based on preferential adsorption of dissolved organic matter fractions onto solid-phase extraction (SPE) sorbents. The use of different SPE cartridges in parallel allows a multidimensional characterization of the polarity of NOM. Three different types of SPE sorbents were used. The C18 cartridge was used to obtain the hydrophobic NOM fraction; the CN SPE cartridge was used to generate the hydrophilic NOM fraction

whilst the NH₂ SPE cartridge was used to produce the transphilic NOM fraction. Sufficient amounts of 0.1 M NaOH were used to elute the HPO and HPI fractions retained by the C18 and CN SPE cartridges, respectively, whilst the TPI fraction was collected as the fraction that passes through the NH₂ SPE cartridge (Nkambule et al. 2012).

PRAM takes advantage of different SPE cartridges to remove a fraction of the organic matter present in the water at actual pH and ionic strength, according to its polarity and charge. Chen et al. (2014) used a combination of PRAM and ultrafiltration to locate the fractions with the strongest contributions to the nitrosamine precursor pool in the effluent organic matter (Chen et al. 2014).

3. CHARACTERIZATION METHODS

3.1. Total Organic Carbon /Dissolved Organic Carbon

Total organic carbon (TOC) includes particulate and dissolved organic carbon (DOC). Inorganic carbon is mainly in the form of carbonates and bicarbonates and may be removed by acidification.

Nowadays TOC-analysers are available and easy to operate, proving fast results and with almost no interferences. In most equipments the carbon dioxide produced after oxidation of the organic matter is measured by infrared spectroscopy.

For DOC analysis of highly saline water samples, ultraviolet absorbance is a good alternative because it provides high precision at low DOC levels and it showed a strong linear correlation between with absorbance at 254 nm (Joseph et al., 2012).

TOC and DOC are the most used parameters to control treatment processes. This parameter has been used in many studies related with treatability of waters (Farre et al. 2013, Joseph et al. 2012, Molnar et al. 2012, Ulu et al. 2014, Wang et al. 2013) and helped to draw some interesting conclusions that are presented below.

Combined coagulation–adsorption treatment using carbon nanomaterials can be effective in removing NOM from various water sources (Joseph et al., 2012).

The degree of HAA precursor removal depends on the dominant oxidation mechanism, which is related to the applied ozone dose and the pH of the oxidation process. Ozonation at pH 10 favours the mechanism of radical NOM oxidation and was the most effective for reduction of HAA formation, which improves with increasing ozone dose (Molnar et al. 2012).

All molecular weight fractions of organic carbon contributed to the DBP formation potential, with the humic rich fractions forming the greatest amount of DBPs, while the low molecular weight fractions forming more brominated DBPs specially when the high bromide to organic carbon ratio is observed (Farre et al. 2013).

The removal of humic compounds by electrocoagulation process was demonstrated through a sufficient reduction of DOC content, absorbance values at 254 and 436 nm, this last for color removal evaluation. The data from absorbance at 254 nm was consistent with the DOC results by Ulu et al. (2014).

3.2. Ultraviolet, and Visible Spectrophotometry

The concentration of a single species or family may be easily evaluated by absorption measurements at a certain wavelength based in the Lambert-Beer's Law, or in certain cases, over a spectral range. In case of NOM, its ultraviolet (UV) absorbance spectra decrease monotonically with increasing wavelength (Hur et al. 2006).

Wavelengths in the range 220 to 280 nm have been considered the most adequate for NOM measurements, although they are dependent of different chromophores present in NOM structure. The carboxylic and aromatic chromophores are detected at 220 nm; aromatic groups with varying degrees of activation are detected at 254 nm (Korshin et al. 2009), being often used as a potential measure of DOC (Lobanga et al. 2013, Matilainen et al. 2011).

Lobanga et al. (2013) assessed NOM removal by adsorption onto granular activated carbon by measuring the ultraviolet (UV) absorbance at 3 wavelengths, namely, 254 nm (UV254), 272 nm (UV272) and 300 nm (UV300) and obtained consistent results.

Besides measurements at wavelengths of 254, 350 and 440 nm measurements are also linearly correlated with DOC concentration, providing a mechanism for its rapid and inexpensive optical determination, in laboratory or in situ. However, this may fail in systems with strong autochthonous, anthropogenic or photodegraded DOC (Spencer et al., 2012).

The ratios between absorbances at different wavelengths have been considered useful in NOM characterization, namely 254/204 nm, 254/436 nm, 250/365 nm and 253/203 nm (Hur et al. 2006, Spencer et al. 2007), being this last correlated with DBP formation (Kim and Yu 2007) and one of the most used (Minor et al. 2014). However, in the range 200 to 230 nm the presence of inorganic ions (e.g., nitrates and sulfates) may interfere (Her et al. 2008). Another possibility is the ratio 465/665 (Minor et al. 2014), which has been considered correlated with O:C atomic ratio, carboxyl content, and total acidity, however it may show problems due to low absorption above background levels at 665 nm (Helms et al. 2008).

