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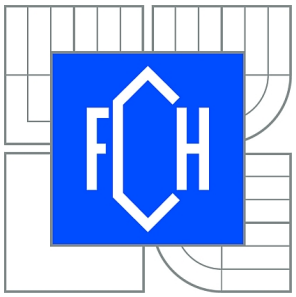

FAKULTA CHEMICKÁ

ÚSTAV CHEMIE MATERIÁLŮ

FACULTY OF CHEMISTRY

INSTITUTE OF MATERIALS SCIENCE

HYDROGELS OF HUMIC ACIDS

HYDROGELY HUMINOVÝCH KYSELIN

DIZERTAČNÍ PRÁCE

DOCTORAL THESIS

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ABSTRACT

Humic acids (HAs) are the main components of the soil organic matter. They are ubiquitous substances with complex chemical and physico-chemical structure. In this study, several modifications of HAs were carried out in order to modify their properties. In the first part, the influences of lignite air oxidation on the yield and physico-chemical character of regenerated humic acids were studied. In the second step, to stabilize the structure of lignite humic acids and improve the water holding capacity, we applied formaldehyde and carbodiimides crosslinking procedures leading to covalent coupling of humic acids moieties. The production of crosslinked structures was motivated by the attempt to design HAs-based systems resembling hydrogels, with the possibility to modify their reactivity and water retention. Samples were analyzed for their chemical composition and physico-chemical properties using various techniques, among the most important were DSC and NMR relaxometry. The chemical composition was studied using FTIR and elemental analysis in order to assess the changes in comparison to pristine humic acids. The stabilities of derivatives were determined by using thermogravimetry. The most important parameter studied in this work was the change in hydration characteristics. For this reason, we developed and applied several new thermoanalytical and NMR relaxometry approaches. In particular, to mimic the situations occurring in nature, we studied sorption of water on humic acids from controlled humidities and monitored qualitative and quantitative aspects of water sorption. Crosslinking by formaldehyde induced a reduction in moisture sorption capacity, which was attributed to the separation of functional groups and a decrease in structural compactness. In addition, the crosslinked humic acids exhibited faster water uptake and approximately three-fold higher water holding capacity than pristine humic acids. In case of carbodiimide coupling (by using water-soluble N-Ethyl-N′-(3-dimethylaminopropyl) carbodiimide (EDC)), the derivatives of humic acids contained only between 14–40 % of original free carboxylic groups. Despite that, they exceeded the moisture harvesting ability of parental humic acids around 10–14 % after their equilibration at 100% relative humidity. Although, they showed also more rigid structure, the EDC derivatives showed also faster swelling kinetics and reached almost the same water holding capacity as original sample after 18 days. However, carbodiimides derivatives began to degrade already after 3–9 days during swelling tests, which subsequently decreased their performance. The results suggest that water holding capacity, swelling kinetics and moisture harvesting ability of humic acids are not influenced exceptionally by the amount of free carboxylic groups or other polar functionalities, but also by their spatial arrangement and the distribution of pore sizes on the surface and inside the humic structure. The knowledge gained in this study is beneficial, among others, for the production of soil humic acids-based conditioners, i.e. remediation agents having required and simultaneously adjustable ability to bind and release water into the surrounding environment. Last, but not least, the presented findings improve the fundamental understanding the hydration processes in pristine and modified humic acids, which is beneficial to elucidate the hydration of complex natural systems and in particular, of natural organic matter.

KEYWORDS

Lignite humic acids, thermal analysis, interactions, hydration, water retention

ABSTRAKT

Huminové kyseliny představují hlavní součást půdní organické hmoty. Jedná se o všudypřítomné sloučeniny s komplexní chemickou a fyzikálně-chemickou strukturou. Předkládaná práce shrnuje několik způsobů modifikací huminových kyselin provedených za účelem úpravy jejich vlastností. V první části byl studován vliv vzdušné oxidace lignitu na velikost výtěžku a fyzikálně-chemický charakter produkovaných regenerovaných huminových kyselin. Druhý krok představoval snahu stabilizovat strukturu lignitických huminových kyselin a zlepšit zádrž vody. Bylo provedeno síťování pomocí formaldehydu a karbodiimidů, které předpokládá vznik nových kovalentních vazeb v různých částech struktury huminových kyselin. Produkce zesíťované struktury byla motivována snahou vytvořit systémy podobné hydrogelům, založené na huminových látkách s možností úpravy jejich reaktivity a retence vody. Chemické složení a fyzikálně-chemické vlastnosti připravených vzorků byly studovány různými metodami, mezi nejdůležitější patří DSC a NMR relaxometrie. Chemické složení bylo studováno pomocí FTIR a elementární analýzy za účelem posoudit a porovnat změny s původním neupraveným vzorkem. Stability vzorků byly stanoveny pomocí termogravimetrie. Nejdůležitějším sledovaným parametrem této práce byla změna hydratačních charakteristik. K jejich studiu jsme vyvinuli a aplikovali několik nových termoanalytických a NMR relaxometrických přístupů. Ve snaze napodobit přírodní procesy byla studována sorpce vody huminovými kyselinami z prostředí s kontrolovanou vzdušnou vlhkostí. Byly pozorovány kvalitativní a kvantitativní hlediska sorpce vody. Zesíťování za použití formaldehydu způsobilo snížení sorpční kapacity vlhkosti. Tato změna je připisována separaci funkčních skupin a menší strukturní kompaktnosti. Zesíťované huminové kyseliny dále vykazovaly rychlejší příjem vody a přibližně třikrát větší kapacitu zádrže vody než původní huminové kyseliny. V případě použití karbodiimidu (ve vodě rozpustného N-Ethyl-N′-(3-dimethylaminopropyl)karbodiimidu (EDC)) obsahovaly výsledné produkty 14–40 % původních karboxylových skupin. I přesto tyto látky překonaly schopnost sorpce vlhkosti původního nemodifikovaného vzorku o 10–14 % po kondiciaci v prostřední se 100% relativní vzdušnou vlhkostí. Navzdory rigidnější struktuře vykazovaly EDC deriváty rychlejší bobtnání a dosáhly téměř stejné kapacity zádrže vody po 18 dnech jako původní vzorek. Vzorky modifikované karbodiimidy vykazovaly známky degradace již po 3–9 dnech což částečně snižuje jejich využitelnost. Získané výsledky naznačují, že kapacita zádrže vody, kinetika bobtnání a schopnost sorpce vlhkosti huminových kyselin nejsou výhradně určeny koncentrací karboxylových skupin, nebo dalších polárních fragmentů, ale také jejich vzájemným rozmístěním a distribucí velikosti pórů na povrchu a také uvnitř struktury. Získané poznatky této studie mohou sloužit například k produkci půdních kondicionérů založených na huminových kyselinách, tj. remediačních preparátů s požadovanými, ale především nastavitelnými schopnostmi poutat a následně uvolňovat vodu do okolního prostředí. V neposlední řadě přispějí uvedené závěry ke zkvalitnění základního porozumění procesu hydratace v modifikovaných a původní vzorcích, což je přínosné ve vztahu k objasnění hydratace komplexních přírodních systémů a to zejména přírodní organické hmoty.

KLÍČOVÁ SLOVA

Lignitické huminové kyseliny, termická analýza, interakce, hydratace, retence vody

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DECLARATION

I declare that the dissertation thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the dissertation thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both supervisor and the dean of the Faculty of Chemistry, BUT.

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1 INTRODUCTION

The last few decades has shown that in the fertile and cultivated areas are soils exhausted, which leads to a decrease in total agricultural production. The long-term use of soil for intensive agricultural practices can influence the infiltration of water, affect the soil by disruption of labile macroaggregate fractions, and change the soil aggregation and structure. As a result, soil water holding capacity is diminished [1].

Soils are complex systems, containing, among others, organic matter including living organisms and inorganic materials such as sand, silt, clay, etc. [2]. Organic matter improves the soil structure and the organic components provide energy for living organisms in the soil and supply nutrients for plant growth [3]. Furthermore, organic matter grants to soils ability to retain water and stimulate plant growth.

For this and other reasons is soil organic matter essential for life on earth. The study of the structure and function of soil organic matter has already been the aim of scientific community, in particular the environmental and agricultural scientists, for long time. However the complexity of soil organic matter hampers its deep understanding, despite the progress in analytical techniques.

Currently, more than one third of the mainland is affected by desertification [4]. This adverse effect is primarily caused by application of poor quality fertilizers, and intensive and long-term use of soils. Depleted topsoils can be quantitatively and qualitatively renewed by adequate using of both artificial fertilizers and manure.

Humic substances are versatile organic carbonaceous compounds naturally occurring in soils. Appropriate application of humic-based material originating from other sources is believed to guarantee the return of natural (stable) carbon back into the soil. Recently [5], these substances were modified in order to obtain compounds similar to hydrogels with improved water retention capacity. It was shown that that humic-based hydrogel represent a promising approach to provide the supply of stable organic carbon and simultaneously impact the water holding capacity of soil.

2 STATE OF THE ART

2.1 Humic substances

Humic substances (HS) are the most widespread and ubiquitous natural nonliving organic materials in aquatic and terrestrial environments, and represent a significant proportion of the organic carbon in the global carbon geochemical cycle [6]. This assembly of organic "leftovers" contributes vital properties to soils, including sequestration, mobilization, and oxidative or reductive transformation of organic xenobiotic molecules, trace gases, and trace metal contaminants [7]. In addition, these organic substances can substantially impact on a variety of geochemical processes, for example, acting in the cycles of nutrient elements like nitrogen and phosphorus [8] or mobilizing heavy metals by complexation [9] and pesticides by adsorption [10], they influence the process of formation fossil fuels (coalification). HS have been documented to interact with over 50 elements from the periodic table including nutrients, toxic metals, radionuclides and the halogens. The effect on toxic metals and micronutrients is unpredictable and often paradoxical, sometimes making them more available to organisms, while at others acting as a sequestering agent so as to reduce their toxicity or beneficial value [2].

They are formed through aerobic and anaerobic decomposition of (mostly) plant detritus, and according to some older theories, also by secondary microbial synthesis. In other words, the formation of humic material is the result of both the degradation of plant residues and the re-synthesis of new molecules taking part during biotic and abiotic processes. During these processes, a selective preservation of plant biomass can affect the isotopic content of biochemical structures [11]. According to new theories, the resynthesis is replaced by other protective mechanisms such as sorption on clay minerals or formation of aggregates [12].

Differences in origin, age, and genesis lead to a high degree of chemical and morphological complexity that makes the compounds difficult to characterize. They generally have a high aromatic content, estimated to range from 20 to 60 % of the carbon present [13]. They are rich in functionalities and include phenols and other alcohols, ketones/quinones, aldehydes, carboxylic acids, amino- and nitro-groups, and sulfur containing entities such as mercaptans, sulfates, and sulfonates [14]. As these substances age, their chemical structure becomes progressively more aromatic and less abundant in polysaccharide and lignin derivatives [2]. Chemical composition of HS is strongly influenced by the coalification process [15]. This can broadly be divided into biochemical and physico-chemical stages (see Fig. 1). The main physical changes are darkening in color and increase in hardness and compactness. The chemical changes represent loss in moisture and volatile contents, and increase in carbon content. During biochemical stage of coalification, also called "peatification", the partial oxidation and hydrolytic decomposition of dead accumulated vegetal matter take place. This microbial and chemical alteration occurs in upper level of soil by aerobic bacteria, actinomyces and fungi. With the increment of depth, these organisms are replaced by anaerobic bacteria. Microbial life is gradually reduced as the easily assimilated substances disappear. Further decomposition by anaerobic bacteria extracts oxygen from organic molecules and results in high concentration of hydrogen. Chemical changes represent mainly condensation, polymerization and reducing reactions. Humification is the most important process during peatification, when HS are formed. The degree of humification depends on the oxygen supply, the peat temperature, and the pH values of water in the peat

swamps. During subsequent geochemical stage of coalification, rising temperatures and pressures, generate hydrocarbons [16], [17].

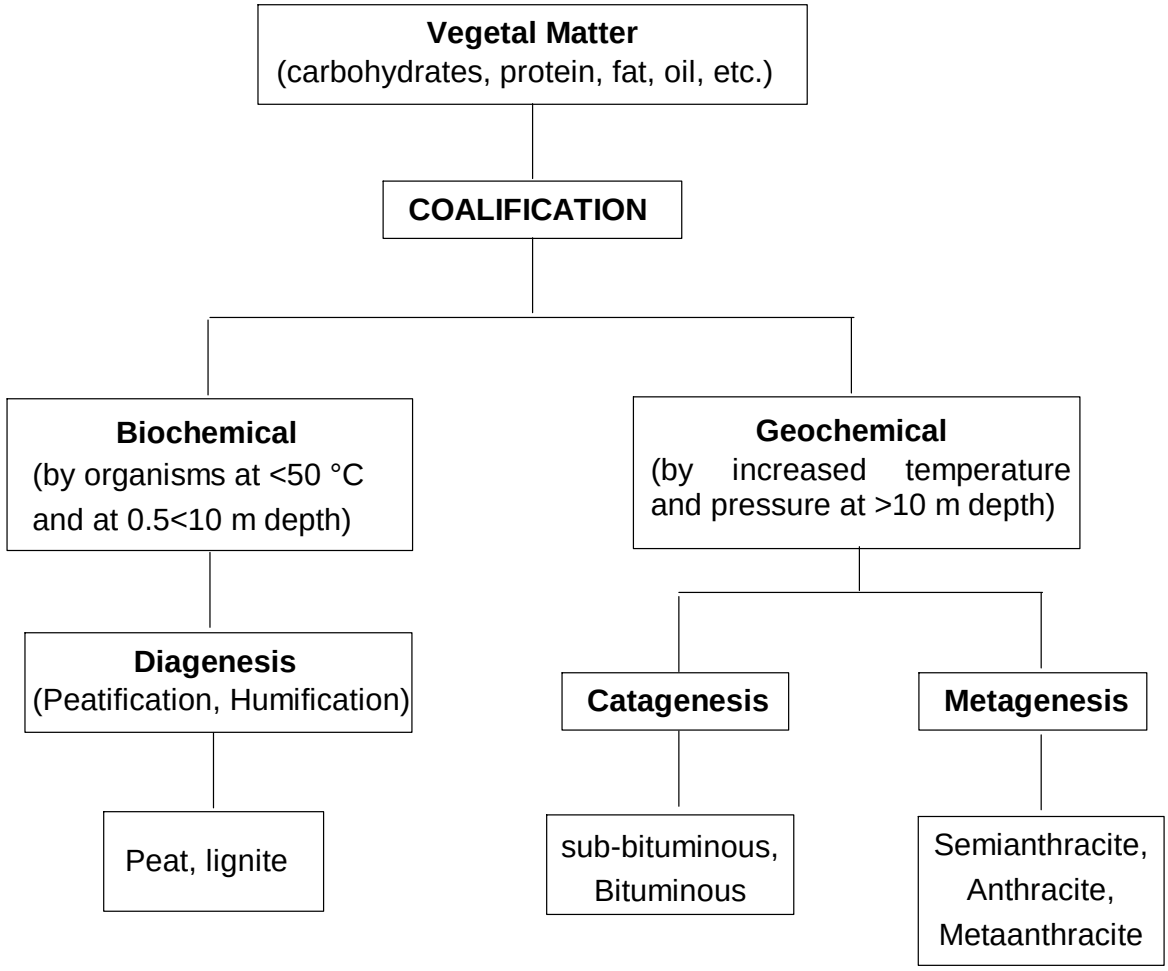

Fig. 1. Simplified formation of coal through „coalification" process of vegetal matter [16]

In lignite deposits, plant tissues are supposed to undergo a first aerobic degradation by fungi and to some extent by bacteria [18], with a preferential utilization of cellulose with respect to lignin. Under specific conditions, a second stage of decomposition can take part. It is anaerobic and driven by anaerobic microorganisms different from those responsible for cellulose degradation. This phase can eventually lead to coal formation and both biotic and abiotic processes are involved [19].

These compounds are now more and more used in fertilizers and soil conditioners. Their role in environmental and health issues is being explored as well [20], [21].

In the early 1980s, the International Humic Substances Society (IHSS) was formed to standardize HS research, among other things. Recently [22], most of the scientists began to concede that humic substances were a mixture of aliphatic and aromatic structure, having both a lignin and polysaccharide origin. New theories are appearing showing a significant contribution of substances of microbial origin [23].

Enormous advances have been made during the last decade due to application of new and modern physico-chemical methods. Nevertheless, despite the great progress [24], the structural chemistry of lignin and HS did not advance so fast as for example the chemistry of animal-originated biopolymers [25].

2.1.1 Formation of humic substances

The formation of humic substances is one of the least understood aspects of humus chemistry and one of the most intriguing. Studies on this subject are of long-standing and of matter of continuous research.

The synthesis of HS (humification – an oxidative process includes aromatization, condensation, phenolic oxidative coupling, demethylation, oxidation of plant polyphenols, etc.) is hypothesized to occur by one of the two pathways: the degradation of plant biopolymers to form the central core of HS, and condensation-polymerization reactions in which plant biopolymers are first degraded to small molecules which then repolymerize [26]. Early workers proposed the abiotic degradation of plant components for the formation of HS, but it soon became apparent that microorganisms played the essential role in humification [27]. According to Maillard [2], humic substances were formed from purely chemical reactions in which microorganisms did not play a direct role except to produce sugars from carbohydrates and amino acids from proteins.

Several theoretical pathways exist for the formation of humic substances during the decay of plant and animal remains in soil, the principal ones are shown in Fig. 2. The classical theory, popularized by Waksman [27], is that humic substances represent modified lignins (pathways 1), but the majority of present-day investigators favor a mechanism involving quinones (pathway 2 and 3) [28]. In practice all four pathways must be considered as likely mechanisms for the synthesis of humic and fulvic acids in nature, including sugar-amine condensation (pathway 4). These four pathways may operate in all soils, but not to the same extent or in the same order of importance. A lignin pathway may predominate in poorly drained soils and wet sediments (swamps, etc.) whereas synthesis from polyphenols may be of considerable importance in certain forest soils. The frequent and sharp fluctuations in temperature, moisture and irradiation in terrestrial surface soils under a harsh continental climate may favor humus synthesis by sugar-amine condensation.

All these proposals have in common the fact that they describe the humification as a complex mechanism involving the breaking of bio-macromolecules into small constituents, and their successive recombination to form an “ex-novo” more complex organic matter [29].

It is currently recognized that biopolymer de- and re-polymerization is not the main process in humification and that the main driving force is the selective preservation of microbial and plant tissues and/or plankton bodies [30]. In soils, this process requires a long time, high decomposition of the degradable fractions (i.e. aliphatic, carbohydrates and proteins) and the preservation of more refractory fraction (i.e. aromatic – e.g. lignin derived molecules) and highly crosslinked lipids (e.g. cutin and suberin from plant) [30]. On the contrary, biomass contains few degraded structures, i.e., carbohydrates, polysaccharides or fatty acids that contribute to the HAs structures [30]. These differences are important as they lead to a different content between hydrophobic and hydrophilic structures and in the quality of the hydrophobic (lipids vs. aromatic) and hydrophilic fractions (non ionic, e.g. carbohydrates, vs. ionic e.g. carboxylic acids) of the HAs molecules, which affect surfactant properties [31].

Reviewing the origin of the soil organic matter, Kögel-Knabner et al. [32] reported that the preservation of bio-macromolecules was due to the biochemical recalcitrance caused by inherent properties of molecular structure. Therefore, it can be concluded that humification can be identified as the simple preservation and conservation of bio-macromolecules in soil,

and that HAs are represented by a heterogeneous miscellanea of alkali soluble biomolecules of both plant and microbial origin.

The heterogeneity of compounds and ambiguities about the characteristics and structure of humic substances provoked many questions whether HS of different soil types are similar to one another or not. All attempts to isolate single pure compounds and to identify their structures have failed [33].

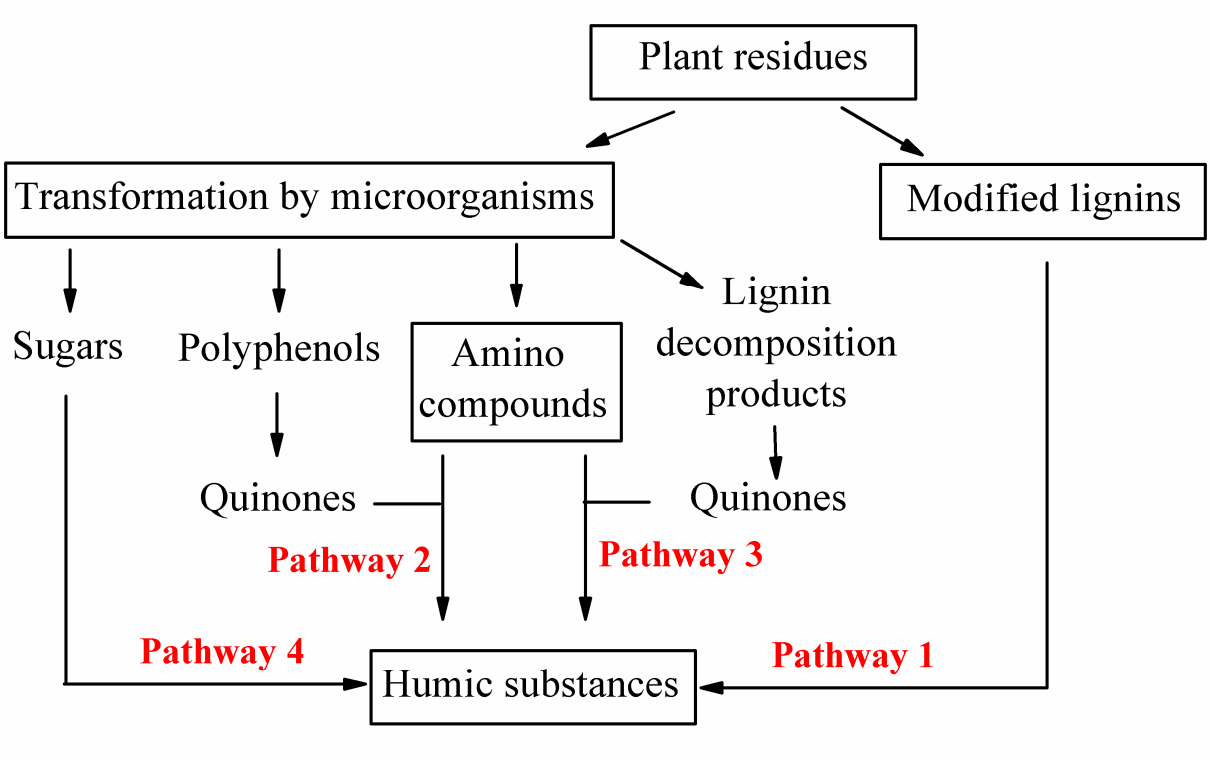

Fig. 2. Suggested mechanisms for the formation of soil humic substances (adapted from [2]).

2.1.2 Chemical composition and extraction of humic substances

The great obstacle in characterization of HS is fact that HS have been obtained in different ways, in different laboratories and results are, therefore, not comparable. A detailed characterization of HS and their manifold processes in the environment requires the application of several analytical tools combined with chemical, spectroscopic and fractionation methods. The most common techniques used to obtain structural and compositional information on HS include elemental analysis, visible, infrared, and ^{13}C NMR spectroscopy, and degradation methods [34].

It was shown that HS are assemblage of large polyfunctional molecules such as lipids, carbohydrates, aromatics, etc. [2], the commonly encountered ionizable functional groups include carboxylic acids, phenols, alcohols, ammonium ions, and thiols. To a lesser extent, sulfonic acids and "active methylene" compounds (with –CO–CH_2–CO– structural moiety) are also encountered [35].

It appears that amide N in peptides is the dominant chemical form of N in humic substances, and that free amino acids are present as well. Positively charged amide groups within humic substances, which have a net negative charge under typical soil pH conditions [2], may have important impacts on humic conformation, interaction with mineral surfaces, and retention of nutrients or contaminants [7]. Supramolecular associations of relatively low-

molecular-mass humic components derived from biological precursors could possess a considerable amount of amide N functional groups [36]. In contrast, the “polymer model,” which posits the destruction of amide groups during the formation of humic macromolecules [2], [36], is inconsistent with the N functional group distributions dominated by amide N that are suggested by extensive experimental evidence.

The major binding sites are attributed to the oxygen-containing functional groups – carboxylic and phenolic groups, which contribute to the total acidity of humic substances. Phenolic groups are usually assumed to be the only weak acidic groups, while the contribution of alcoholic groups in carbohydrate entities and enols is usually neglected [35].

The diversity among humic substances makes their classification difficult. It is customary to divide them into three operationally defined classes, broadly based on their solubility characteristics. Thus humin (black in color) is the fraction that is insoluble in both acid and base, humic acid is the fraction generally soluble at pH > 2, and fulvic acid (FAs) is soluble at all pH values (see Fig. 3) [2]. FAs remain in solution after removal of HAs by acidification and tend to be more aliphatic. FAs are light yellow to yellow-brown in color.

Although chemical and physical differences do underlie these variations in solubility, the separation of humic substances into three fractions is operational, and does not indicate, for example, the existence of three distinct types of organic molecule [33].

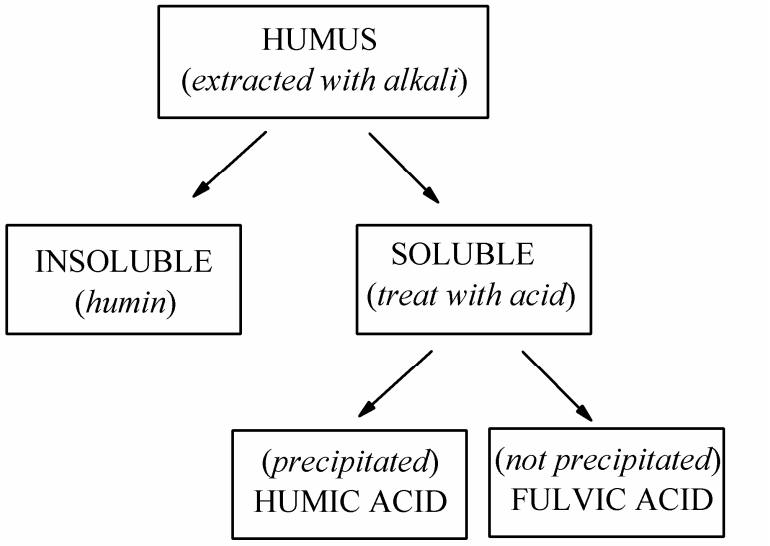

Fig. 3. Scheme for the fractionation of humic substances (adapted from [2]).

Many biomolecular fragments are intimately associated-even covalently bonded-with the humic fraction and cannot be separated effectively without significant alternation of the chemical properties of the fraction [7].

HAs are the most important and relevant substances in direction of our research, therefore in next part of the dissertation thesis the attempt is paid directly to study of HAs.

2.2 Humic acids

The name humic acid is derived from its early source humus. The concentration of HAs in soil and waters is dependent on many factors such as climate, pH, substrate material, topography and time. HAs are bio-material highly functionalized (carboxylic, phenolic, alcohol, amine, amide, carbonyl, quinones, etc) and carbon rich environmental material which

gives soils their brown-black color [2]. Due to their crucial role in many physico-chemical processes occurring in the global ecosystem, HAs are fascinating and their study is multidisciplinary, being very important for many environmental terrestrial, marine and river processes [2], [29].

Diversity and heterogeneity of the HAs might be due to varying proportions of gymnosperms versus angiosperms in the lignite-forming vegetation, varying contributions of algal or microbial biomass, different extents of organic matter degradation, the effects of bacterial C cycling and/or climatic changes during the lifetime of the lignite [19].

Regarding the coal-derived humic acids, it can be deduced that, as the coalification rank increases, the composition of HAs change, increasing their aromatic content. Furthermore, during the humification process, HAs lose all features of plant residues, and moreover, that C skeletons containing aromatic units increase with rank. In fact, the presence of high quantities of lignin derivatives in lignite can survive only in early coalification [37].

2.2.1 Humic acids chemical composition

The existing concepts about the chemical structure of HAs depend on the methods used for their characterization [38]. As follows from previous text, humic substances, rather than been constituted by macromolecular polymers (random polymers of a variety of biological monomers [39] as traditionally believed) may be better described as self-assembled supramolecular associations of relatively small and chemically diverse heterogeneous molecules (possibly lower than 2000 Daltons). The supramolecular nature implies that humic molecules, instead of being covalently interlinked, are then randomly associated and stabilized mainly by weak dispersive forces (van der Waals, π–π, CH/π) into apparently high molecular dimensions [22], [40]. The low-molecular view is gaining a growing interest and it is supported more and more supported experimentally [7].

The supramolecular understanding of HAs suggests a very important fact, which is the possibility of increasing the size of humic molecules by polymerization reactions. This modification can improve some HAs properties such as stability, water holding capacity and others.

Hydration, due to hydrophobic effect separates hydrophobic and hydrophilic microregions in the HAs. ‘‘hydrophobic scaffold’’ is formed resembling a quasiporous structure with rigid hydrophobic moieties surrounded by flexible, swollen hydrophilic moieties, which penetrate the pore system. This separation effect is furthermore supported by latest results of molecular modeling, which showed that hydrophobic supramolecular structures are more stable in hydrophilic environments and hydrophilic structures, vice versa, are more stable in hydrophobic environment [41].

2.2.2 Proposed structural formulas of humic acids

Basically, the contemporary structural models suggest the presence of a biologically relatively resistant and highly substituted core consisting of aggregated aromatic structures with presence of heterocycles [42]. The core units are linked to each other via a net of alkyl chains of different lengths [43], which are highly substituted with functional groups such as carboxylic, aliphatic, aromatic, hydroxyl, and amino groups [44].

The primary structure reflects the conditions of formation, such as parental material, climate conditions, and character of present microorganisms. In soils, the role of aggregates

is, among others, closely linked with soil stability, sorption processes, water holding capacity, and cell biology of soil living organisms [2].

During decades, several authors have attempted design hypothetical structures of HAs. Using the technological advances and mainly computational resources, Schulten [42], proposed a 3-dimensional HAs structure (Fig. 4).

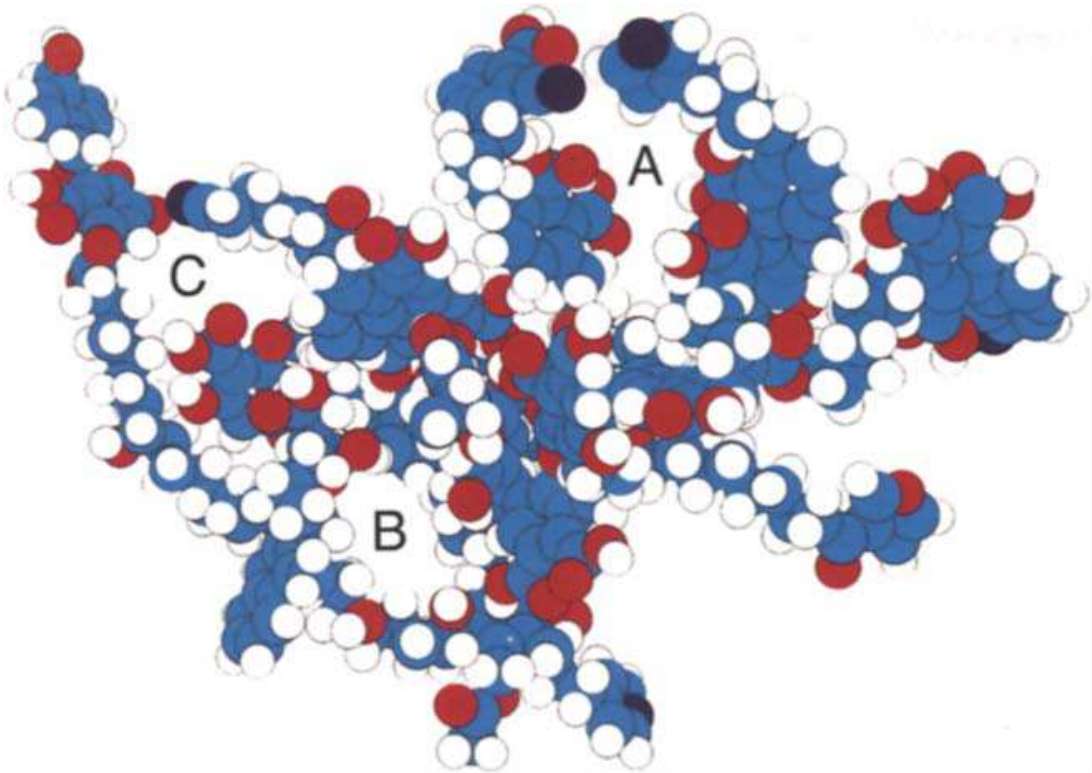

Fig. 4. Geometrically optimized three-dimensional (3D) structure of HAs [42]; Element colors are: carbon = light blue; hydrogen = white; nitrogen = dark blue; and oxygen = red. For the better display the main voids are marked A, B and C.

One of the most interesting features of the proposed HAs structure (Fig. 4) is the presence of voids of various dimensions, which can trap and bind other components such as carbohydrates, proteinaceous materials, lipids, biocides, as well as inorganics such as clay minerals and hydrous oxides. For the development of this HAs structure has been assumed that carbohydrates and proteinaceous materials are adsorbed on external surfaces and in internal voids. The oxidative degradation of this structure would produce the benzene carboxylic, phenolic, and aliphatic carboxylic acids which have been isolated and identified repeatedly as major oxidation products of HAs [43].

This preliminary, energetically still unoptimized structure shows clearly the atomic distances and angles *(Sticks)* and gives a first three-dimensional structure information. Even this rough display indicates already the importance of aliphatic chains as link between the aromatic moieties and prerequisite for the formation of gaps and voids in the HAs structure [45].

More recently, Fetsch et al. introduced a hypothetical HAs structure based on study [46]. In Fig. 5, all previous knowledge and hypothesis are included in the proposed model. Furthermore, the presence of nitrogen and sulfur linkages is stressed. In fact, the main

functional groups involved in metal ions complexation are shown, whereas the inner structure, still unknown is represented by circles.

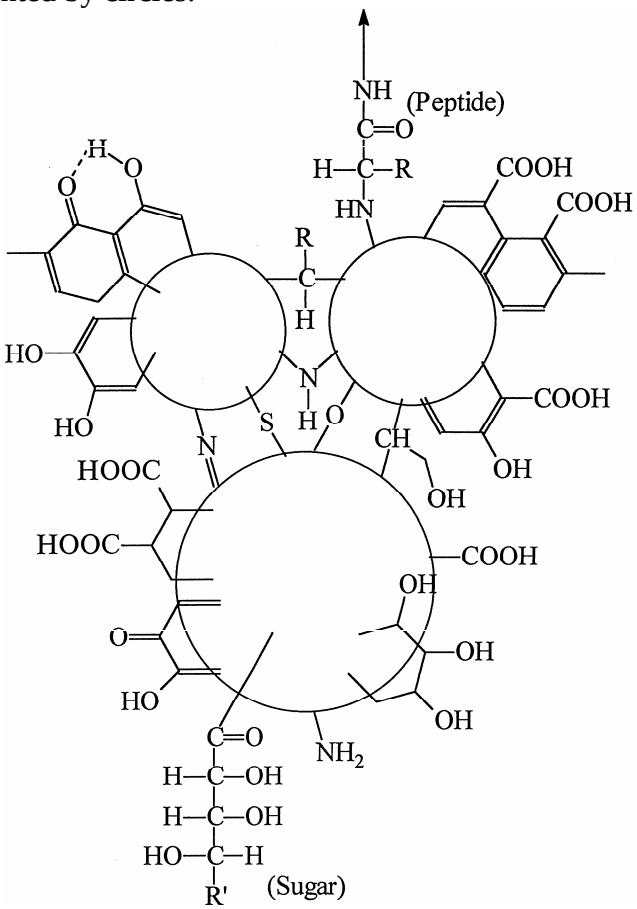

Fig. 5. Fetsch et al. hypothetical model of HAs structure [46].

An alternative model, conceived by A. Piccolo and co-workers [47], HAs is considered not to consist of long polymer chains, but rather of small units that are held together by weak intermolecular forces. In principle, this appears to be akin to the associative model, and it may even be argued that the idea was implicit in Wershaw's original suggestion that HAs molecules associate to form micelles in aqueous solution. However, the stipulation that the aggregated species are small in size was not made in that model, and subsequent developments of the idea generally retained the polymer concept [48].

Despite the growing evidence of humic acids being aggregates small molecules, the research is still in progress. The detail knowledge of HAs structure might help to explain their remarkable properties. One of the main problems to be addressed is the notion that HAs are intractable mixtures and complex analytical problems must be solved before HAs can be fully exploited, e.g. it is difficult to fully understand how aggregate is related to humic substances when the characteristics of humic substances and their compounds are still unclear.

