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# 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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# TABLE OF CONTENTS

# 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].

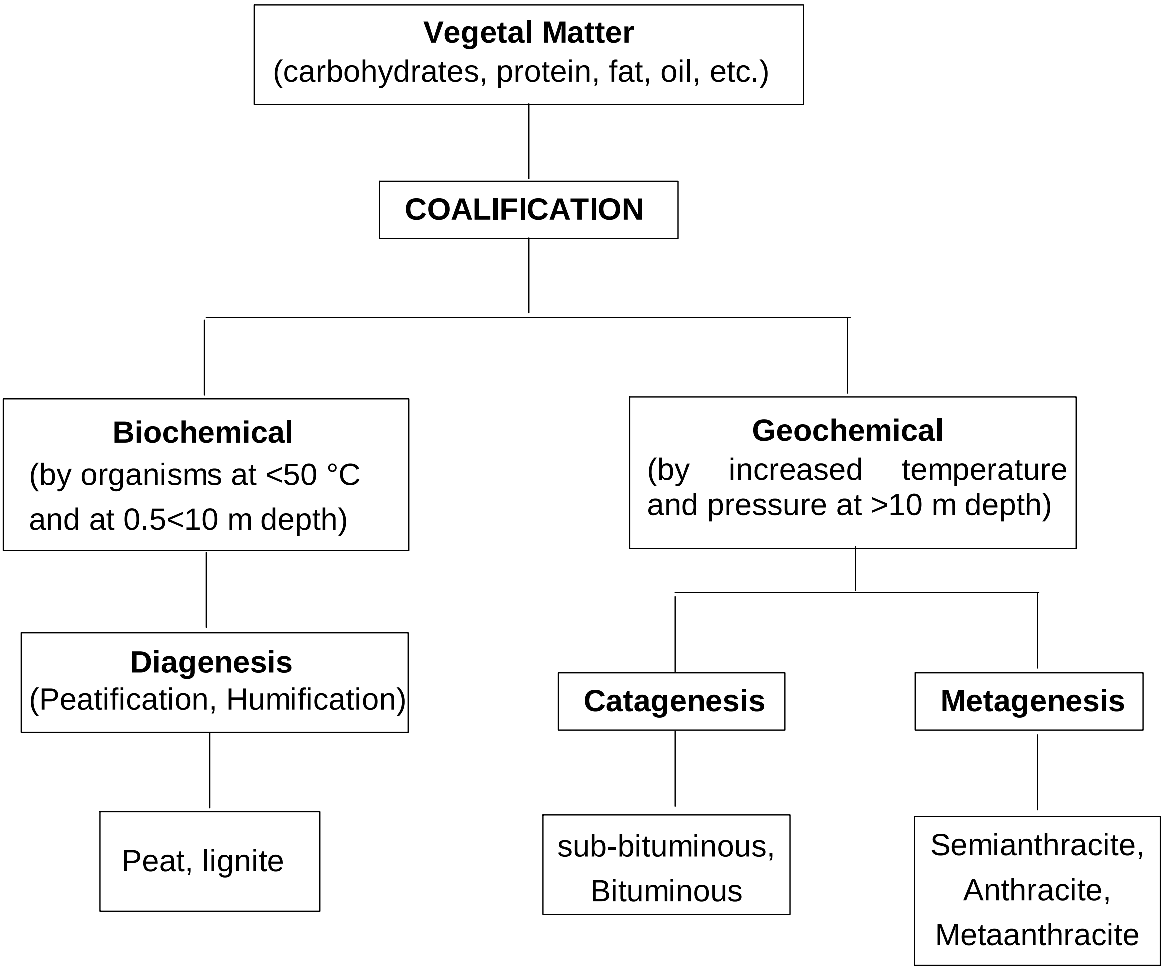

*Fig. 1. Simplified formation of coal through „coalification“ process of vegetal matter [16]*

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 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 [26].

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 [27].

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 [28]. In contrast, the “polymer model,” which posits the destruction of amide groups during the formation of humic macromolecules [2], [28], 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 [27].

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. 2) [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 [29].