Specific ultraviolet absorption at 254 nm (SUVA₂₅₄) defined as UV absorption at 254 nm divided by the DOC concentration (in mg/L) is often used as indicator of NOM hydrophobicity and aromaticity (Weishaar et al. 2003), when the values are higher than 4 and suggest a hydrophilic character for values lower than 3 (Edzwald and Tobiason, 1999). This parameter is well correlated with the ratio of absorbances 254/204 nm (Hur et al., 2006) but it may not predict the reactivity of NOM towards DBP formation because simple spectroscopy may not be able to detect small differences (location of functional groups, as hydroxyl and carboxylate) in similar structures but it is still the most used surrogate for DOM and DBP precursors (Chow et al. 2005; Wang et al. 2013).

The NOM fraction with average molecular weight less than 1 kDa, consisting of hydrophilic compounds, was considered the major THM precursor although this fraction had the lowest SUVA values (< 2 L/mg m) (Ozdemir 2014).

SUVA₂₅₄ has been used to study the formation of DBP after disinfection (by chlorine and bromine) of 13 source waters from China. The different SUVA₂₅₄ values measured, prior to disinfection between 0.9 and 5.0 L/(mg·m), suggested that the organic compounds from different sources exhibited different reactivities with chlorine. After disinfection four THM and seven HAA were evaluated, ranging respectively from 29 to 259 µg/L and from 20 to 448 µg/L, suggesting higher risk of HAA formation than THM. Chloroform and bromodichloromethane were the major constituents of THM. Trichloroacetic acid and dichloroacetic acid were the most abundant HAA species. When disinfection is performed

with Br, the brominated THM species presented higher concentrations than brominated HAA species, being of special concern when the bromide concentration is higher than 100 $\mu\text{g/L}$ (Zhang et al. 2011).

SUVA measurements in individual NOM fractions may enhance the NOM characterization provided through resin fractionation and HPSEC, although in the first it has been observed some variability in the absorbance of the compounds separated in the HIN fraction (Kent et al. 2014).

An alternative approach, the differential absorbance at 254 nm, which is the change in absorbance after a given action (e.g., disinfection) was also tested, but has shown no correlation with THM and HAA formation (Ates et al. 2007).

In order to overcome these limitations differential absorbance at 272 nm was proposed. It represents the extension of destruction of aromatic rings and DBP formation and has shown strong correlation with THM and HAA formation (Roccaro and Vagliasindi 2009).

3.3. Fluorescence

Fluorescence spectroscopy is based on excitation of molecules at a specific wavelength, by energy absorption, and then re-emitted energy at a different wavelength. The amount and wavelength of the emitted energy depend on both the fluorophore (component that causes a molecule to absorb energy) and the chemical environment of the fluorophore.

This characterization has typically involved the use of excitation-emission wavelength pairs to identify fluorophores based on the location of peaks on F-EEM contour plots (Coble 1996). These peaks have been used to distinguish between humic like NOM, with longer emission wavelengths (> 350 nm), and protein-like NOM, with shorter emission wavelengths (< 350 nm). The major fluorescence peaks for natural water samples are referred in a recent work by Sillanpää et al. (2015).

This method has received increased attention due to its potential application as a monitoring technique in the water industry, namely in NOM characterization (Bierozza et al. 2009, Matilainen et al. 2011, Peiris et al. 2010b). The fluorescence emission of NOM is governed primarily by the identity and concentration of aromatic functional groups in NOM molecules, but nitrogenous groups might have a significant contribution as well.

Since fluorescence presents better sensitivity and selectivity compared to traditional methods (Bierozza et al. 2009, Peiris et al. 2010a) and is a function of structure and functional groups in molecules, it can be used to give information about the chemical characteristics of NOM (Baghoth et al. 2011a, Peiris et al. 2010a). This technique also has other advantages, is a simple, relatively inexpensive and very sensitive tool that requires little or no sample pre-treatment (Baghoth et al. 2011a). Fluorescence spectroscopy has also been used as a technique for classifying and distinguishing between humic substances of various origins and natures (Chen et al. 2002).

Three-dimensional fluorescence excitation–emission matrix (EEM) spectroscopy provides a total intensity profile of the sample over the range of excitation and emission wavelengths scanned (Markechova et al. 2013). Therefore, provides more detailed information in comparison to the traditional single-scan techniques, allowing to distinguish different types and sources of DOC in natural waters (Baghoth et al. 2011a).