2.2.3 Physico-chemical properties of humic acids

As it can be expected, the physical properties of humic compounds highly depend on their chemical structures. Due to their polyfunctionality, HAs are one of the most powerful chelating agents among natural organic substances. They are able to complex heavy metals,

inorganic anions [49], halogens, organic acids [50], aromatic compounds, pesticides and herbicides [51], etc.

Aliphatic chains give HAs flexibility, while the aromatic units are rigid. This is why HAs can interact with so many different solutes.

Sorption of hydrophobic organic compounds occurs by partitioning/dissolution into an amorphous phase or interaction with rigid units such as stacked aromatic rings [52]. Water and bound metals evidently play major roles in HAs-solute interactions and in HAs aggregation/disaggregation [42].

HAs are excellent sorbents because of their hydrophilic, hydrophobic and highly functionalized nature. All this characteristics qualified them as amphiphiles [53] that lower the surface tension of water and form micelles that can sequester hydrophobic molecules (such as pyrene) and adsorb polar compounds in the same time.

HAs form gels containing up to 98 % water under some conditions, but different gel drying methods give solids with different morphologies [54] that may never re-wet to form the same gel.

The concentration of HAs in solution should relate to its macromolecular configuration and aggregation [53].

It should be noted, that interactions of HAs vary with regard to the type of cation considered. Group I cations ("hard" ions) are likely to undergo electrostatic interactions with HAs functional groups, while the bonds formed by "soft" Group III cations are more covalent in character. Group II cations, including Cu^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+}, Pb^{2+}, and Ni^{2+}, behave in an intermediate fashion [14].

The differences between FAs and HAs are necessarily based on chemical differences and can be explained by variation in number of functional groups and proportion between polar and non-polar parts. While the two classes of compounds share many structural features, including an abundance of carboxy, hydroxy, phenolic, and ketonic groups, FAs has a lower molecular weight, a higher functional group density, and higher acidity than HAs. The total acidities of FAs (9–14 meq g^{-1}) are considerably higher than for HAs (4–8.7 meq g^{-1}). Molecular weights for FAs are in the 500±2000 Da range, while they extend from 2.0 to 1300 kDa for HAs. The oxygen content is reported as 32.8±38.3 % for HAs, and 39.7±49.8 % for FAs [14].

Another important difference is that while the oxygen in FAs can be accounted for largely in known functional groups (COOH, OH, C=O), a high portion of the oxygen in HA seems to occur as a structural component of the nucleus. The HAs/FAs ration usually, but not always, decreases with increasing depth of soil.

Carbohydrates have been reported to account for about 10 % of the HAs weight and a similar value has been suggested for proteinaceous materials in HAs [55]. Thus, it has been assumed that one HAs molecule traps within its voids approximately 10 % carbohydrates and 10 % proteinaceous materials. The resulting HAs mixture has then an elemental composition of $C_{342}H_{388}O_{124}N_{12}$ and an elemental analysis of 61.8 % C, 5.9 % H, 29.8 % O and 2.5 % N. If more carbohydrates and/or proteinaceous materials are added, the C content decreases and the O content increases [45].

2.2.4 Micell-like and pseudomicell structure theory

In solutions, both HA and FA aggregate are forming complex assemblies [14], [56]. According to a polymer view, the amphiphilic HAs molecules are considered to "aggregate" both intra- and intermolecularly [57], [58]. The former is made possible by the chain length and flexibility of the humic polymers, which allow them to fold and coil in a manner that directs hydrophilic (consisting mainly of ionic groups such as carboxylic acids, and nonionic polar portions such as phenols, alcohol, aldehydes, ketones, amides and amines) groups outward and keeps more hydrophobic (consist mainly of hydrocarbon chains coming from relatively unaltered segments of plants polymers) moieties isolated in the center. This process, which could in principle occur with a single polymer strand, produces an entity that is operationally similar to a conventional micelle, albeit more structurally constrained. Like a micelle, it has a hydrophobic interior and a more hydrophilic surface, giving it distinct solubilizing powers for nonpolar solutes Fig. 6. Briefly, hydrophobic regions form under acidic conditions, but under basic conditions disperse either completely [7] or partly [59]. To indicate both similarities and differences with normal surfactant micelles, these HAs structures have been referred to as pseudomicelles. A portion of a generalized structure, which may be visualized as a "knot" in a HAs polymer "string" is shown in Fig.7 [14].

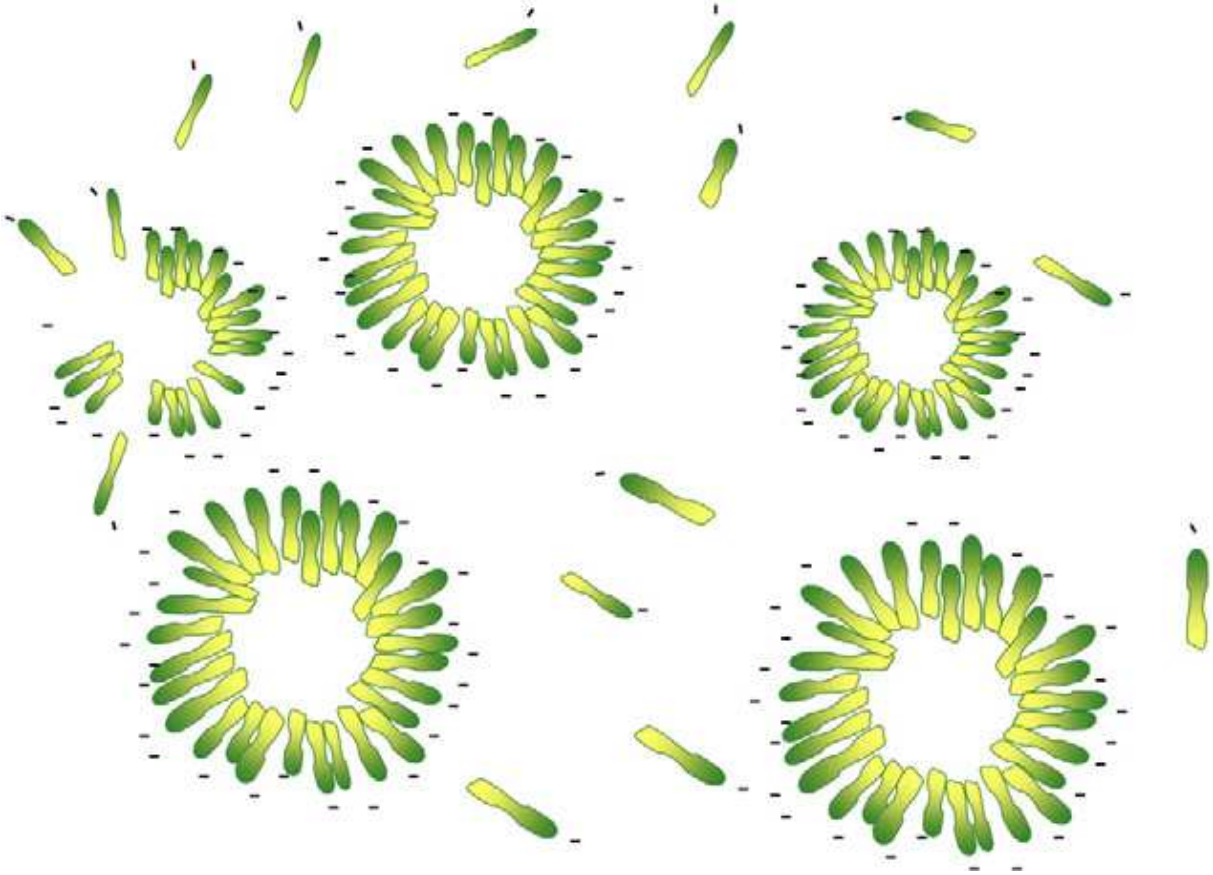

Fig. 6. Representation of humic acid micelles: in a given natural water system one may consider HAs as a membrane like-structure with a hydrophobic interior regions and a charged-hydrophilic surfaces [56].

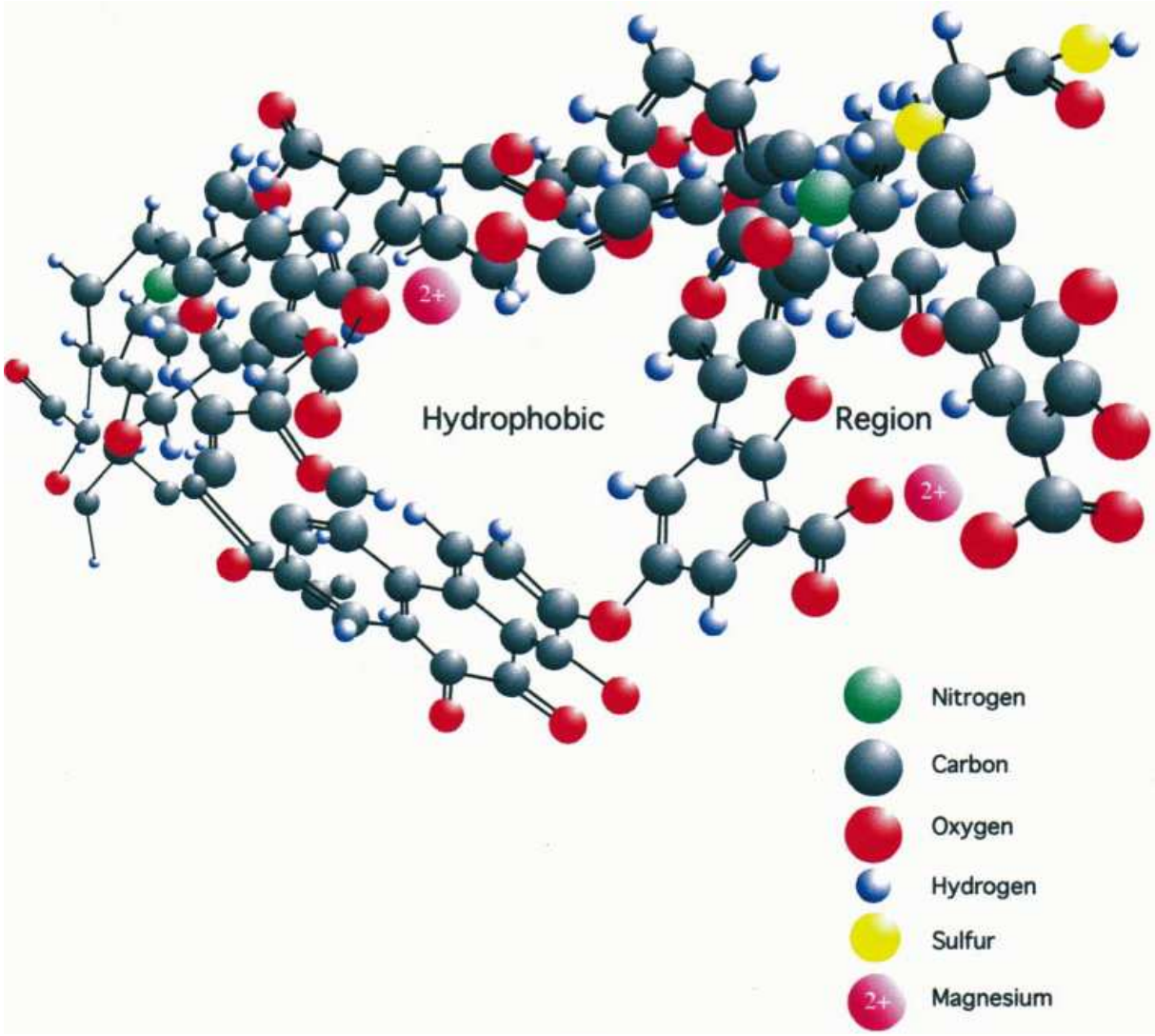

Fig. 7. Portion of proposed „pseudomicell type“ structure of HAs [14], [58].

The structures are considered to exist at all low HAs concentrations in aqueous solution, although certain variations in composition must be anticipated. It is, for instance, likely that intermolecular aggregation supplements intramolecular coiling in pseudomicelle formation, and that this depends on both the concentration and polydispersity of the solute. The proposed assembly thus consists of coiled humic polymer chains, interspersed with smaller HAs fragments [14].

The ability of HAs to arrange themselves so as to have their internal hydrophobic groups (hydrocarbon) and hydrophilic portions outwards, such as carboxyl, phenols and hydroxyl groups, depends on the length and flexibility of their constituent chains. Intramolecular aggregation of HAs plays an important role in the micelle formation, and long flexible HAs polymers are considerably the best sequestrating agents [60] observed the formation of a micellar structure for dissolved HAs, in which the humic polymers formed largely intramolecular domains of relatively low polarity with the ability to sequester the small hydrophobic species into them.

Higher content of alkyl chains increases the hydrophobicity of a HAs, increasing the free energy of the water/surfactant-like system that can be reduced anticipating the process of micellization. Therefore, micelle formation seems to be due, only, to the hydrophobic and/or amphiphilic fractions of HAs [61].

Aggregation of humic polymers proceeds in continuous fashion from micelle-like assemblies to macroscopic precipitates–as the salt concentration is increased. Intermolecular interactions depend on the polydispersity of humic acid solutions [57].

Engebretson et al. supported the view that molecules of some minimum size and flexibility must be present for humic acids to exhibit a significant increase in pseudomicellar behavior upon the addition of cations. In addition, their observation suggests the existence of an intermolecular component operative in the formation of hydrophobic regions that are capable of sequestering nonpolar compounds. This is consistent with observations to assume that it involves interplay between smaller and larger humic acid components [56], [58].

The extent to which humic acid associations occur appears to depend on molecular size, flexibility, polydispersity, and solution conditions. While the last can be controlled, the first three variously contribute to total association. For completeness, the nature and distribution of functional groups on humic acids must also be included among elements that undoubtedly affect the association [58].

2.2.5 Effect of pH on humic acids

Humic acid contains a large number of carboxyl and phenolic hydroxyl groups [2] and is thought to exist as a polyelectrolytic macroion in aqueous solution. The degree of dissociation of these functional groups depends largely on the pH of the solution. At higher pH, the mutual repulsion of the negatively charged sites causes the polyelectrolyte to adopt a stretched configuration. For the flexible macromolecular structure proposed for certain humic acids [62], this means that there is a reduced tendency to form hydrophobic microenvironments, or pseudomicelles, at elevated pH [57], [58]. It also implies that the hydrophobic domains in the humic acid molecule are relatively unstructured. According this view, as the pH of a humic acid solution is lowered and some of the charged sites are neutralized, a reduction in intramolecular repulsion is predicted. This results in a contraction of the polymer chain with concomitant expulsion of some water of hydration. The anticipated consequence of these structural changes is an increase in the number, structure, and microviscosity of the hydrophobic domains in humic acid. At a given pH, temperature, and ionic strength, the number and nature of the hydro- phobic association sites in an aqueous humic acid solution are determined by the type and concentration of humic acids [57], [58].

Increasing the H^+ concentration causes the protonation of the HAs carboxy groups, which eventually leads to precipitation, which usually begins at pH ~ 3–2 and reaches completion at pH ~ 2–1. This should again be thought of as a continuous process, which begins at an intramolecular level, proceeds through intermolecular aggregation, and ultimately produces a precipitate. As it promotes aggregation at the molecular level in its earlier stages, the process again enhances the detergent character of the HAs, through the establishment of hydrophobic domains [14].

A HAs sample at pH 7 has a larger number of dissociated acidic functions than at pH 4.7 [2]. By loosening the inter- and intramolecular hydrogen bonds, which stabilize the conformational structure at pH 4.7, the humic association at pH 7 has a lower molecular size and is stabilized by only dispersive forces [40].

At pH 7.0 when the negative charges arising from the dissociation of humic carboxyl groups disrupt the hydrophobic associations and humic constituents may become available to react by a free-radical mechanism. At higher pH, the increased hydration of humic molecules enhances their mobility in solution and the probability of a concomitant contact among each other, the oxidant and the catalyst [63].

The influence of a high pH on humic acid configuration is anticipated to be strongest for the most flexible polyanionic macromolecules [57].

As the pH decreased and the ionic strength increased, the humic aggregate changed from a more expanded to a more collapsed configuration, as proposed by Swift [64].

2.2.6 Humic acids applications

Nowadays, the approach to the use of renewable resources increases the interest in the ability of HAs to sequester organic pollutants during soil decontamination [65] or to substitute petrol-derived surfactants in textile dying and washing [31]. To handle contamination by persistent organic pollutants, recently attention has been focused on the possibility to desorb pollutants from contaminated soils through the action of water helped by the addition of pollutant-solubilising agents, i.e. non-ionic or anionic surfactants [65]. Due to the persistence of the synthetic surfactants in the environment an interesting alternative of soil remediation technologies is to consider the ability of HAs, extracted from different sources, to act as bio-surfactant agents.

Humic acids can be used as natural surfactants and they can be proposed for technological applications [56].

The humates have been used as soil additives and plant fertilizers influencing the quality of plants. Moreover, mixtures of humate solutions with metals have yield in trace-element fertilizers and also as possible desert soil-enrichments [3].

Cosmetic and pharmaceutical preparatives on the base of soil humus extract have been also reported and also excellent applications in hydrotherapy and balneotherapy have been described [66]. In the last decade there has been an increasing interest in the employment of humic materials in medicine and biology, because of their antiviral, profibrinolytic, anti-inflammatory and estrogenic activities [20]. Medical studies show the power of humic materials to protect against cancer and related cancer-causing viruses using special humic substances therapies [67].

2.3 Chemical modification of humic acids

Interest in the chemical modification of humic acids of different origin is primarily caused by the possibility of obtaining preparations with valuable properties, which are superior to the initial humic acids. The necessity of modification with the minimum destruction of the initial structure of humic acids plays an important role in the selection of a modification method, because it was found that humic acid molecule as a whole are responsible for the biological effect [68].

Low mechanical strength, strong swellability in water and aqueous acidic solutions, partial solubility in water and complete dissolution in alkaline solutions hinder wide use of humic acids. Modification of HAs can enhance their reactivity, thermal and chemical resistance, and sorption properties and affect their solubility [69]. It was found that the modified products surpass HAs in some respects and show promise for industrial use.

Point of interest for Lebedova et al. was to modify the HAs structure by converting some functional groups into groups responsible for bioactivity (phenolic and carboxy) [70]. In this case, it was undesirable to use such destructive methods as oxidation with nitric acid or peroxide, etc., since oxidative degradation leads to a considerable decrease in the molecular weight of HAs.

In addition (natural processes), the apparently unstable conformation of humic superstructures could be stabilized by increasing the number of intermolecular covalent bonds via an oxidative coupling reaction catalyzed by a phenoloxidase enzyme [71]. The extent of covalent polymerization should be a function of the amount of humic molecules, mainly phenolic or benzenecarboxylic acids derived from lignin and microbial biosynthesis [2], which may undergo oxidative coupling reactions [72].

2.3.1 Chemical crosslinking

Special type of modification the chemical structure of HAs is crosslinking. Every chemical modification process (included crosslinking) involves the reaction of one functional group with another resulting in the formation of a covalent bond. Of the hundreds of reagents described in the literature [73] or offered commercially, most utilize common organic chemical principles that can be reduced to a few primary reactions. An understanding of these basic reactions is a requirement for the application of crosslinking reagents to relevant questions in chemistry [74].

Crosslinking is believed to play a role in the humification of soil/sediment organic matter (SOM) and was confirmed to be involved in the diagenesis of organic geopolymers. Crosslinking increases molecular weight, creates network structures, reduces solubility, inhibites biodegradation, increases matrix rigidity, decreases free volume and influences the sorbent properties of the solid towards organic compounds [75]. Such changes may be important for the ecological function of humic substances, yet the role of crosslinking in these substances has poorly been investigated [5], [76].

Only limited evidence exists for effects of crosslinking on properties, biological stability and sorptive properties and function of organic matter and humic acids. Crosslinking is thought to be one way in which organic matter is stabilized and protected in soil [77], because it increases molecular weight and decreases bioavailability, but direct evidence for this effect is unavailable.

The carboxyl and hydroxyl function groups are abundant in humic substances. Therefore, for the modification of HAs many of researchers applied different organic crosslinking agents known to link hydroxyl and carboxyl groups in polymers [5], [69], [76].

In particular, HAs can be crosslinked with formaldehyde and urea [69]. In the case of formaldehyde, the reaction mechanism is similar to that by which phenol-formaldehyde oligomer is formed. If curing by the resol type is intended, then the highest content of formaldehyde is possible and desirable [69]. Addition of formaldehyde may cause redistribution of the electron density in the condensed HAs system, enhancing the acid properties of weak acid groups and thus increasing the static exchange capacities [69].

2.3.1.1 Zero-length crosslinker

Specific and commonly used crosslinkers are so-called “Zero-length crosslinkers”, the smallest available reagent systems for chemical crosslinking. These compounds mediate crosslinking between two molecules by creating a bond without an intervening linker [74].

Zero-length crosslinking reagents do not incorporate any atoms to the reactive crosslinked species due to the lack of a spacer arm. This provides a major advantage in the form of providing more precise distance constraints as the crosslinkable groups must be within appropriate distances in order to react [78].

Zero-length crosslinks offer several key advantages over their longer counterparts. As mentioned above, the primary advantage is more precision on the derived distance constraints [78]. Moreover, because the reaction can only occur between residues that are roughly within specific distance of each other, residue interactions captured by this method are more likely to describe direct contact sites rather than simply sites that are in close proximity [79].

Zero-length crosslink experiments are also less likely to generate „self-linked“ products that occur, when interacting reactive groups are on the same molecules [79], as both reactive groups would have to be on the same molecules and directly interact with each other.

In addition, zero-length crosslinking reagents are less likely to generate „dead-end“ products, which occur when only one of the reactive groups is able to react with a site on a matrix and the second site reacts either with water or a quenching reagent, compared to other crosslinkers [80].

2.3.1.2 Carbodiimides crosslinkers

Carbodiimides (RN=C=NR) are unsaturated compounds with an allene structure. Since their first synthesis from thioureas at the end of the last century, carbodiimides have been widely used in organic synthesis and biotechnology. Their synthetic methods as well as their physical and chemical properties are summarized in review article [81]. The condensation of carboxylic acids with a variety of nucleophiles in the presence of dialkylcarbodiimides becomes to be one of the most commonly used synthetic methods despite the development of new reagents. The main applications of carbodiimide coupling remain the formation of amide bonds. In addition, extensive studies have been also devoted to reactions of carbodiimides with water, alcohols, amines, phenols and carboxylic acids. Therefore, this approach is also highly useful for the preparation of anhydrides, esters and thiosters. Applicability of carbodiimides in both organic and aqueous solvents contributes to the wide spectrum of possible conjugation reactions [82].

This substance class is probably the most widely used type of zero-length crosslinkers, which are applied to mediate amide bond formation between a carboxylate and an amine group (or phosphoramidate bond formation between a phosphate and an amine) [83].

To adjust carbodiimide couplings to multiple applications a number of coupling catalyst and additives have been proposed, e.g. N-hydroxybenzotriazole and N-hydroxysuccinimide, widely used for the preparation of the corresponding active esters capable of the efficient acylation of amino groups in amino acids. Acylation catalysts, mainly pyridine derivates e.g. 4-dimethylaminopyridine, are used to increase the reactivity of electrophilic species through carbodiimide couplings [82].

Most of the studies are based on amide formation under very mild conditions between carboxylic acids and amines in aqueous and organic systems in the presence of carbodiimides.

Carbodiimide-mediated amide formation occurs effectively between pH 4.5 and 7.5 [74]. The reaction of carbodiimide with carboxyl groups proceeds most rapidly in the pH range from 3.5 to 4.5. This indicates that both of protons and dissociated carboxyl groups are necessary for the reaction of carbodiimide [84].

Bioconjugation by carbodiimides has also been widely performed, because no residues remain in modifying and crosslinking proteins (zero-length crosslinker [78], [84].

N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide (EDC) is mostly applied in combination with sulfo-NHS (N-hydroxysulfosuccinimide) [85], which hydrolyzes slowly in aqueous solution [86]. This crosslinker creates an unstable reactive acylisourea ester. The intermediate ester can then interact with a primary amine to form a peptidyl bond with the elimination of a water molecule. Ordinarily, this intermediate species has a lifetime of several seconds; but addition of a second reagent, N-hydroxysuccinimide (NHS) or its water-soluble sulfo-derivative (sulfo-NHS) greatly extends the stability of this reactive intermediate, and thus significantly enhances the extent of crosslinking [86], [87]. EDC/sulfo-NHS-coupled reactions are highly efficient and usually increase the yield of crosslinking in comparison with that obtained by merely using EDC [86]. EDC is quite stable at the neutral and higher pH regions, at least, for 5 h at 25 °C. On the other hand, the activity of EDC decreased at low pH [84]. In this case the reactive groups need to be accessible to the aqueous phase. As with most other types of crosslinkers, the specificity of zero-length crosslinkers such as EDC is a double-edged sword [78].

The intermediate ester is relatively unstable and reverts back to the unmodified carboxyl if it does not react with an amine. As a result, EDC dead-end adducts have only been observed at high concentrations of crosslinker [80]. In contrast, dead-end products are common and can often have higher stoichiometries than actual crosslinks for other crosslinking chemistries. Dead-end products occur when only one of the reactive groups is able to react with a site on a protein and the second site either reacts with water or a quenching reagent.

N,N'-dicyclohexylcarbodiimide (DCC) has both biochemical and synthetic applications. This reagent can be used to couple primary amines to carboxylic acids functional groups. DCC is soluble in many organic solvents. DCC have been used to form ester linkages or amides with the corresponding carboxylic acids at high efficacy in anhydrous solutions [85]. Carboxyl groups can be also linked by hydroxyl by DCC and DMAP (4-dimethylaminopyriine)/nucleophilic catalyst can works as mediated acylation, i.e Steglich esterification.

2.4 Humic substances analysis

A serious analytical problem arises, when HS from different origins have to be characterized and identified in relation to their original parent sources. Different origin and diagenesis of nature deposites, differs radically from the next, depending on plant residues and coal generating conditions make difficult unification of studies these substances [88].

In fact, the only way to characterize such HS is to apply a multiple-technique approach, which can generally provide a more coherent picture of this complex molecular system. Although these approaches are powerful in the investigation of HS, there are certain limitations regarding e.g. the duration of machine analysis required to obtain a good signal-noise ratio (e.g. NMR, spectroscopy) and the tedious and lengthy procedure of pretreatment (purification, extraction, required sample form preparation). Moreover, even if the treatment dramatically reduces impurities, it can cause structural alterations and artifacts in HS [89].

To overcome the limitations inherent to the traditional analytical approach, there is need for fully instrumental methods of determination, which are accurate, rapid and free from interference. In this way, a direct analysis of HS without chemical pretreatment could improve the understanding of their geochemistry and identify their origin. The use of physical

together with chemical procedures is attractive. In fact, almost all existing experimental techniques have been proposed to study separation, fractionation, chemical characterization and physico-chemical behavior of HAs.

2.5 Selected methods of humic acids characterization

These several of short chapters have the purpose to enlighten the fundamentals of function principles of instrumental analytical techniques used during the experiment part of work on this thesis. These paragraphs are appropriately concise and do not attempt to explain the analytical techniques completely. Most of the techniques applied to study HAs have either intrinsic or practical limitations. Therefore, suitable combination is necessary. Hence, in this work, the attempt is paid to combine and discuss the application of two apparently different methods such as thermal analysis and nuclear magnetic resonance (NMR) relaxometry.

2.5.1 Thermogravimetric analysis

Thermal analytical techniques are useful to obtain information on physical and chemical properties of a given sample as a function of time or temperature. The information obtained from simple, inexpensive and rapid application of thermoanalytical techniques to organic samples can provide valuable data concerning to an ecosystem, and infer orientation for the use of the land for agricultural practices.

Thermogravimetric analysis (TGA) is a technique of thermal analysis, which examines the mass change of a sample as a function of temperature or time under defined atmosphere. Usually, it is used to characterize the decomposition and thermal stability of materials under a variety of conditions and to examine the kinetics of physico-chemical processes occurring in the sample [90]. Thermogravimetry has become popular for studying such properties of solids with the advantage in using small samples with temperature control for continuous and simultaneous measurement of weight loss at high sensitivity. The results are essentially quantitative and influenced by heating rate, mass of samples, gas flow rate, sample packing and the gaseous atmosphere.

TGA and DTA (differential thermal analysis) analyses have been proposed as methods to characterize the genesis of coal [91] and HS from different environments [92]. In addition, they have furnished also important information concerning the structural composition of humic molecules [93], [94].

In studies of humic material, TGA has been applied to both quantify the moisture and ash contents [95] as well as to observe and characterize structural changes in the material during heating [96]. This technique can contribute to understanding the heating effects on HAs components such as loss of water, organic matter combustion and dehydroxylation, in order to compare the relative abundance of more and less labile carbon sources [97]. Due to the intrinsic molecular complexity of these materials, however, the interpretation of the thermal curves and related processes is not yet completely clear.

In generally, the first endothermic peak on the TGA-DTA curves of HAs appears usually between 90 and 120 °C with a mass loss typically up to 10 % (depending on the storage conditions) corresponding to the dehydration of the samples [5]. The water molecule elimination occurred from the external sphere and not from the internal layers of the matrices and did not cause modification of the structure. This process is associated with a reversible phenomenon and depends not only on the thermolabile mineral content, but also on the

organic components [97]. The mechanisms of water release indicate that these molecules are weakly-bonded.

An intense first exothermic reaction occurs commonly around 400 °C with a second at 550 °C. The first exothermic peak is considered to be the result of thermal combustion of thermolabile fragments HAs as polysaccharides, decarboxylations of acidic groups and dehydration of hydroxylate aliphatic structures [98], [99]. Ioselis et al. also observed the decomposition of carboxylic, phenolic, carbonyl and alcoholic groups in HS at about 300 °C [100].

The second exothermic peak is related to the combustion of aromatic structures and cleavage of C–C bonds [93], [99]. In the analytical conditions of TGA and DTA analysis (with a heating rate of 10 °C min^{-1}) aromatic structures can be formed from cyclic structures. The cyclic structures can be both, present in the original composition of the HS or be generated by cyclization of aliphatic chains, upon heating [34]. In addition, the presence of long chain hydrocarbons and N compounds can contribute to thermal reactions over 450 °C.

The combustion reaction of the first exothermic peak is constantly lower than the second one, suggesting the presence of a small proportion of carbohydrates and hydroxylated aliphatic structures [101], [102] (or the combustion heat of these fraction is smaller). The more intense combustion reaction of the second peak is due to the cracking of higher molecular weight polynuclear systems [103]. Higher degree of polymerization caused higher thermal stability and resistance to heating [104].

The last thermal effect is assumed to be related to high temperature combustion accompanied by a polycondensation reaction [105], involves the condensation of the higher molecular weight polynuclear systems into coke [103].

The variability of exothermic peaks found between the HAs from the same origin is related to the provenance of the humic acids deposits, the environmental conditions and type of organic material that occur during humification. All these variables markedly alter the structure and physico-chemical features of humic substances [106].

The organic matter decomposition at high temperature is an indicative behavior that could be associated with its corresponding macromolecular domain, with an expected increase in structural stability [104].

In case of thermal degradation, residual fractions of HAs can be represented by aromatic heterocycles. These macromolecular compounds resistant to thermal degradation explains their long permanence times in soils [107].

Ishiwatari et al. showed that the thermal profile of HAs can be influenced by the accumulation of recent C such as cellulose and non- “typical” lignin structures [108]. The presence of these components in the HAs structure can be justified considering the mainly anaerobic environment in which humic materials originates. In these conditions, also the cellulose components are slowly decomposed and plant residues may be accumulated and their structure may be preserved during the humification process. It was found that the ionization of carboxylic and phenolic groups increased the thermal stability of HS [93].

2.5.2 Differential scanning calorimetry

The differential scanning calorimetric (DSC) is a technique in which the temperature or heat flow into the sample is monitored as a function of the chosen temperature program [109]. DSC method is a rapid technique to give characteristic curves, whose variation in enthalpy is

associated with phase changes in sample, reflecting events related to structures and chemical compositions [110].

Standard phase transitions of water are realize usually in the temperature scale achievable be DSC equipment. Therefore, DSC measurement is frequently used for studying of water interaction, hydration properties, distribution and state of water in many different materials [111], [112] included humic acids [5], [113].

Water present in hydrogels and hydrogen-like systems exists in a continuum of states between the extreme non-freezing and freezing forms. The strength of the interaction of water with polar groups is different. It is known that interaction of water molecules with the amine group is weaker than with the hydroxyl one [114].

DSC is an indirect probe of water behavior in the sense that it monitors the heat capacity associated with a phase transition induced by a temperature change. Information is extracted from (i) changes in heat capacity, (ii) the magnitude of the heat given out (exothermic) or taken in (endothermic) during the thermal event, (iii) the shape of the endotherms or exotherms and (iii) the temperature at which the thermograms occur. The recorded data are sensitive to experimental conditions, in particular, to the heating/cooling rates [115].

The amount of different type of water can be estimated by DSC, however, obtained information is limited [109], [116].

2.5.3 Low field NMR relaxometry

In general, NMR is a direct probe of the molecular environment, orientation and motion of resonant nuclei (most commonly, ^{1}H, ^{19}F, ^{2}D, ^{13}C, $^{17}0$). The technique is sensitive to molecular motions within a frequency range that typically spans some decades, depending on the particular NMR experiment. The ability of NMR to distinguish characteristically different molecular environments and motions of a resonant nucleus in a polymer is a unique, site-specific, feature of the technique [115].

The NMR relaxometry is less known than the conventional high-field spectroscopy. The magnetic field is too low to resolve chemical shifts so that all protons are observed at the same resonance frequency. They are discriminated by variations in their relaxation time [117].

There are two types of relaxation processes occurring simultaneously. First, spin-lattice relaxation (longitudinal relaxation) characterized by spin-lattice relaxation time constant T1 (enthalpic character). Second, spin-spin relaxation (transverse relaxation) is characterized by transversal relaxation time constant T2 and on the contrary has an entropic character [118]. In general, transverse relaxation times T2 are used to study the swelling, because of the much faster determination compared to longitudinal relaxation times T1 [117], [119], [120].

NMR relaxometry experiments employ several pulse sequences, most frequently the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence to measure the transverse relaxation time T2. This pulse sequence involves application of a 90° pulse, followed by a train of 180° pulses separated by a delay time (τ). This method is popular, because it allows rapid collection of data. The relaxation time is dominated by the motion of the water molecules so that it is long (s) when molecules are free to rotate as they are in the bulk liquid. When water molecules are bound to pore walls or confined in very small pores, relaxation times can become very short (ms). It is important to recall that bound-water molecules may exchange rapidly on the NMR time scale with neighboring bulk water (chemical exchange). A group of such fast-exchanging waters will produce a single weighted average NMR signal [117].

2.5.3.1 Study of swelling by NMR relaxometry

Many macromolecular substances, such as synthetic polymers or biopolymers, swell when placed in contact with fluids. The swelling is characterized as sorption of the liquid into the macromolecular solid phase, which in turn, increases its volume. The amount of swelling depends both on the nature of fluid and sorbent. Strongly crosslinked materials swell less than weakly crosslinked humic materials [119].

Swelling kinetics experiments of soil samples using NMR relaxometry have shown that the swelling process is linked to the migration of the peak relaxation times towards smaller relaxation times and the increase of the amount of water protons relaxing at smaller relaxation times [117], [120]. These findings were attributed to a redistribution of water during swelling. Todoruk et al. [117] identified two processes with time constants of about 1 day for a fast process and up to 22 days for a slow process for different soils, which were comparable to the values reported by Schaumann [120]. The result suggested diffusion processes and chemical reactions, such as ester hydrolysis [117]. The slow process was attributed to the formation of gel phases and to the slow water intrusion into micropores, which re-opened during swelling.

2.5.4 Elemental analysis

Elemental analysis is based on the classical principle of high temperature combustion. The formed gases are quantitatively determined on a thermo-conductivity detector. An attached computer calculates the element concentrations from detector signal and sample weight.

Due to their complexity, specific information on HS elemental composition are not very conclusive and, because of this, the respective atomic ratios (H/C and N/C) have been preferentially employed to establish the organic matter source, the degree of condensation, the diagenetic transformations and the environmental conditions under which the HS were generated [121].

C/N ratio is indicative of a stage of coalification [122]. As the coalification process goes on, the increase in C/N ratio observed is probably due to a further loss of proteins which, together with the carbohydrates, are the most easily destroyed [37]. These changes could be interpreted by different chemical and microbiological processes involved in the coalification process from peat to lignite.

How the N can persist in organic materials is an important question. Knicker et al. [123] demonstrated the existence of a resistant form of N in HAs attributing its presence to heterocyclic N compounds such as porphyrins and metalloporphyrins. Heterocyclic N is a less significant contributor to humic composition. Nitrogen, the central element of porphyrin structures, was involved mainly in the complexation of metals. The presence of these N forms could represent a potential use of coal HAs as an N reservoir in soil.