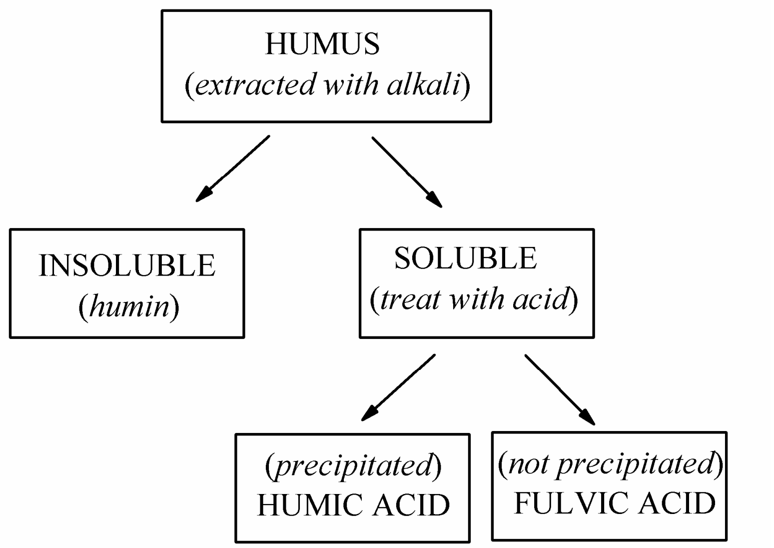

*Fig. 2. Scheme for the fractionation of humic substances (adapted from [2]).*

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], [30].

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 [31].

### 2.2.1 Humic acids chemical composition

The existing concepts about the chemical structure of HAs depend on the methods used for their characterization [32]. As follows from previous text, humic substances, rather than been constituted by macromolecular polymers (random polymers of a variety of biological monomers [33] 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, $\pi$–$\pi$, CH/$\pi$) into apparently high molecular dimensions [22], [34]. 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 [35].

## 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 [36].

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 [37]. 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) [38]. 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 [39]. 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 [40].

### 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 [41] 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 [42].

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 [43]. 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], [44].

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 [45], 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], [37], [44].

In particular, HAs can be crosslinked with formaldehyde and urea [37]. 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 [37]. 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 [37].

#### *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 [42].

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 [46].

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 [46]. 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 [47].

Zero-length crosslink experiments are also less likely to generate „self-linked“ products that occur, when interacting reactive groups are on the same molecules [47], 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 [48].

### *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 [49]. 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 [50].

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) [51].

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 [50].

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 [42]. 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 [52].

Bioconjugation by carbodiimides has also been widely performed, because no residues remain in modifying and crosslinking proteins (zero-length crosslinker [46], [52].

N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide (EDC) is mostly applied in combination with sulfo-NHS (N-hydroxysulfosuccinimide) [53], which hydrolyzes slowly in aqueous solution [54]. 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 [54], [55]. EDC/sulfo-NHS-coupled reactions are highly efficient and usually increase the yield of crosslinking in comparison with that obtained by merely using EDC [54]. 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 [52]. 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 [46].

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 [48]. 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 [53]. 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 [56].

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 [57].

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 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 [58], [59].

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 [60].

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 [61].

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 [58], [59]. 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 [61].

Water molecules may exhibit both plasticizing [62] and anti-plasticizing [63], [64] 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 [65] or the strong interactions of the water molecules with the polar groups of hydrophilic polymer [66] 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 [67]. A hierarchy of interactions has been proposed in order of decreasing energy: ion-ion > water-ion > water-polar = polar-polar = water-water > water-hydrophobic [68].

### 2.5.1 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 [61]. 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 [69]. 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 [60].

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 [70]. 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 [71].

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 [61].

The mechanism of formation of non-freezing water in polymers is not explained [72], [73]. 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 [74].

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 [61].

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 [61].

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 [60]. 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 [60].

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 [61]. The quantity of freezing water is generally characterized by the integration of the endotherm (melting of ice) [75] since the exothermic curve may not be detected during cooling in some cases [76]. 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 [77].

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 [78], [79]. 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 [79]. The maximum error in the calculated amounts of freezing water is expected to be only 6.6 % [61]. 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. [80].