Peiris et al. (2010a) used EEM approach for characterization of the major membrane foulants (HS-like, protein-like and colloidal/particulate matter) present in natural water and during membrane cross-flow ultrafiltration operation of river water, using a multivariate data analysis (PCA- methods principal component analysis). These researchers concluded that the proposed method has proved to be suitable for identifying the major foulant components and their contribution to reversible and irreversible membrane fouling, illustrating its potential for monitoring and controlling membrane fouling in drinking water treatment applications.

Carstea (2012) had evaluated some potential methods of the fluorescence spectroscopy, to characterize different water systems and different sources of water pollution. These methods are very useful in establishing the natural characteristics of the water body, the quantity and quality of DOM and the relative proportions of terrestrially and microbially derived components.

Fluorescence analysis was used to characterize the different NOM in groundwater contaminated with ^{60}Co and ^{137}Cs (Caron et al. 2014). The fluorescence signals are reported as humic-like, fulvic-like and protein-like. This analysis, on the other hand, indicated a contrasting behavior between the clean and contaminated sites, with a dominance of protein-like material, a feature usually associated with human impacts.

Markechova et al. (2013) describe some recent applications of fluorescence EEM combined with statistical methods using parallel factor analysis (PARAFAC) in drinking water industry and believe that it is an established tool of organic matter fingerprinting. It could provide valuable information that can be used to determine contaminants in drinking water to characterize organic matter and its removal in drinking water treatment. They conclude that the main advantages of fluorescence spectroscopy are rapidity, simplicity, sensitivity, and selectivity. However, the disadvantage is its dependence on environmental factors (temperature, pH, ionic strength, etc.).

The study focused on current approaches to the chemical structural characterization of DOM, also relates that EEM with PARAFAC is applicable to bulk samples and in situ analyses (Minor et al., 2014).

Other methods include: fluorescence regional integration (FRI) (Chen et al. 2003); multivariate data analysis (e.g., principal component analysis, PCA, and partial least squares regression, PLS) (Persson & Wedborg, 2001); and multi-way data analysis using parallel factor analysis (PARAFAC) (Stedmon et al. 2003). PARAFAC has been used to decompose F-EEMs into individual components some of which have been attributed to protein-like or humic-like) (Baghoth et al. 2011b).

3.4. FTIR/FTIR-MS

Fourier transform infrared (FTIR) spectroscopy is a powerful analytical tool for characterizing and identifying organic molecules. The wavelengths at which an organic molecule absorbs infrared (IR) radiation give information about chemical bonds and molecular structure of organic compounds. FTIR therefore provides an overview of transiently polar covalent bonds within a sample, including organic functional groups, the C = O bonds in carbonate species, and the Si–O bonds in silicates (Minor et al., 2014).

FTIR has been used to characterize NOM, in which the resulting absorption spectrum is a unique fingerprint of compounds, allowing the identification of both inorganic and organic

functional groups (Matilainen et al. 2011). However, as mentioned by Sillanpää et al. (2015) the interpretation can be complicated, because of severe overlapping of characteristic spectral features due to the complexity and polyfunctionality of NOM. They elaborated a list of the main characteristics of NOM for the interpretation of spectra.

FTIR analysis is useful for monitoring the inorganic composition of NOM isolates, since it can detect the presence of bicarbonate, carbonate, nitrate, phosphate, silicate and sulfate (but not inorganic halides). By the same motive (due to the interference of chloride salts) if the sample has not been efficiently desalted, the signal from the organic constituents cannot be discerned (Sillanpää et al. 2015).

Samples can also be analyzed by attenuated total reflectance (ATR), a sampling technique used in conjunction with infrared spectroscopy, where the sample is pressed against a crystal of higher refractive index. IR light penetrates through the crystal and into the sample, creating an evanescent wave in the sample if the sample interacts with this wave, less light will end up being reflected back to the detector (Minor et al. 2014). ATR generally allows qualitative or quantitative analysis of samples with little or no sample preparation, easy-to-use, fast, and non-destructive. It is used for analysis of solids, liquids, semi-solids and thin films (Swathi et al. 2014).

Recently, electrospray ionization (ESI) coupled to Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS), has emerged as a very promising technique in the characterization of DOM from different water environments (Zhang et al. 2012) and has recently been developed for NOM characterization. More than 4000 NOM components could be resolved in one sample using this technique (Sillanpää et al. 2015).

The study that employed ESI FT-ICR MS to characterize the low molecular weight DOM along conventional drinking water treatment plant (Zhang et al. 2012), showed that this tool provides the power to examine individual molecules within DOM and enables the study of the behavior of individual molecules of DOM during drinking water treatment processes.