By using elemental analysis, it is known that atomic ratios indicate the humification status, e. g. the H/C ratio of a HAs sample can range from 2 (aliphatic, as in a lipid) to < 1 (naphtalene, $C_{10}H_8$, and other polyaromatic hydrocarbons). Unsubstituted aliphatic chains (high H/C) might be responsible for the poorer solubility of HAs during the acidification step [34]. The O/C ratio could range from 0 to 2 (in CO_2) and will be higher for wet samples. However, the majority of soils HAs have a narrow range of these ratios.

In evaluation and interpretation of elemental analysis should be considered, however, that HS are complex materials and that differences observed among them could be related not only to the source-specific influences but also to the mechanisms of humus genesis, including the

bacterial metabolism typical of the specific environment, the degree of humification and the physico-chemical characteristics of the surrounding areas [34].

2.5.5 Fourier Transformed Infra-Red spectroscopy

FTIR relies on the fact that the most molecules can absorb light in the infra-red region of the electromagnetic spectrum. Specific molecules absorb light energy at specific wavelengths, known as their resonance frequencies. This absorption initiates transitions between vibrational states of bonds contained within the molecule. The resultant absorption spectrum from the bond natural vibration frequencies indicates the presence of various chemical bonds and functional groups present in the sample.

In fact, the polar chemical functionalities absorbing infrared radiation such as phenolic and alcoholic hydroxyls, aromatic and aliphatic carboxyls and carbonyls, aliphatic C-H and amides, might be present in all the studied HS, regardless of their origin. Such interpretation is in line with the concepts attributed to humus mixtures, according to which they might consist of associations of molecules, with a "universal average formula unit", held together via supramolecular interactions [50], [124]. The relative contents of these groups, on the other hand, can differ from one sample to another and might also be dependent on the extraction procedures. In addition, the variations in the spectra may come from slight differences in their composition. Only a careful analysis of the spectra can reveal specific characteristics.

In humic substances, all these types of typical vibration (OH stretching appears around at 3560–3410 cm^{-1}, C–H band at around 2920–2850 cm^{-1}, amidic C=O and amide II band at 1550–1510 cm^{-1}, COOH at around 1200–1220 cm^{-1}, C=O at 1720 cm^{-1}, C=C stretching at 1640–1615 cm^{-1}, C–O of polysaccharide bands at 1040–1030 cm^{-1}) are expected to exist and their corresponding frequency signals might be overlapped in this region, the shifts to the right or left might therefore be results of the relative influence of each group [34], [125].

2.6 Water

Various natural and synthetic polymers, which have hydrophilic groups such as hydroxyl, carboxyl and carbonyl, show strong or weak interaction with water. Through this interaction, physical properties such as thermal properties of both, polymers and water are markedly influenced [109], [116].

Water molecules adsorbed by a polymer containing different types of polar groups form layered clusters with different characteristics depending on their microenvironment. Several factors play an important role in determining the intensity of these interactive forces, such as electrostatic fields, the nature of the polar groups (strong or weak acids), and the distance between the water molecule and the polar group [126].

The ion composed of a carboxylate anion on the polymer chains and a mobile counter cation is more or less dissociated in the presence of water. Both the ion pair and the dissociated ions have their own ability to attract water molecules, depending on their strength and their dissociation degree. However, the dissociated ions always have larger hydration ability than the corresponding ion pair [111].

The NMR study has shown that water in the system is in a state ranging from non-rigid solid to viscous liquid and that the motion of counter ions in the system is profoundly influenced by the molecular motion of water [109], [116]. However, the molecular behavior of the water-polyelectrolyte system has not yet been fully understood.

Due to the modification of the structure of polymer materials after water sorption, the mechanical and the physical properties of hydrophilic polymers can vary significantly with water activity in the surrounding medium. Upon water absorption, small or less perfect polymer crystallites can be destroyed [111].

Water molecules may exhibit both plasticizing [127] and anti-plasticizing [128], [129] properties in isolated humic substances. The „anomalous“ behavior of water in polymer materials can be attributed to the effect of capillary condensation, the confinement of water clusters by polymer chains [130] or the strong interactions of the water molecules with the polar groups of hydrophilic polymer [131] either directly or via other water molecules.

In summary, the behavior of water is influenced by physical-spatial as well as chemical interactions. Size effects imposed by boundaries and interfaces reduce the number of degrees of freedom of the water molecules, and therefore, the natural order observed in pure water in the bulk is diminished; this is particularly true at low temperatures, when the host polymer matrix is rigid, or nearly so. Chemical effects include hydrogen-bonding to binding sites such as ion clusters, ester, amide, carboxyl and polar sites [132]. A hierarchy of interactions has been proposed in order of decreasing energy: ion-ion > water-ion > water-polar = polar-polar = water-water > water-hydrophobic [133].

2.6.1 Water phase transitions

Water represents an important medium and is required for life-giving processes. Therefore, for better understanding of living pathways is necessary to deeper clarification of the water properties and behavior. Generally speaking, the investigations on the states of water in a polymer must give valuable information on the sorption, diffusion and permeation properties of molecular species in hydrophilic polymers [111]. One of the simplest approaches to study the hydration of water is focused on the exploration of water phase transition.

DSC studies have shown that water molecules, directly associated with the ionic group, do not display a first-order phase transition [134]. One reason for not finding any first-order phase transition with DSC could also be that not enough water molecules are present. The freezing-melting phenomenon is macroscopic, thus demanding a larger number of water molecules present to take place [126].

NMR relaxation studies have also suggested that the molecular motion of water molecules around the ionic groups is markedly restricted, compared with that of free water [135]. Such fractions of water are referred as non-freezing water. Non-freezing water is usually the first 1–3 layers of water molecules (depend on the type of material) adjacent to a surface and does not freeze because the motion of water structures is severely limited by the close association with the surfaces [136].

Crystallization as well as melting of water in hydrophilic polymers are complex processes. At low water content, all the sorbed water molecules are non-freezable (no melting point detected). The water begins to crystallize only when the water content is above a characteristic threshold. As far as the nature and the structure of polymer are concerned, the maximum quantity of non-freezing water is characteristic value. It should reflect on the ability of the polar groups to interact with water molecules in the polymer matrix. This ability would depend on the physico-chemical affinity of the polymer groups to water, the free space available in the vicinity of the sites as well as the local elastic resistance of the chains to a swelling deformation stress [111].

The mechanism of formation of non-freezing water in polymers is not explained [137], [138]. It is generally accepted that non-freezing water is formed by the hydrogen bonds between water molecules and polar groups in the polymer. Liu et al. analyzed the origin of non-freezing water in gelatin/water samples by DSC, positron annihilation lifetime spectroscopy and NMR spectroscopy and proposed that nanocavity in polymers is an important reason for the formation of non-freezing water [139].

The existence of non-freezing water in the polymer also suggest that the interactions between a water molecule and small fraction of the polar carboxyl groups are strong enough to prevent water to move to other places to form ice crystals when the sample is cooled down to low temperatures. These interaction would be well-oriented hydrogen bonds between water (H-bond donor) and the carboxyl groups (H-bond acceptor), which were locally in a favorable configuration [111].

It should be emphasized that the cause of non-crystallizability of water molecules absorbed in polymer should not be the same for hydrophilic polymers as for hydrophobic ones. For the latter, the part of non-freezing water is very small and corresponds to a random dispersion of the water molecules (due to an entropy effect) throughout the entire polymer matrix. On the contrary, in hydrophilic polymers, water molecules are so strongly bound to specific polar sites by hydrogen bonds that they cannot gather with other molecules to form crystallizable water. The removal of water molecules from the sites would require a large driving force (chemical potential), which cannot be provided by surrounding medium during cooling. However, there should also be a dynamic exchange of water molecules between sites and hydration layers. Apparently, these exchanges did not prevent the molecules from being under the polar-site interaction field, which kept the water molecules non-freezable [111].

Less closely associate water fractions exhibit melting and crystallization, showing considerable super cooling and significantly smaller enthalpy than that of bulk water. These water fractions are defined as freezing water. These freezing water molecules cannot form normal ice structures due mainly to the influence of the polar groups. The effect of the polar groups on the water molecules is thought to be realized through several layers of water molecules. The mobility of the water molecules thus increases with increasing distance from the polar group [126]. The structure will be perturbed, giving the water non-bulk-like thermodynamic parameters. These water molecules are more weakly influenced by the ionic groups, which gave less ordered water molecules and which, in turn, lead to a higher mobility than that of the bulk water. It is, however, not certain that the interaction forces from the ionic sites are the only reason for the formation of nonfavoured ice structures. Another contributing factor could also be physical packing problems on the surface [126].

The amount of freezing water in a hydrophilic polymer depends on the hydrogen bonding ability of the polymer polar groups. The higher resistance of the matrix to a swelling action, the lower total quantity of water (i.e. that of freezing water) absorbed in the polymer [111]. The quantity of freezing water is generally characterized by the integration of the endotherm (melting of ice) [140] since the exothermic curve may not be detected during cooling in some cases [141]. The melting characteristic of the water is strongly affected by the sample matrix. The broad shape of the onset may be due to the binding forces for water molecules within this boundary phase, which change strongly with increasing distance from the surfaces. At a certain distance from the surfaces and outside this boundary phase, the water molecules are still affected by the surfaces, but in a more uniform way [119].

This procedure for freezing water calculation does not take into account the difference in the melting enthalpies of ice of different crystal structure and existence of amorphous ice. Freezing water content must have a certain kind of crystalline structure, which may be the same as the structure of natural ice. There are nine polymorphic forms of ice possible, such as ice I, Ic, II, III, IV, V, VI, VII and VIII reported [142], [143]. However, the structures from ice IV to ice VII (and other specific ice polymorphs) are found only at very high pressures. Accordingly, in the usual experimental conditions that treat the above kinds of water sorbed on polymers, the possible structures of ice for freezing water can be considered to be ice I, Ic, II and III. The maximum value of melting enthalpy of ice (ice I) is estimated as 334 J g^{-1}, and the minimum value of melting of ice (ice III) is estimated as 311 J g^{-1} from the phase diagram of water [143]. The maximum error in the calculated amounts of freezing water is expected to be only 6.6 % [111]. Increasing water content causes either decrease in heterogeneity of ice structures or occurrence of additional processes during the ice melting (e.g., transition from Ic (cubic) to I (hexagonal) ice, see e.g., ref. [112].

The sum of the freezing and non-freezing water fractions is the bound water content [109], [116]. The nomenclature of water fractions varies, however, among authors. The term "bound" is used to indicate that this water is influenced by the polar groups of the polymer. The presence of bulk-like water (with ice melting point of ca. 0 °C) can be explained by the formation of water clusters in water-saturated system. An alternative to this explanation is that water molecules were dispersed mono-molecularly in the polymer, but upon cooling, water molecules, which are close enough to each other, agglomerate to form small ice crystals. This would be eventually possible due to weak polymer-water interactions [111].

The quantity of bulk water depends greatly on the nature of the polymer matrix. If there are no physical or chemical crosslinks between polymer chains, a hydrophilic polymer can absorb more and more water when the external water activity increases, and ultimately dissolves in liquid water [111].

The total water content, Wc, is usually defined as Wc (g g^{-1}) = (water, g)/(dry polymer, g) [140].

Above mentioned approach is widely applied to study of system humic material/water [5], [111], [113]. At very low water content, all water molecules are present in the form of non-freezing water, i.e., all of them are restricted by the intimate contact with the humic molecules surface and thus they cannot participate in ice formation [113], [119]. Freezing water represents a kind of water/ice whose structure is affected by the interaction with humic molecules, mainly by polar groups and by the distribution of pores in the physical structure [119].

2.6.2 Hydration of organic matter

Hydration is the crucial factor playing role in biological function of molecules in both living and natural systems. Water represents an important medium for nutrient transport, cell membrane processes, induces biologically active conformation of biomolecules, etc.

Hydrated polymer systems have been widely investigated owing to the effect of water on the performance of commercial polymers and the crucial role played by water-polymer interactions in biological processes. In the presence of excess water, a polymer may become swollen, exhibiting major changes in mechanical and chemical properties. Water can plasticize the polymer matrix or form stable bridges through hydrogen bonding, resulting in an anti-plasticizing effect. The behavior of water can be transformed in the presence of a

polymer, depending on the degree of chemical or physical association between the water and polymer phases [116].

Kučerík et al. confirmed the observation that during hydration not only new surfaces are wetted, but also the change in the physical structure of HS takes part. This causes the existence of ‘‘restricted’’ water, which is reflected by appearance of cold crystallization on DSC exotherm [113]. The results an increase in heterogeneity in the sample, potentially caused by an increase in the distribution of inner places and surfaces in humic matrix, such as cavities and holes in which water molecules experience a variety of interactions and physical states [113].

The motion of water confined in polar cavities or bound on the polar surface is more restricted and therefore the formation of ice is associated with formation of less perfect crystals or even amorphous ice than in case of bulk water or water on hydrophobic surface [144].

The recent statement introduce that the wetting of organic matter proceeds in two steps [145]. The first step includes wetting of the surfaces and after that the water is distributed to the pore volume. Kinetics of the movement of the water from one compartment to another determined on „relaxation“ assumption shows a fast process, occurring within 24 hours, and a slow process, which requires significantly longer (up to 22 days) [117]. During the wetting a small pores are opened by water (pore expansion processes) subsequent migration of water into these pores results in an apparent decrease in T2 relaxation time since restrictions on the molecular motion increase[117]. Essentially, this results in disruption of hydrogen bonds, ionic interactions, and possibly covalent bonds between and within clay and organic matter. One can not exclude that this process may be accompanied by a hydrolysis reaction. As bonds break, reoriented organic matter and released organic matter and clay may then take up water [117]. In contrast, Jaeger et al. described hydration of peat as a three step process including breaking of hydrogen bonds, water diffusion, and reorientation of molecular chains during hydration [119]. These differences are most probably due to significantly larger heterogeneity of the whole peat sample in contrast to the purified HS [113].

Due to the porous structure of HAs matrix, water is present in different states influencing the ice melting enthalpy. The step-like dependency indicates the mechanisms of hydration: water penetrates the cavity and increases its volume by swelling, while the water still remains largely freezable, and the melting enthalpy increases with increasing water content. As soon as the water content exceeds the capacity of the cavity, weak interactions stabilizing the domain are broken and water can penetrate further and wet another set of surfaces, which are most probably more hydrophobic as they are wetted only at higher water content. Kucerik et al. assumed that the spatial separation between hydrophilic and hydrophobic hotspots of the HAs is at least partially induced by the hydration process itself; as a result of the hydrophobic effect, hydrophobic molecules are thermodynamically separated and ‘‘packed’’ away forming porous scaffold. The scaffold stabilizing the structure is preserved and potentially strengthened during hydration [113].

2.6.3 Water evaporation and sorption

Thermal behavior of water can be investigated by vaporization technique if the appropriate experimental condition is selected [5], [146].

Water molecules evaporation ordinarily terminates at a temperature higher than 100 °C, previously established intermolecular bonding between the hydrophilic groups and water

molecules are broken[116], [147]. In generally, it is considered that the higher order structure of matrix molecules seems to affect the vaporization temperature, when water molecules are strongly restrained [146].

Among various factors affecting the vaporization behavior of restrained bound water, atmospheric conditions and heating velocity are considered to be most important [147].

In the drying process of hydrophilic polymers, it is difficult to attain a completely dry state under usual conditions. Hence, the dryness of the polymers is not concretely defined due to strong hydrogen bonding established between the hydrophilic groups and water molecules [146].

Water molecules at low temperatures are preferentially located or partitioned at hydrophilic equilibrium sites with high enthalpies of adsorption and high barriers to translation. The average thermal energy of the system at elevated temperatures exceeds that due to any localized polarization, thus diminishing the probability of strong site interactions because of the increased average energy of the system as a whole. As the temperature increases towards ambient and above, polymer-water interactions become comparable to water-water interactions in the bulk liquid, dipolar interactions are not appreciably different for monomeric or polymeric species and dissociation of water involved in hydrogen-bonding becomes more facile [115].

Johnson et al. for example, determined the amount of water forming as clusters in the microcavities of hydrophobic polymers [148]. This is the simplest case for determining the amount of water since in hydrophobic polymers it forms a separate phase and consequently has no plasticizing effect. An increase in hydrophilicity leads to a more complicated picture of the state and amounts of water in the polymer.

Recently, Rault and coworkers published paper dealing with water in various hydrophilic polymers [149]. They did not use the concept of bound and unbound water but designate the system polymer/water, in which the water acts as a plasticizer. A critical weight fraction C* of the water in the system was defined: below C* water does not crystallize, whereas above C* it crystallizes partially, forming ice crystals.

The following schematic way of the progress adsorption of water molecules for a polar group has to been designed. The first nonfreezing water molecules are probably tightly adsorbed in the perimeter of the ion pair. As the adsorption proceeds, more and more water molecules are slowly adsorbed, progressively enabling a dissociation of the ion pair. The adsorbed water will form clusters around the polar groups as the adsorption proceeds. Within these clusters, different layers of adsorbed water will be formed. There is no clear distinction between the different layers within the clusters. The characteristics of the polar group have a great influence on the size of the cluster and on the amount and type of water adsorbed. The adsorbed water will eventually attain bulk-like water properties when the distance from the polar groups is sufficiently large. This water will then form normal water-water hydrogen bonds undisturbed by the polar groups [126].

It was observed that sorption at a given compound activity was strongly increased upon hydration (or solvation by strongly interacting organic solvents). This solvent-assisted sorption was conceived to result from formation of new sorption sites upon solvation of the organic matter. This mechanism involves solvent-assisted penetration of organic sorbates into noncovalently linked moieties that are not available for compound sorption in dry state due to strong intra- interactions (e.g., H-bonding, proton-transfer phenomena, bridging via metal cations) [150].

The interplay between solvent-assisted penetration into SOM contacts and sorbate/solvent competition for new sites at those disrupted contacts determines the overall solvent effect on sorption. Tradeoff between solvent-assisted penetration of organic compound molecules into polar contacts and competition between sorbate and solvent molecules for sorption sites is strongly affected by the interaction potential of a sorbate molecule and its ability to compete with water. When a compound is sufficiently effective in competing with water molecules for sorption sites of a given SOM, there is a chance for newly formed sites to contribute to increased sorption of a compound in the hydrated sorbent. When a compound is not sufficiently competitive, not only is hydration-assisted sorption absent, but even a reduction in sorption may be expected due to water competition for sorption sites accessible in the original dried SOM. Therefore, multiple sorption scenarios are anticipated including hydration-assisted sorption (benzyl alcohol and m-nitrophenol on peat and humin), hydration-reduced sorption (acetophenone and nitrobenzene on humin), and apparent lack of a hydration effect (acetophenone and nitrobenzene on peat) [150].

2.6.4 Pore size distribution

Pore properties such as pore shape, pore volume, and pore size distribution are important features of all porous media. These parameters affect the swelling capability that is directly related to mechanical and optical properties.

Pore analysis is not straightforward and difficult to define by a single number. For a complete analysis, pore dimension, geometry, topology, connectivity, and number of isolated pores should be considered. This, however, is not realistic and, in many cases, pores are assumed to be cylindrical in shape. Based on this, pore size distribution has been determined by various methods [151].

Water held in the capillaries of porous materials has a depressed melting temperature because of the lower pressure at a curved interface in cavities. The melting temperature depression has a reciprocal relationship with the pore diameter and thus the pore size distribution can be evaluated. Therefore, DSC method “thermoporosimetry” can be used to investigate the pore size distribution at different moisture ratios [151].

Another approach uses water evaporation. In case of study the cellulose, with the drying, water is evaporated from the pores and the pores collapse due to the capillary forces with the high surface tension of water. When the water starts to leave the pore, a very low pressure can exist in the water left in the pores, pulling the cell wall together as shown in Fig. 8. [151].

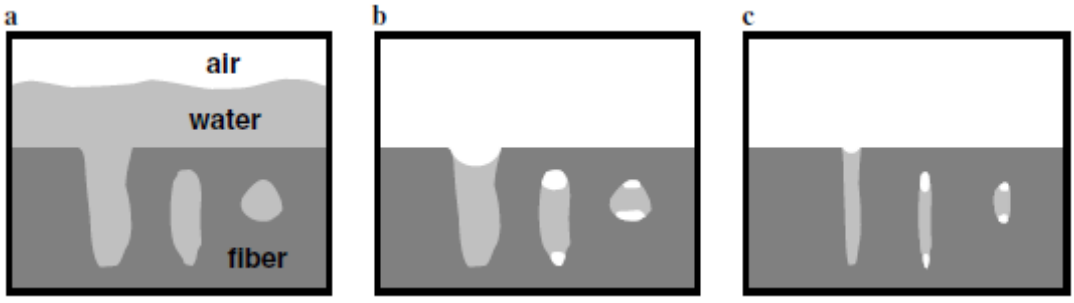

Fig. 8. Conceptual model of pore closure. When water is evaporated (a → b → c), surface tension pulls the cell wall together and the pore size becomes smaller [151].

Which pores collapse depends on two primary factors. First, the size of the pores affects the internal pressure of the pore liquid. Smaller pores will have smaller radii of curvature, generating lower pressures within the pore liquid and thus larger pore closure forces. The second factor is the ability of the pore wall to resist collapse. Larger pores should have less resistance in collapse. In fact the collapsing pressure of a tube is related to the inverse of the tube diameter [152].

2.7 Hydrogels

Hydrogels are three-dimensional and hydrophilic polymer networks capable of swelling in water or biological fluids and retaining a large amount of fluids in the swollen state (usually more than 20 % of the total weight). Three-dimensional networks are usually formed by chemical or physical crosslinking of hydrophilic polymer chains. In chemical gels, polymer chains are connected by covalent bonds, but in physical gels they are held together by molecular entanglements, noncovalent bonds, such as van der Waals interactions, ionic interactions, hydrogen bonding, hydrophobic interactions, traces of crystallinity and multiple helices [153].

Physical and chemical properties of hydrogels depend on their molecular and supramolecular structure, water content and state of water.

2.8 Soil amendments

Organic amendments are used in agricultural soils to improve their physical and chemical properties. Several studies have shown that supply of exogenous organic matter can modify not only the amount of indigenous organic matter, but also the quality of bulk soil [154]. Soil organic matter includes several different components and it is not clear, which one is modified when organic inputs are used. In order to evaluate the quality of organic matter, fulvic or humic fractions are widely used to define the complexing properties of organic matter in soils. For example, Rivero et al. used humic and fulvic acids to show that compost inputs during three years in tropical conditions significantly raised the humic fraction over the fulvic fraction, suggesting an increase in functional groups and aromaticity [155].

The use of organic soil amendments has been associated with desirable soil properties including higher plant available water holding capacity and cation exchange capacity and lower bulk density, and can foster beneficial microorganisms [156]. Benefits of compost amendments to soil also include pH stabilization and faster water infiltration rate due to enhanced soil aggregation [157].

Organic amendments provide advantages beyond the benefits of increased organic matter content on soil physical and chemical properties since nutrients that are seldom applied by farmers (e.g. manganese, zinc, and sulfur) are added as insurance against potential yield limitations. Furthermore, nutrients that are normally applied in commercial fertilizers (e.g. potassium) and liming sources (i.e. calcium, magnesium) are supplemented in organic amendments and permitted to accrue in the soil [158].

Limited field studies have been conducted to determine the impact of soil amendments on microbial communities in actual organic and conventional production systems in the fields [156]. However, it has been shown that microbial activity and biomass is higher in fields with organic amendments than fields with conventional fertilizers [156].

Inappropriate crop cultivation and harvesting, erosion and natural degradation contribute to reduce gradually the amount of SOM [159]. Nowadays, organic soil amendment is the most

common practice for restoring, maintaining and/or improving the physical, chemical and biological performances of SOM [160].

Since humic substances, of which humic acids are a major fraction, are the most abundant and important components of SOM, detailed information on the amount and quality of the HAs-like fractions contained in soil organic amendments, and on the effects that these materials may exert on the status, quality, chemistry and functions of native soil HAs are of intrinsic importance for the agronomically efficient and environmentally safe use of organic amendments in soil [160].

When evaluating the use of such products as conditioners for sandy soils, one has to take into consideration the improvement of the hydrophysical properties and the nutritional status of the soil, the increase in yield and the saving coasts of irrigation water and fertilizers on one side and the coasts of the product itself and coasts of the conditioning process on the other side [161].

Specific hydrophilic organic polymeric products, when mixed with sandy soils, associated quickly with irrigation water to form gels resulting in an increase of the soils capacity to store water. The water stored in this way is available to plants for some considerable time. Due to the bonding effect of hydrogel molecules with soil particles and their swellability, an improved and stable structure of the soil is obtained. Besides, beneficial changes in soil porosity, particularly the amount of the water retaining pores, were achieved by the conditioning process [162]. Moreover, the germination process, the plant growth, the nutrients uptake, the yield and both the water and fertilizer use efficiency were beneficially increased by mixing the plant pits in sandy soil with hydrogels [161].

The variations in hydrogel effects and plant responses seem due to differences in the type of hydrogels and soils. Therefore, information regarding the effects of a given gel type and species responses under specific soil conditions is necessary before field applications. Furthermore, timely supplies and the cost of hydrogel are important factors in the success and economics of projects envisaging the rehabilitation of sandy and arid areas through increased plant establishment [163].

3 AIM OF THE WORK

The objective of this work is to (1) test the possibility to modify the South-Moravian lignite by air oxidation and learn, which impact have the oxidative conditions on extracted humic acids and define the conditions for the highest yield of the extractable fraction (2) prepare chemically crosslinked HAs material by using both specific and unspecific ways of crosslinking reaction, (3) characterize obtained samples in terms of physical and chemical properties (4) characterize samples for their properties, mainly their ability to hold water. This should lead to fundamental understanding the changes induced in lignite humic acids after crosslinking. Furthermore, we want to study the effect of the content specific functional groups on the hydration and water holding properties, or in other words, how the performed specific/unspecific changes of HAs influence the interaction between HAs and water. We assume that the understanding the processes occurring in HAs/water system are crucial for designing of tailored HAs structure. In fact, the application of carbonaceous amendments and fertilizers is one of the ways, how to improve soil quality and fertility, but their application may not be only beneficial, as often claimed. The amendments such as humic acids or charcoal do not have a very pronounced affinity to water and thus their application can cause also negative effects such as soil repellency or a decrease in soil porosivity, thereby changing the hydraulic properties. For this reason, we hypothesize that affinity to water is an important factor in designing such amendments and should be one the most important decisive factor for their application. However, the properties of water in those amendments are still not well understood. This work addresses this issue and suggests several alternative methods for its better understanding.

4 OVERVIEW OF RESULTS AND DISCCUSION

In this section, we summarize and comment the obtained data, which were either already published in or submitted to scientific peer review journals.

Lignite is a low-rank coal mainly used for energy production. This is a highly inefficient exploitation way of lignite, because it has a low caloric value due to low coalification degree (high O/C ratio) and high water content. However, lignite can be used as a versatile material for several purposes due to its specific properties, composition and favorable content of humic acids. The simplest and cheapest way is the direct application of natural lignite after milling to suitable particle sizes by spreading or ploughing as a source of stable carbon in soils or in the other environmental applications [164]. In case of using lignite as a raw material, no specific molecules and some ballast fractions (e.g. arsenopyrite) are supplied to the environment. Therefore, the modification of lignite or extraction of more-specific parts should improve the positive effect.

Humic acids occur naturally in lignite and can account for an important fraction (10 to 80 %), depending on the maturity of organic matter in lignite. Numerous studies have shown that the content of humic acids in lignite can be significantly increased using different oxidation agents and procedures [102], [165], [166]. Increasing of humic acids fraction in lignite up to 60 % was recommended to be carried out in solution by using strong oxidation agents as nitric acids or hydrogen peroxide [165]. On the other hand, this intensive oxidation attack is connected with a loss of significant amount of carbon. The extractions procedures connected with necessary purification parts are relatively expensive and therefore, may represent a potential barrier in environmental or agricultural applications. Moreover, the final product obtain huge amount of ash.

4.1 Humic acids obtained by air oxidation of lignite

Literature overview shows the possibility to increase the content of humic acids also by air oxidation of coal [167]. It was found out that using oxygen or air, the fragmentation reaction can be controlled, carbon losses can be minimized and almost any coal can be converted in high yields into HAs [168]. Oxidation procedures represent an ideal process resulting in higher content of extractable humic matter Fig. 9 [167]. Oxidation at temperatures as high as 180 °C was started the most simplest way of production so-called regenerated humic acids (RHAs – oxidation products obtained from coals by pre-treatment with some of oxidation agents) [167]. However, some of humate processed at higher temperature can exhibit genotoxic effect probably caused by accelerated formation of reactive oxygen species such as superoxide anion [169]. In addition, using of such high temperature is relatively demanding with respect to the price of technologies as well as with respect to release of enormous amount of (mainly) carbon dioxide. Using of air represents a cheap oxidation agent and has great potential from ecology/economics point of view.

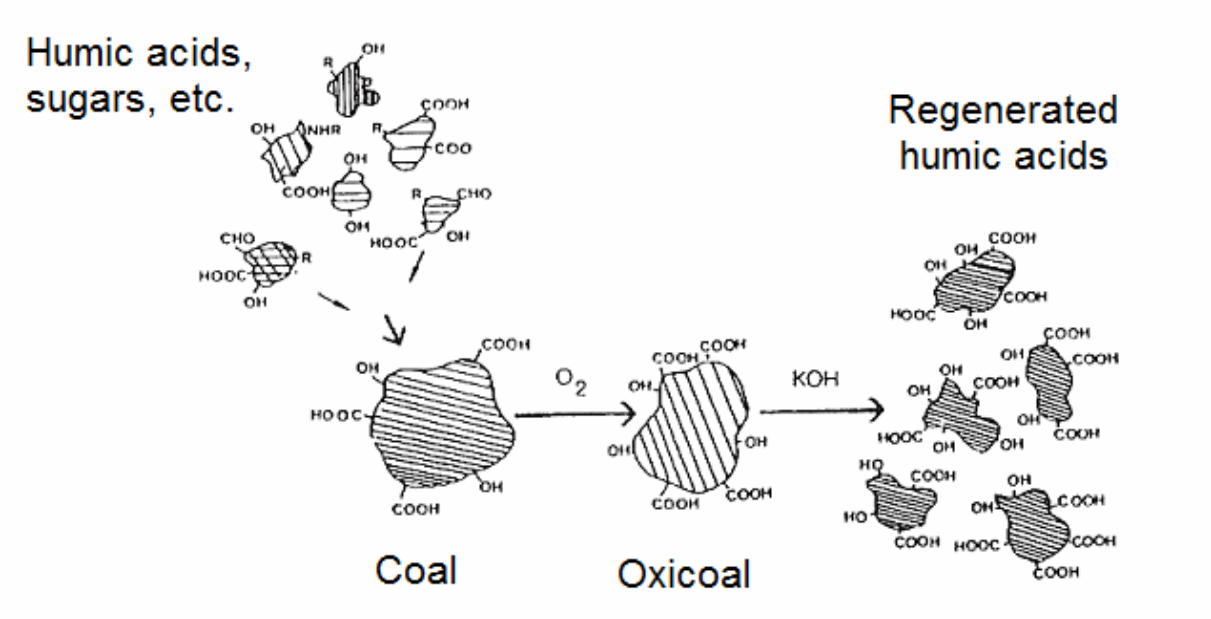

Fig. 9. Conceptual model of coal oxidation as an ideal inverse diagenetic process, reprinted with kind permission from [167].

Therefore, in the first work (Appendix I) we investigated the influence of lignite air oxidation on the yield and physico-chemical character of respective RHAs. The conception was based on the premise that relatively mild conditions could lead to a significant increase of the HAs yield. In addition, another advantage of this approach is a nondestructive effect on the chemical properties of forming HAs. Therefore, we focused on the possibility to oxidize lignite by air in both stationary and fluidized bed reactors under different temperatures (i.e. within 25 and 85 °C) and at different time periods in order to increase the extractable fraction content. Obtained results suggested that this approach lead to an increase in the yield of the HAs. However, apparently, in the first step, air oxidation caused the oxidation of COOH groups of HAs responsible for their solubility. Kinetics of oxidation of other structures associated with formation of new COOH groups was evidently slower, which was indicated by the yield in the time. That means that lignite was relatively resistant to the oxidation attack under these conditions and the kinetics of oxidation of carbon skeleton was relatively slow. Followed alkali extraction was used to obtain regenerated humic acids, while the yield was compared with the content of humic acids in non-treated lignite sample. Generally, both higher temperatures and increasing time period resulted in higher yield of RHAs (first a decrease and after a certain period an increase in the yield), which could be explained by the changed chemical character of oxidized lignite, especially an increase in the content of soluble oxidized structures.

Despite the difference in the yield of RHAs (extractable part of lignite increased up to 75 % after oxidation), their chemical character was in all cases fairly similar – the reason was probably the kinetics of oxidation processes as well as in the strength of oxidation agent (the air is a mild oxidation agent). FTIR showed slight chemical changes represented by an increase in number of ester bonds in RHAs produced mainly at temperatures above 80 °C causing a significant increase in apparent molecular weight as evaluated by HPSEC. In addition, the modification increased mutual affinity of HAs molecules to form aggregates via intermolecular interactions. This structural change is supposed to be responsible for the formation of gel-like structure with increased water holding capacity.

Thus, concurrently, the aim of the second work was additional evaluation and characterization of changes in prepared RHAs samples structure induced by air oxidation of lignite (see Appendix 2). Samples were tested for their thermo-oxidation stabilities and degradability reflecting mostly the character of their supramolecular structure.

The formation of ester bounds (described in Appendix 1) and the capacity of surfaces areas (strength of polar groups) was evaluated as a moisture uptake/release after 21 days studied by TGA. Water content of samples placed in moisture container (100% relative humidity RH) for 21 days increase with increasing time of parental lignite oxidation and was typically detected in range from 10 to 13 %. Moisture content was higher in samples, which were prepared in static reactor at lower temperatures and also increased at longer period of oxidation at 85 °C. The highest moisture content was observed for RHAs prepared in fluid reactor at 50 °C for 72 hours and RHAs prepared in static reactor at 85 °C for 456 hours.

Comparison of the results obtained from thermo-oxidative degradation of samples prepared in the static reactor indicated an increase in labile component content and simultaneously a decrease of stable fraction. Evidently, during oxidation the partial decomposition of stable humic part occurred, and therefore, the humic acids extracted from oxidized lignite contained larger pool of labile molecules. The amount of mass decomposed in the TGA first step (labile part) increases with the time oxidation. That increase reached the limit at 240 hours of oxidation, when the lignite structure was completely changed a no further conversion could be obtained by extended period of oxidation (under specific the conditions [102]). Results from DSC supported the observation from TGA (i.e. increased amount of labile parts). Therefore, we assumed that hydration properties can be modified before the extraction step by air oxidation pre-treatment of natural lignite.

This study showed that air oxidation did not caused a remarkable modification of HAs structure. On the other hand, the results indicated that the content of polar functional groups is not the main factor driving hydration, but that water holding capacity depends also on physical structure. For this reason, in third section, we focused on chemical specific modification of lignite humic acids.

4.2 Crosslinking of humic acids by using formaldehyde

The purpose was to covalently crosslink the HAs using different methods as illustrated in Fig. 10, and to characterize the properties of products. Thus, crosslinking by formaldehyde (see Appendix 3) and by carbodiimide coupling (see Appendix 4) was tested and studied in detail. Presumed target sites for crosslinking are the hydroxyl and carboxyl groups, which are the most abundant groups in humic substances.

Despite the apparent straightforwardness of the crosslinking approach, crosslinking was always a trial-and-error process for heterogeneous materials such as HAs. Identification and characterization of crosslinking products is hampered by the complexity of the crosslinking reaction mixture. To overcome these difficulties, a number of strategies were employed to facilitate the detection of the crosslinking products.

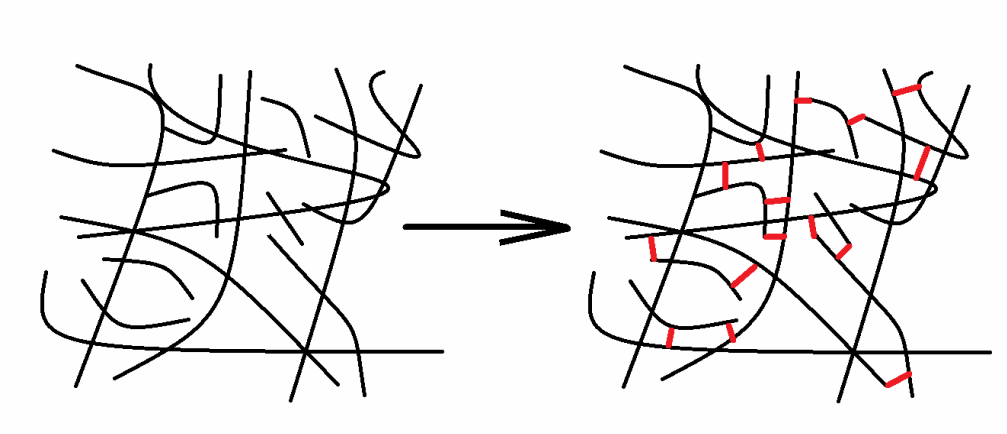

Fig. 10. Illustration of chemical modification of HAs structure. Red lines represent new chemical bounds.