The sum of the freezing and non-freezing water fractions is the bound water content [58], [59]. 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 [61].

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 [61].

The total water content, Wc, is usually defined as Wc (g $g^{-1}$) = (water, g)/(dry polymer, g) [75].

Above mentioned approach is widely applied to study of system humic material/water [5], [61], [81]. 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 [77], [81]. 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 [77].

### 2.5.2 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 [59].

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 [81]. 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 [81].

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 [82].

The recent statement introduce that the wetting of organic matter proceeds in two steps [83]. 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) [84]. 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 [84]. 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 [84]. 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 [77]. These differences are most probably due to significantly larger heterogeneity of the whole peat sample in contrast to the purified HS [81].

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 [81].

### 2.5.3 Water evaporation and sorption

Thermal behavior of water can be investigated by vaporization technique if the appropriate experimental condition is selected [5], [85].

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 [59], [86]. 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 [85].

Among various factors affecting the vaporization behavior of restrained bound water, atmospheric conditions and heating velocity are considered to be most important [86].

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 [85].

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 [87].

Johnson et al. for example, determined the amount of water forming as clusters in the microcavities of hydrophobic polymers [88]. 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 [89]. 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 [60].

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) [90].

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) [90].

## 2.6 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 [91].

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

## 2.7 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 [92]. 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 [93].

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 [94]. Benefits of compost amendments to soil also include pH stabilization and faster water infiltration rate due to enhanced soil aggregation [95].

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 [96].

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 [94]. However, it has been shown that microbial activity and biomass is higher in fields with organic amendments than fields with conventional fertilizers [94].

Inappropriate crop cultivation and harvesting, erosion and natural degradation contribute to reduce gradually the amount of SOM [97]. Nowadays, organic soil amendment is the most common practice for restoring, maintaining and/or improving the physical, chemical and biological performances of SOM [98].

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 [98].

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 [99].

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 [100]. 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 [99].

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 [101].

# 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 [102]. 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 [103], [104], [105]. 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 [104]. 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 [106]. 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 [107]. Oxidation procedures represent an ideal process resulting in higher content of extractable humic matter Fig. 3 [106]. 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) [106]. 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 [108]. 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.