3.5. Detectors used in Liquid Chromatography

Several detectors may be coupled to liquid chromatography systems achieving different and also complementary information.

Liquid Chromatography-Organic Carbon Detection (LC-OCD) system in which NOM is oxidized to CO₂ before it is measured by infrared detection can be employed to examine the NOM fractions in more detail (Kennedy et al. 2005).

Besides the organic carbon detector, it is possible to incorporate an UV detector, which may be used to assess the aromaticity, as well as the humic fraction, by computing the respective SUVA values, and a dissolved organic nitrogen (DON) detector (Baghoth et al. 2011a).

A LC system coupled with organic carbon and organic nitrogen detectors (LC-OCD-DON) may be used to analyse NOM and quantify: biopolymers (such as polysaccharides, polypeptides, proteins and amino sugars); humic substances (fulvic and humic acids); building blocks (hydrolysates of humic substances); low molecular weight humic substances and acids; and low molecular weight neutrals (such as alcohols, aldehydes, ketones and amino acids) (Baghoth et al. 2011b).

Baghoth et al. (2011a) showed that there were significant correlations ($p < 0.01$) between sample DOC concentration, UVA₂₅₄, and maximum fluorescence intensities for the seven PARAFAC components and DOC concentrations of the five LC-OCD fractions (Baghoth et al., 2011a).

An electrospray ionization (ESI) MS is available for fast characterization of NOM matrices. During ESI-MS and pyrolysis GC/MS analyses, the NOM compounds are likely converted into multiply charged molecules or decomposed to fragments, respectively. Therefore, analysis on mass spectrometry of environmental samples is more focused on low molecular ions (Matilainen et al. 2011). On the other hand, matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) is favorable for the analysis of NOM compounds, especially for macromolecules, due to its unique advantages: (i) it is a relatively soft ionization technique to avoid significant fragmentation of large molecules; (ii) it facilitates interpretation of the spectral data since only singly charged ions are generated (Wang et al. 2014).

The aromatic molecular structures in hydrophobic NOM are more likely to absorb the laser energy and ionize in LDI-MS analysis. Therefore, humic and fulvic acids could be readily ionized under mild laser power, transferring the energy within analytes through proton donation. The charged molecules induce repulsive effects and desorption thereafter. So, humic and fulvic acids in NOM could perform as matrices for themselves in LDI-MS (Wang et al. 2014).

The time-of-flight matrix assisted laser desorption/ionization mass spectrometry (LDI-TOF-MS) technique was successfully introduced to the analysis of aquatic NOM. The analysis variables such as solution concentration, additive and laser power were properly adjusted using the HPO NOM from Suwannee River as a model sample. Aquatic-phase NOMs were better analyzed in positive mode by preparing sample solution in acid solvents. Dilute solutions were favorable in detecting small molecules, while large molecules exhibited stronger mass peaks with sample spots prepared with concentrated solutions. The mild laser power of 27.8 μJ per shot was effective in characterizing molecules below 5000 Da in the samples, but higher laser power of 39.0 μJ per shot should be applied for ionization of large molecules up to 15 000 Da. In the comparative study of HPO NOM from terrestrial origins and domestic waste water, it was found that the compounds in waste water NOM contrasted from those of nature waters. Meanwhile, common mass peaks were also observed in all the NOM samples. LDI-MS was efficient in measuring the molecular size distributions of macromolecules and/or aggregates formed within humic substances.

Mass peaks beyond 2000 Da were found in rivers, but in the reservoir water and treated waste water only were found smaller molecules. The river waters were more likely to form membrane foulant, while the waste water and reservoir that had HPO NOM are unable to form large aggregates (Wang et al. 2014).

3.6. NMR

Nuclear magnetic resonance spectroscopy (NMR) is a technique used to determine a compound's unique structure, which has been used to study the functional groups in NOM. The NMR phenomenon is based on the fact that nuclei of atoms have magnetic properties. This spectroscopic method it is the only one for which a complete analysis and interpretation

of the entire spectrum is normally expected. NMR is an extremely powerful tool for the structural characterization of organic molecules, such as components of humic substances and contaminant transformation products (Cardoza et al. 2004). The heterogeneity and complexity of NOM limit the practical value of the NMR data (Sillanpää et al. 2015). Despite its lower sensitivity, NMR has nonetheless proved its utility in investigating environmental interactions between NOM and contaminants (Cardoza et al. 2004).

Different NMR techniques, ^1H and ^{13}C and ^{15}N NMR, have been used in NOM research. The ^{13}C NMR technique, can be performed on solid or liquid sample and is useful for determining carboxylic structures (as aliphatic, alkoxy, aromatic and carbonyl carbon). The ^{13}C NMR is less sensitive to carbon than ^1H NMR to hydrogen (Sillanpää et al. 2015).