The strategy of crosslinking using by formaldehyde is based on the condensation between phenolic fragments of humic aromatic core producing a methylene bridge. Therefore, the carboxylic groups are preserved during this reaction and can contribute to water uptake. Surprisingly, the elemental composition of HAs was not dramatically changed after modification. On the other hand, the process of crosslinking by formaldehyde caused a slight increase in aliphatic content, which was expectable. The results from HPSEC showed an increase in molecular weight of modified HAs samples, which verified the introduction of new covalent bonds. However, the results could also indicate a leakage of some fraction during the preparation, which is unfortunately a common problem in chemistry of humic substances and can hardly be avoided.

The stability tests implied that crosslinking decreased the thermo-oxidative stability of produced HAs. It was explained by increasing in porosity and decreasing in material compactness, allowing better diffusion of reactive atmosphere.

This modification brought about a reduction in moisture sorption capacity at low RH and an increase at higher RHs. Results also showed that the surface of crosslinked HAs materials is generally more susceptible for wetting at higher RH. This was attributed to the increase in distance between polar functional groups responsible for condensation of water (formation of larger water nano-droples on surface) and a decrease of structural compactness after crosslinking.

Furthermore, we also hypothesized that the physical and physico-chemical properties of humic acids samples changed during volumetric swelling at constant temperature and moisture conditions. These changes were slow and affect the state as well as the amounts of different water types inside the HAs. These can be observed through changes in mobility and thermal behavior of the involved water molecules, which can be determined by NMR relaxometry and DSC.

NMR relaxometry experiments focused on the study of hydration behavior revealed that crosslinking caused increase in molecular rigidity and unification of relaxation times distribution of water protons. Water mobility was largely affected by the dimension of pores and newly introduced chemical bonds. In light of NMR relaxometry results, the crosslinking caused structural changes leading to the unification of pores size in HAs. Therefore, water could easily penetrate into the crosslinked structure, because it had to overcome less

restrictions connected with wetting and swelling of cavities of small dimensions. This was reflected in larger water holding capacity and faster hydration kinetics.

DSC was shown as a powerful technique in characterization hydration of HAs via freezing-melting processes. The decrease in melting enthalpy of water/ice in crosslinked HAs revealed faster water uptake. Approximately three-fold higher water holding capacity showed sample oxidized by 5% nitric acids and crosslinked by formaldehyde. We assumed that the separation of functional groups in crosslinked samples partially supports wetting of inner pores.

The NMR relaxometry and DSC experiments suggested that a layered bound water phase existed inside the HAs. This phase consisted of several layers of non-freezing and freezing water, whereas the binding forces and, consequently, the water structuring decreased with increasing distance from the HAs surfaces resulting in higher mobility of the water molecules. This layered bound water phase included water entrapped in HAs structures/pores. During swelling, the amounts of bound water as well as the mobility of the water molecules inside the bound water phase increased, which indicated a water intrusion into the HAs matrix as well as a reorientation of HAs chains into the pore space.

The increasing amounts of non-freezable and as well as the reduction of the translational and rotational mobility of water molecules in the modified HAs samples indicated changes of the physical and physico-chemical properties of HAs during swelling at constant temperature and moisture conditions.

4.3 Crosslinking of humic acids by using carbodiimide coupling

The second tested cross-linking strategy was using carbodiimide coupling to bound specific HAs moieties. The carbodiimides are zero-length crosslinkers, a thus the direct conjugation is performed without incorporation of crosslinking agents between targeted molecules.

The introduction of new ester bonds and thereby affirming the chemical change of the humic structure after crosslinking was confirmed by using FTIR spectra.

The slight differences in EA of samples were attributed to changes in structures of HAs originated again from purification (washing out of polar molecules/partial extraction of matrix, the rest of crosslinker and/or catalyst moieties in the system). However, due to the complexity and heterogeneity of tested materials, the abundance of individual elements alone appeared insufficient in evaluation of structural changes and thus, the crosslinking was verified by using relative ratios of the elements.

Thermo-oxidative stability tests performed by TGA revealed that the crosslinking reactions caused mostly an increase in the mass loss in the second degradation step and an increase in respective peak temperature with some exceptions. This destabilization could be caused by the increasing surface pore size facilitating the access of air and thereby accelerating the thermo-oxidative degradation. This result and results from formaldehyde crosslinking clearly showed that the interpretation of TGA records should be done carefully, taking into account the fact that the oxidative/degradative reactions take part onto gas/solid interface and the porosity of both systems play an important role.

In carbodiimide crosslinking, the carboxyl groups of HAs are involved in crosslinked reaction, and thus their residual concentration was used as an indicator of crosslinking conversions. We used pH titrations and found that all modified samples had lower degree of deprotonization than untreatment sample. These results clearly indicated formation of

crosslinked structure and preferable involvement of strong carboxylic groups in crosslinking. In particular, samples modified by EDC contained mainly weak acidic carboxyl groups. Therefore, we concluded that EDC is more selective and presumably more effective with HAs samples containing stronger carboxylic acids.

The higher molar ratio between crosslinker DCC and relevant functional groups in HAs caused a decrease in the water affinity (lower amount of water harvested in appropriate time), partially because the amount of hydrophilic functional groups capable to bind water molecules is lower due to their involvement in the crosslinking. On the other hand, the EDC samples (pre-hydrated for 24 hours and 5 days) showed higher water holding capacity than the ORIG sample.

The interaction of water molecules with HAs structure were studied also by DSC, in this case as changes in evaporation heats. The original sample gave the lowest heat in comparison with the crosslinked derivatives. In all crosslinked samples, at 76% RH, the equilibrium water content is smaller, but more energy is needed to evaporate water from their structure. This indicated the preferential sorption of water on the surface and a shorter distance between functional groups, which decreased size of water molecule bridges and increased the energy necessary to evaporate adsorbed moisture.

These results let us to conclusion that water harvesting capability depends on the amount of suitable polar groups only partially, and their spatial distribution is an important parameter as well. These micro-structural aspects are frequently overlooked in humic acids, but they have a great influence on bottom-up modeling of properties of used amendments and their role in ecosystem.

The NMR relaxometry was used in order to study change in transverse relaxation times of water protons in HAs that were progressively hydrated (water was first adsorbed from surroundings atmosphere and later added by a pipette). Two proton transverse relaxation times were determined, which indicated two distinct water fractions. A slower relaxing pool changed during the hydration relatively moderately, and it corresponded to the fraction related in part to the slower motion of protons both in HAs structure as well as water molecules attracted to the surface of the HAs. The second, faster relaxation time pool derives predominantly from relaxation of water trapped molecules in different hydration level and bound in the HAs structure by different interactions, in environments of different pore sizes. In fact, different hydration profiles were observed by NMR relaxometry in prepared derivatives. In particular, the low relaxation time in the initial phases indicated a stronger binding of moisture either on the surface or in the inner pores. Upon pipette addition of water, untreated sample showed a progressive swelling, while in the DCC derivatives, water penetrated very slowly as indicated by very high relaxation time. In EDC derivatives, the relaxation of faster component increased also regularly, but relatively slowly, which indicated more rigid structure than in untreatment sample.

In order to monitor the changes in water holding capacity during the hydration we used, similarly as in previous case, freezing/thawing DSC experiments. In untreated sample, we determined a stable increase of NFW content up to largest value in 18 days, while in crosslinked samples some maxima (typically around 9^{th} day) were observed. In all cases the crosslinked humic acids showed lower water holding capacity, which was related to the reduction in number of hydrophilic functional groups in crosslinked humic acids and increase in crosslink degree.

The maxima of NFW contents indicated the existence of a parallel process taking part during the hydration. We assumed that this could be caused by the partial hydrolysis of HAs structures, in particular of newly formed bonds. The lowest resistance showed the EDC derivatives, which were hydrated very fast up to 80–90 % of untreatment sample at 18th day, but already between 3–9 days, the structure started to collapse. The fast wetting of these samples correlates well with explanation that EDC samples have larger size of surface pores, which facilitates the water penetration into the structure. The DCC samples showed similar maxima too, and lower NFW contents.

The EDC derivatives showed higher water holding capacity and harvesting ability and in contrast to DCC derivates, did not show the rests of trapped crosslinking agents in the structure. The crosslinking by carbodiimides seems to introduce the new covalent bonds that are probably destabilized within several days after water addition. The observations implied that for the water holding capacity of HAs both the number of polar groups and pore-size distributions were important parameters. The HAs pores system and distribution of polar groups played a crucial role for processes of wetting, moisture harvesting and later also for the strength of water binding.

4.4 Conclusion

One of the purposes for crosslinking of humic substances is to enhance their resistance to further biotic or abiotic degradation. An increased chemical stability of humic material may be of paramount importance in improving the control of fundamental ecological and environmental processes such as organic carbon stabilization in soils to reduce both CO_2 emission to the atmosphere and soil physical degradation leading to erosion and desertification. However, as shown in our work, the crosslinking decreased thermo-oxidative stability, which is in contrast to expectations. It is shown, that stability is largely influenced by system porosity, which changes in crosslinked derivatives. Therefore, this issue should be investigated more in detail in further studies.

Next, the relative importance of intermolecular crosslinking HAs under different conditions still needs to be further understood. This knowledge is required to predict the effects of individual functional groups and resulting state of structure on environmental functions and properties of HAs affecting stability, adsorption, bioavailability, water management and transport of organic chemicals in soils. Chemical composition, physical structure of HAs are still not known satisfactorily enough to deduce the parameters important to predict precise crosslink structure formation and their mutual effects on HAs supramolecular structure as well as HAs stability and behavior, in generally.

In this context, specifically hydration/dehydration mechanisms as well as HAs-water interactions and distribution of water molecules in matrice need to be studied in order to appropriately judge new crosslinked structures of HAs.

The findings of this study are of environmental importance for helping to optimize renaturation and rewatering of commercially used humic acids based remediation agents and to better understand sorption/desorption and transport processes of pollutants and nutrients in natural organic matter rich soils. It is unlikely that crosslinking using formaldehyde or carbodiimide coupling of humic molecules may naturally occur in the environment. However, the technologically-induced polymerization of humus may become important to improve control of the organic carbon cycle in the biosphere or the fate of contaminants in the environment by using carbonaceous amendments. In addition, the particular crosslinking

agents and reaction conditions used in this study may serve as models for crosslinking reactions occurring during the natural aging of organic matter. The products may be used in future studies as a basis for characterizing the effects of crosslinking of organic matter in geosolids on its properties, including, among others, biological stability and sorptive potential. Moreover, further experiments may identify other types of crosslinking reactions involving organo groups occurring under environmental conditions

Knowledge of the molecular-chemical structure of humic substances and soil organic matter appears to be of fundamental importance for understanding and predicting the composition, dynamics, and reactivity of natural and anthropogenic organics in soil and water. The presented results and procedures of HAs properties are expected to give a positive impact in this direction.

Further efforts will have to be made in synthesizing novel crosslinking reagents, in understanding better the kinetics of crosslinking reactions in HAs and finally in developing novel strategies for facilitated detection of crosslinking products.

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6 LIST OF ABBREVIATIONS AND SYMBOLS

Da	=	Dalton
DCC	=	N,N'-dicyclohexylcarbodiimide
DMAP	=	4-dimethylaminopyridine
DSC	=	Differential scanning calorimetry
DTA	=	Differential thermal analysis
EA	=	Elemental analysis
EDC	=	N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide
FAs	=	Fulvic acids
FTIR	=	Fourier transformed infrared spectroscopy
HAs	=	Humic acids
HPSEC	=	High performance size exclusion chromatography
HS	=	Humic substances
IHSS	=	International Humic Substances Society
NFW	=	Non-freezing water
NHS	=	N-hydroxysuccinimide
NMR	=	Nuclear magnetic resonance
RHAs	=	Regenerated humic acids
RH	=	Relative humidity
SOM	=	Soil organic matter
TGA	=	Thermogravimetric analysis
WaMB	=	Water molecule bridges
Wc	=	Water content

All the symbols and magnitudes are explained in the text.

7 LIST OF APPENDICES

- **Appendix A**

Kučerík, J., Cihlář, Z., Vlčková, Z., Drastík, M.: Regenerated humic acids obtained by the air oxidation of South Moravian lignite. Part. 1. Production and characterization. *Petroleum and Coal*, 2008, vol. 50, no. 3, pp. 49–55. ISSN: 1335-3055.

- **Appendix B**

Cihlář, Z.; Kučerík, J.: Regenerated humic acids obtained by the ait oxidation of south Moravian lignite. Part. 2. Thermoanalytical characterization of products. *Petroleum and Coal*, 2010, vol. 52, no. 4, pp. 49–55. ISSN: 1335- 3055.

- **Appendix C**

Cihlář, Z.; Vojtová, L.; Conte, P.; Nasir, S.; Kučerík, J.: Hydration and water holding properties of cross-linked lignite humic acids, *Geoderma*, 2014, vol. 230–231, no. 1, pp. 151–160. ISSN: 0016- 7061.

- **Appendix D**

Cihlář, Z.; Kučerík, J.; Vojtová, L., Michlovská, L.: Preparation and hydration characteristics of carbodiimide crosslinked lignite humic acids. Manuscript submitted.

7.1 Appendix A

Kučerík, J., Cihlář, Z., Vlčková, Z., Drastík, M.: Regenerated humic acids obtained by the air oxidation of South Moravian lignite. Part. 1. Production and characterization. *Petroleum and Coal*, 2008, vol. 50, no. 3, pp. 49–55. ISSN: 1335-3055.

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REGENERATED HUMIC ACIDS OBTAINED BY THE AIR OXIDATION OF SOUTH MORAVIAN LIGNITE. PART. 1. PRODUCTION AND CHARACTERIZATION.

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Abstract
The influence of lignite air oxidation on the yield and chemical/physical character of regenerated humic acids (RHA) has been studied. RHA were produced under different experimental conditions, i.e. both in static and fluid reactor. Oxidation in the fluid reactor brought first a decrease and after a certain period increase in the yield of RHA. The same observation was done in case of static reactor. It has been verified that higher temperature during the oxidation supports the production of RHA. Despite the difference in the yield of RHA (increase up to 75%) their chemical character was in all cases fairly similar. Some tiny differences were revealed using FTIR which showed the increase in number of ester bonds in RHA produced mainly at temperatures above 80°C causing a significant increase in apparent molecular weight as evaluated by HPSEC.

Key words: lignite, regenerated humic acids, air oxidation

1. Introduction

Humic substances are ubiquitous natural products of microbial degradation of dead plant tissues and animal bodies and represent the major biosphere pool of natural organic matter [1]. In the course of their genesis they underwent processes of humification and in case of lignites also partly coalification. Major components of humic substances are considered to be recalcitrance of plants and algae, including materials derived from lignins, tannins, sporopollenins, and large aliphatic molecules such as algaenans, cutans and suberans. From the chemical point of view, they represent a complicated mixture of distinct chemical compound classes such as lipids, waxes, carbohydrates, polyphenols, proteins and nucleic acids derivates [2]. Their role in nature is vital for sustainable development of life on the Earth. In soils they help to prevent drying and shrinking, improve moisture retaining properties of soils, permit exchange of gases, stabilize structure, enhance availability of micronutrients to higher plants, increase cation exchange capacity, modify application rate of pesticides for effective control, have a direct effect on plant growth [3]. In this respect, they influence the micro algae growth, seed germination, plant growth and development affecting some metabolic processes as respiration, nutrient uptake and inducing morphofunctional changes in the root architecture. In many cases these effects are similar to those induced by plant growth regulators as auxins, gibberellins and cytokinines [4,5].

Operationally humic substances are divided into three groups according to their solubility, i.e. humic acids (HA) soluble in alkali media, insoluble humin (HU) and fulvic acids (FA) soluble at all values of pH [3].

In the past, HA were posited to consist solely of a system of coiled macromolecules having molecular weights in the range of tens to hundreds of thousands of Da [3]. In basic or low-ionic strength solution they had elongated shapes, but became coils in acidic or high-ionic strength solutions. Later, Wershaw [6] proposed that HA consist of ordered aggregates of amphiphilic molecules, composed mainly of relatively unaltered plant polymer segments possessing acidic functionality and held together by hydrophobic (π-π, charge transfer) bonds and H bonding interactions and the hydrophobic parts of the molecules are in the interior, with the hydrophilic part making up the exterior surfaces. Ordered aggregates of humus in soils were depicted as existing as bilayer membranes coating mineral grains and as micelles in solution.

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Recent research brought a new insight into molecular structure of humic substances. As demonstrated, despite the polymer theory which has never been unambiguously proved, the humic mixture consists of various molecules assembled together via weak interactions (π-π, CH-π, van der Waals and H-bonds) forming aggregates of apparently large molecular weight [7]. Low bonding energy implies the possibility of easy separation of distinctive classes of molecules allowing better characterization followed by a potential technological utilization. Based on the polarity of solvents (among others), the sequential extraction techniques allow the separation of free lipids, bound lipids, saccharidic and aromatic fraction (polyphenols, heterocycles) [8].

Coals of variable degree of coalification possess variable amount of extractable humic acids. Use of oxidation procedures represents an ideal inverse diagenetic process (Fig. 1) resulting in a higher content of extractable humic matter [9]. Oxidation products obtained from coals by pre-treatment with nitric acid, potassium manganate or by air oxidation have been reported as regenerated humic acids (RHA) [9].

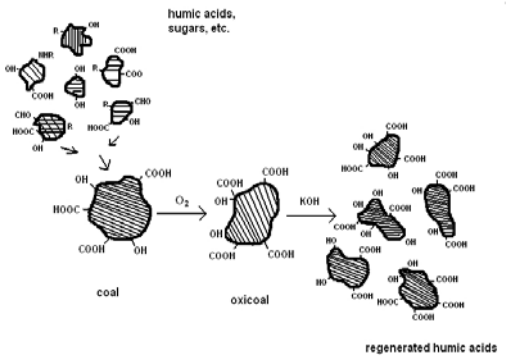

Fig. 1. Coal oxidation as and ideal inverse diagenetic process, adopted from ref. [9]

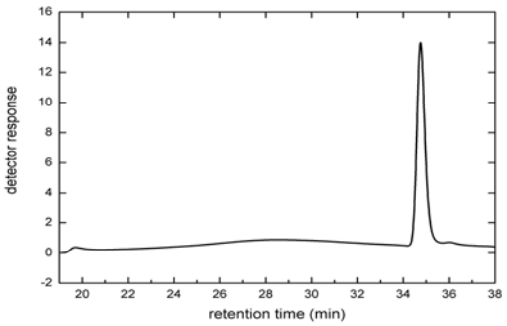

Fig 2. HPSEC analysis of humic acid extracted from parental lignite as detected by RI detector.

Generally, RHA have similar chemical-physical properties as humic substances which were precursors of coal formation. The elemental composition, functional groups content and distribution depends on the conditions of RHA formation and on the chemical properties of parental coal material. In their structure higher content of aromatic structures can be found in comparison with those humic acids extracted from parental coal. RHA also possess more semichinoidal radicals in their structure, i.e. 2×10^{18} spin g^{-1}. That content is minimally twofold higher in comparison with non treated humic samples [3]. Semichinoidal structures are easily oxidizable forming chinones which increases the rate of reduction reactions in soils. Such reactions are responsible for transport of metal ions in soils and support their adsorption by plant roots [9]. Thus technological/agricultural application of RHA bears promising potential.

Air oxidation of coal has already been studied and described in detail [9]. It has been supposed that coal structure contains same “weak” points that can be broken to depolymerize the original structure (Fig. 2). Oxidation at temperatures as high as 180°C was stated the most simplest way of production of RHA [9]. On the other hand, such approach is relatively demanding with respect to the price of the technological performance as well as with respect to the evolution of huge amount of carbon dioxide and other gases.

Since HA as well as RHA represent an interesting raw material useful in technological, agricultural and environmental applications the knowledge on the production is of a great interest [10]. Thus the aim of this work was to investigate the potential of air as a cheap oxidation agent of South Moravian lignite and to assess the chemical-physical character of obtained regenerated humic acids.

2. Experimental

Extraction of HA and production of RHA

Lignite from the Mikulčice locality (Czech Republic) was used in this work. Detailed information on the lignite chemical-physical properties were already reported elsewhere [11,12]. Using several screens, lignite particles were separated according to the particle size into three groups: 1-0.5 mm, 0.5-0,125 mm and fraction <0.125 mm.

RHA were extracted from air pretreated lignite. Lignite was treated by two ways. In the first case, lignite was placed into a container and stream of air made lignite particles “fly” and therefore provided maximal contact with air. Therefore, kind of fluid reactor was used for this purpose. Temperature was

constant either at 25°C or 50°C. The second way of oxidation was carried out in the oven in which the lignite sample was let to be oxidized put on the floor at 85°C for the chosen time period.

Humic substances were extracted from pretreated and parental lignite according to the procedure described earlier[13]. Briefly, a lignite fraction was mixed with the aqueous 0.5 M NaOH and 0.1M $Na_4P_2O_7$ solution (1:10) and stirred for 3 hours. After centrifugation, the supernatant was treated with the concentrated HCl until pH was about 1 in order to precipitate the humic acids (fraction HA). HA were then treated with 0.5% (v/v) HCl-HF solution overnight to remove residual ashes, dialyzed (Spectra/Por® dialysis tubes, 1000 *Mw* cut-off) against distilled water until chloride-free. Obtained products were dried in the oven to constant weight.

All humic samples (50 mg) were suspended in CO_2 free distilled water (60 ml) and titrated to pH 7 with 0.1M NaOH solution employing an automatic titrator as described earlier [14]. The resulting sodium humates were freeze-dried and homogenized in an agate mortar for further HPSEC analyses.

High performance size exclusion chromatography (HPSEC)

The HPSEC system Agilent consisted of a pump equipped with two detectors in series: a UV/VIS detector set to 280 nm for humic analyses, and a refractive index detector (RI). An automatic injector, with a 100 µl sample loop, was used to load HPSEC solutions and a Phenomenex Biosep S2000 (600 × 7.5 mm) column was used for size exclusion separations. The column was preceded by a Biosep Guard column and a 0.2 µm stainless-steel inlet filter. Flow rate was set to 0.6 ml min^{-1} while the HPSEC eluent was a 50 mM $NaH_2PO_4.H_2O$ solution adjusted to pH 7 with 1M NaOH. The buffer concentration was chosen to have a constant ionic strength of 50 mM in order to minimize ionic exclusion or hydrophobic interactions with the column.

All solutions were filtered through quartz filters (Glass Microfibre Filter Whatman International, LTD) before injection. The HPSEC analyses were conducted in duplicate and no significant differences were observed between measurements. For the calculation of weight average molecular weight (*Mw*) following equation was used:

$$M_w = \frac{\sum_{i=1}^{N} (h_i M_i)}{\sum_{i=1}^{N} h_i}$$

where M_i and h_i are the molecular weight and the height of each i-th fraction in the chromatogram, respectively.

Standards of known *Mw* such as polysaccharides (PS) of 186, 100, 23.7 and 12.2 kD, (Polymer Sciences Laboratories, UK) and sodium polystyrenesulphonates (PSS) of 169, 123, 30.9 and 6.78 kD (Polymer Standard Service, Germany) were used for column calibration. Calibration curves were semi-log linear over the range defined by standards and were used to obtain the molecular weights of humic samples.

FTIR spectroscopy

Conventional KBr pellet technique was used. Humic samples previously dried at 105°C for 2 hours and cooled and stored in desiccator were mixed in agate mortar with KBr (1:200 w/w). For this purpose Nicolet Impact 400 was employed.

3. Results and discussion

Production of RHA extracted from South Moravian lignite has been already published [12]. It has been demonstrated that the content of RHA can be increased from 20% to 60% using nitric acid or 45% using hydrogen peroxide. Nevertheless, such approach was associated with a loss of a huge amount of carbon due to the intensive oxidation attack.

3.1 Influence of particle size on the yield of humic acids

In the first step, the attempt was to evaluate the influence of particle size on the yield of extraction of humic acids from lignite. For this purpose 3 particle size fractions mentioned in Experimental were used. Table 1 reports the results of the experiment.

Table 1 Yield of extraction of humic acids from lignite of various particle sizes

Fraction (mm)	1-0.5	0.5-0.125	0.125>
Yield (%)	12.0	23.0	22.0

One can see that the particle size is an important parameter for the production of humic acids. While large particle diameter provided a relatively low yield, particles smaller than 0.5 mm gave yield above 20%. It is logical since lower particle size is connected with larger area and it seems that the extraction agent could not reach to the particles interior and extracted solely the humic material from the particle surface. Additional conclusion can be made considering that humic acids are more abundant in particles with lower diameter. This assumption can be justified by the fact that in the structure of lignite, there can be identified parts of different degree of coalification as well as non-decomposed moieties of parental phytomass having different mechanical properties.

Therefore, in the next experiments, different fractions were used to test the efficiency of suggested oxidation methods.

3. 2 Oxidation in a fluid reactor

This part of the work has been devoted to the evaluation of influence of air oxidation on the yield of RHA extracted from oxidized lignite. For this purpose the fraction 1-0.5 mm was chosen. The obtained data are summarized in Table 2.

Table 2 Oxidation of fraction 0.5-1 mm in the fluid reactor

Oxidation parameters	24 hours at 25°C	72 hours at 25°C	120 hours at 25°C	72 hours at 50°C
Yield* (%)	-15.63	-7.97	75.42	5.46

**Increase (+) or decrease (-) with respect to the value given in Table 1*

As can be seen in Table 2, the oxidation in fluid reactor did not give rise to the yield increase in all cases. We assume that it is caused by the composition of the particles having diameter 0.5-1 mm. In fluid state particles are supposed to be under the mechanical stress (milling) and thus the particle size is decreasing (verified by a parallel experiment). Such processes should lead to the increase in the yield of humic acids. However, it seems that such way of oxidation preferably supports the oxidation of COOH groups of humic acids responsible for their solubility. Kinetics of oxidation of other structures, resulting in formation of other carboxylic groups is evidently slower which is indicated by the yield after 72 hours at 25 and 50 °C and after 120 hours.

3.3 Oxidation in stationary phase

In this part of the work we have tested the influence of air oxidation on the yield of humic acids. Since oxidation in fluid reactor was not too sufficient for fraction 0.5-1 mm, we used the fraction 0.5-0.125 to see whether in this case the yield increase will be more intensive. Temperature 85°C was chosen with respect to the results reported recently, which indicated that at temperature above 80°C lipid fraction present in humified matter forming a specific domain is melted [15] and, thus, some parts of lignite are better accessible for oxygen diffusion. Again, similar trend as in case of fluid reactor has been observed (Table 3). Oxidation of lignite brought about a decrease in the yield of RHA with the minimum after 120 hours of oxidation. Further, an increase was observed which resulted in 18% abundance after 456 hours. It seems that lignite is relatively resistant to the oxidation attack under these conditions and the kinetics of oxidation of carbon skeleton is relatively slow. Although the increase of HA content using such simple and chemically mild methodology is low, the next part is devoted to the characterization of selected humic acids obtained during above-mentioned procedures.

Table 3 Oxidation of fraction 0.5-0.125 mm in the static reactor

Oxidation period (hours)	72	120	240	456
Yield* (%)	-22.94	-43.65	-5.51	17.98

**Increase (+) or decrease (-) with respect to the value given in Table 1*

3.4 Characterization of HA and RHA

Selected samples were characterized for their elemental and ash content. Obtained values are reported in Table 4. Values are given in weight %. Chemical properties of humic acids extracted from fraction 1.0-0.5mm did not practically differ from RHA prepared for 24 hours at 25°C, i.e. RHA1, so the latter is used as a reference. Samples produced in fluid reactor were denoted as follows: RHA2 and RHA3 are regenerated humic acids produced for 72 and 120 hours, respectively, and RHA4 stands for regenerated humic acids produced 72 hours at 50°C. Samples RHA5, RHA6, RHA7 and RHA8 were prepared at 85°C in the static reactor for 72, 120, 240, 456 hours, respectively. Sample HA9 represents humic acid extracted from parental lignite.

Table 4 Elemental composition, ash and moisture content of parental and regenerated humic acids, ash content in all samples up to 2.2%, traces of sulphur are included in O content.

	C (%)	H (%)	N (%)	O*(%)	C/H	C/O	Moisture
RHA1	56.07	4.82	1.28	37.22	11.75	1.52	8.34
RHA2	57.37	4.80	1.36	36.47	11.95	1.57	7.63
RHA3	56.62	4.89	1.34	37.15	11.58	1.52	7.85
RHA4	56.81	4.45	1.29	37.45	12.76	1.52	8.16
RHA5	56.91	4.66	1.37	37.06	12.21	1.54	7.61
RHA6	57.21	4.97	1.29	36.53	11.51	1.57	7.98
RHA7	57.72	4.72	1.26	36.30	12.22	1.59	7.03
RHA8	57.42	4.84	1.29	36.45	11.86	1.58	7.48
HA9	57.21	4.62	1.05	37.22	12.38	1.53	n.d.

**calculated by difference*

Decreasing C/H ratio appeared to be related to decreasing aromaticity or degree of unsaturation [13]. Basically, the higher C/H value the higher is the content of aromatic structures in humic material. Ratio C/O indicates the proportion between oxygenated carbons and indirectly also the content of COOH groups.

In fact, the elemental content and its ratios did not show any remarkable changes in composition of RHAs. As mentioned previously, the reason can be seen in the kinetics of oxidation processes as well as in the strength of oxidation agent.

Fig. 2 shows a representative HPSEC of humic acid extracted from parental lignite detected by RI detector, Table 5 reports the respective *Mw* of all HA and RHAs based on sodium salt polystyrene sulfonates (PSS) and polysaccharides (PS) calibration. Although the comparison of *Mw* among each other has an importance, the values should be taken as apparental since there is no humic material which can be used for the molecular weight calibration [16]. Further, as mentioned before, humic acids should be considered as a mixture of relatively small molecules and the data reflect more the aggregation properties of humic acids. Aggregates are stabilized by weak interactions (π-π, CH-π and van der Waals and by H-bonds) which depends predominantly on the pH value and ionic strength [17]. In our case, analysis resulted in two distinctive peaks. As demonstrated recently, largest molecular size fractions excluded from column first contain aromatic, alkyl and potentially carbohydrate-like content. The carbohydrate-like and the alkyl chain length seem to decrease with decreasing molecular size. Progressive reduction of aromatic carbon atoms was also observed with decreasing molecular size of the separated fractions. Further, the abundance of hydrophilic molecules have been reported in low molecular fraction [16,18].

Table 5. HPSEC based molecular weight calculated from UV and RI records

	PSS calibration (UV)	PS calibration (RI)
RHA1	9232	9444
RHA2	12646	10760
RHA3	13404	13710
RHA4	7279	8442
RHA5	15213	15030
RHA6	10323	10840
RHA7	12881	13540
RHA8	17460	16940
HA9	9961	nd

In contrast to the elemental analysis the HPSEC analysis revealed better the differences among humic sample (Table 5). Although there is a shift between RI and UV calibrations, the mutual comparison within HA and RHA is the same. Comparison of samples in the first set prepared in the fluid reactor shows the increase in *Mw* with increasing time of oxidation. On the other hand, elevated temperate of oxidation (50°C) brought a significant decrease in *Mw*. It means that the oxidation of lignite progressively liberates the soluble fractions with larger dimensions while temperature can cause their size reduction.

The oxidation in stationary phase at 85°C showed also increasing tendency. The exception is RHA6 which gave a lower value of *Mw* than RHA5, although such value is still higher than that of HA extracted from non-treated lignite. It is noteworthy, that *Mw* of sample RHA8 is almost two-fold larger than the original HA9.

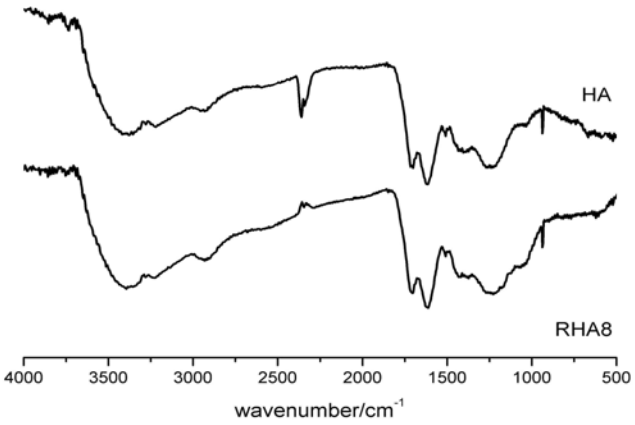

Fig. 3. Comparison of FTIR spectra humic acid extracted from parental lignite (HA) and regenerated humic acid (RHA8)

FTIR spectroscopy measurement of humic acids and RHA showed typical spectra published and described by many authors [3,12]. In this work only the FTIR spectra of HA and RHA8 are reported (Fig. 3). First, some notes to the attribution of peak wave numbers to chemical bonds vibrations. Fig 3 shows an intensive peak within the range 3400–3420 cm^{-1} which can be attributed mainly to the hydrogen bonded –OH; weak peaks in the region 2850–2960 cm^{-1} (aliphatic C–H stretching), strong sharp peaks around 1710 cm^{-1} (C=O of COOH) and 1620 cm^{-1} (C=C stretching, C=O stretching of COO^{-}, ketonic C=O and aromatic C=C conjugated with COO^{-}), a peak around 1510 cm^{-1} (N-H deformation, C-N stretching vibration, C=C aromatic bounds) 1450–1400 cm^{-1} (aliphatic C–H bending, and COO^{-} asymmetric stretching, and possibly C=C and C=N plane vibrations of heterocycles), peaks around 1250 cm^{-1} (aromatic C, C-O stretch), a weak peak around 1050 cm^{-1} (C-O of polysaccharides and Si-O) were observed. The comparison of relative intensities of aliphaic and aromatic bands gave similar results as those reported for elemental analysis. Further, deeper analysis and comparison of peaks in the area 1710 cm^{-1} revealed the slight increase in abundance of esters in samples with higher molecular weight. Therefore, it can be assumed that oxidation of parental lignite led to oxidative disruption of weak points in lignite resulting in progressive increase in yield of extractable humic material. It is likely that higher temperature together with air atmosphere support the reactions between COOH and OH- alcohol groups, i.e. esterification. The increase in molecular weight, i.e. increase in number of ester is significant especially for samples developed at temperatures above 80°C when lipidic fraction is melted and recombination reactions can occur. Such conclusion is in agreement with the ability of regenerated humic acids to form gels having high water holding capacity [9]. However, in this work, gel formation was attributed to the higher ability of regenerated humic acids to form H-bridges due to the increase in content of COOH groups. To shed light on the influence of ester groups on water holding capacity is beyond the scope of this paper and it will be investigated in a special article.

4. Conclusion

The influence of lignite air oxidation on the yield and chemical/physical character of regenerated humic acids (RHA) has been shown. RHA were produced under different experimental conditions, i.e. both in static and fluid reactor. Oxidation in the fluid reactor brought first a decrease and after a certain period increase in the yield of RHA. The same observation was done in case of static reactor. It has been verified that higher temperature during the oxidation supports the production of RHA. Despite the difference in the yield of RHA (increase up to 75%) their chemical character was in all cases fairly similar. Some tiny differences were revealed using FTIR which showed the increase in number of ester bonds in RHA produced mainly at temperatures above 80°C causing a significant increase in apparent molecular weight as evaluated by HPSEC.

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7.2 Appendix B

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REGENERATED HUMIC ACIDS OBTAINED BY THE AIR OXIDATION OF SOUTH MORAVIAN LIGNITE. PART. 2. THERMOANALYTICAL CHARACTERIZATION OF PRODUCTS

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Abstract

A sample of humic acid prepared from parental lignite and seven regenerated humic acids prepared from oxidized ones were extracted from South Moravian lignite. First, the samples were tested for their stabilities and degradabilities using thermogravimetry. It was shown that oxidation of lignite in static reactor at 85 °C produced regenerated humic acids with an increasing content of labile part. The dependency of labile content was exponential-like and reached a constant value after 240 hours of oxidation. Increasing content of labile part in regenerated humic acids was also confirmed by measuring of heat accompanying the thermo-oxidative degradation using differential scanning calorimetry. The previously reported increase in number of ester bonds and the character of surface of prepared humic material was evaluated by moisture uptake measurement. Using thermogravimetry, the determination of the content of moisture adsorbed on the surface of humic samples after 21 days was carried out. In accordance with previous conclusions based on elemental analysis and yield of regenerated humic acids, the highest moisture content was observed for humic sample prepared in fluid reactor from lignite oxidized at 50 °C for 72 hours and a sample extracted from lignite oxidized in static reactor at 85 °C for 456 hours.