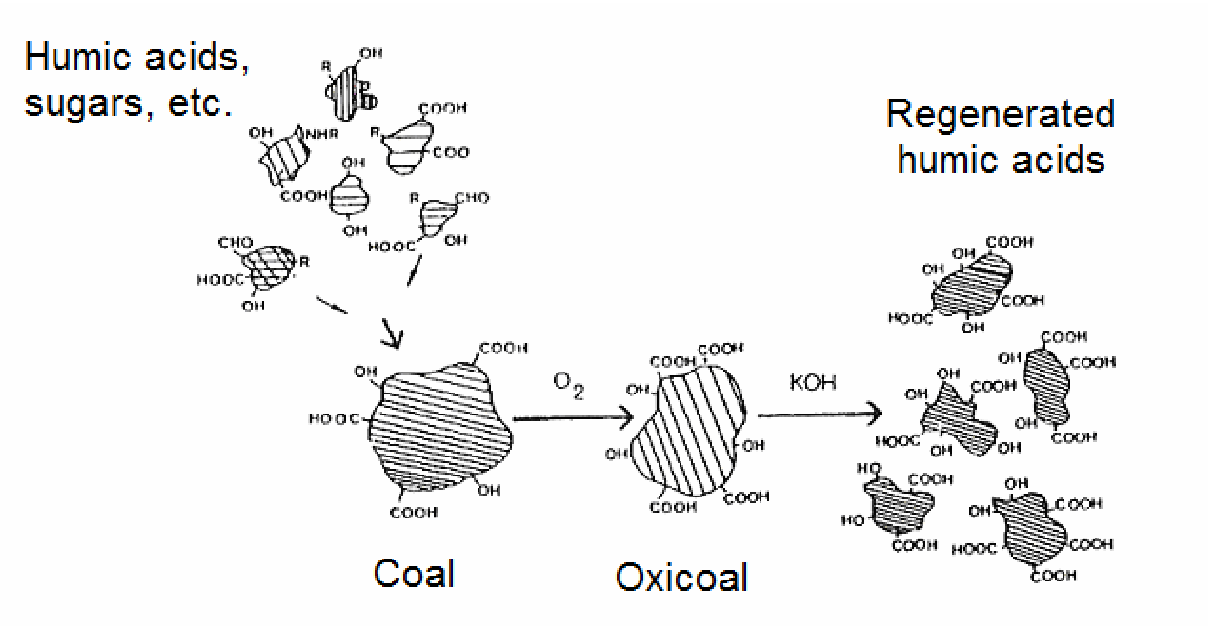

*Fig. 3. Conceptual model of coal oxidation as an ideal inverse diagenetic process, reprinted with kind permission from [106].*

Therefore, in the first work [105] we investigated the influence of lignite air oxidation on the yield and chemical-physical 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 [103]). 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 [105]) 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 [103]). 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. 4, and to characterize the properties of products. Thus, crosslinking by formaldehyde (see [5]) and by carbodiimide coupling (see [109]) 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.

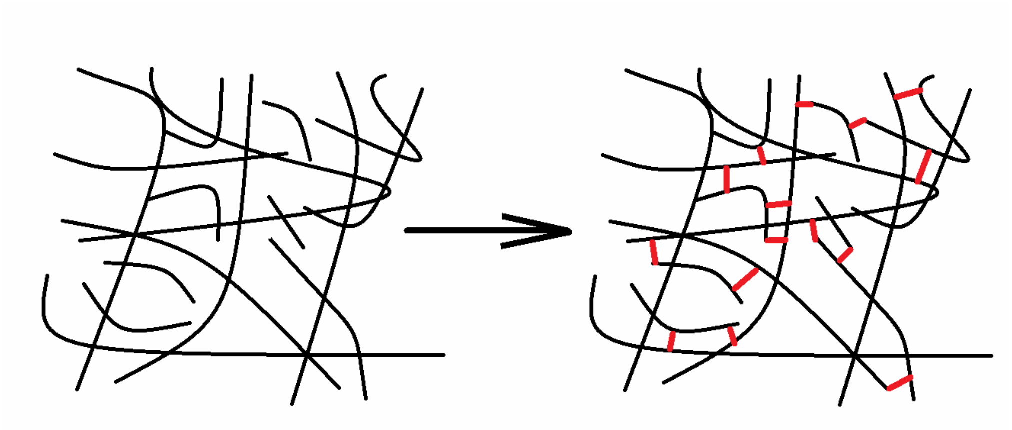

*Fig. 4. Illustration of chemical modification of HAs structure. Red lines represent new chemical bounds.*

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 caboxylic 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 9th 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 PUBLICATIONS AND ACTIVITIES

**Peer-reviewed publications:**

- 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.

- 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.

- 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.

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

**Conference contributions:**

- Bursáková, P., Vlčková, Z., Cihlář, Z., Kučerík, J.: Hydration of regenerated humic substances. *Chemické listy,* 2008, vol. 102, no. S, pp. 1135–1136. ISSN: 1213-7103.

- Bursáková, P., Průšová, A., Cihlář, Z., Kučerík, J.: A comparative study on hyaluronan and humic substances hydration. In *VIII. Pracovní setkání fyzikálních chemiků a elektrochemiků.* Brno: Masarykova Univerzita, 2008. pp. 19–20. ISBN: 978-80-210-4525-5.

- Cihlář, Z.; David, J.; Kučerík, J. *Vývoj hydrogelů na bázi huminových kyselin.* Chemické listy. Pardubice: Česká společnost chemická, 2010. pp. 513–513. ISSN: 0009- 2770.

- Průšová, A., Cihlář, Z., Kučerík, J. *Advanced DSC experiments as a background for teaching of thermal analysis at Brno University of Technology.* Bratislava: Slovak University of Technology, Bratislava, SK, 2011. pp. 56–56.

- Cihlář, Z.; Kučerík, J. *Chemical crosslinking of lignite humic acids.* Chemické listy. Brno: Česká společnost chemická, 2011. s. s905 (s905 s.)ISSN: 0009- 2770.

# 8 CURRICULUM VITAE

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