The general assignments for structural features of NOM, obtained by solid-state, are referred by Matilainen et al. (2011). The identification of carbons bonded to nitrogen can be identified by ^{15}N NMR techniques data (Sillanpää et al. 2015). ^1H NMR provides basic structural information on non-exchangeable structural proton distributions in NOM and can support elemental data interpretation in the quantification of aromaticity and degree of humification (Matilainen et al. 2011). Although, the interpretation of spectrum can be limited, it presents high sensitivity and is a powerful tool especially together with ^{13}C NMR in structural analysis (Sillanpää et al. 2015). Structural features of NOM obtained this way are also listed by Matilainen et al. (2011).

A combination of spectroscopic techniques, including ultraviolet–visible, ^{13}C -nuclear magnetic resonance, Fourier-transform infrared, fluorescence, and electron paramagnetic resonance, were employed by Chen et al. (2002) for characterization of the structural and functional properties of NOM.

3.7. Py-GCMS/TMAH-GCMS

Tetramethylammonium hydroxide (TMAH) thermochemolysis gas chromatography-mass spectrometry (TMAH GC-MS), has been used to investigate the structural composition of organic matter such as humic substances, lignin (Chefetz et al. 2000), and NOM (Wetzel et al., 1995). TMAH GC-MS is a one step reaction that is principally a degradative technique and secondarily a derivatization technique. TMAH labile C-O bonds such as esters, amide bonds, some ether bonds (β -O-4 bonds in lignin) and some extent glycosidic bonds are cleaved by the TMAH thermochemolysis reaction and result in fragments. This degradation occurs mainly through a base catalysed hydrolysis reaction at elevated temperatures. Simultaneously, functional groups containing acidic protons, such as carboxylic acids and phenols, are methylated whereas esters are converted to the corresponding methyl esters (Fillely et al. 1999). The resulting products are volatile enough to be separated by GC and analyzed by MS (Frazier et al. 2003).

Pyrolysis GC-MS has also been useful for structural studies of NOM (Bruchet et al. 1990). Substantial amounts of CO and CO₂ are produced during pyrolysis. These may be retained with the TMAH GC-MS technique since sub-pyrolysis temperatures are used (250°C) and since methylation reduces the polarity and the tendency to undergo thermal transformations. Because sub-pyrolysis temperatures can be used, this degradative technique has been referred to as a thermally assisted chemolysis or thermochemolysis (McKinney et al.

1995). These aspects of the TMAH thermochemolysis GC-MS procedure may be advantageous when studying the structural composition of NOM (Frazier et al. 2003).

A well-studied degraded lignin sample was analyzed using the TMAH thermochemolysis GC-MS procedure to substantiate the quantitative recovery of TMAH thermochemolysis products. In addition, four freshwater NOM samples were also analyzed to demonstrate that this technique is useful for structural investigations of NOM. The TMAH procedure has been optimized herein to represent a greater fraction of the NOM with quantitative determination of product yields (Frazier et al. 2003).

Previous studies (Wetzel et al. 1995) indicated that the TMAH treatment of NOM produces a suite of products that include fatty acid methyl esters (FAME) and lignin-derived aromatic products, aromatic products from other sources (Frazier et al. 2003).

It looks that the following compounds are TMAH products that are less specific in origin with regard to particular biomolecules and appear ubiquitously as TMAH products from NOM: 3-Methoxy pentane; Butanoic, butyric, and Pentanoic acid methyl esters; Benzyl methyl ether; N,N-dimethyl benzamine; Benzoic acid, Methyl ester; peak 24; Dimethoxy benzenes; Trimethoxy benzenes; 4,6-Dimethyl-3,5-dioxo-2,3,4,5-tetrahydro triazine; 1,3,5-Trimethyl-1,3,5-triazine-2,4,6-trione; and Caffeine. However, there is a benefit in including these compounds in comparisons of NOM since the relative peak areas for these compounds vary between NOM samples and can be useful for ‘fingerprinting’ (Frazier et al. 2003).

FAMES are the most predominant TMAH products observed in the chromatograms of the NOM samples as identified by the mass spectral fragmentation pattern with authentic standards.

FAMES have both microbial and plant origins and range in size from the Octanoic acid, Methyl ester (8:0 FAME) through Tetratricontanoic acid, Methyl ester, where Hexadecanoic acid, Methyl ester (16:0 FAME) and Octadecanoic acid, Methyl ester (18:0 FAME) are often the most prominent peaks.