Key words: lignite oxidation; regenerated humic acids; moisture uptake; thermal degradation.

1. Introduction

Humic substances (HS), the end product of decayed organic matter, are described as refractory, dark colored, heterogeneous organic compounds [1] which arise from dead plants, organisms and animal tissues [2]. In the next stage, they are supposed to undergo chemical degradation as well as biological degradation by microorganisms, to produce complex chemical structures which are more stable than original materials. As an important fraction of the natural organic matter, HS are present in all natural waters, soils, sediments, peat, bogs and other ecosystems [3, 4]. Physical properties of HS highly depend on their chemical structure, consequently on source materials and isolation conditions. Basically, the published structural models suggest the presence of a biologically relatively resistant and highly substituted core of aromatic nature with presence of heterocyclic structures [5]. The core units are linked to each other via a net of alkyl chains of different lengths, which are highly substituted with functional groups such as carboxylic, aliphatic, aromatic, hydroxyl, and amino groups [6, 7]. In contrast, the recent theory describes humic acids as a mixture of relatively small molecules stabilized predominantly by weak interactions [8]. In the last decade, due to application more sophisticated analytical techniques and approaches, such concept gains a growing attention.

HS play a significant role in environmental and ecological processes by controlling transport and transformation of toxic chemicals in soil and water, metal complexation, nutrient availability, maintenance of soil structure, and carbon and nitrogen exchange between soil and atmosphere [9, 10, 11]. The same function should have HS added into the system artificially, i. e. naturally occurring HS can be supplied by other sources or prepared synthetic reactions. Another option is the application of the so-called regenerated humic acids (RHA) that represent a pool of humic compounds produced by oxidation of lignites or coals of different quality. In comparison with natural HA, RHA are more stable, aromatic, and contain more carboxylic and phenolic groups [12].

The transformation of coal into RHA can be achieved by means of the oxidation using various chemical agents or with air or oxygen [13]. Using oxygen or air, the fragmentation reaction can be controlled, carbon losses can be minimized and almost any coal can be converted in high yields into HA [14]. This method of preparation is very cheap, profitable and mild compared to oxidation in suspension. Despite the differences in fate of RHA and original HA, they have similar characteristics and chemical behavior. They are water insoluble, black, solubility is pH dependent and the dissolution at high pH is related to the presence of many acidic functional groups such as carboxylic and phenolic groups which are responsible for the behavior and performances of these substances in solution [12]. The oxygenated fragments which have been generated (RHA) are held strongly together by hydrogen and/or covalent bonds (e. g. ester groups) [12].

The chemical character of RHA, produced by air oxidation of South Moravian lignite has already been described [15]. It has been verified that higher temperature during the oxidation supports the production of RHA. Despite the difference in the yield of RHA (extractable part of lignite increase up to 75 % after oxidation) their chemical character was, in all cases, fairly similar. In fact, the elemental content did not show any remarkable changes in composition of RHA. As it was mentioned previously [15], the reason can be seen in the kinetics of oxidation processes as well as in the strength of oxidation agent. Some small differences were revealed using FTIR which showed the increase in number of ester bonds in RHA produced mainly at temperatures above 80°C. As a result a significant increase in apparent molecular weight evaluated by High Performance Size Exclusion Chromatography (HPSEC) was observed [15]. Change in the number of ester bonds caused a change in supramolecular structure of HS, which supported formation of humic aggregates. This structural change is supposed to support the formation of gel-like structure with increased water holding capacity.

Methods of thermal analysis such as thermogravimetry (TGA) and differential scanning calorimetry (DSC) represent a very attractive approach in the study of HS [16, 17]. TGA has been used mainly for quantification of the moisture and ash contents [6] In addition, some authors used this technique also for characterization and study of structural changes in HS [12]. It was concluded that the difference in the thermal decomposition curves of HS samples are caused by different degrees of humification [18] and by different biological and chemical pathways which influence the HS supramolecular structure [19].

The aim of this work is additional evaluation of changes in humic acids structure induced by air oxidation of South Moravian lignite. First, the formation of ester bounds described in the recent paper [15] is evaluated as a moisture uptake studied by TGA. Further, the stability and degradability of humic substances reflecting the character of their supramolecular structure is evaluated by DSC and TGA.

2. Experimental

2.1 Production of RHA

Detailed information on production RHA characterized in this work has already been reported [15]. Briefly, South Moravian lignite of different particle sizes was oxidized by the air flow in either fluid or static reactor at different temperatures and for different time intervals. Further, from oxidized lignite, regenerated humic acids were extracted by alkali extraction, purified and freeze-dried. Unlike in reference [15] in this work, only selected samples were studied. Those are listed in Table 1. Sample denoted as HA represents humic acids extracted from parental lignite, samples produced in fluid reactor are abbreviated as RHA 1–3, and samples prepared in the static reactor as RHA 4–7.

Table 1 List of samples with their respective conditions of preparation.

Mark	Fraction [mm]	Temperature [°C]	Time of oxidation, [hrs]
HA	HA from parental lignite		
RHA 1	1–0.5	20	72
RHA 2			120
RHA 3		50	72
RHA 4	0.5–0.125	85	72
RHA 5			120
RHA 6			240
RHA 7			456

2.2 Moisture affinity evaluation

The samples were stored in a moisturizing container, under controlled 100% relative humidity condition at 25 ± 2°C for 21 days. According to the literature [20], after 21 days the equilibrium between HS and water atmosphere should be achieved. Moisture content in HS was measured by TGA.

2.3 Thermal analysis

Shimadzu differential scanning calorimetr DSC-60 was employed in order to determine the heat of thermo-oxidative decomposition of humic samples. The temperature and heat scale were calibrated using In and Zn standards. Measurements were carried out in open aluminum crucibles at heating rate 10°C min^{-1}, from the room temperature to 600°C. An empty pan was used as a reference. Samples mass was typically around 1.5 mg. The dynamic air atmosphere with the flow rate of 20 ml min^{-1} was used as a reactive gas. Prior the measurement, humic samples were carefully homogenized in an agate mortar. All records were corrected by the baseline subtraction (a run without pans under same conditions). Experimental data were processed by means of Shimadzu TA60 software.

Thermogravimetrical analysis TA Instruments Q5000IR with the dynamic nitrogen atmosphere was used for determination of water content in HS samples previously kept in the moisturizing container. The flow rate of nitrogen was set at 5 mL per minute and the heating rate was 10 °C per minute from the room temperature to 250°C. As a sample holder the open platinum pan was used. Typically, mass of samples was around 5 mg.

Next experiments were conducted again on TGA in order to determine the thermo-oxidative stability and degradability of studied samples. The conditions of measurements were as follows: the heating rate 10 °C per minute from the room temperature to 650°C, samples mass about 5 mg, air was used as a reactive gas with the flow rate of 25 ml min^{-1}.

All obtained results were evaluated by means of TA Universal Analysis 2000 software.

3. Results and discussion

As shown in our previous paper [15], the air oxidation of parental lignite leads to the change of physical-chemical properties of respective regenerated humic acids which was revealed by HPSEC. In contrast, other analysis indicated only small changes in their chemical composition. Therefore, it seems that the differences among samples can be associated rather with their physical (supramolecular) than chemical structure. There exist a range of techniques allowing determination of several parameters useful for study of physical structure of humic substances. In this work, physical structure was assessed by two methods of thermal analysis such as TGA and DSC. Further, moisture uptake as another parameter associated with the character of obtained humic acids was determined.

3.1 DSC thermo-oxidative degradation heat and degradation steps

It is a general agreement that the process of HA thermal or thermo-oxidative degradation proceeds in two or three exothermal degradation steps [21]. Those are preceded by moisture evaporation associated with evaporation and/or sublimation of small amount of volatile humic parts [22]. Next, the exothermal reactions associated with rapid mass loss of samples occur. In the lower temperature range, aliphatic molecules (methyls and other saturated and unsaturated moieties), many functional groups (carboxylic, methylene, alcoholic), polysaccharide C–O bonds and simple aromatics (biodegradable components) are degraded or evaporated [25]. In this step occurring in the temperature range ~200–300°C, recombination reactions complicating the simple determination of individual components may take place [23]. For temperature higher than 300°C the decomposition of carboxylic, phenolic, carbonyl and alcoholic groups from HS occurs [18, 25]. The third step (>400°C) reflects the degradation of aromatic, polyaromatic and further also polyheterocyclic structures (highly humified components and recombination byproducts) [24]. The individual stages of degradation are not always separable, mostly, using thermo-analytical approach, steps are overlapping and only two steps are visible. Hence, it is probable that the character of processes occurring during the first step has a strong influence on the second and third one and the total combustion heat can reveal the differences between specific humic samples [23].

This part of the work was devoted to the evaluation of stability of HA and extracted RHA. The obtained data are summarized in Table 2. The DSC analysis of HA and RHA studied in this work confirmed previous observations concerning the thermo-oxidative degradation of HA from different sources. A typical DSC record of thermo-oxidative degradation of HA is

shown in Figure 1. It can be seen two (or three) pronounced separated peaks generally attributed to the thermo-oxidative degradation of labile (~250–440°C) and stable (~445–510°C) humic acids constituents. The resulting heat is proportional to the peak area and it is a result of both degradation and recombination reactions [26] .

Table 2 The summary of results obtained from DSC and TGA (*recalculated for dry part).

		TGA results Δm [%]			
Samples	Combustion heat [kJ g^{-1}]	moisture content	1. step*	2. step*	ash content*
HA	11.3	6.65	77.8	21.1	1.09
RHA 1	10.8	6.44	75.9	23.5	0.58
RHA 2	10.7	6.61	70.0	29.2	0.78
RHA 3	10.3	6.82	75.6	23.8	0.62
RHA 4	11.4	7.87	73.3	25.6	1.18
RHA 5	12.7	7.44	75.5	23.4	1.10
RHA 6	11.8	7.23	79.9	18.9	1.24
RHA 7	11.7	7.16	80.5	18.5	1.00

As can be seen in Figure 2 all the humic samples gave similar DSC records. Nevertheless, some differences appeared comparing the degradation onsets, peak temperatures and peak area (proportional to combustion heat). It seems that increased amount of labile parts as detected by TGA caused the increase in the DSC peaks areas (Figure 2). It can be seen that the peak area around 250–400°C increased confirming the results from TGA. However, it is noteworthy, that the peak increase from 400–500°C occurred as well, but, this region is attributed to the degradation of stable part which was, according to TGA results, partly decomposed by regeneration. Therefore, it implies that the processes in the first step are mostly exothermal recombination of labile part than its degradation.

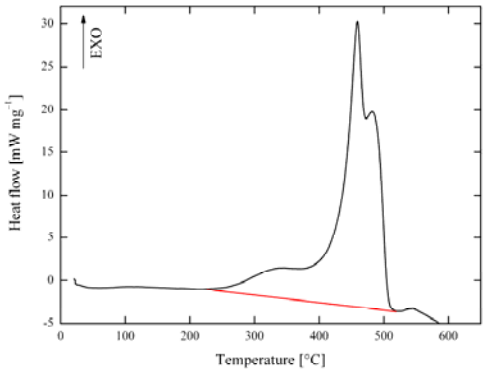

Fig. 1. DSC record of thermooxidative degradation of RHA 4

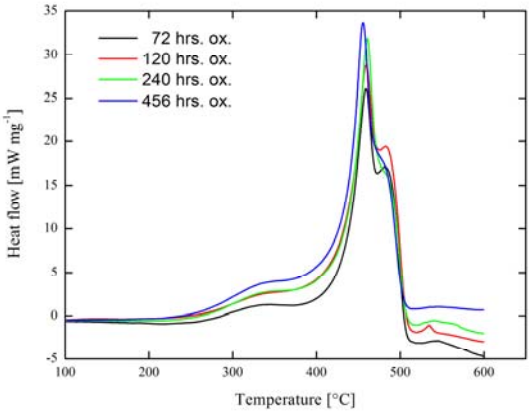

Fig. 2. DSC record of thermooxidative degradation of samples RHA 4–7

Fig. 3 shows a typical TGA record including the first derivative (DTG). In all samples, the progressive mass loss which occurred up to 125°C was caused mainly due to the loss of adsorbed water. In general, the first derivative of thermogravimetric curves showed two degradation rate peaks. One peak occurred between 300 and 440°C and is associated with a mass loss from 70 to 80 % of the total sample mass. A second peak occurred between 480 and 520°C and gave 18 to 30% of mass loss. Thermogravimetric results conformed results obtained by DSC measurements.

The parameters obtained from the DTG, TGA and DSC measurements are summarized in Table 2. HA analyzed in this paper were intensively purified by HCl and HF to reduce the ash content. As it can be seen, the ash content was relatively low, in all cases it was determined about 1%. Moisture content was determined in the interval from 6.6 to 7.9%. It seems that the greater moisture content had samples prepared in the static reactor (RHA 4–7). This increase can be associated with the physical and chemical changes in humic samples as a result of structure alteration which occurred in the course of oxidation periods. TGA measurement of humic samples further showed two mass loss steps. The first step can be related to decomposition of labile parts and the second one to the stable parts of humic acids. Comparison of the values from samples prepared in the static reactor

(RHA 4–7) indicates an increase in labile component content and simultaneously a decrease of stable fraction. Evidently, during oxidation the partial decomposition of stable humic part occurred and therefore the RHA extracted from oxidized lignite contained larger pool of labile molecules. Further decomposition is then easier which can be related to the change of stabile/labile parts ratio. Nevertheless, these results are not confirmed by DSC measurement. Total heat evolved was obtained by the DSC peak integration (Fig. 1). The largest value of 12.7 kJ g^{-1} was observed for sample RHA 5. In this case, no significant correlation was observed.

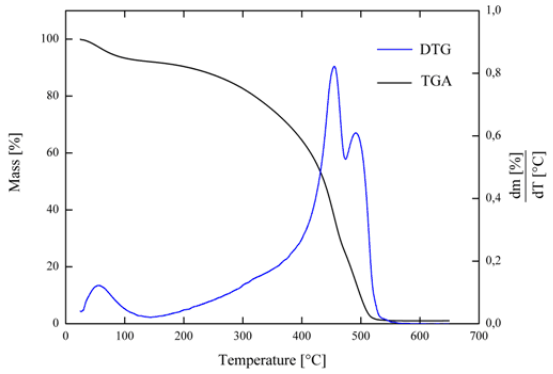

Fig. 3. TGA and DTG record of RHA 4

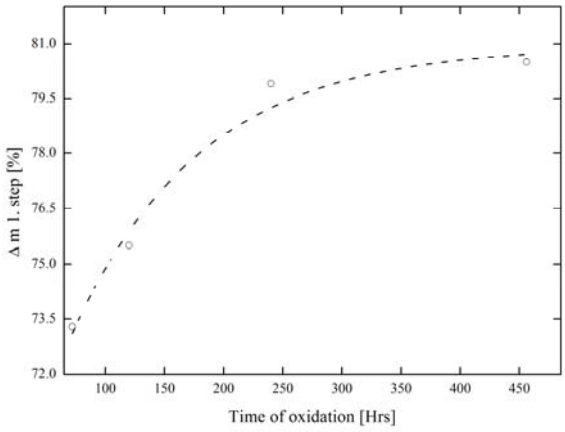

Fig 4. Dependence of the loss of mass in the first step of RHA 4–7 on the time of oxidation

Figure 4 shows the correlation between the mass loss of humic acids in the first step and the time of respective lignite oxidation. It seems that amount of mass decomposition in the TGA first step increases with the time of oxidation. Increasing of labile parts in RHA is also confirmed by DSC measurement as mentioned above. As it can be seen the increase reaches the limit which implies that at approximately 240 hours of oxidation the lignite structure was completely changed a no further conversion can be obtained by extended period of oxidation. Apparently, that statement is valid only for conditions used in this work, i.e. for South Moravian lignite and oxidation by air at 85°C.

3.2 Moisture uptake

Some differences in chemical character among samples have already been revealed using FTIR which indicated a small increase in the number of ester bonds in RHA [15]. Results of HPSEC showed the increase in the apparent molecular weight with increasing time of lignite oxidation of respective samples prepared in the fluid reactor [15]. Thermal analysis techniques such as DSC and TGA confirmed the difference in physical structure. In order to evaluate the changes in detail, the capacity of surface area to uptake moisture was tested using moisturizing container.

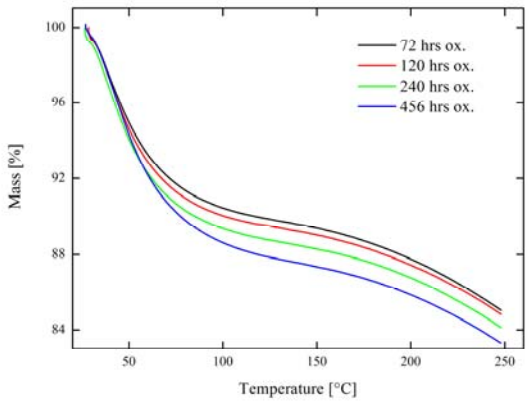

Fig. 5. Evaporation of moisture from samples RHA 4–7

Table 3 Moisture content in RHA samples

Sample	Δm – moisture content [%]
HA	12.9
RHA 1	12.9
RHA 2	11.8
RHA 3	13.5
RHA 4	10.0
RHA 5	10.8
RHA 6	11.2
RHA 7	13.1

Fig 5 shows selected TGA curves, which resulted from the evaporation of moisture adsorbed on the surface of samples RHA 4–7, placed into the moisturizing chamber for 21 days. As

it can be seen all the records gave similar curve shapes which imply similar mechanisms of moisture release. Water content was determined as a mass loss recorded up to 125°C. The results regarding the determination of moisture content in RHA samples are summarized in Table 3. Amount of evaporated moisture was determined in the range from 10 to 13%. In fact, water content increases with increasing time of lignite oxidation. The evaporation of water molecules from RHA is governed by the strength of polar groups on the surface of humic material. The greatest rate of evaporation occurred at the beginning of the experiment where the records had steepest slopes, while at approximately 100°C the kinetics decreased and at between 150 and 200°C degradation started.

As could be seen, moisture content was higher in samples which were prepared at lower temperatures and also increased at longer period of oxidation at 85°C. This trend corroborate with yield of humic substances extracted from oxidized and parental lignite published recently [15] and can be probably attributed to the content of COOH and phenolic OH groups in humic material.

4. Conclusion

Thermal analysis was used to study properties of humic acids and RHA produced from lignite oxidized by air under different conditions. The influence of these conditions on moisture uptake and thermal stability were studied. Our recent results indicated an increase in number of ester bonds in RHA [15]. Further, by means of HPSEC it was demonstrated the increase in the apparent molecular weight with increasing time of oxidation for samples prepared in static and the fluid reactor.

It has been verified that higher temperature during the oxidation supports the production of more "cross-linked" RHA. Moisture content in samples placed into the moisturizing chamber for 21 days (relative humidity is 100%) increased with increasing time of parental lignite oxidation. Comparison of the TGA values from samples prepared in the static reactor (RHA 4–7) indicates an increase of labile component content, and, simultaneously, a decrease of a stable fraction. The increase reached the limit which implies that at approximately after 240 hours of oxidation the structure was completely altered a no further transformation of humic matter took place by further oxidation (for conditions used in this work, oxidation by air at 85°C). No significant correlation was observed for samples extracted from lignite oxidized in fluid reactor. Increasing content of labile pool in RHA was also confirmed by a DSC measurement.

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7.3 Appendix C

Cihlář, Z.; Vojtová, L.; Conte, P.; Nasir, S.; Kučerík, J.: Hydration and water holding properties of cross-linked lignite humic acids, *Geoderma*, 2014, vol. 230–231, no. 1, pp. 151–160. ISSN: 0016- 7061.

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Hydration and water holding properties of cross-linked lignite humic acids

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ABSTRACT

Lignite and lignite humic acids, which are used as soil amendments sometimes, are supposed to improve soil properties such as water holding capacity. The structure of those materials is composed of various organic molecules stabilized mostly by weak interactions. Therefore, excess of water causes only partial swelling, but most of the physical structure is destabilized. This accelerates the desiccation and hampers their application as natural hydrogel-like substances. In order to stabilize the structure of lignite humic acids and improve the water holding capacity, we applied formaldehyde cross-linking procedure based on covalent coupling of aromatic humic acids moieties. By combining the ^{1}H NMR relaxometry and methods of thermal analysis, the kinetics and degree of hydration, water distribution and moisture uptake were investigated. It was found that cross-linking induced a reduction in moisture sorption capacity at low relative humidity and an increase at higher relative humidity, which was attributed to the separation of functional groups and decreasing of structural compactness after cross-linking. As a result, the cross-linked humic acids, exhibited faster water uptake and approximately three-fold higher water holding capacity in comparison with the parental sample. The distribution of relaxation times of water protons in swollen humic acids revealed the unification of pore size distribution upon cross-linking. Although the improved hydration of cross-linked lignite humic acids already resembles the hydration of some hydrophilic polymers, the water holding capacity is still below the capacity of classical hydrogels. Nevertheless, the low price of lignite, sorption properties and its overall positive affect on soil quality and productivity give a promise in application of this material both in agriculture and remediation technologies.

1. Introduction

Intensive agricultural practices together with needs to meet the increasing global food demands are responsible for the desertification processes occurring in the hottest and Southernmost world countries (Kefi et al., 2007). In order to keep soil fertility, both organic and inorganic fertilization practices are traditionally applied. Nowadays, new organic amendments are under consideration. In fact, they are supposed to increase both soil fertility, soil physicochemical characteristics and carbon sequestration, thereby favoring crop productions and preventing emissions of greenhouse gases to the atmosphere (Lehmann and Joseph, 2009). Last decade, among the most studied organic amendments belong chars (or biochars)(Madari et al., 2012), which are carbon-rich products obtained through carbonization of biomasses as it occurs, for example, during pyrolysis and pyrogasification ((Cimò et al., 2014; Conte et al., 2013b; De Pasquale et al., 2012; Knicker, 2010; Knicker et al., 2013). However, also other "cheap and available" carbon-containing materials deserve special attention. One of the most prominent is lignite, which unlike charcoal, has lower structural integrity, contains high amounts of volatile components and has relatively low carbon/oxygen ratio. The latter is caused by the presence of original, not coalified wooden rests, high content of mineral inclusions and relatively high content of humic substances (HS) (Honek et al., 2009).

Humic substances are versatile materials caused mainly by their polyfunctionality (Nasir et al., 2011b). The functions include a wide range of interactions (Sutton and Sposito, 2005), biological activity (David et al., 2014; Jindo et al., 2012), water holding capacity (Stevenson, 1994). However, the positive effect of humic substances-based amendments in environmental and agricultural applications can be easily reversed. For example, the *S. cerevisiae* D7 tests showed the positive antimutagenic effect of native sodium and potassium salts. However, a short heating of those samples induced a negative effect (Marova et al., 2011). Thus, the humic acids preparation and modification requires a special attention.

Numerous studies have shown that humic substances are capable of altering both the chemical and the physical speciation of the ecotoxicants and affect their bioavailability and toxicity (Simpson and Hatcher, 2004).

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The potential utilization of humic substances originating either from lignite or from other sources in agriculture, in environmental technologies and industry has been reported several times (Bakajova et al., 2011; Ctvrtnickova et al., 2011; Nasir, 2011; Nasir et al., 2011a; von Wandruszka, 2000). Using nitric acid and/or hydrogen peroxide (Kucerik et al., 2003), air (Kučerík et al., 2008) or air oxidation under elevated temperature (Calemma et al., 1994), in lignite, the content of humic acids can be significantly increased. However, the unpredictable behavior and structural heterogeneity are (among others) factors reducing the wider applications of humic substances. Their chemical modification is one of the possibilities, how to enhance and control their reactivity, thermal and chemical resistance, sorption properties and solubility (Ryabova and Mustafina, 2003). In this case, the procedures known from polymer science are frequently applied. For example, modification of humic acids with formaldehyde decreases the solubility, increases sorption capacity for calcium cations, whereas the average ionization constant remains unchanged. Formaldehyde crosslinking causes also redistribution of the electron density in the condensed HA system, thereby enhancing the acid properties of weak acid groups and thus increasing the static exchange capacity (Ryabova and Mustafina, 2003). Another promising direction for controlled cross-linking of humic substances is curing of humic phenol-formaldehyde segments, which enhances their detoxifying ability towards heavy metals (Kovalenko et al., 2006). Using those reactions, additional phenolic and quinonoid moieties can be incorporated into humic backbone (Perminova et al., 2005).

Because the modification of humic substances represents additional costs, it has to improve the targeted properties significantly. One of the most prominent properties of humic substances is the water retention capacity, which is connected with their partial hydrophilicity and porous character (Jaeger et al., 2010). At low water content, the water molecule bridges (WaMB) connect and stabilize the segments of soil organic matter (Aquino et al., 2011; Schaumann and Bertmer, 2008; Schaumann and LeBoeuf, 2005). Increasing water content improves sorption capacity of organic matter towards the non-polar compounds (Borisover, 2013; Borisover and Graber, 2004), which underlines the importance of non-covalent polar links in stabilization of soil organic matter supramolecular structure. Excess of water content breaks those interactions (Kucerik et al., 2012) thereby decreasing the structural compactness. This might cause problems in humic-substances based amendments due to their fast desiccation and uncontrolled water release. From the polymer chemistry point of view, this situation can be improved by introduction of covalent bindings, i.e. cross-linking connecting loosely bound molecules.

The cross-linking plays very likely also a role in the humification of natural organic matter and the diagenesis of organic geopolymers (Grasset and Ambles, 1998; Grasset et al., 2002; Pignatello, 2012). This structural stabilization may be important to the ecological function of humic substances, but this issue has been poorly investigated yet (Schneckenburger et al., 2012). Therefore, the investigation of properties of cross-linked humic substances is beneficial both for understanding of their function and their application potential.

The objective of this study was to test the chemical and physicochemical properties of pre-oxidized and formaldehyde cross-linked humic acids obtained from South Moravian lignite. Further, the detail study on the water holding capacity, hydration mechanisms and water distribution in parental and cross-linked humic acids is presented. Obtained information has the relevance in fundamental research, because it brings new information about the hydration mechanisms of humic acids as well as in technologies related to their industrial and agricultural applications.

2. Materials and methods

2.1. Extraction, oxidation and modification of humic acids

South Moravian lignite mined in Mikulcice (mine Mir, near Hodonin, Czech Republic) was used as a source of humic acids. Humic acids were isolated by alkaline extraction. Air-dried lignite fraction was mixed with the aqueous solution of 0.5 mol L^{-1} NaOH and 0.1 mol L^{-1} $Na_4P_2O_7$ and shaken for 3 hours. The suspension was centrifuged and the supernatant was treated with concentrated HCl to reach pH 1 in order to precipitate the humic acid. HA were then treated overnight with a 0.5% (v/v) HCl-HF solution to remove residual ashes, dialyzed (Spectrapore dialysis tubes, 1000 M_w cut-off) against distilled water until chloride-free, and freeze-dried.

Oxidation of HA was performed as follows: 3 g of humic acids were mixed with 60 mL of different oxidizing agents (H_2O_2 or HNO_3) with corresponding concentrations (1 and 3% and 1 and 5% for hydrogen peroxide and nitric acids, respectively) and intensively stirred for 30 min at room temperature. Furthermore, the mixture was 3 times washed by 100 mL of distilled water in order to remove excess of oxidizing agent, filtered and freeze-dried.

Formaldehyde polycondensation was conducted under the conditions reported by (Ryabova and Mustafina, 2003). 0.3 g of humic acids were dispersed in 200 mL distilled water and the pH was adjusted to 7 by 0.1 mol L^{-1} NaOH. Further, the catalytic amount of NaOH (0.2 mL, 1 mol L^{-1}) was added followed by 1 g of a 35% aqueous solution of formaldehyde. The mixture was stirred for one hour at 60 °C under reflux. The product was treated with the concentrated HCl until pH about 1 in order to precipitate the humic acids. Those were then dialyzed (Spectra/Por® dialysis tubes, 1000 M_w cut-off) against distilled water until chloride-free and freeze-dried. The cartoon of the reaction for formaldehyde cross-linking inspired by the works of (Perminova and Hatfield, 2005; Ryabova and Mustafina, 2003) is reported in Fig. 1 and the obtained products are listed in Table 1.

The abbreviation of samples has been chosen in this way: original sample without any treatment is abbreviated “ORIG” and its formaldehyde cross-linked derivative is “ORIG_FM”. The modified samples are marked as “OX”, the suffix “P” stands for hydrogen peroxide and “N” for nitric acid. The number indicates the concentration of respective oxidizer and suffix “_FM” means that the modified HA sample was cross-linked as well.

For analyses carried out in liquid state, humic acids were converted into soluble sodium salts. 50 mg of freeze-dried humic acids were suspended in 50 mL of distilled water. Automatic titrator Titroline alpha plus was employed to stepwise titrate the suspension until the humic acids were dissolved and pH reached 7.4. After that the system was let to equilibrate additional 60 minutes, then filtered and freeze-dried. In this way the sodium humates of original, oxidized and modified samples were obtained.

2.2. Chemical and physical characterization of humic acids

2.2.1. Fourier transformed infrared spectrometry

FTIR spectroscopy was used to assess the changes in chemical composition of both modified and cross-linked humic samples with respect to the composition of original sample. Conventional KBr pellet technique was employed. From 0.5 to 1 mg of humic samples, previously dried at 105 °C for 4 hours in the oven, were mixed with KBr (1:200 w/w) in an agate mortar. Obtained mixture was squeezed to form a pellet and measured in the FTIR spectrometer Nicolet iS10. FTIR spectrometer was set to 128 scan and resolution was 4 cm^{-1} in air dry atmosphere. All obtained FTIR records were elaborated using Omnic 8.0.342 software. For the comparison of intensities of selected bands, the spectra were normalized to overlay the intensities (baselines) at 4000 and 500 cm^{-1}, as indicated in (Ctvrtnickova et al., 2011).

2.2.2. Elemental analysis

Elemental analyses (carbon, hydrogen, nitrogen, and sulfur; the content of oxygen was taken as a difference from 100 %) were performed on a Perkin Elmer Series II CHNS/O Analyzer 2400 at Engineering Test Institute Brno. Table 1 shows the results of the organic elements on ash- and

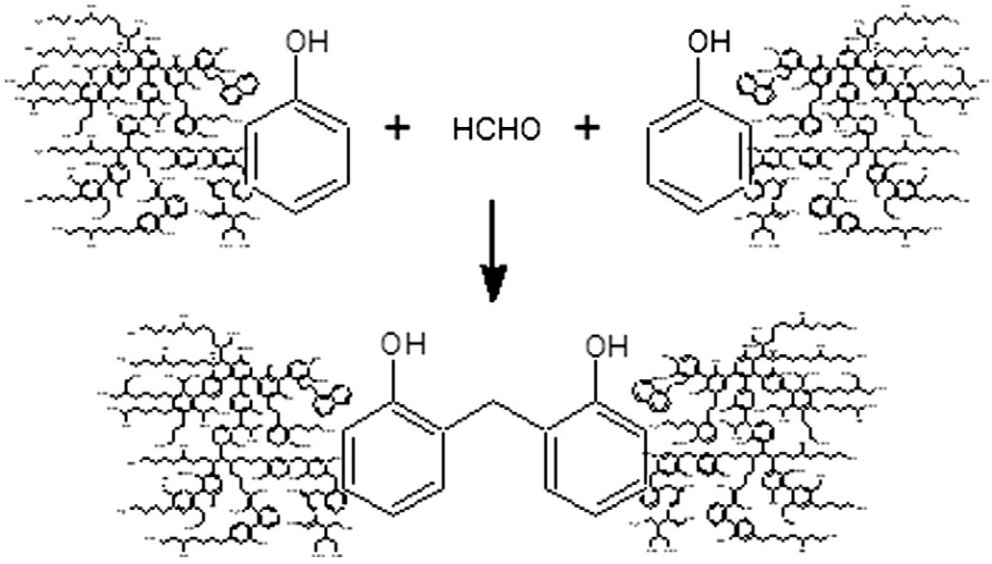

Fig. 1. Crosslinking of humic material using formaldehyde based on paper, repainted from (Perminova and Hatfield, 2005) with kind permission of the publisher.

moisture-free basis for the solid lignite humic acids samples. The deviation of measurement never exceeded 0.2%.

2.2.3. High performance size exclusion chromatography (HPSEC)

Molecular size distributions of humic samples were assessed by an Agilent HPSEC system with a solvent pump, a Biosep S2000 column from Phenomenex (600 x 7.5 mm), UV detector (UVD, 280 nm) and a refractive index detector (RID). The column was preceded by a Biosep Guard column with a 0.2 μm stainless-steel inlet filter. Flow rate was set to 0.6 mL min^{-1}. Samples were dissolved in the mobile phase to achieve the concentration of 0.6 mg mL^{-1}, while the HPSEC eluent was a 50 mM $NaH_2PO_4.H_2O$ solution adjusted to pH 7 with 1 M NaOH in order to keep constant ionic strength and minimize potential ionic exclusion or hydrophobic interactions with the column stationary phase. All solutions were filtered through quartz filters (Glass Microfibre Filter Whatman International, Ltd.) before injection. Samples were loaded by the automatic injector with a 100 μL sample loop. For the calculation of weight-average molecular weight (M_w) Eq. (1) was applied:

$$\overline{M_w} = \frac{\sum_i n_i M_i^2}{\sum_i n_i M_i} = \frac{\sum_i A_i M_i}{\sum_i A_i} \quad (1)$$

In Eq. (1) M_i are the molecular weights, n_i are the number of molecules and A_i are the heights of each i-th slice in the chromatogram, respectively. The HPSEC analyses were conducted in duplicate and the standard deviations of calculated M_w never exceeded 4 % for UVD and 5 % for RID.

Standards of known M_w were used for column calibration. Polysaccharides (186, 100, 23.7 and 12.2 kg mol^{-1}) from Polymer Science Laboratories (UK) were chosen to calibrate the RI detector whereas sodium polystyrenesulphonates PSS (169, 123, 30.9 and 6.78 kg mol^{-1}) from Polymer Standards Service (Germany) were used to calibrate the UV detector. Calibration curves were semi-log linear over the range defined by standards and were used to obtain the molecular weights of humic samples. Each sample was measured twice and the standards were randomly distributed in the autosampler to check measurement reproducibility and state of the filter and the column. The deviation from repeated measurements never exceeded 5%. Water was used to determine the total volume of the column (22.8 mL), whereas blue dextran (2000 Da) was used to measure the void volume (10.5 mL). The two detectors were employed were employed according to (Conte and Piccolo, 1999). First, the UVD, which determines the molecular absorptivity of chromophores at the wavelength of 280 nm. Second, the refractivity index detector (RID). Although the RID is less sensitive than UVD, it is a nass-sensitive instrument independent of the chemical nature of the sample. In fact, whereas UVD can monitor only UV sensible molecular system (i.e. unsaturated molecules), RID provides information on the overall mass size distribution of humic samples (Conte and Piccolo, 1999). The RID responses equally to polymer concentration in all

Table 1
Elemental composition (atomic %) of parental and pre-treated lignite HA samples and their respective ratios.

Sample	treatment	[atomic. %]*						[wt. %]*		TGA[b,c]	FTIR
		C[b,c]	H[b,c]	N[b,c]	O[a,b,c]	C/H	C/O	w[d]	Ash[b]	1 / 2	1436/1508
ORIG	original	39.9	43.9	0.67	15.5	0.91	2.57	6.9	0.9	0.52	1.15
ORIG_FM	formaldehyde	43.2	40.8	0.76	15.3	1.06	2.83	5.8	1.1	0.56	1.24
OX_P_1	1% H_2O_2	43.4	40.8	0.75	15.0	1.06	2.88	5.1	0.9	0.52	1.15
OX_P_1_FM	1% H_2O_2; FM	42.2	41.4	0.74	15.7	1.02	2.69	5.8	1.0	0.58	1.22
OX_P_3	3% H_2O_2	42.5	41.9	0.76	14.9	1.01	2.85	4.5	0.9	0.51	1.16
OX_P_3_FM	3% H_2O_2; FM	41.7	42.2	0.81	15.3	0.99	2.73	5.4	0.6	0.70	1.19
OX_N_1	1% HNO_3	43.3	40.9	0.75	15.1	1.06	2.86	4.2	0.6	0.55	1.13
OX_N_1_FM	1% HNO_3; FM	42.2	42.0	0.76	15.1	1.01	2.80	6.2	0.8	0.52	1.14
OX_N_5	5% HNO_3	43.0	41.9	0.74	14.4	1.03	2.98	4.2	0.6	0.55	1.14
OX_N_5_FM	5% HNO_3; FM	42.0	42.0	0.73	15.3	1.00	2.75	6.5	0.6	0.67	1.24

[a]calculated by difference, [b]dry sample, [c] ash free, [d]moisture content.

molecular weight regimes with the exception of low molecular weights, where the polymer end-groups represent a non-negligible portion of the molecules' mass and do change the refractive index (Wu, 2004).