TMAH thermochemolysis GC-MS is demonstrated to be effective for investigating the lignin, carbohydrate, and lipid signature of aquatic NOM. Furthermore, the quantitative assessment of product yields for this procedure adds a new dimension to the comparison of NOM samples within and between laboratories. If the sources and yield of TMAH products of unknown origin can be determined, then they could be combined with products of understood molecular origin to provide bulk compositional characteristics of NOM. The comparison of data gathered by various analytical techniques such as TMAH GC-MS, ^{13}C and ^1H NMR, and more sophisticated mass spectroscopy techniques could provide a more accurate and comprehensive picture of NOM chemistry. This advance in the structural understanding of NOM chemistry can expand efforts in molecular modeling of NOM and modeling of environmental processes in which NOM composition is involved (Frazier et al. 2003).

3.8. Biological Methods for Biodegradable OM Quantification

Dissolved organic carbon can be divided into nonbiodegradable and biodegradable dissolved organic carbon (BDOC) according to microorganisms utilization efficiency. The BDOC component that can be used by bacteria to generate a cell body is known as assimilable organic carbon (AOC). AOC and BDOC are closely related to the growth of

heterotrophic bacteria in the drinking water distribution system, which is the focus of the biological stability analysis of drinking water. AOC is an organic matter component that can be easily assimilated by bacterial organisms; BDOC is the substance and energy source used by bacteria and other microorganisms in the water for metabolic processes, such as assimilation and alienated consumption (Wang et al. 2015).

Several biological tests have been developed to assess the level of biodegradable organic matter in water. These bioassays are generally based on two concepts: (i) the AOC that measures the growth of bacterial inoculum in response to the amount of nutrients in the water and (ii) the BDOC that measures the fraction of DOC assimilated and mineralised by heterotrophic microbes (Matilainen et al. 2011).

The AOC is believed to be mostly composed of low molecular weight compounds, which are the most difficult to remove. They are also readily degradable, thus indicating the potential for bacterial growth in the distribution system (Matilainen et al. 2011).

BDOC is the fraction of the DOC which can be metabolized and assimilated by heterotrophic microflora. The determination of the biodegradability of DOC consists of the measuring the DOC consumption by microorganisms (Servais et al. 1987). BDOC concentration is determined as the difference between the initial DOC concentration and the minimum DOC concentration observed during the incubation period (Trulleyova and Rulik, 2004).

A number of methods have been developed to measure the biodegradability of aquatic organic matter. One approach to BDOC measurements involves the use of plug-flow biofilm reactors. These reactors are composed of glass columns filled with glass beads that support an active biofilm. The sample flows through a bioreactor and BDOC is determined as a difference between DOC concentration in the initial solution and the solution that has passed through the bioreactor (Kaplan and Newbold 1995). Once the reactor is stabilized, the time required for analysis is dramatically shortened compared with batch methods. However, bioreactors require a long time (several months) for colonization, large amounts of the test solution and expensive experimental equipment (Yano et al. 1998).

Another method for BDOC measurement involves sterile filtration of the sample, reinoculation with a natural assemblage of bacteria and measurement of the decrease of DOC concentration. In these methods, suspended bacteria with the same origin as the sample are used to inoculate the water sample (Servais et al. 1987). In another procedure, bacteria fixed onto inorganic particles are used as the inoculum (Korth et al. 2002). The incubation times range between 20 and 35 days. Several studies have dealt with a comparison of suspended bacteria and attached bacteria to assess their effect on the final BDOC result or to assess the effect of operational conditions (Block et al. 1992, Volk et al. 1994). The major drawback of batch experiments is the rather long incubation time. However, long-term incubations with frequent subsampling can provide a picture of the time course of DOC decomposition (Trulleyova and Rulik 2004).

Different types of bacterial inocula for batch methods were compared and the results indicate that the most suitable method for BDOC determination in samples of both low and high BDOC concentration is inoculation with bacteria attached to sediment. Metabolically active bacteria attached to natural sediment particles or to artificial substrata can metabolize and assimilate aliphatic saturated compounds more quickly than free (suspended bacteria) and in addition, they are able to utilize high-molecular-weight unsaturated compounds. These compounds are not biologically accessible for other inocula although the abiotic adsorption of

DOC molecules onto inorganic surfaces and biofilm surfaces also contributes to this result. The stream bed is the primary site of DOC processing in small streams, so it is supposed that the biodegradability of DOC should be predominantly determined by means of a batch method using biofilm bacteria attached to sediment particles or another inert support. As bacterial biofilms, which colonize drinking water distribution and storage systems, are well adapted to the quality and quantity of DOC in drinking water the BDOC determination in drinking waters should use as inoculum autochthonous biofilm on artificial supports previously exposed in distribution or storage systems (Trulleyova and Rulik 2004).

The original batch BDOC method (Servais et al. 1987) uses a highly acclimated inoculum originated from the same environment as the water sample (indigenous inoculum). To satisfy the requirement, the water sample is filtered and inoculated with the sample itself. This procedure has several drawbacks. Most water samples do not contain sufficient initial number of microorganisms and therefore, an incubation period of 28 days is required. Furthermore, the inoculum may not provide maximum possible BDOC exertion due to limited microbial diversity. BDOC results of different samples cannot be compared since they are based on different inocula.

Compared with the biochemical oxygen demand (BOD) method, the BDOC procedure is relatively new but is quite similar in principle. Both procedures rely on the use of mixed cultures as an inoculum; the only key difference is that instead of dissolved oxygen, DOC is measured before and after incubation in the BDOC procedure. BDOC is considered more accurate, precise, and sensitive than BOD (Khan et al., 1998). Due to its limited sensitivity, BOD is not applicable to water with low organic concentrations such as drinking water and unpolluted natural water. BDOC has a wider range of applications from tap water to wastewater (Khan et al. 2005).

Due to the similarities of the BOD and BDOC tests, it may be possible to use commercial BOD seeds for BDOC determination or even standard inocula. Commercial BOD seeds contain a collection of micro flora that are capable of using a variety of substrates and are commonly found in water environment. For example, a commercial one consists of *Pseudomonas*, *Nocardia*, *Streptomyces*, *Bacillus*, and *Micromonospora* genera (Fitzmaurice and Gray, 1989) while another two contain *Pseudomonas*, *Bacillus*, and *Enterobacter*, and a yeast strain (Paixao et al. 2000). The possibility of using commercial BOD inocula as seeds in the BDOC procedure was evaluated, for three types of samples, and the BDOC exertion trends of the three commercial seeds were quite identical (Khan et al. 2005).

In another study a mixed liquor suspended solids seed provided faster exertion kinetics than other four seeds evaluated, which had comparable first-order rate constants. All five seeds were not much different in term of the accuracy and precision. Similarities among the commercial seeds suggest that they probably share common microbial composition and are equivalently effective, being potential alternatives as BDOC inocula. Nevertheless more research, especially on different types of water and wastewater samples, should be conducted before it can be ensured that in general the commercial seeds are acceptable as replacements for the indigenous sample seed. Otherwise, the equivalency of the two seed types has to be verified on a case-by-case basis (Khan et al. 2005).

BDOC measurements were applied in several studies and were helpful in order to obtain some interesting conclusions. Preozonation increases the biodegradability of NOM and BDOC increases with increasing ozone dosage (Rodriguez et al. 2011). Activated carbon adsorption of the commercial humic acids (hydrophobic and aromatic high molecular weight

compounds) is improved after pre-ozonation at the range of doses typical in drinking water treatment. However, hydrophilic and low molecular weight compounds were not significantly affected by pre-ozonation (Rodriguez et al. 2011). Due to the complex nature of NOM, and especially humic substances, these results cannot be generalized but they are useful in drinking water treatment when intermediate ozonation is used (ozone applied prior the filtration stage) for the evaluation of biological filters, in which adsorption and biodegradation processes occur together (Rodriguez et al. 2011).

3.9. Combination of Methods

Several combinations of methods have been tested in order to achieve a better quantitative and qualitative characterization of NOM. A combination of fractionating techniques and analytical methods increase the sensitivity and improve the reliability namely HPSEC with multiple on-line detectors, UV, Fluorescence and quantitative DOM measurement (Allpike et al. 2005, Chow et al. 2005).

The couple of multiple detectors, UV detector followed by a TOC module and a MS detector, with SEC seems a promising system for NOM characterization (Matilainen et al. 2011).

The advantages associated with combining different approaches for the characterization of NOM have been demonstrated. (Kent et al., 2014) have combined complimentary NOM characterization techniques, resin fractionation, HPSEC/DOC and UV 254 absorption. This combined methodology addresses the analytical limits of each tool and drove to interesting conclusions: the HOA fraction was dominant and mostly composed of high molecular compounds, followed by the HIN fraction that was mostly composed of low MW compounds and by the HIA fraction, which had MW values distributed within the range of HOA and HIN compounds. The majority of HIN compounds were not detected by the HPSEC analysis. The SUVA measurements allow a better interpretation of HPSEC data since they give an indication of the amount of DOC detected (Kent et al. 2014).