2.2.4. ^{1}H NMR relaxometry experiments

The HA samples were analyzed in a wet state. The hydration procedure consisted in the addition of 0.5 mL of water to 1 g of each HA sample. Then, the samples were left 24 hours to equilibrate. Finally, the excess of water was allowed to slowly evaporate in standard desiccators for several days. Afterwards samples were stored in a desiccator with a vessel filled by water at 25 ± 2 °C for 21 days, to prevent the water desiccation and achieve equilibration. According to the literature (Hurraß and Schaumann, 2007), after 21 days the equilibrium between HS and water atmosphere can be considered achieved.

Proton longitudinal (T_1) relaxation times were acquired on a Stelar Spinmaster-FFC-2000 Fast-Field-Cycling Relaxometer (Stelar s.r.l., Mede, PV–Italy) at a constant temperature of 20 °C.

The fundamentals of FFC-NMR relaxometry are reported elsewhere (Conte and Alonzo, 2013; Ferrante and Sykora, 2005; Kimmich and Anoardo, 2004). Briefly, the technique is based on the cycling of the Zeeman magnetic field (B_0) through three different values usually indicated as B_{pol} (polarization field), B_{relax} (relaxation field), and B_{acq} (acquisition field). B_{pol} is applied for a period of time during which magnetization saturation and sensitivity enhancement are achieved. Then, the magnetic field is switched to a new one, B_{relax}, applied for a period (τ) during which the intensity of the magnetization relaxes towards a new equilibrium condition. Finally, the application of a magnetic field B_{acq} together with a ^{1}H 90° pulse makes the magnetization observable. B_{pol} was not applied for the present study.

The experimental relaxometric setting consisted of: 1. acquisition of the longitudinal magnetization evolution at value of a relaxation magnetic field of 464 mT (i.e. proton Larmor frequency of 20 MHz) applied for a period of time (τ) arrayed with 64 values varying from 1 to 800 ms. τ array was chosen in a geometrical progression in order to cover the entire relaxation curve of interest; 2. a recycle delay of 4 s; 3. a 380 mT (ω_L = 16.3 MHz) acquisition field with a ^{1}H 90° pulse of 9 μs in order to obtain observable magnetization and reveal free induction decay (FID) with a time domain of 100 μs sampled with 512 points. 2 scans were accumulated. All the experimental data were acquired with the AcquNMR V95® software provided by Stelar.

The recovery curves retrieved by applying the sequence outlined above were elaborated by the UPEN algorithm (Alma Mater Studiorum, Università di Bologna, Italy) (Borgia et al., 1998; Borgia et al., 2000) with the aim to obtain the T_1 distributions and, therefore, information on pore distributions and water interactions (Bayer et al., 2010; Conte and Alonzo, 2013).

2.3. Thermal analysis and sample preparations

2.3.1. Differential scanning calorimetry (DSC) measurement

TA Instruments Differential Scanning Calorimetry (DSC) Q200 equipped with a cooling accessory RCS 90 was employed in order to determine the melting enthalpy of freezable water into HA structure (Kucerik et al., 2012). The temperature and heat scale were calibrated using distilled water and In standards. An empty pan was used as a reference during the experiments. Thermal (freezing/thawing) experiment started at 40 °C, then the sample was cooled down to −70 °C at 3 °C min^{-1}, isothermally kept at −70 °C for 2 min. Then the heating run from −70 °C to 40 °C at 10 °C min^{-1} was conducted. Experiments were carried out under dynamic atmosphere of nitrogen, flow rate was 50 mL min^{-1}.

Sample of approximately 1–4 mg was placed in aluminum sample pan (TA Instruments, Tzero® technology) and the excess of water (milli-Q) was added to HA sample. Surplus water was allowed to evaporate at room temperature until the desired water content was obtained (1 g water/1 g HA). Then the pan was hermetically sealed and left to additional equilibration at room temperature for 1 day. Prepared samples were then measured repeatedly after 1, 5, 8, 14 and 21 days of their preparation. Each sample was measured 2–3 times, standard deviations never exceeded 4% of average volume.

2.3.2. Thermogravimetrical analysis (TGA)

TA Instruments thermogravimetric analyzer Q5000IR with the dynamic nitrogen atmosphere 5 mL min^{-1} was used in order to monitor the mass losses at 40, 50, 70 and 90 °C. In this way the quantitative information on the water fractions adsorbed by different binding energies were obtained. In addition thermogravimetry was also used to get total water content in humic acids represented by a mass loss in the temperature interval 25–150 °C determined under dynamic atmosphere of nitrogen 25 mL min^{-1} (Table 1). The heating rate was 10 °C min^{-1} from the room temperature to 250 °C. This information was further used also for the precise determination of water content during HA/water sample preparation.

In order to determine the thermo-oxidative stability and degradability of studied samples the TGA was used under the dynamic atmosphere of air with the flow rate of 25 mL min^{-1} with the heating rate 10 °C per minute from the room temperature to 650 °C. The samples were measured in triplicates, the standard deviation of mass losses never exceeded 2%.

In all TGA analyses the open platinum pan was used as a sample holder. Typically, mass of samples was always around 5 mg. Prior to the analysis the device was calibrated on temperature and mass change using procedures recommended by provider. All of the experimental dates were processed by means of TA Universal Analysis 2000 software.

Prior to the moisture content analysis, humic acids were stored in moisturizing containers, under controlled different relative humidity conditions at 25 ± 2 °C for 21 days. The atmospheres were kept by appropriate supersaturated water/ salts systems. For this purpose, $Na_2Cr_2O_7$, $(CH_3COO)_2$ Mg and $(NH_4)_2SO_4$ were used and 55, 61 and 79% relative humidity (RH) atmosphere were obtained, respectively.

3. Results and discussion

3.1. Chemical and physicochemical properties of humic samples

3.1.1. Elemental analysis

Table 1 shows the list of samples, the type of oxidation, the chemical modification, the elemental composition of each sample and the results extracted from thermogravimetry and FTIR spectra. Carbon content was moderately increased after all pre-treatments. The lowest content of carbon was measured in sample ORIG, while the largest C content was determined in samples OX_P_1. Higher carbon content in modified samples can be explained by the incorporation of methylene groups (–CH_2–) into humic acids structure as follows from the scheme reported in Fig. 1. Nitrogen content slightly increased in all samples with respect to the ORIG sample. This is caused by nitrification of the structure in case of nitric acid treatment (Kucerik et al., 2003). The increase in relative nitrogen content after crosslinking is quite peculiar since no N atoms are supposed to be introduced into the structure during cross-linking reactions. In line with the literature data about the influence of small molecules on physical structure of humic acids (Conte et al., 2007; Kucerik et al., 2007; Simpson, 2002), it can be assumed that presence of formaldehyde influences the physical structure of humic acids, causing possible leakage of some fractions during purification after synthesis. The loss of some fractions can artificially increase the content of N in cross linked humic acids. The oxygen content was almost the same for all samples, while samples OX_P_3 and OX_N_5 represented samples with the lowest oxygen content. Those samples were oxidized by stronger oxidizing agents than the OX_P_1 and OX_N_1, which probably caused either the decomposition of humic parts and/or an increase in number of highly soluble molecules during sample manipulation. The air oxidation of lignite reported recently (Kučerík et al., 2008) brought

similar results and revealed that oxidation of lignite consists of several phases, in which the yield of regenerated humic acids can even decrease, when weaker oxidative conditions are used due to the preferential oxidation of less stable moieties (typically aliphatic molecules) and oxygen-containing functionalities in parental lignite. This is reflected also in the C/O ratio, which indicates the hydrophilicity degree of a humic matter. Lower value of this ratio is associated with higher content of oxygen functional groups and consequently also higher hydrophilicity. The C/O ratio indicated that a significant difference caused the nitric acid treatment, the highest value was observed for sample OX_N_5, which indicates an intensive decomposition of oxygen containing groups during the oxidation. The crosslinking reactions caused scattered changes in this value, which is caused probably by interaction of formaldehyde with the structure causing above-mentioned leakage during purification.

3.1.2. FTIR spectroscopy

FTIR spectroscopy of humic acids showed the typical spectra of lignite humic acids already present in literature e.g. (Tatzber et al., 2007). In this study, as an example reported in Fig. 2, only the FTIR spectra of ORIG_FM, OX_N_5 and OX_N_5_FM are reported (Fig. 2). To improve their readability, the spectra in Fig. 2 are intentionally shifted.

Fig. 2 shows two intensive peaks around 2926 and 2848 cm^{-1}, which are attributed to $-CH_2-$ and $-CH_3$ vibrations; strong sharp peaks at 1715 cm^{-1} (C = O of COOH) and 1605 cm^{-1} (C = C stretching, C = O stretching of COO^-, ketonic C = O and aromatic C = C conjugated with COO^-); a peak around 1508 cm^{-1} (N-H deformation, C-N stretching vibration, C = C aromatic bounds); a weak peak at 1436 cm^{-1} (aliphatic C–H bending, and COO– asymmetric stretching, and possibly C = C and C = N plane vibrations of heterocycles); an intensive peak around 1215 cm^{-1} (aromatic C, C–O stretch); and a weak peak around 1050 cm^{-1} (C–O of polysaccharides and/or Si-O).

The spectra of the remaining samples had similar patterns with small quantitative differences. Table 1 shows the comparison of the intensities corresponding to the aliphatic C-C (1436 cm^{-1}) and aromatic C = C (1508 cm^{-1}) moieties. The increasing ratio between these two bands indicates the introduction of new aliphatic bonds, thereby confirming the chemical change of the humic structure after crosslinking. The comparison of other peak intensities was not possible due to the high heterogeneity of humic acids, which causes overlapping of the bands super-positions.

3.1.3. Thermogravimetry

The influence of the modification on the thermo-oxidative stability was tested using thermogravimetry. Fig. 3 shows a typical

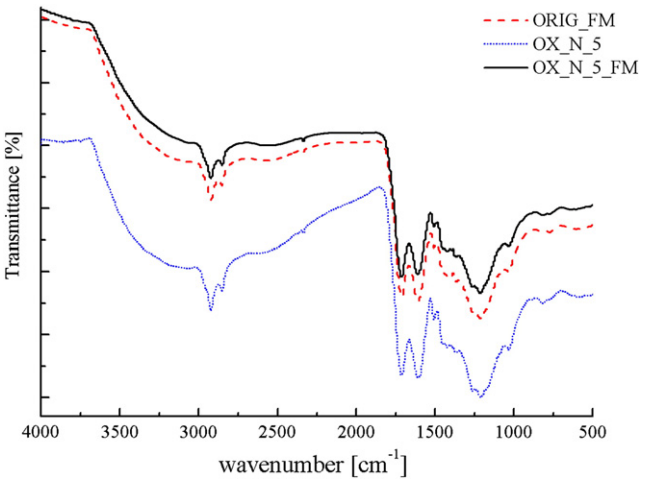

Fig. 2. Comparison of transmittance FTIR spectra for selected samples of HA samples. Y-axes is artificially shifted to increase the readability of the records.

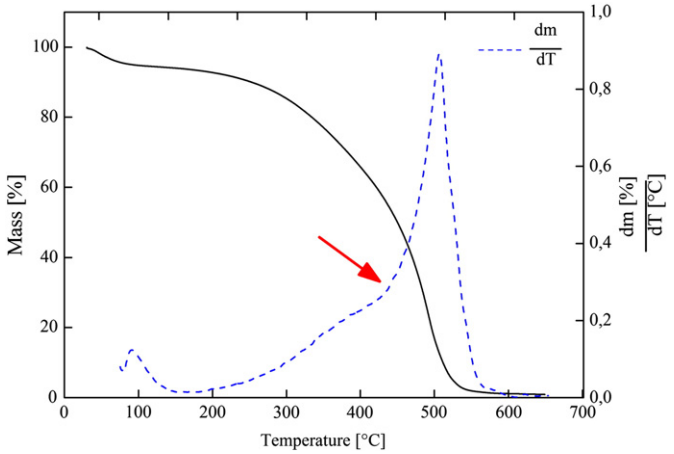

Fig. 3. TGA and DTG record of the sample ORIG_FM, the arrow indicates the change in mechanisms, i.e. the onset of the second degradation step.

thermogravimetry record including its negative first derivative (DTG). The incombustible rest of the mass after the experiment, i.e. the ash content, was approximately 1 % (Table 1). In all samples, the progressive mass loss, which occurred up to 160 °C, was caused mainly due to the loss of adsorbed moisture. For all samples, the total equilibrium moisture content was determined in the interval from 4.2 to 6.9 %. The first derivative of thermogravimetric curves of humic samples showed two peaks in the temperature interval between 180–600 °C, which confirmed a two-step-degradation of lignite humic acids also observed elsewhere (Kucerik et al., 2004). As shown in Fig. 3, first step occurred between 180–450 °C. It is associated with larger mass loss connected with decomposition of labile humic components, typically aliphatic moieties and functional polar groups (Kucerik et al., 2004). The second step can be seen in temperature interval between 450–600 °C. The determined mass losses and their ratio are reported in Table 1. All samples showed either the same or higher ratio between mass losses than ORIG sample. Similarly the crosslinking reactions caused mostly increase in the mass loss ratio with the exception of OX_N_1_FM sample, which showed a slight decrease. Thus both types of modification, i.e. oxidation and crosslinking brought an increase in the labile pool with respect to original samples. This confirms the increase in content of aliphatic structures in cross-linked materials.

The thermogravimetry analyses indicate that both oxidation treatment and crosslinking caused structural destabilization of humic samples. The decrease upon oxidation is in line with literature data, it was explained as the consequence of oxidative disruption of the chemical structure (Kucerik et al., 2005). In case of crosslinking, the decreased stability can be related to the changes in physical structure caused by the aliphatic bridges cross-linking. The increased system porosity supports diffusion of air and enhances the segments mobility due to lower structural compactness. Both cases, i.e. the increase in the reactive surfaces and the decrease of structural compactness, would decrease the temperature of thermo-oxidative degradation onset. This explanation is supported by hydration studies reported in following paragraphs.

3.1.4. High performance size exclusion chromatography

The influence of the pre-oxidation and cross-linking of humic acids was studied by using HPSEC. Fig. 4 reports the results obtained from both UVD and RID, in both cases a bimodal distribution was detected.

The intense peak at the retention time of 23 min was due to the largest components of the humic sample. Conversely, smaller HA components were detected at the largest retention times. It has been already shown that the largest molecular size fractions are made mainly by aromatic, alkyl and potentially carbohydrate-like systems. The length of the carbohydrate-like and the alkyl chains decrease as molecular size decreases. Progressive reduction of aromatic carbon atoms was also

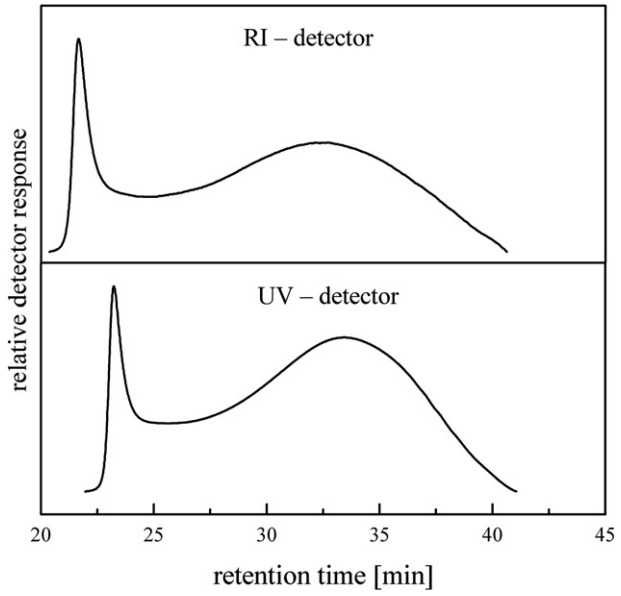

Fig. 4. Exemplary HPSEC chromatograms of the 1% HNO3 pre-treated sample (OX_N_1) detected by UV detection (UVD) at 280 nm and refractive index (RID).

observed with decreasing molecular size of the separated fractions. Finally, the largest amount of hydrophilic molecules has been reported in the low molecular fractions (Conte et al., 2006).

Weight-average molecular weights (M_w) determined from both detectors are reported in Table 2. The comparison between original and modified humic acids shows that both oxidation and formaldehyde modification induced an increase in the molecular size of humic acids. From this point of view, the cross-linking of humic molecules resulted in formation of higher molecular weight polymer-like structures, which verifies the introduction of new covalent bonds or indicates a leakage during the procedure. The comparison of the parental sample ORIG with humic acids oxidized using nitric acid and hydrogen peroxide showed an increase in M_w as well. This is in line with previous discussion about several stages of oxidation favoring oxidation of specific parts of humic acids (Kučerík et al., 2008). The change in molecular weight indicates that those parts were mostly aliphatic moieties containing functional groups, which have lower tendency to form aggregates and thus, their degradation during oxidation caused an increase in M_w. The exception from this explanation seems to be the 5% nitric acid, which is strong enough to degrade also other parts than only aliphatic moieties, because the increase in M_w is insignificant in comparison with weaker hydrogen peroxide or lower concentrated 1% nitric acid.

Table 2
Weight-average (Mw) of parental and pre-treated lignite HA samples. UVD stands for data obtained from UV detector at 280 nm and RID means refractivity index.

Sample	M_w (UVD) [kg.mol^{-1}]	M_w (RID) [kg.mol^{-1}]
ORIG	19.6	54.1
ORIG_FM	26.0	73.4
OX_P_1	22.0	60.8
OX_P_1_FM	30.0	85.2
OX_P_3	23.3	63.3
OX_P_3_FM	25.7	71.0
OX_N_1	24.9	64.6
OX_N_1_FM	23.0	65.9
OX_N_5	20.5	57.8
OX_N_5_FM	23.3	64.6

3.2. Water uptake and water holding capacity of cross-linked materials

3.2.1. Moisture adsorption

The water uptake and water holding capacity belong to the most important properties of humic substances. In natural systems, there are several ways how humic substances can come to the contact with water. To mimic those processes, the samples were first exposed to the controlled atmosphere in a moisturizing container.

Fig. 5a shows an example of the TGA curves measured at four different temperatures obtained for sample OX_N_5_FM, placed into the moisturizing chamber with 55 % relative humidity (RH) for 21 days. Mass losses correspond to the moisture evaporation. Slopes of the curves show that the rate of evaporation of water is the largest at the beginning of the process. Approximately after ten minutes the evaporation is almost finished, i.e. the mass loss is close to zero. The determined decrease in mass corresponds to the amount of water adsorbed on specific sites in HA bond by weaker or stronger forces, depending on the temperature used in the experiment. Fig. 5b shows the quantitative differences of adsorbed water under three different RH conditions. Amount of evaporated moisture was determined in the range from 5 to 10 % for all samples.

The changes in the moisture contents, i.e. difference between water contents on parental and cross-linked samples, are reported in Fig. 6. Here, the y axis shows the change in percentage related to the total sample mass including the dry part. Conversely, the small numbers show this change as a percentage related solely to the water content. Therefore, the positive values of the numbers given in Fig. 6 indicate higher moisture adsorption of samples without cross-linking, and conversely, negative values indicate higher moisture adsorption on cross-linked samples. We do not report the results obtained for 61 % RH, because their values were always between results obtained for RHs 55 % and 79 %. Results for 55 % RH in Fig. 6a shows that the cross-linking induced mostly the decrease in moisture adsorbed by humic samples. The exception is sample OX_N_5, which showed an increase for all adsorbed fractions. The highest difference between cross-linked and its parental substrate 27.4 % at 90 °C for OX_P_3.

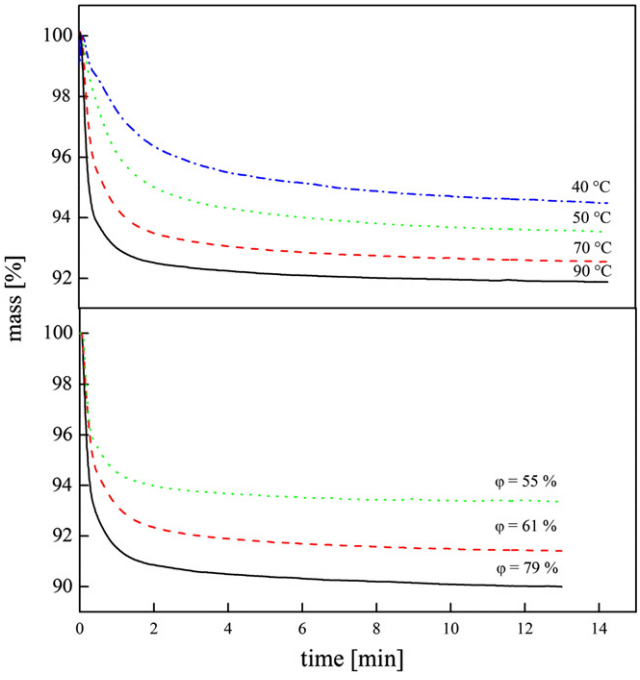

Fig. 5. Exemplary TGA record of pre-treated sample (OX_N_5_FM): Above, moisture evaporation from sample placed for 21 days in 55 % relative humidity chamber and detected under indicated isothermal conditions; Below, moisture evaporation at 70 °C for sample previously stored in chamber with 55, 61 and 79% relative humidity.

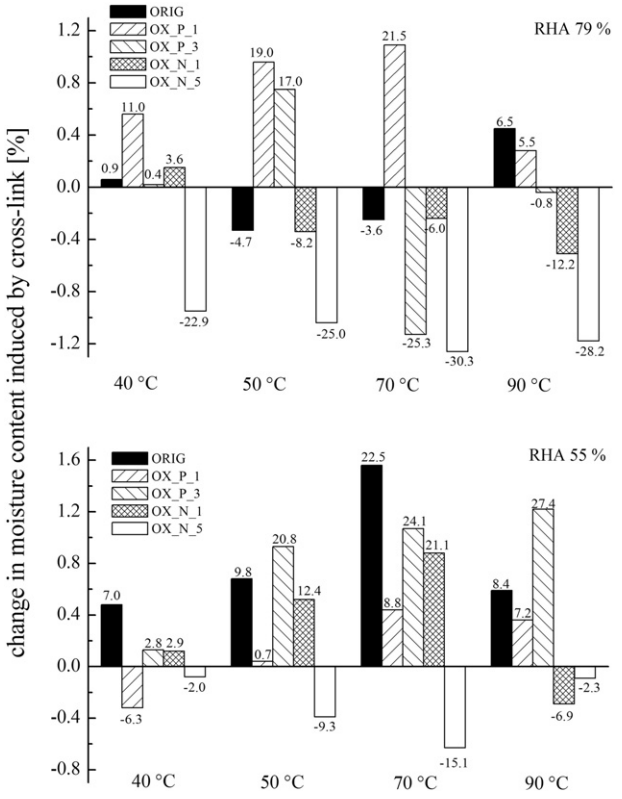

Fig. 6. Moisture content determined by TGA for HA samples stored under specific atmosphere with relevant relative humidity (RH 55 % and 79 %, respectively).

The situation has significantly changed, when the samples were exposed to 79 % RH. In this case, also the sample OX_N_5 adsorbed larger portion of moisture after crosslinking, while the other samples gave mixed results. The increase in this case was around 30 % of total moisture content.

This shows that the surface of cross-linked materials is generally more susceptible for wetting at higher RH. Because the number of polar groups did not change significantly (see results of elemental analysis and FTIR spectra), it can be concluded that cross-linking caused a change in the physical structure of humic acids, thereby supporting the water condensation at higher partial water pressure. In carbonaceous materials, water molecules adsorb onto oxygenated surface sites and act there as nuclei for the formation of larger three-dimensional water clusters or water molecule bridges (WaMB). Under appropriate conditions (e.g. high total pressure or partial water pressure), these clusters connect, either along the surface of across the pore, and pore filling can occur (Brennan et al., 2002; Liu and Monson, 2005; McCallum et al., 1999). Therefore, the determining factor for water condensation is the polar groups density (Brennan et al., 2002). In other words, the distance of the functional groups is a decisive factor in water condensation at different RHs.

It is known that water in soil organic matter can form under appropriate conditions nanodroplets that bridge and stabilize soil organic matter segments (Aquino et al., 2011). Our results indicate that in parental humic acids, the polar groups are close to each other, whereas in the cross-linked humic acids the structural rigidity prevents this kind of aggregation. For this reason, groups are more separated in the latter system Distance between functional groups explnais the difference in structural compactness retrieved by thermogravimetry. At low RH, the condensation in parental humic acid occurs as a bridging of multiple functional groups, already forming the nanodroplets. Conversely, in cross-linked material water condenses at separated functional groups, which are in a large distance to be bridged by the formation of nano-droplets. At higher RH, the water condensation continues and supports the conditions for the formation of nanodroplets also in cross-linked material. Due to the separation of functional groups, the nanodroplets are larger in cross-linked humic acids. The formation of nanodroplets between separated functional groups and their dependency on the distance have been reported recently, based on the experimental and modelling approach dealing with cation-water-molecule bridges in sapric histosol (Kunhi Mouvenchery et al., 2013).

3.2.2. Distribution of water in swollen samples

The other possibility to mimic the natural hydration processes is the direct addition of water excess to humic acids. It is recognized that after wetting stage, which can take from minutes to months in some organic substrates (Jaeger et al., 2010), water molecules penetrate and distribute into the structure causing its swelling (Schaumann et al., 2005). This is accompanied with the interruption of weak interactions stabilizing the supramolecular HS structure (Diehl, 2013; Jaeger et al., 2010; Kucerik et al., 2012).

Fig. 7 reports distribution of longitudinal relaxation times obtained by applying the UPEN algorithm (see Materials and Methods). It must be stated that the weaker the interaction between water and the porous material, the higher is the water molecular mobility, thereby leading to longer T_1 values. Conversely, as the interactions are stronger, water mobility is more restricted, and longitudinal relaxation time is shorter (Bayer et al., 2010; De Pasquale et al., 2012; Jaeger et al., 2010). Water mobility restriction is affected by the dimension of pores in a solid system. In fact, water confined in small sized pores is more constrained than that moving in large sized porous (De Pasquale et al., 2012). On the other hand, chemical bonds also affect molecular mobility and, hence, T_1 values. As an example, water in contact either with carbonaceous materials (Conte et al., 2013b), or inorganic solids (Conte et al., 2013a), may form unconventional hydrogen bonds, which reduces water mobility, thereby producing shorter longitudinal relaxation times.

All samples, except for ORIG_FM, showed only one T_1 component (Fig. 7). It is important to note that the oxidized samples showed broader T_1 distributions as compared to the modified samples. This suggests that cross-linking caused structural changes leading to the unification of pores in humic acids. In fact, the hydration of humic acids is connected with progressive disruption of weak interactions stabilizing their physical structure during which the processes such as dissolution, swollen cavity disruption followed by water redistribution, take place (Kucerik et al., 2012). In some humic acids this redistribution might be non-uniform (Kucerik et al., 2012). Introduction of covalent bonds stabilizes the structure in terms of formation of permanent pores and cavities, which cannot be easily disrupted upon swelling. This observation, as well as the increase in pores size, indicates that water can easily penetrate into the cross-linked structure, because it has to overcome less restrictions connected with wetting and swelling of cavities of small dimensions. This should be reflected in larger water holding capacity and faster hydration kinetics.

3.2.3. Water holding capacity and kinetics of water uptake

In order to quantify the water holding capacity and rate of water penetration, the amount of hydration water was successively determined. In principle, water molecules located in the intimate contact with humic acid do not freeze due to their lower mobility, which prevents their participation in ice formation (Kucerik et al., 2012). Fig. 8 reports the DSC thermograms explaining this experiment. In principle, humic acid with certain water content was cooled and heated and the formation of ice was monitored as the enthalpy of ice melting. Ice is formed from freezable water, its content can be expressed as $W_{freezable} = W_{total}\text{-}W_{non\text{-}freezing}$. Total water content W_{total} is constant in the course of the experiment while the non-freezing water content $W_{non\text{-}freezing}$ is progressively increased. This causes a decrease in content of freezable water $W_{freezable}$, which is proportional to its melting peak area. Therefore, as a

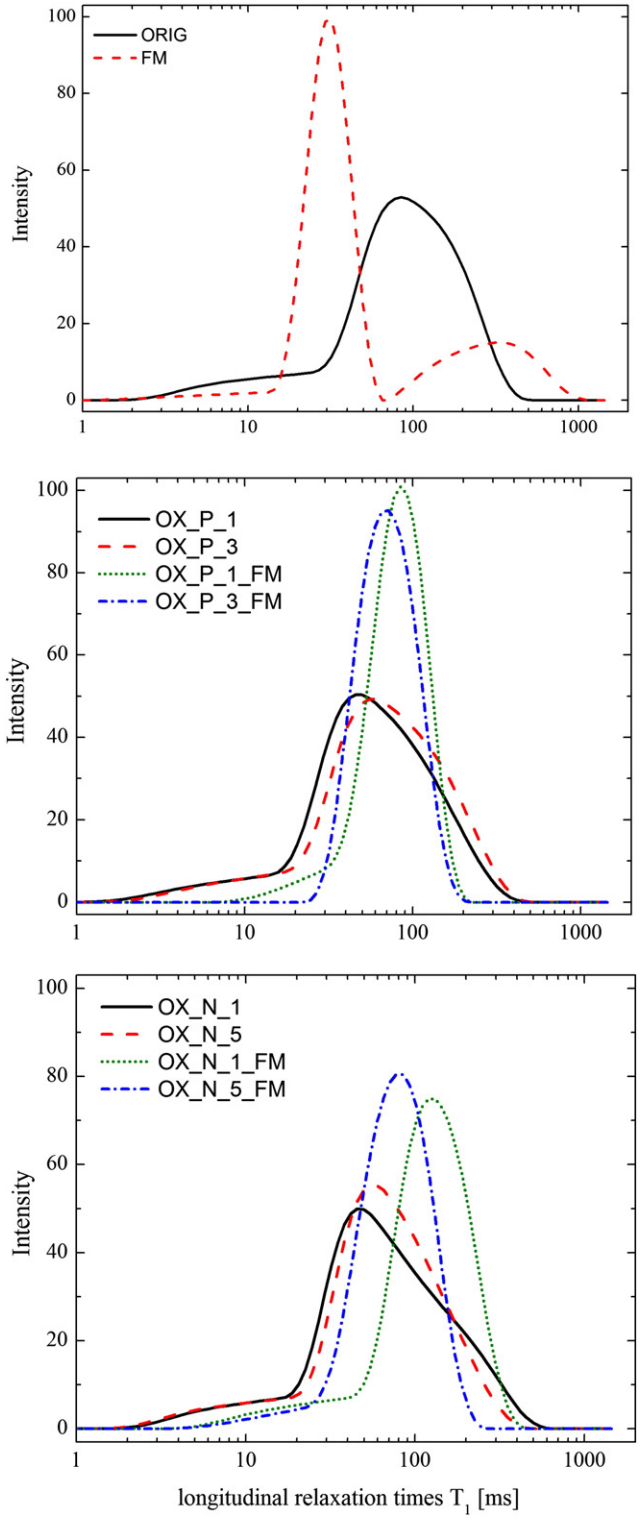

Fig. 7. Representative distributions of T_1 relaxation times curves of water in wetted HA.

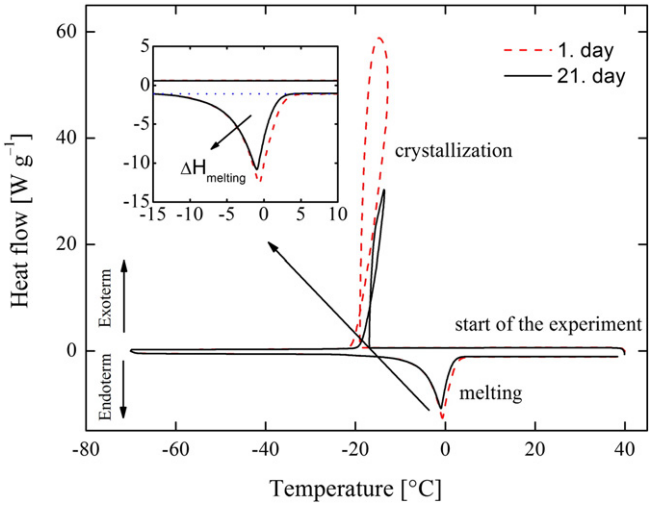

Fig. 8. Comparison of peaks corresponding to melting of ice formed by freezable water in OX_N_5_FM, measured by DSC 1st and 21st day after preparation.

measure of the progressive hydration, the decreasing peak area corresponding to the melting of ice formed by freezable water fraction was used. As it can be seen in Fig. 8 the peak area is reduced after 21 days of hydration.

Table 3 summarizes the comparison of enthalpies of ice melting in all samples (~1 g water/1 g HA) over the period of 21 days. Column 0 day represents theoretical values of melting enthalpy for water added to humic acids and assuming no interaction between water and structure of HA, i.e. situation before swelling of the humic structure takes part. It can be seen the initial steep decrease after 1st day followed by a moderate decrease in the melting enthalpy. This moderate trend decelerated around 8th day of hydration experiment. After this time, only a small difference in melting enthalpy can be observed. The values of enthalpy were almost constant after 21 days and only small changes occurred afterwards. The results reveal different kinetic process related to the uptake of water molecules onto and into humic structure.

The last column in Table 3 reports the relative affectivity of cross-linking with respect to the water holding capacity determined after 21 days of hydration. It can be seen that both oxidation of parental humic acids as well as cross-linking increases the ability of humic structure to accommodate water. The term "Hydration" value has the meaning of the non-freezing water, i.e. water, which does not freeze when cooled down, distributed either in small pores or as the fraction located in the intimate contact with surface of humic acids. In all cases, the crosslinking caused the increase in water holding capacity. The highest water holding capacity showed sample OX_N_5_FM, which gave value 92%. In other words, the hydration of this sample was 0.92 g_{H2O}/g_{HA}. Comparison of original humic acids "ORIG" with humic and fulvic acids collected from different localities shows that lignite humic acids investigated in this work have naturally lower abilities to hold water (Kucerik et al., 2012). Modification caused the three fold increase in water holding capacity, which is value comparable with hydrophilic polysaccharides, e.g. (Mlcoch and Kucerik, 2013; Prusova et al., 2010, 2013). We hypothesize that this is partially caused by the separation of functional groups in cross-linked humic acids, which supports wetting of inner pores.

The anticipated increase in hydration kinetics can be observed in the column 0-1. day. Results in Table 3 show that the modified samples had significantly faster water uptake in comparison with their parental samples. This confirms the previous conclusions about increase in pore size after modification and easier penetration of water into the structure.

4. Conclusions

South Moravian lignite humic acids were oxidized using nitric acid and hydrogen peroxide and then cross-linked using formaldehyde. The chemical and physical structures of products were analyzed using analytical and physical-chemical methods. The elemental composition of humic acids was not dramatically changed after modification and the process of cross-linking caused the slight increase in aliphatic content and apparent molecular weight size. Thermo-oxidative tests revealed that cross-linking decreased the thermo-oxidative stability of cross-linked products, which was ascribed to the increase in porosity

Table 3
The summary of the results obtained from DSC experiments.

Sample	Melting enthalpy [J g^{-1}]							Change of melting enthalpy [J g^{-1}]			
	0.day[a]	1st day	5th day	8th day	14th day	21th day	H_2O [wt. %]	0 – 1st day[a]	1 – 21th day[a]	0 – 21th day[a]	[b]Hydration [g_{water}/g_{HS}]
ORIG	355	269	258	252	251	251	106	86	18	104	29
ORIG_FM	372	244	228	228	223	218	111	128	25	154	41
OX_P_1	366	220	208	201	199	192	109	145	29	174	48
OX_P_1_FM	344	179	173	168	167	164	103	165	15	181	53
OX_P_3	361	214	211	203	197	197	108	147	17	164	45
OX_P_3_FM	397	247	234	214	212	210	119	150	37	187	47
OX_N_1	320	195	191	189	189	189	96	125	6	131	41
OX_N_1_FM	366	195	188	180	179	177	110	171	19	190	52
OX_N_5	373	218	213	200	200	200	112	155	18	174	47
OX_N_5_FM	334	168	135	71	39	26	100	166	142	308	92

[a] theoretical values, [b]decrease of enthalpy after 21 days related to 0 day *100.

of cross-linked humic acids and a decrease in material compactness. The experiments dealing with the interaction of water with obtained humic acids indicated a decrease in moisture sorption capacity at low relative humidity and increase at higher relative humidity. This was attributed to the cross-link-induced increase in the distance between polar groups responsible for condensation of water in humic acids. The hydration study revealed that cross-link, together with an increase in molecular rigidity, caused unification of relaxation times distribution of water protons and facilitated the water penetration into the structure. It was showed that oxidation of parental humic acids followed by formaldehyde-based cross-linking induced a 3 fold increase in the water holding capacity of parental lignite humic acids. This is, however, still below the capacity of synthetic soil hydrogel amendments.

The experiments clearly showed that chemistry of humic substances is connected also with their porosity, which is sometimes overlooked, especially in sorption experiments. In fact, the results showed that only a small change in chemical structure of humic acids can have a great influence on physical structure and thereby on physical properties. In other words, a small change of humic acids, representing soil organic matter, on microscopic scale can have large consequences in the macro world, which is still important to keep in mind in planning of environmental, agricultural and soil protection strategies.

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7.4 Appendix D

Cihlář, Z.; Kučerík, J.; Vojtová, L., Michlovská, L.: Preparation and hydration characteristics of carbodiimide crosslinked lignite humic acids. Manuscript submitted.

Preparation and hydration characteristics of carbodiimide crosslinked lignite humic acids

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Abstract

Polar functional groups in humic acids represent reactive hotspots, which can easily form covalent bonds with other organic molecules. Such reactions can lead to the chemical crosslinking of humic acids that changes dramatically their original physicochemical properties. Water holding capacity and moisture harvesting ability are assumed to be directly linked with the content of unsubstituted polar groups and belong among the most important natural functions of humic acids. In order to produce humic acids with controlled properties and to investigate the major influence of free carboxylic groups on hydration of humic acids, we extracted lignite humic acids, synthesized eight crosslinked lignite humic acids derivatives and tested their hydration properties. The crosslinked derivatives were produced using either water-soluble N-Ethyl-N’-(3-dimethylaminopropyl)carbodiimide (EDC) or water-insoluble N,N'-dicyclohexylcarbodiimide (DCC) under various conditions.

It was found that in terms of hydration properties, more beneficial was the use of EDC than DCC. Furthermore, better performance was reached, when the humic acids were prewetted for at least 24 hours prior to the synthesis. Although the EDC derivatives of humic acids contained only between 14–40 % of original free carboxylic groups, they exceeded the moisture harvesting ability of parental humic acids around 10–14 % after their equilibration at 100 % relative humidity. Despite their more rigid structure, the EDC derivatives showed also faster swelling kinetics and reached almost the same water holding capacity as original sample after 18 days. However, both the EDC and DCC derivatives began to degrade already after 3-9 days during swelling tests, which subsequently decreased their performance.

The results suggest that water holding capacity, swelling kinetics and moisture harvesting ability of humic acids are not influenced exceptionally by the amount of free carboxylic groups or other polar functionalities, but potentially also by their spatial arrangement and the distribution of pore sizes on the surface and inside the humic structure.

Key words: lignite humic acids; swelling; carbodiimide crosslink; moisture harvesting; carbonaceous soil amendment.

1. Introduction

The application of carbonaceous amendments such as humic acids,(von Wandruszka, 2000) biochar (Conte, 2014) and composts (Scotti et al., 2013) has been gaining a growing attention due to their (mostly) positive effect on the stability of soil organic matter and thereby mitigation of soil organic carbon mineralization. The progress and advances in analytical chemistry allowed the detail insight into their molecular structure (Grasset and Ambles, 1998; Grasset et al., 2002; Knicker et al., 2013; Nebbioso and Piccolo, 2011; Stenson et al., 2003), but the relationship between their chemical composition, physical structure and biological activity is still incomplete. This relationship is, however, beneficial for their appropriate use in terms of dosage, effect on soil biota, structure and water holding capacity and overall shifts in biogeochemical cycles of amended soils.

Humic substances (HS) are organic materials naturally occurring in soils, peat, lignite, stream, river, wetland, lake, ground waters, sediments, and natural waters developed as a decomposition product of plant and animal residues (MacCarthy, 2001). Humic acids (HAs) represent a fraction of HS, which is insoluble in water under acidic conditions, but becomes soluble and extractable in alkali solutions. HAs are a mixture of molecules rich in carboxyl groups (-COOH), hydroxyl groups (-OH), amino groups (-NH_2), quinonyl groups (-$C_6H_3O_2$) and others (Stevenson, 1994). This grants them unique properties and versatile environmental functions, e.g. the transport, bioavailability, and solubility of heavy metals and organic molecules, water holding capacity (Stevenson, 1994), regulation of the global carbon and nitrogen cycles(Filip et al., 2011), and biological activity (Jindo et al., 2012).

Currently, HAs are produced on a commercial scale, mostly by extraction from peat or some types of low rank coals. Apart from the agriculture, they are used also in veterinary and human medicine and in some branches of industry (e.g. additives for curing rubber, additives for coloring and increasing the stability of macromolecular substances).(Pena-Mendez et al., 2005) In most cases, the native HAs are used, but a perusal of literature shows an increasing trend to modify their properties in order to improve some of their physicochemical properties (Ctvrtnickova et al., 2011; David et al., 2014; Kovalenko et al., 2006; Perminova et al., 2005; Ryabova and Mustafina, 2003; Schneckenburger et al., 2012).

The variability of functional groups implies that HAs can undergo a large span of chemical reactions to modify their physicochemical properties. Crosslinking is one of the possibilities to enhance and/or control their reactivity, thermal and chemical resistance, sorption properties and solubility (Ryabova and Mustafina, 2003). Indeed, the crosslinking is a natural process in stabilization

of soil organic matter (Smejkalova et al., 2006), playing also a role in diagenesis of organic geopolymers (Grasset and Ambles, 1998; Grasset et al., 2002; Pignatello, 2012; Schneckenburger et al., 2012).

So far, several models of HAs molecular structure have been discussed. A traditional model describing humic acids as a mixture of polymers (Stevenson, 1994), a bilayer model describing HS as a system of amphiphilic macromolecules (Wershaw, 1986), a supramolecular model of small molecules stabilized by non-covalent interactions (Piccolo, 2002; Piccolo and Spiteller, 2003), or a mixture of molecules of various sizes (Schaumann, 2006a; Schaumann, 2006b). Some of these models are in strong contrast, but there is a general consensus that the larger molecular weight or crosslinking of protect soil organic matter against mineralization (Monreal et al., 2010).

In fact, the crosslinking reactions are well known in polymer science. The crosslinking increases molecular weight, creates a network structure, reduces solubility, inhibits biodegradation, increases matrix rigidity, decrease free volume and influences the sorbent properties towards organic compounds (Pignatello, 2012). Ordinarily, the large polymer swellability is accompanied by its poor mechanical properties. Therefore, increasing the crosslinking agent density is a way to improve the mechanical properties, but this in turn affects adversely the swelling. In agriculture, the crosslinked polymer amendments has shown promising results; they have been observed to help reduce irrigation water consumption and the death rate of plants, improve fertilizer retention in the soil, and increase plant growth rate (Puoci et al., 2008), (Huttermann et al., 1990). Our aim is to find a way how to replace these petroleum-based polymers by natural compounds, such as lignite humic acids.

For this reason, in this work, we continue our research (Cihlar et al., 2014), which is aimed to (i) develop a method for production of stable humic acids with controlled water holding and harvesting capacity and (ii) contribute to the fundamental understanding the relationship between crosslinking of humic acids and their physicochemical properties. In fact, the effects of covalent crosslinking on the physical-chemical properties, biological stability and sorption properties of HAs are still poorly understood(Schneckenburger et al., 2012). The presumed target sites for crosslinking are the abundant groups such as hydroxyl, carboxyl and amino groups. Carbodiimides are zero-length crosslinkers; they cause a direct conjugation of carboxylates (–COOH) to primary amines ($–NH_2$), or to esters without becoming part of the final crosslink (amide or esters bond) between targeted molecules (Wong, 1991). In this work, we employed the most readily available and commonly used carbodiimides such as water-soluble N-Ethyl-N’-(3-dimethylaminopropyl)carbodiimide (EDC) for crosslinking HAs in aqueous solutions and water-insoluble N,N'-dicyclohexylcarbodiimide (DCC) for crosslinking HAs by non-aqueous organic synthesis methods(Wong, 1991; Dombekova et al., 2008) Accordingly, a set of carboniimide crosslinked humic acids was produced under different conditions

and tested with respect to their chemical properties, water holding capacity, hydration mechanisms and moisture harvesting ability.

2. Materials and methods

2.1 Chemicals

N-Ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N,N'-dicyklohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS) and 4-dimethylaminopyridine (DMAP) were purchased from Sigma-Aldrich (Germany). Ultrapure water (type II according to ISO 3696) was prepared on Elix 5 UV Water Purification System (Millipore, Merck spol. s r. o.). Dichloromethane (DCM, p.a.), dimethylformamide (DMF, p. a.) and hydrochloric acid (HCl, 36 %) were purchased from Lach-ner (Czech Republic). Ethanol (96 %) was purchased from Moravian distillery, Kojetín, CZ. In addition, sodium hydroxide and hydrofluoric acid were purchased from Lach-Ner Ltd., Neratovice, CZ, while sodium pyrophosphate from Sigma Aldrich Co., Steinheim, Germany).

2.2 Extraction of humic acids

Humic acids were isolated from South Moravian lignite (kindly provided by Lignit s.r.o.), which was mined in Mir mine in Mikulčice, nearby Hodonín, Czech Republic, by alkaline extraction. Air-dried lignite fraction was mixed with the aqueous extractions solution of 0.5 mol L^{-1} NaOH and 0.1 mol L^{-1} $Na_4P_2O_7$ (1:10) and stirred for 3 hours. After centrifugation (15 min, 2612 RCF), the supernatant was treated with concentrated HCl to reach pH 1 precipitating the humic acid. Consequently, HAs were treated overnight with a 0.5% (v/v) HCl-HF solution to remove residual ashes, dialyzed (Spectrapore dialysis tubes, 1000 Mw cut-off) against distilled water until chloride-free, and freeze-dried at –55 °C and 15 Pa for 24 hours to obtain black-brown powder form of HAs.

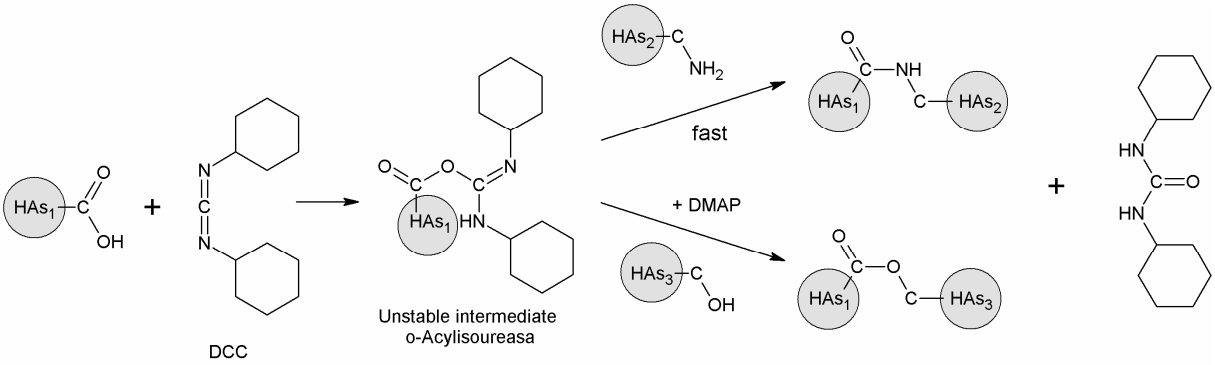
2.3 Synthesis of crosslinked humic acids by EDC

0.5 g of HAs was added directly into the 50 mL of crosslinking agent composed of a mixture of EDC and NHS having concentration of 50 and 25 mmol L^{-1}, respectively. A mixture of ultrapure water and ethanol in a ratio of 1:1 was used as a solvent. The crosslinking reactions proceeded at room temperature for 2 hours under air atmosphere. Thereafter, surplus solvent was evaporated. The obtained conjugate was purified from unreacted reagents and reaction by-products by triplicate rewashing with the excess of distilled water followed by the centrifugation (2612 RCF, 10 min). Finally, prepared suspension was frozen and subsequently freeze-dried using lyophilizer ALPHA-4 at –55 °C and 15 Pa for 24 hours. The powder form of produced crosslinked HAs was analyzed as described in next paragraphs.

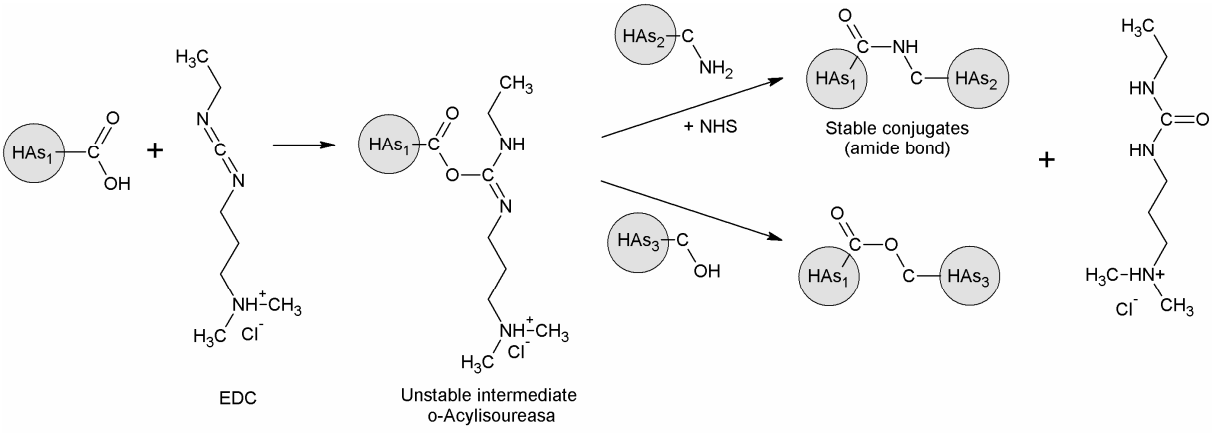
2.4 Synthesis of crosslinked humic acids by DCC

Crosslinking of HAs by DCC was performed according to following procedure: 0.5 g of HAs was mixed with 2 mL of previously distilled dichloromethane (DCM) from CaH_2 in a two neck flask under an inert atmosphere of N_2 using high vacuum apparatus. Further, DCC (in equivalent amount to content of OH and/or COOH groups in HAs) and the catalyst DMAP (10 mol %) were dissolved in 3 mL of DCM and stirred for 20 min. Subsequently, these dissolved reagents were quantitatively transferred under nitrogen by cannula to the HAs suspension in DCM. Carbodiimide coupling was finished in 24 hours under inert conditions. In order to remove the crosslinker, catalyst and by-products all samples were purified. Firstly, the mixture was three times washed with ethanol excess and centrifuged (2612 RCF, 10 min). Supernatant was poured off and the suspension was repeatedly rinsed with distilled water. After centrifugation (2612 RCF, 15 min), the sample was frozen overnight at –18°C and subsequently freeze-dried using lyophilizer ALPHA-4 at –55 °C and 15 Pa for 24 hours to obtain powder form of crosslinked HAs. The reaction mechanisms of both syntheses are reported in Fig. 1.

The abbreviation of samples was chosen in following way: original sample without any treatment is abbreviated as “ORIG”. The crosslinked samples are marked either as “DCC” or “EDC” in respect of used crosslinker. Further, in case of DCC, the presented number indicates a molar ratio between crosslinker and relevant functional groups in HAs, in case of EDC the number represents the period of pre-hydration. The suffix “_DMF” and “_w” denote HAs samples crosslinked in dimethylformamide and ultrapure water, respectively. Finally, the suffix “_w_C” representative sample crosslinked without using catalysis, when different mechanisms and products are expected. The products and their names are summarized in Table 1.



a)



b)

Fig. 1 Schematic reaction of crosslinking of humic acid with carbodiimides a) using EDC b) using DCC. HA stands for humic acids assemblies.

Table 1 List of samples with their respective conditions of preparation

Sample	Treatment
ORIG	original Has
DCC_0.5	HAs crosslinked by DCC in ratio (1:0.5) in DCM, cat. DMAP
DCC_1	HAs crosslinked by DCC in ratio (1:1) in DCM, cat. DMAP
DCC_2	HAs crosslinked by DCC in ratio (1:2) in DCM, cat. DMAP
DCC_0.5_w_C	HAs crosslinked by DCC in ratio (1:0.5) in DCM without cat. DMAP
DCC_0.5_DMF	HAs crosslinked by DCC in ratio (1:0.5) in DMF, cat. DMAP
EDC_24_hrs	HAs pre-hydrated 24 h, crosslinked by EDC in H_2O/Et-OH (1:1), cat. NHS
EDC_5_d	HAs pre-hydrated 5 days, crosslinked by EDC in H_2O/Et-OH (1:1), cat. NHS
EDC_w	HAs without pre-hydration, crosslinked by EDC in H_2O, cat. NHS

DCC: N,N'-dicyclohexylcarbodiimide DCM: dichloromethane
NHS: N-hydroxysuccinimide C = DMAP: 4-dimethylaminopyridine
EDC: N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide
DMF: dimethylformamide

2.5 Characterization of humic acids

2.5.1 Attenuated Total Reflectance Fourier transformed infrared spectroscopy

ATR-FTIR spectroscopy was used to evaluate the changes induced by crosslinking with respect to the structure of original sample. Humic samples were previously dried at 105 °C for 4 hours in the vacuum oven to reduce the water content, which is required to suppress the influence of moisture on FTIR spectra. Measuring was performed on Bruker Tensor 27 with diamond ATR crystal in a range between 4000–600 cm^{-1}. All spectra (128 scans at 4 cm^{-1} resolution) were recorded at laboratory temperature in air dry atmosphere and evaluated by OPUS software. For the comparison of

intensities of selected bands, the spectra were normalized to overlay the intensities (baselines) (Ctvrtnickova et al., 2011).

2.5.2 Elemental analysis

The contents of carbon, hydrogen, nitrogen, sulfur were determined by using an Elementar Vario Micro cube. The content of oxygen was taken as a difference from 100 % (total content of determined elements). The samples of approximately 2 mg (accuracy ± 0.0001 mg) were weighted in tin vessels (4x4x11 mm; Elementar Analysen systeme GmbH) and loaded in the integrated carousel. Sulfanilamide (Art-No.: 15.00_0062, Elementar analyser systeme GmbH) was used several times during measuring as a standard for correction and control of obtained results. Table 2 shows the results of the organic elements on ash- and moisture-free basis for the solid lignite humic acids samples. Measuring was carried out in triplicates and the deviations never exceeded 0.2 %.

Table 2 Elemental composition (atomic %) of parental and crosslinked HAs samples and their relevant ratios

	[atomic. %]							[wt. %]
Sample	C[b,c]	H[b,c]	N[b,c]	S[b,c]	O[a,b,c]	C/H	C/O	Ash[b]
ORIG	39.8	41.7	0.95	0.36	17.2	0.95	2.31	3.3
DCC_0.5	37.5	47.1	1.77	0.25	13.4	0.80	2.80	3.6
DCC_1	36.6	49.2	2.67	0.23	11.3	0.74	3.22	3.3
DCC_2	37.8	49.2	2.67	0.20	10.1	0.77	3.75	3.1
DCC_0.5_w_C	37.4	46.9	1.62	0.25	13.8	0.80	2.70	4.0
DCC_0.5_DMF	39.9	43.8	1.39	0.31	14.6	0.91	2.73	4.3
EDC_24_h	39.4	41.3	1.53	0.39	17.4	0.95	2.27	1.4
EDC_5_d	39.9	40.8	1.28	0.40	17.6	0.98	2.26	1.6
EDC_w	39.4	43.9	1.40	0.31	15.0	0.90	2.63	2.2

[a]calculated by difference, [b]dry sample, [c] ash free

2.5.3. Stability test carried out using thermogravimetrical analysis (TGA)

Simultaneous Thermal Analyzer NETZSCH STA 449 F3 Jupiter coupled with Quadrupole Mass Spectrometer QMS 403C Aëolos was used in order to determine the thermo-oxidative stability of produced materials. Following conditions were used: dynamic atmosphere of synthetic air with the flow rate of 50 mL min^{-1}, heating rate of 10 °C per minute from the ambient temperature to 1000 °C. The samples were measured in triplicates, the standard deviation of mass losses never exceeded 3 %. In all TGA analyses, the open alumina pan was used as a sample holder. Typically, mass of samples was in a range of 6–10 mg. Prior to the analysis, the device was calibrated on temperature and mass change using procedures recommended by provider.

2.5.4 Determination of free carboxylic group contents

The pH titrations of HAs were conducted to assess the amount of free COOH groups that were untouched by crosslinking reactions. To determine carboxyl groups content, NaOH has been used for titration (Masini et al., 1998). Equivalent points were chosen at pH 7.0, which is usually considered as the end of the titration of carboxyl groups(Khil'ko et al., 2011).

The applied technique consisted in the following steps. The accurately weighed samples (approximately 40 mg) of HAs and their derivatives were placed in flasks and dispersed/dissolved in 10 mL of distilled water. The contents were intensively shaken for 24 hours at 20 °C, to pre-wet and pre-hydrate the HAs structure in order to support the deprotonization of COOH groups and distribution of sodium cations in the HAs structure. Then, the pH values of obtained suspensions were measured.

Furthermore, under constant stirring, the standardized 0.1 mol L^{-1} of NaOH was added stepwise to the HAs suspension. After a fast increase in pH followed a slow decrease, which took typically several hours to reach an equilibrium value. Afterwards, a new dose of titrant was added again. In this way, the final pH 7 was reached. The total titration time was typically 7 days.

The pH values of solutions were measured using a multi-parameter analyzer Consort C 863, (Belgium) equipped with a glass combined electrode. All titrations were performed in absence of background electrolyte. Each solution was titrated at least three times, the reported data representing the average values. The standard deviations of the consumption of NaOH never exceeded 7 %.

2.6. Study of water behavior in HAs

2.6.1 Assessing hydration state

The ^{1}H NMR relaxometry was carried out to monitor the progress of moisture adsorption and hydration connected with swelling. For this purpose, two subsequent experiments were conducted. First, we studied the kinetics of moisture harvesting. 200 mg of humic acids samples were stored at 19 °C in a moisturizing chamber. The atmosphere was saturated by continuous evaporation of water from the vessel situated at the bottom of the container, which assured ~100% relative humidity (RH) conditions. Each 48 hours, HAs samples were analyzed for their water content (by weighing) and for the dynamics of protons (by using ^{1}H NMR relaxometry). As soon as the water content was not increasing anymore, the second “direct hydration” experiment was started. In fact, the samples from the previous experiment were hydrated further, this time by addition of several µL of water using a micropipette. After each addition, the samples were left for 24 hours to equilibrate.

^{1}H NMR measurements were performed using a Minispec mq 7.5 NMR Analyzer (Bruker Co., Germany) instrument, operating at the proton Larmor frequency of 7.5 MHz. The measurements were carried out at 25 °C. The low frequency is sensitive mainly for relaxation of protons in water

(Conte and Alonzo, 2013). Their concentration was progressively increased, which caused a change in the relaxation times of water presented in HAs structure. T2 (spin-spin) transverse relaxation times were obtained by applying the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence. For the samples with different water content, the interecho spacing (0.1 ms) was kept constant, whereas the number of echoes and repetitions was changed depending on the water content in order to obtain a good signal-to-noise ratio. The repetition time between scans was five times T1 (spin-lattice relaxation time, determined separately by inversion recovery sequence) to avoid the T1 weighting. To calculate T2 values, the transverse relaxation decay curves were fitted by biexponential function Eq. 1 using RIWinFit software (Version 2.4, Resonance Instrument Ltd., Oxfordshire, United Kingdom):

$$F(t) = A_1 \exp\left(\frac{-t}{T_{2,1}}\right) + A_2 \exp\left(\frac{-t}{T_{2,2}}\right) \quad 1$$

Where t is time, $A_{1,2}$ are amplitudes, and $T_{2,1}$ and $T_{2,2}$ are spin–spin relaxation times of present water fractions.

2.6.2. Hydration study

Differential scanning calorimetry (DSC) measurements were performed in order to analyze properties and behavior of the hydrated system water/HAs. TA Instruments DSC Q1000, equipped with cooling accessory RCS90 was used and data were elaborated by TA Universal Analysis 2000 software. The temperature and enthalpy calibration of the device were carried out using In and Sn standards. An empty pan was used as a reference during the experiments.

The upper temperature limit for DSC experiment, i.e. the decomposition temperature was determined using evolved gas analysis, as described in 2.4.3.

Freezing/thawing experiments were carried out in order to determine the enthalpy of ice melting, which was formed from the freezable water present in the HAs sample. The enthalpy was determined from the area of the endothermic peak occurring typically in the temperature interval from −30 °C to 10 °C.

Sample of approximately 1–4 mg (weighed with an accuracy of ± 0.01 mg) was placed in aluminum sample pan (TA Instruments, Tzero® technology) and required amount of ultrapure water was added to HAs sample. Five concentrations within Wc from 0.5 to 3 g water/g HAs were prepared for each sample. The pans were hermetically sealed and left for additional equilibration at room temperature for 1 day. Afterwards, the samples were measured repeatedly during 18 days.

The DSC experiment started at 30 °C, then the sample was cooled down to –60 °C at 3 °C min^{-1}, isothermally kept at −60 °C for 1 min. Then the heating run from −60 °C to 30 °C at 5 °C min^{-1} was

conducted. Experiments were carried out under dynamic atmosphere of nitrogen with flow rate of 50 mL min^{-1}.

Selected samples were prepared in triplicates and no significant difference in the DSC record and respective melting enthalpy was observed. In addition, some measurements ran through three immediately subsequent identical cycles to test reproducibility of ice melting and to investigate potential changes in the hydrated HAs structure due to the freezing-melting cycles. In this way, it was also verified that sample pans were well hermetically sealed, thus no water was evaporated and that formation of ice, due to its volume expansion, does not cause the HAs supramolecular destruction as mentioned in ref.(Kucerik et al., 2012)

2.6.3. Strength of water binding determined by DSC

The heat necessary for evaporation and/or desorption of water from HAs were determined in order to evaluate a change in the strength of water binging caused by modification of humic acids.

2–4 mg of HAs, equilibrated for 8 weeks at 76% relative humidity (saturated NaCl solution), were placed in an aluminum pan and hermetically sealed (Tzero® technology, DSC Q1000 TA Instruments). Prior the measurement, the hermetic lid was carefully perforated using a sharp tool and the measurement was carried out straightway (Prusova et al., 2010). The following thermal protocol for the measurement of desorption enthalpy was used: started at 20 °C; fast cooling to −40 °C. The next step was heating from −40 °C to 200 °C at heating rate 5 °C min^{-1}. The flow rate of the dynamic nitrogen atmosphere was 5 mL min^{-1}.

Selected samples were measured in triplicate to determine the statistical significance. Standard deviation never exceeded 7 %; typically it was below 5 %. Weighing samples before and after experiment allowed a precise determination of water content evaporated during the measurement and used for the normalization of measured enthalpy.

3. Results and discussion

3.1. Chemical and physicochemical properties of humic samples

3.1.1. Chemical changes in crosslinked humic acids

The exemplary FTIR spectra of ORIG, DCC_2 and EDC_w are reported in Fig. 2. To improve their readability, the spectra are intentionally shifted. The spectra of the remaining samples had similar patterns with only small quantitative differences. The spectrum of ORIG has bands typical for HAs from other source, namely an intensive peak within the range between 3400–3000 cm^{-1}, which can be attributed mainly to the hydrogen bonded –OH; two weak peaks in the region 2920–2850 cm^{-1} (aliphatic C–H stretching), strong sharp peaks around 1710 cm^{-1} (C=O of COOH) and 1610 cm^{-1} (C=C stretching, C=O stretching of COO–, ketonic C=O and aromatic C=C conjugated with COO^{-}), a weak

peak around 1510 cm^{-1} (N-H deformation, C-N stretching vibration, C=C aromatic bounds), peaks around 1250 cm^{-1} (aromatic C, C-O stretch), a weak peak around 1040 cm^{-1} (C-O of polysaccharides and/or Si-O)(Tatzber et al., 2007).

In crosslinked HAs a new sharp absorption peak appeared around 3330 cm^{-1}. This can be attributed to a change of symmetric and anti-symmetric stretching of the primary amine to the stretching vibration of the secondary amine.(Xu et al., 2009) The content of saturated aliphatic structures is reflected in 2940–2850 cm^{-1} absorption range. Two peaks (around 2934 and around 2847 cm^{-1}) correspond to the asymmetrical and symmetrical stretching of methylene (-CH_2-) groups, respectively, and are characteristic for presence of aliphatic and non-strained cyclic hydrocarbons (Silverstein et al., 1991). In addition, peak around 2940 cm^{-1} can be attributed to the asymmetrical C-H stretching of methyl (-CH_3) groups (Tatzber et al., 2007). Higher intensity in this region for samples modified by DCC is presumably caused by the rest of crosslinker and/or catalyst moieties in the system. Next systematic difference is observed from 1580 to 1720 cm^{-1}. First, the carboxyl C=O stretching absorption peak (around 1718 cm^{-1}) is slightly stronger in the ORIG than in the EDC_w, but weaker than in the DCC_2. This could be expected, due to the involvement of carboxyl groups in the chemical modification. The aim of the modification was the formation of ester and/or amide bonds through carbodiimide coupling. The FTIR spectra confirmed the introduction of new ester bonds, thereby affirming the chemical change of the humic structure after crosslinking.

For DCC_2, on the other hand, the peak around 1630 cm^{-1} increased its intensity with respect to the carboxyl peak. The bands in this range of the infrared spectrum may have several reasons, including aromatic C=C stretching, amide group ($RCONH_2$) C=O stretching and amide -NH bending. Carbon-carbon stretching usually appears at about 1650 cm^{-1} for doubled bonds, but is shifted to about 1600 cm^{-1} by conjugation. Substituted or unsubstituted amides, on the other hand, show the C=O and -NH bands in the 1640–1690 cm^{-1} and 1600–1640 cm^{-1} regions, respectively (Silverstein et al., 1991).

The comparison of other peak intensities was not possible due to the high heterogeneity of humic acids, which causes overlapping of the bands super-positions.

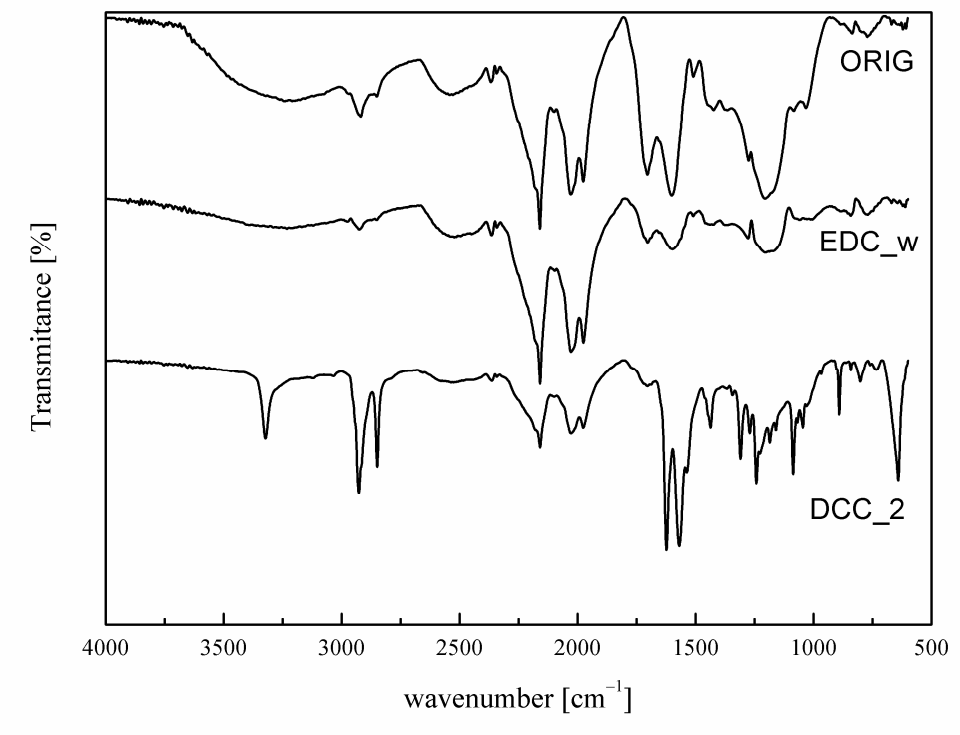


Fig. 2 Comparison of transmittance ATR-FTIR spectra for selected samples of HAs. Y-axe is artificially shifted to increase the readability of the records.

3.1.2. Elemental analysis

Table 2 summarizes the elemental composition of investigated HAs. Due to their complexity, the elemental composition of HAs alone is not helpful in evaluation of changes. Therefore, in addition, the respective atomic ratios (C/H and C/O) have been calculated to assess the relative shifts in their ratios. In all cases, carbon content was slightly decreased after the crosslinking. The lowest content of carbon was measured in sample DCC_0.5_w_C. It is rather peculiar, because no new C atoms are supposed to be incorporated into the structure during crosslinking reactions as follows from the scheme reported in Fig. 1. We assume that the lower or identical carbon content in modified samples was caused by the partial extraction of some HAs fractions during purification. In contrast, the content of nitrogen slightly increased in all modified samples, especially for samples crosslinked by DCC (reagent insoluble in water). This could be caused by the presence of some rests of previously used couplings agents in the samples, which indicate an partially insufficient purification procedure or strong physical sorption of DCC on HAs. The oxygen content was lower for all samples, except for samples EDC_24_h and EDC_5_d. A decrease of oxygen was expected, because H_2O is eliminated during carbodiimide coupling (Fig. 1). Samples DCC_1 and DCC_2 had the lowest oxygen content, which support the hypothesis about washing out of polar molecules. This is confirmed by the C/O ratio, which indicates the hydrophilicity degree of a humic matter. Higher value of this ratio is

associated with lower content of oxygen functional groups and consequently denotes lower hydrophilicity. The C/O ratio reveals a significant difference caused by the DCC treatment, the highest value was observed for sample DCC_2, which suggests an intensive formation of esters bounds during the modification. Ratio C/O indicates, among others, indirectly the content of COOH. The C/H ratio is an indicator of aromaticity or unsaturation (Stevenson, 1994). In most cases, the ratio decreased, which can be ascribed to above-mentioned dissolution of oxygen rich molecules. The decrease in C/H indicates that they were mostly the aliphatic molecules. Similar washing-out problems with aliphatic water-soluble molecules have been reported for crosslinking of HAs using formaldehyde (Cihlar et al., 2014).

3.1.3. Thermo-oxidative stability

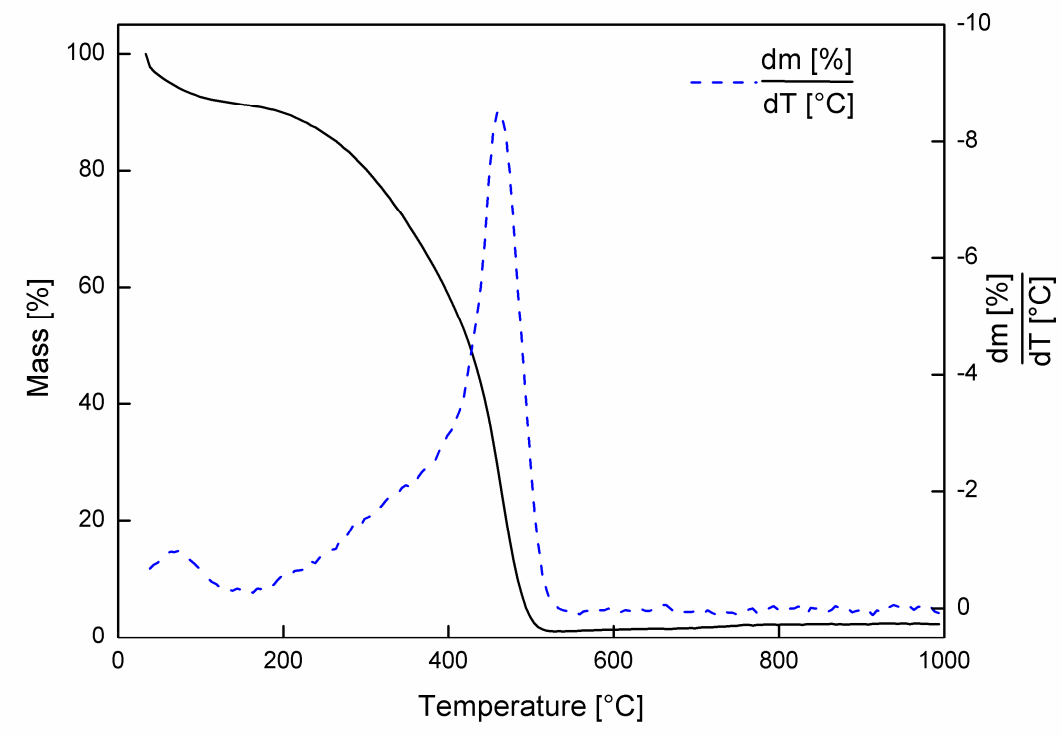
The effect of the crosslinking on the thermo-oxidative stability of HAs was analyzed by using thermogravimetry. Fig. 3a shows a typical thermogravimetry record (TG) including negative first derivative (DTG). In all samples, the progressive mass loss, which occurred up to 160 °C, was caused mainly due to evaporation/desorption of moisture. The first derivative of thermogravimetric curves of humic samples displayed two broad intensive peaks in the temperature interval between 180–1000 °C, which confirmed two-step-degradation process of lignite humic acids, typical for humic acids from different sources (Rotaru et al., 2008). The individual stages of degradation are not perfectly separable, and likely, some recombination reactions take place. At low temperature degrade or evaporate labile humic components, typically aliphatic molecules, many functional groups, polysaccharide C-O bonds and simple aromatics moieties. The second step is connected with the degradation of aromatic, polyaromatic and further also polyheterocyclic structures (highly humified components and recombination byproducts)(Kucerik et al., 2004). The incombustible rest of the mass after the thermal experiment, i.e. the ash content, was up to 4.3 % (Table 2).