The combination of methods is the key to achieve a complete characterization of organic matter that is specially important in treatability studies of water reuse. The water sources in some regions of limited water supplies include the reuse of wastewater and sometimes there is an indirect reuse of wastewater when the treated wastewater is discharged upstream of the captation of water for treatment and supply. The characteristics of effluent and natural organic matter and respective treatabilities by coagulation and biodegradation were investigated and differentiated using a combination of fractionation and analytical methods: XAD resin fractionation, size-exclusion chromatography in combination with a dissolved organic carbon detector, fluorescence spectroscopy (excitation-emission matrix and fluorescence index), biodegradable dissolved organic carbon testing, ultraviolet absorbance at 254 nm, and dissolved organic carbon measurements (Nam et al. 2008). It was shown that effluent organic matter exhibited lower SUVA, higher content of nonaromatic hydrophilic organic matter and higher fluorescence index, higher total dissolved polysaccharide and a clear protein-like peak (SEC-DOC results), when compared with NOM. The distinct properties of effluent and natural organic matter explain the lower efficiency of coagulation in the removal of effluent organic matter due to its hydrophilic character, because coagulation tends to remove more efficiently hydrophobic organic matter. Oppositely, biodegradation of effluent organic matter was

preferential (and faster), namely hydrophilic substances (protein-like and polysaccharide groups).

HP-SEC fractionation was performed with a chromatographic system coupled with an UV absorbance detector at 260 nm and it was compared with asymmetrical flow field-flow fractionation system coupled to seven angle laser light scattering and UV/VIS spectrophotometer by (Alasonati et al. 2010) showing that the methods are consistent and confirm the same trends.

An organic carbon detector for size-exclusion chromatography that uses conventional UV-persulfate oxidation method to convert organic carbon to carbon dioxide, followed by a mass spectrometer presented lower limits of detection and quantification than Fourier transform infrared spectroscopy 'lightpipe' detector (quantification based on phthalate standards calibration) (Warton et al. 2008). The use of MS allows further characterisation of NOM by measuring the stable carbon isotope composition of DOC in the different fractions obtained by SEC, which can provide information on the biogeochemical origins of DOC in these fractions.

Instrumental methods like NMR, infrared and mass spectrometry allow the determination the entire structure of a molecule. An enhanced characterization of NOM was obtained by resin fractionation into broad chemical classes before gathering information on chemical composition (by techniques such as pyrolysis GC-MS or ^{13}C NMR) and molecular size (by SEC) or field-flow fractionation (Wong et al. 2002). Consistent information were obtained when comparing resin fractionation with SEC results (Wong et al. 2002): the SHA fractions have a higher aliphatic and unsaturated carbon content and less alkoxy and carbonyl carbon than the weakly hydrophobic acids; the high SUVA values observed for the SHA fractions corresponds to high levels of unsaturation; the aliphatic and alkoxy carbon regions are wider and more undistinguished than other spectral regions due to larger residual carbon-proton dipolar bonds (higher proton concentration) and possibly larger dispersion due to high molecular weight and broad molecular weight distribution.

The same authors (Wong et al. 2002) tried to relate the information gathered about NOM structure with treatability and concluded that: the ready removal of hydrophobic and charged hydrophilic fractions is desirable due to their interactions with coagulants or ion exchangers; an enhanced removal of the neutral hydrophilic fraction is needed because it may contribute to the formation of disinfection byproducts, bacterial regrowth in distribution systems and membrane fouling.

CONCLUSION

Given the negative impact of the presence of NOM in water treatment systems, the capacity to group NOM in different fractions allows the identification and quantification of each one, which is the first step to know which kind of compounds are more hazardous. Researchers are interested in natural DOM composition, reactivity and its interaction with anthropogenic compounds.

Prior to characterization, sampling planning should also be a concern, multiple samples and seasonal studies are suggested. Due to the complexity and variability (in time and space) of NOM, special care must be used in order to get representative water samples.

Each method has its strengths, weaknesses, and biases nevertheless the combination of multiple structural characterization techniques provide a more comprehensive view of DOM composition. Coupling structural information with stable C and N and/or radiocarbon isotopic information can provide key constraints on DOM sources and reactivity.

Between fractionation methods, nowadays HPSEC is one of the most popular methods for size fractionation of NOM has the possibility of being combined with different detection procedures.

The development of FTIR-MS has enhanced the molecular analysis of complex NOM structures. NMR spectroscopy has been widely used to investigate the structures and other properties of NOM. Recently methods, like multidimensional NMR techniques together with FTIR-MS, are promising for characterizing the structure and functional features of the major fractions of NOM.

The significance of MS technique has increased due to coupling with HPLC, allowing direct analysis without prior destruction or derivatization of the NOM molecules. It has been recently introduced a promising technique to the analysis of aquatic NOM, the LDI-TOF-MS.

The evolution of fractionated and characterization methods of NOM is bringing new knowledge to the scientific community. This contribute will help managers and operators of water treatment facilities to better understand and improve the efficiencies of the process concerning NOM removal, which is a key point to achieve an even better drinking water quality.

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