The determined mass losses and peaks temperatures are reported in Table 3. The crosslinking reactions caused mostly an increase in the mass loss in the second degradation step and an increase in respective peak temperature with some exceptions. The decrease was observed mainly for samples crosslinked in water namely EDC_24_hrs and EDC_5_d and DCC_0.5_w_C, which was prepared in dichloromethane. We have two possible explanations. It can be related to the washing-out some hydrophilic fraction from the HAs structure, which cannot be afterwards involved in coupling reaction. This hypothesis is supported mainly by the sample of EDC_5_d that was in contact with water longest time and showed the most intensive destabilization, but it is in contrast to C/O ratio reported in Table 2, which did not change. Thus, the more probable is the second hypothesis that the destabilization can be also caused by the increasing surface pore size facilitating the access of air and thereby accelerating the thermo-oxidative degradation.

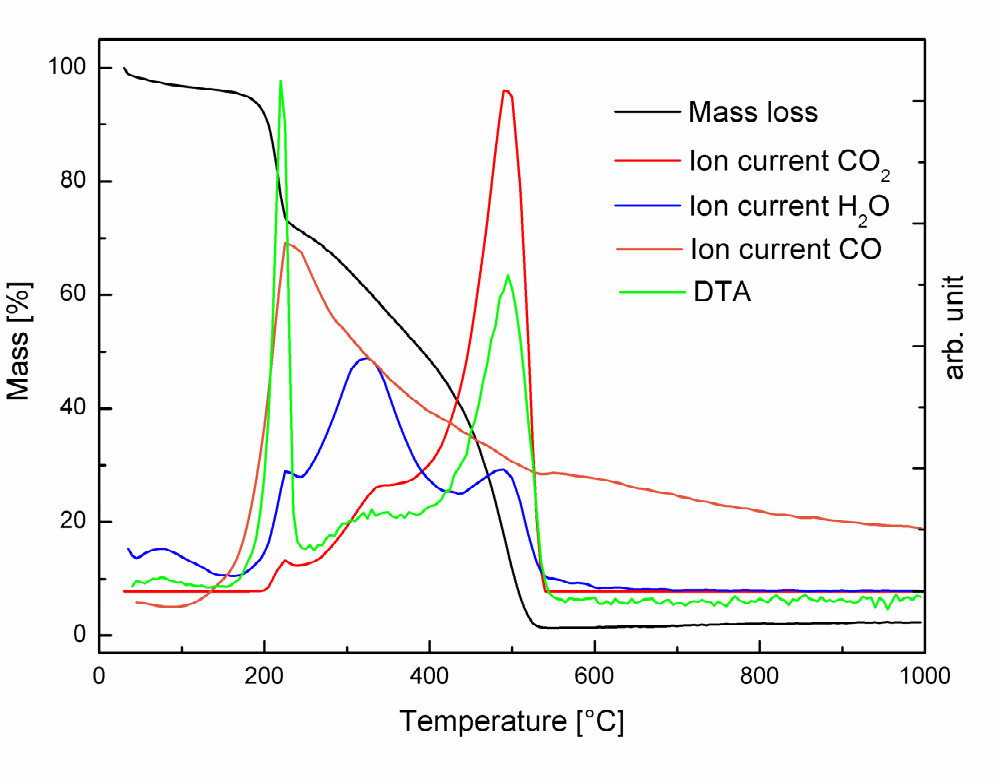
The TG records of DCC samples showed an additional mass loss step around 200 °C. Fig. 3b shows an exemplary record for DCC_2. It can be seen that the temperature region up to 160 °C is associated only with the evaporation of moisture. The analysis of the gases typical for HAs degradation, such as CO and CO_2, reveals that the HAs degradation started after the sudden mass loss occurred. This indicates that the elevated temperatures caused a liberation of physically occluded residual crosslinked agents trapped in the HAs structure that was not removed during purification.

Table 3 Temperature intervals and corresponding mass loses recorded by thermogravimetry

	Peak I.		Peak II.	
Sample	mass loss [wt. %]	peak T [°C]	mass loss [wt. %]	peak T [°C]
ORIG			71.7	460
DCC_0.5	12.4	208	72.7	451
DCC_1	12.5	211	77.3	440
DCC_2	11.6	208	80.0	487
DCC_0.5_w_C	13.1	208	67.4	448
DCC_0.5_DMF			73.6	478
EDC_24_hrs			68.7	437
EDC_5_d			69.2	425
EDC_w			71.5	478



a)



b)

Fig. 3 TG and DTG record of (a) ORIG sample and (b) of DCC_2 sample together with evolved gases CO_2, CO and H_2O signals

3.1.4. Content of residual carboxylic groups

The residual carboxylic groups' content was used as an indicator of the degree of HAs crosslinking. Table 4 summarizes the obtained data. The first column corresponds to the concentration of dissociated H^+, which was calculated from the pH of the suspension obtained by mixing of HAs samples with water. Thus, the value reflects the amount and strength of dissociated (mostly) carboxylic groups. In addition, the value is partially related also to the rigidity of the swollen HAs structure, because in crosslinked system, water diffusion is restricted. Table 4 shows that except for sample DCC_0.5_DMF, all samples had lower degree of deprotonization than ORIG sample, while the lowest initial proton concentration was determined for sample DCC_2. These values, therefore, indicate formation of more rigid crosslinked structure and preferable involvement of strong functional groups in crosslinking. Therefore, a decrease in proton concentration is one of the indicators of the formation of new covalent bonds.

Values in second column have a meaning of total concentration of carboxylic groups in HAs. In all cases the values are lower than for original HAs, which confirms that the amount of carboxylic groups decreased upon the crosslinking. The extent of their use is reflected in the third column, which reports the percentage differences between original HAs (100 %) and crosslinked ones. The data

show that the DCC caused higher degree of crosslinking than EDC. However, the data in first column indicate that DCC samples contained still many strong acidic carboxylic groups (in terms of tendency to deprotonize), while EDC samples contained mainly weak ones. This indicates that EDC is more selective and presumably more effective with HAs samples containing stronger carboxylic acids.

The behavior of DCC_0.5_DMF sample is unique because it showed the highest concentration of H^+ ions and the same time 50 % of original carboxylic groups were involved in crosslinking. We have no explanation of such properties.

Table 4 Concentration of H^+ in HAs aqueous suspensions (calculated from pH of the suspension) and the total amount of COOH groups (calculated from NaOH consumption to pH 7).

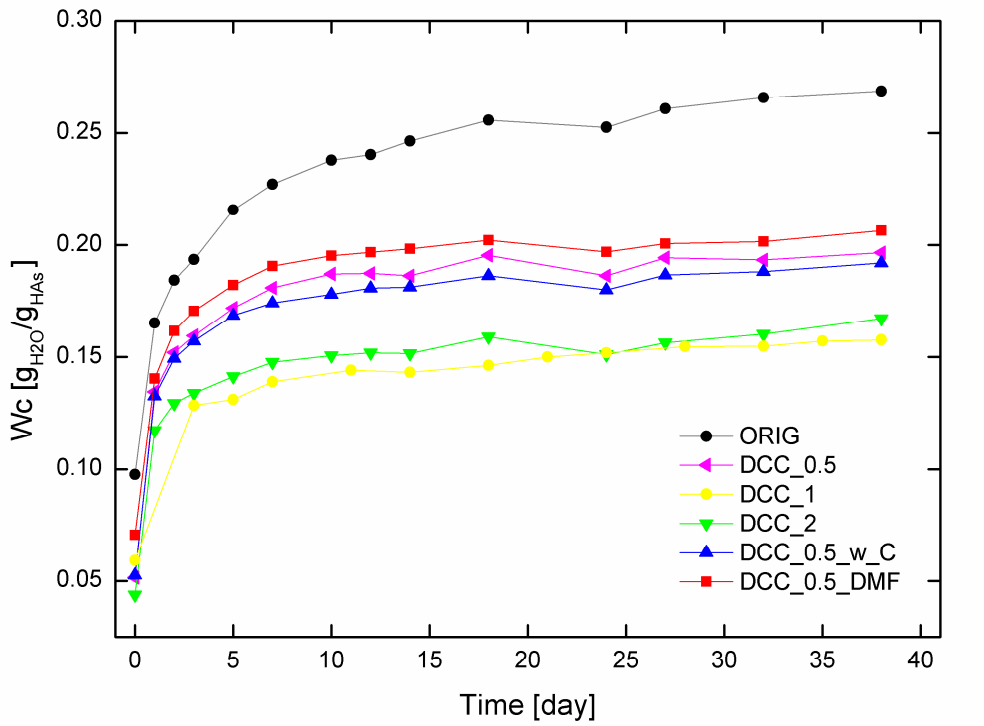
	c H^+ [$mmol_{H+}/mg_{HAs}$] * 10^{-3}		
Sample	Initial concentration	Titrated to pH 7	% of ORIG c H^+
ORIG	7.89	1.47	100
DCC_0.5	2.86	0.62	42.2
DCC_1	0.58	0.22	15.0
DCC_2	0.37	0.36	24.5
DCC_0.5_w_C	5.30	0.67	45.6
DCC_0.5_DMF	10.10	0.74	50.3
EDC_24_hrs	3.02	0.87	59.2
EDC_5_d	2.96	1.26	85.7
EDC_w	1.89	1.01	68.7

3.2. Water uptake properties of crosslinked samples

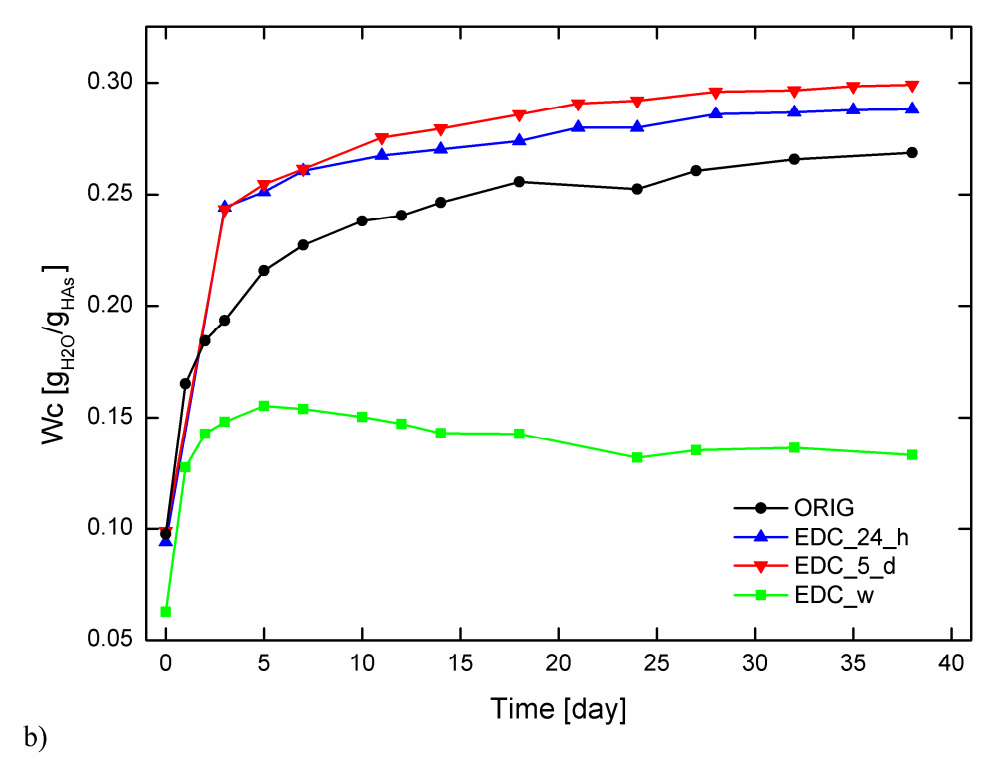
Water holding and retention capacities belong among the most pronounced properties of HAs. Atmospheric moisture condenses mainly on polar groups forming a nucleus for condensation of additional water molecules (Cihlar et al., 2014). Eventually, a water cluster bridging soil organic matter segments can be formed (Kucerik et al., 2014), depending on the distance between polar groups (Aquino et al., 2011; Cihlar et al., 2014; Kunhi Mouvenchery et al., 2013). Although this water harvesting ability is not frequently studied, we assume that this is one of the crucial parameters of soil amendments, especially when applied in water-limited ecosystems. HAs structure can hold relatively high water content similarly to swollen hydrogels. However, in some cases, water excess can cause the structural collapses due to a break of H-bonds stabilizing HAs segments (Kucerik et al., 2012). This leads to opening, hydration of less accessible parts of HAs and change in HAs reactivity. In this work, the polar groups in HAs were involved in crosslinking, which on the one hand increases the structural rigidity, but on the other hand, assumingly decreases the affinity of derivatives to water. Therefore, we focused mainly on the investigation of water – HAs interactions.

3.2.1. Moisture harvesting capacity

In the first step, we focused on the ability of prepared samples to harvest atmospheric humidity. Fig. 4 shows the dynamics of water harvesting as an amount of water increase in time. As expected, higher concentration crosslinking agent DCC caused a decrease in the water affinity, because the amount of hydrophilic functional groups capable to bind water molecules is lower (table 4) due to their involvement in the crosslinking. However, the EDC samples (pre-hydrated for 24 hours and 5 days) showed higher water holding capacity than the ORIG sample. It is rather peculiar behavior, especially because the amount of carboxylic groups decreased more than 60 % after crosslinking. As aforementioned, the formation of water molecule bridges is not directly proportional to the amount of functional groups, but it is governed by their spatial arrangement (Aquino et al., 2011; Cihlar et al., 2014; Kunhi Mouvenchery et al., 2013). In other words, when the groups are too separated, moisture is adsorbed only on them. At increasing relative humidity, the bridges can be formed also between more distant groups increasing the amount of adsorbed moisture.



a)



b)

Fig. 4 Equilibrium water contents in investigated humic acids samples, a) HAs crosslinked by DCC b) HAs crosslinked by EDC

3.2.2 Strength of water binding

In order to study and quantify interaction of water molecules with HAs structure, we determined the heat of water vaporization/desorption. In principle, the measured heat is a sum of the interaction energy between water adsorbed on and in the HAs structure. Two types of interactions between water molecules and HAs are important. The first mechanism includes the water-polar group interactions. The second one is hydrophobic interactions that is hypothesized to be important for the above-mentioned water molecular bridges (WaMB) stabilization (Aquino et al., 2011). The adsorbed moisture occurs mostly on the surface, where the evaporation enthalpy corresponds to interaction energy. On the contrary, the evaporation of water from the structure requires additional energy due to diffusion (Kucerik et al., 2011). The additional energy demands, such as possible reorganization of HAs physical structure, are weak and therefore, they can be neglected.

Table 5 summarizes the measured evaporation heats and amount of water in samples equilibrated at 76 % RH. The ORIG sample gave the lowest heat in comparison with the crosslinked derivatives and also lower value than heat of pure water evaporation (2.25 kJ g^{-1}). This implies that the overall binding of water in this sample is weaker than in pure water. Accordingly, not all interactions between water and HAs are strong polar interactions, a part of them are probably hydrophobic ones. Simultaneously, at 76% RH the ORIG sample had the largest amount of water. However, at 100% RH

the content was lower in comparison to 24 hrs and 5 days for EDC samples (Fig. 5). Recently, a similar shift in water content at different RHs was observed for formaldehyde crosslinked HAs and it was attributed to the narrower distance between polar functional groups in formaldehyde crosslinked samples (Cihlar et al., 2014). This distance is decisive for the size of WaMB (Aquino et al., 2011; Kunhi Mouvenchery et al., 2013), which is formed at appropriate RH. In other words, if the distance is larger, than the WaMB is formed at higher RH.

The second parameter that is influenced by larger distance between polar groups is the accessibility of inner pores of the HAs allowing the moisture deeper penetration into the HAs structure. The hypothesis about larger pores on the surface of EDC samples is supported also by stability tests (Table 3) in which the EDC samples showed lower thermo-oxidative stability.

Accordingly, one can conclude that the average distance between functional groups in ORIG samples is lower than in EDC 24 h and 5_d samples. As a consequence, in EDC samples, at 76% RH, the equilibrium water content is smaller, but more energy is needed to evaporate water from their structure (Table 5).

The DCC samples, on the other hand, had lower water content in both cases and generally higher evaporation enthalpy. This indicates the preferential sorption on the surface and a shorter distance between functional groups, which decreases a size of WaMB and increases the energy necessary to evaporate moisture. An exemption seems to be sample DCC_1 that showed very low water content and low strength of binding, which indicates a weak surface sorption.

It is worth to say, that the obtained contents of adsorbed moisture correlate (R^2 = 0.77) with the amount of deprotonized functional groups in samples suspended in pure water (Table 4). This supports our conclusions that water uptake capability depends on the amount of suitable polar groups only partially, and their spatial distribution is an important parameter as well.

Table 5 Desorption/evaporation heat of water from HAs samples exposed to 76% RH; heat of pure water evaporation was determined 2.25 ± 0.05 kJ g^{-1}

Sample	Heat [kJ g^{-1}_{water}]	SD	Wc g/g	SD
ORIG	**2.08**	0.02	0.134	0.004
DCC_0.5	2.26	0.00	0.081	0.001
DCC_1	2.19	0.08	**0.065**	0.001
DCC_2	2.38	0.10	0.086	0.004
DCC_0.5_w_C	2.50	0.15	0.085	0.000
DCC_0.5_DMF	2.26	0.11	0.091	0.006
EDC_24_hrs	2.38	0.01	0.134	0.001
EDC_5_d	2.32	0.09	0.127	0.001
EDC_w	2.29	0.04	0.082	0.002

Those conclusions are in accordance with the NMR relaxometry results. Fig. 5 shows the change in transverse relaxation times of water protons in four selected HAs that were progressively hydrated. In fact, water was first adsorbed from surroundings atmosphere and later added by a pipette (see the arrows in Fig. 5). Using Equation 1, two proton transverse relaxation times were determined: a slower one T2A, related in part to the slower motion of protons both in HAs structure as well as water molecules attracted to the surface of the HAs. Therefore, T2A changed during the hydration relatively slowly. In contrast, the T2B derives predominantly from relaxation of water trapped molecules in different hydration level and bound in the HAs structure by different interactions.

In the initial phase of hydration, the ORIG sample showed the T2B below 5 ms and a moderate increase after addition of water by pipette. In contrast, sample DCC_2 showed the T2B above 5 ms in the initial phase and a very rapid increase after addition of water by pipette. In this way behaved also the other DCC samples except for DCC_1 that showed lower T2B in the initial phase and slower increase upon addition by using pipette. Last, sample EDC_5_d revealed T2B lower than 5 ms in the initial phase and a very slow increase after pipette addition. Sample EDC_24_hrs behaved similar as EDC_5_d.

Those records illustrated four different hydration profiles. In fact, the low relaxation time in the initial phases indicated a stronger binding of moisture either on the surface or in the inner pores. Upon pipette addition, ORIG and DCC_1 samples showed a progressive swelling, while in the DCC_2, water penetrates very slowly as indicated by very high relation time. In EDC_5_d sample the T2B increases also regularly, but relatively slowly, which indicates more rigid structure than in ORIG sample.

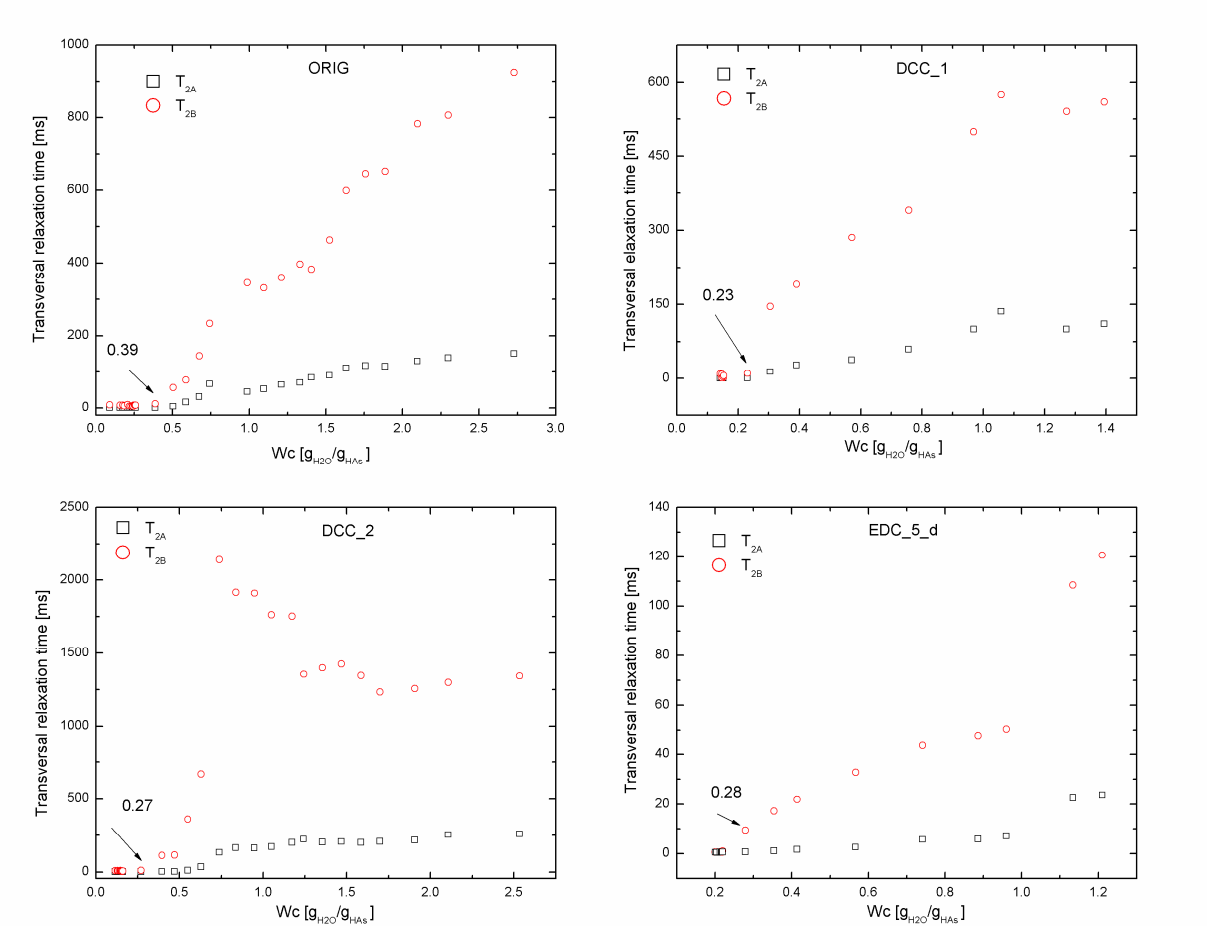


Fig. 5 T_2 relaxation times versus respective water fraction for ORIG, DCC_1, DCC_2 and EDC_5_d representing the hydration progress in all samples. Neither the standard deviation of the fitting nor the repeat measurements are shown as in all cases, the deviations were smaller than the symbol size.

3.2.3 Water holding capacity and kinetics of water uptake

To complete and support the NMR results, we determined the changing water holding capacity during the hydration. The hydration of organic molecules is frequently studied by means of freezing/thawing DSC experiments (Kucerik et al., 2012; Prusova et al., 2010). This approach allows the categorization of water into several fractions according to the melting enthalpy of ice formed in the HAs structure upon cooling. In fact, the water fraction in intimate contact with HAs surface or distributed in small pores does not freeze due to its lower mobility preventing its participation in ice formation under experiment conditions, thus it is called “non-freezing water” (NFW). In contrast, water in sample, which freezes and melts upon heating, is called “freezable” water (Kucerik et al., 2012).

The typical DSC melting curves of ice presented in HAs samples and the evaluation procedure are shown in Fig. 6. In principle, HAs samples with certain water content were cooled and melting

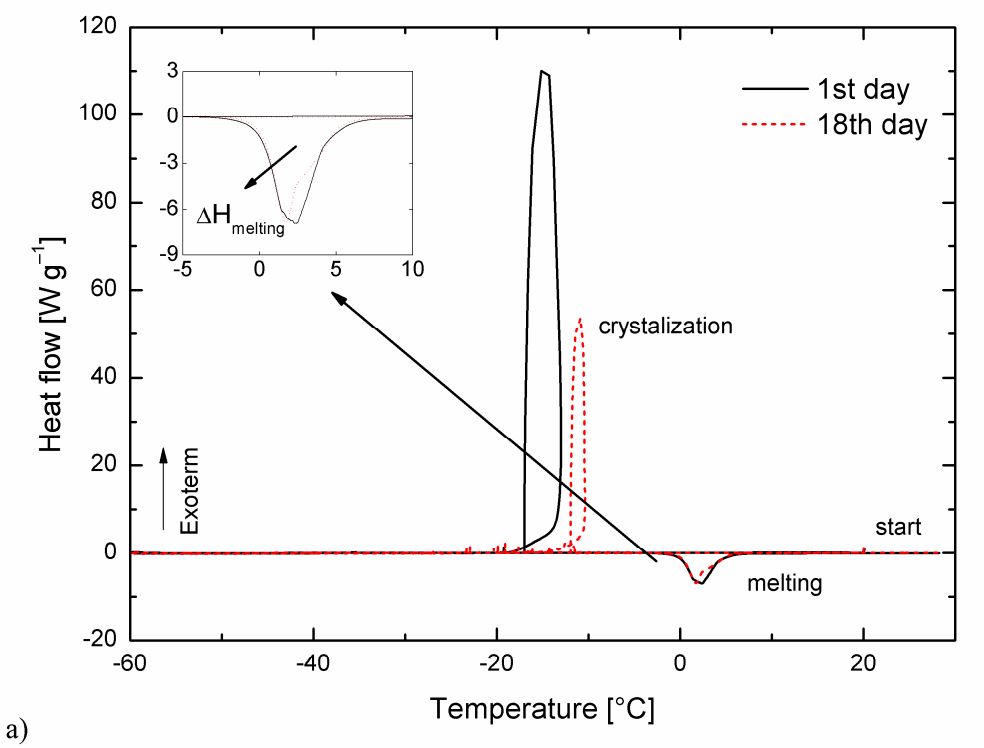
enthalpy of ice was measured at heating run. Melting enthalpy of freezable water is proportional to its melting peak area, and it was plotted against respective values of water fractions (Prusova et al., 2010). The extrapolation of melting enthalpy to zero enthalpy revealed that the amount of non-freezing water (hydration) changed in time.

Table 6 compares the NFW contents in all samples over the period of 18 days. In ORIG sample one can see a stable increase of NFW up to largest value in 18 days, while in crosslinked samples some maxima can be observed (highlighted). In particular, the maxima were observed mainly around 9th day. The results reveal different kinetic process related to the uptake of water molecules into humic structures. In fact, water can cause the breaking of H-bonds stabilizing HAs segments (Jaeger et al., 2010) and successive solubilization of polar molecules and swelling of the structure (Kucerik et al., 2012). It can be seen that in all cases the crosslinked humic acids showed lower water holding capacity, which is related to the reduction in number of hydrophilic functional groups in crosslinked humic acids. Furthermore, the resultant structure of the hydrogel is less flexible and deformable, which decreases the capacity of crosslinked HAs to absorb larger amount of water.

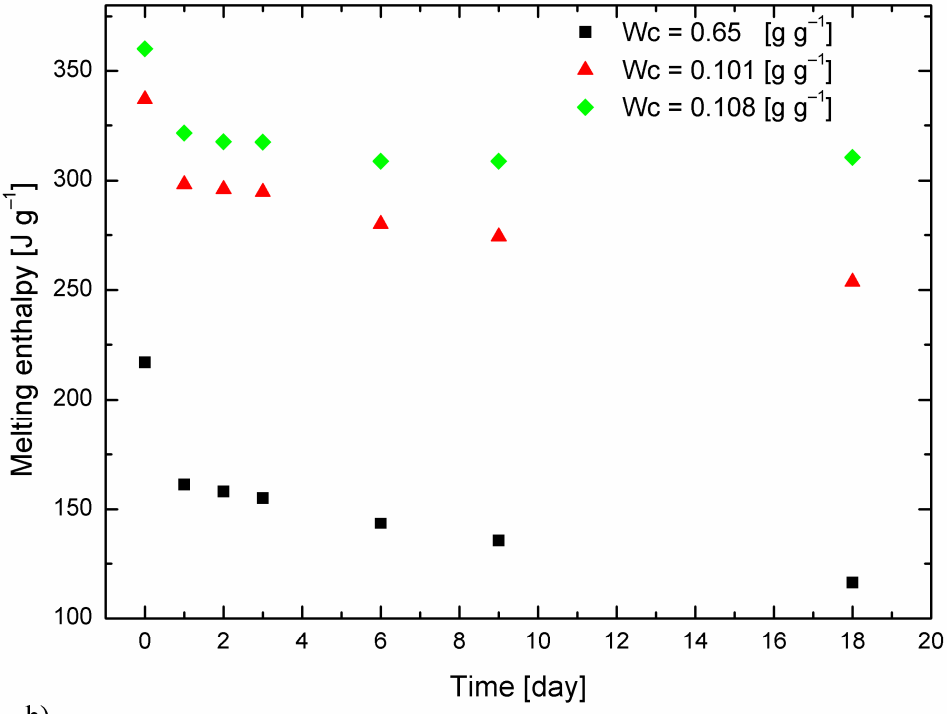
Of particular importance are the maxima of NFW contents, which indicated the existence of parallel processes during the hydration. We assume that this can be caused by the hydrolysis of HAs structures. The lowest resistance showed the sample EDC_5_d, which was hydrated very fast up to 90 % of ORIG sample at 18th day, but already in 3 days, the structure started to collapse. EDC_24_h was stable till 9th day and showed 83 % of hydration of the ORIG sample at 18th day. The fast wetting of these samples correlates well with the above-explanation that EDC samples have larger size of surface pores, which facilitates the water penetration into the structure.

The DCC samples showed the maxima too, and lower NFW, except for DCC_1, which was hydrated fast and showed maximal NFW content at 9th day similar to the ORIG at 18th day. This is in contrast to its poor adsorption on the surface and indicates larger pore size in this sample.

Noteworthy, our conclusions about role of pore system on water holding cannot be easily proven by an experiment. In particular, there is a great difference between pore size distribution in dry and swollen state of HAs´ powders. Second, as shown in Table 6, the change in pore size distribution of swollen HAs is a time-dependent process, which can be easily followed by monitoring of its change, but the absolute values are uneasy to obtain. Third, assumingly, the distribution of pore size is spanning from nanopores to macropores. To the best of our knowledge, there is no a single method providing the distribution of pores in that large span. A combination of method would be more feasible, but then the comparison of results would depend also on the inherent problems of these techniques.


Heat flow [W g^{-1}]
Temperature [°C]
1st day
18th day
crystalization
melting
start
Exoterm
ΔH melting

a)


Melting enthalpy [J g^{-1}]
Time [day]
Wc = 0.65 [g g^{-1}]
Wc = 0.101 [g g^{-1}]
Wc = 0.108 [g g^{-1}]

b)

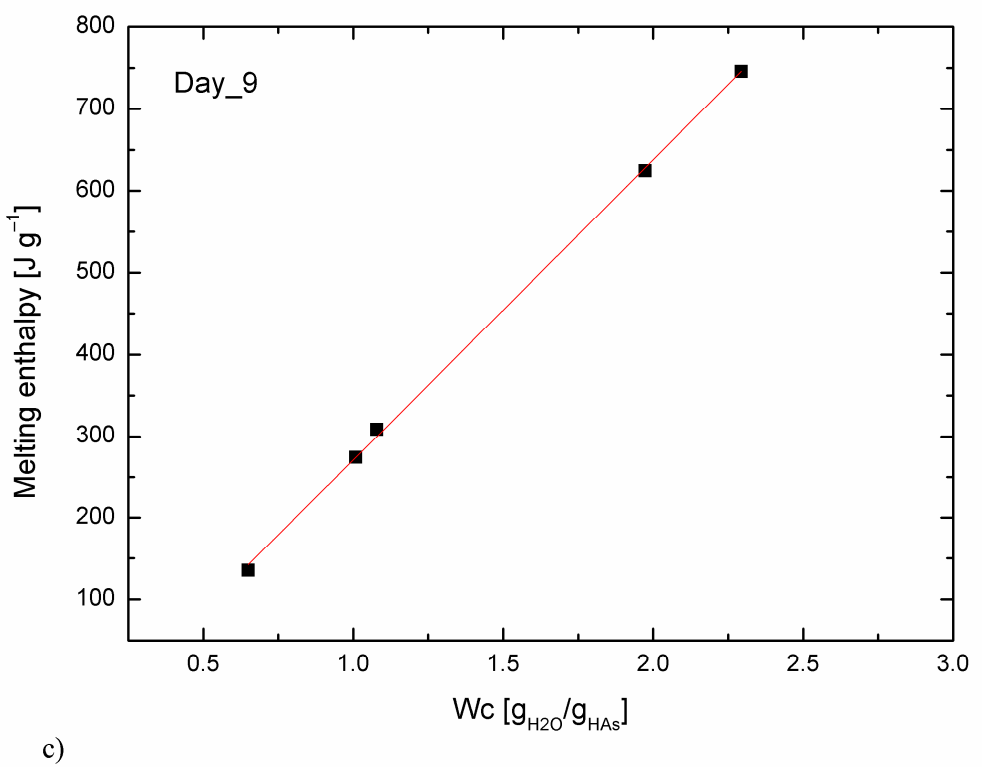


Fig. 6 a) Comparison of peaks corresponding to melting of ice formed by freezable water in DCC_2, measured 1th and 18th day after preparation (Wc = 1 g/g). The lowest confidence coefficient R^2 in determination of NFW content was 0.81. b) Change in the melting enthalpy of ice in hydrated DCC_2 as a function of hydration time for three different water contents. c) Dependence of the melting enthalpy of ice in hydrated DCC_2 after 9 days of hydration on water content.

Table 6 Development of non-freezing water content in HAs samples

	NFW [g_{H2O}/g_{HAs}]				
Time [day]	1	2	3	9	18
ORIG	0.394	0.396	0.416	0.551	**0.587**
DCC_0.5	0.238	0.246	0.280	**0.301**	0.284
DCC_1	0.416	0.457	0.495	**0.554**	0.482
DCC_2	0.235	0.235	0.236	0.262	**0.263**
DCC_0.5_w_C	0.298	0.280	0.305	**0.305**	0.264
DCC_0.5_DMF	0.400	0.424	0.429	**0.487**	0.471
EDC_24_hrs	0.281	0.416	0.433	**0.489**	0.444
EDC_5_d	0.461	0.511	**0.532**	0.512	0.438
EDC_w	0.313	0.351	0.358	0.365	**0.490**

4. Conclusions

Lignite humic acids were crosslinked by carbodiimide coupling using either EDC or DCC. Both procedures provided derivatives differing in their water holding capacity, kinetics of hydration and moisture harvesting.

From this "practical" viewpoint, the EDC crosslinking seems to be more beneficial, because the samples showed higher water holding capacity and harvesting ability and did not show the rests of trapped crosslinking agents in the structure. A very important factor seems to be the pre-wetting of pristine HAs before the crosslinking (at least 24 hours), which seems to be crucial for increasing the crosslinking degree. This procedure resulted in an increased pore size of HAs that allowed to water molecules to penetrate the structure faster. In both cases the crosslinking seems to introduce the new covalent bonds that are probably destabilized within several days after water addition.

Furthermore, the results indicate that for the water holding capacity of HAs both the number of polar groups and pore-size distribution are important. The HAs pore system plays a crucial role for processes of wetting, moisture harvesting and later also for the strength of water binding.

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