VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ
BRNO UNIVERSITY OF TECHNOLOGY

FAKULTA CHEMICKÁ
ÚSTAV FYZIKÁLNÍ A SPOTŘEBNÍ CHEMIE

FACULTY OF CHEMISTRY
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REMEDIATION POTENTIAL OF HUMIC ACIDS

DIZERTAČNÍ PRÁCE
DOCTORAL THESIS

AUTOR PRÁCE Ing. ANNA UHROVÁ
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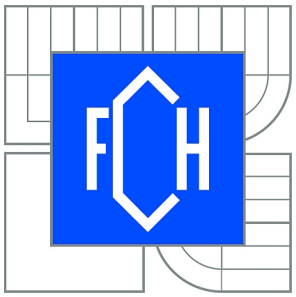

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Remediation Potential of Humic Acids

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To analyze obtained products for their chemical and physicochemcial structure
To analyze the impact of products on biological activity of maize grains
To analyze their impact on soils properties

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ABSTRACT

In this work, we tested the modified lignite humic acids for their remediation capability of agricultural soils. Prior to the extraction of humic acids, the parental raw lignite was modified by ten organic acids. The pre-treatment was aimed to simulate similar processes that occur in rhizosphere, i.e. small-chain organic acids induce the reconformation of soil organic matter thereby releasing biologically active aggregates/molecules promoting the plant growth. In the first step, the produced modified humic acids (MHA) were characterised for their physico-chemical properties and molecular structure of by using elemental analysis (EA), Fourier-transformed infrared (FTIR) spectroscopy, high-pressure size exclusion chromatography (HPSEC), surface tension measurement and gas chromatography-mass spectrometry (GC-MS) analysis. In the second step, the parameters of biological activity were obtained from experiments oriented to both higher plants and remediation of microbiological activity in used soil. The biological activity towards higher plants was conducted on maize seeds and represented by total mass and length increments of roots, root division and sugars and protein contents. The influence on used soil was determined by laboratory soil incubation (CO_2 production) and soil water repellency measurement (contact angle). All results were subjected to statistical analysis using Pearson´s correlation coefficient to find relationship between physico-chemical properties and biological activity of studied HAs samples. The results showed correlations between biological activity and physico-chemical properties of humic acids. On the contrary, the surface properties did not show any correlations with physical-chemical properties of studied HAs. The most efficient modifier in terms of biological activity was 20% formic acid and the less efficient was 20% propionic acid.

KEYWORDS

modified humic acids, surface activity, biological activity, soil remediation

ABSTRAKT

V předložené práci byly testovány modifikované lignitické huminové kyseliny za účelem zjištění jejich schopnosti remediace zemědělské půdy. Před samotnou extrakcí jednotlivých huminových kyselin byl lignit modifikován jednou ze série deseti organických kyselin. Cílem modifikace byla simulace procesů vyskytujících se v rizosféře, tj. procesů, kdy malé organické molekuly způsobují změny ve struktuře půdní organické hmoty, během nichž dochází k produkci biologicky aktivních agregátů/molekul podílejících se na růstu rostlin. Prvním krokem této práce bylo zkoumání fyzikálně chemických vlastností vyprodukovaných huminových kyselin a jejich molekulové struktury prostřednictvím elementární analýzy, infračervené spektroskopie s Fourierovou transformací (FTIR), vysokoúčinné gelové chromatografie (HPSEC), metodou měření povrchového napětí a plynovou chromatografií s hmotnostní spektrometrií (GC-MS). Dalším krokem bylo studium parametrů biologické aktivity, získaných z experimentů zaměřených jak na vyšší rostliny, tak na remediaci mikrobiologické aktivity zemědělské půdy. Biologická aktivita vůči vyšším rostlinám byla zkoumána na základě experimentu s kukuřicí, byla měřena délka a hmotnost kořenů, rozvětvení jejich laterálních kořenů a obsah sacharidů a proteinů. Z experimentů cílených na půdu se jednalo o měření množství uvolněného CO_2 při laboratorní inkubaci ošetřené půdy a půdní hydrofobicitu (metoda měření kontaktního úhlu). Na závěr byly výsledky podrobeny statistické analýze s využitím Pearsonova korelačního koeficientu s cílem nalézt vztahy mezi fyzikálně chemickými vlastnostmi a biologickou a povrchovou aktivitou studovaných huminových kyselin. Korelace byly zjištěny mezi biologickou aktivitou a fyzikálně chemickými vlastnostmi huminových kyselin. Naopak, nebyly zjištěny mezi povrchovými a fyzikálně chemickými vlastnostmi. Nejefektivnějším modifikačním činidlem z pohledu biologické aktivity byla 20% kyselina mravenčí, nejméně efektivním 20% kyselina propionová.

KLÍČOVÁ SLOVA

modifikované huminové kyseliny, povrchová aktivita, biologická aktivita, půdní remediace

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DECLARATION

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

PROHLÁŠENÍ

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CONTENT

1 INTRODUCTION

Carbon is the fourth most abundant element in the Universe (after hydrogen, helium, and oxygen). On Earth, carbon cycles through the land, ocean, atmosphere, and the Earth's interior in a major biogeochemical cycle (the circulation of chemical components through the biosphere from or to the lithosphere, atmosphere, and hydrosphere). Biology also plays an important role in the movement of carbon in and out of the land and ocean through the processes of photosynthesis and respiration. In addition to the natural fluxes of carbon through the Earth system, anthropogenic (human) activities, particularly fossil fuel burning and deforestation, are also releasing carbon dioxide into the atmosphere [1].

Soil represents a huge fixed carbon reservoir, which is in permanent contact with other environmental compartments such as waters and air. Thus, its potential pollution can directly expand through the surface, ground waters and air [2]. Soil pollution may be caused by industrial accidents as well as by anthropogenic activities, and represents a long-term source of environmental contamination. While the contaminants are bind by weak interactions to soil compartments [3, 4], at any time, they may be easily released back to the environment and affect the human food chain [5, 6].

Thereupon, processes of decontamination of polluted soils are largely desired. The soil remediation technologies, and relatively inexpensive alternative, the soil washing technologies represent useful tool for transformation and detoxification of pollutants [6]. For example, bioremediation enables permanent elimination of pollutants by in situ remediation, however, is limited by the correct selection of active microbes, the appropriate soil conditions for microbial activity, recalcitrance of pollutants to biodegradation, and formation of metabolites, which may be even more toxic than the parent contaminant [7].

Humic substances, naturally occurring surfactants, are recognized as a possible aid in soil remediation techniques [6]. For example, the bioavailability of polychlorinated biphenyls (PCB) and polycyclic aromatic hydrocarbons (PAH) appeared to be increased by addition of oxygenous humic substances to contaminated soils [8, 9]. Nowadays, one of the main sources of humic substances represents lignite and peat, both in the past used mainly as a not very effective fuel in power plants. Lignite represents the youngest type of coal with the age belonging between peat and brown coal. From the physical chemistry point of view, lignite can be described as a system with a variable and unique surface morphology. From the chemical point of view, lignite is a very heterogeneous composite – except humic substances, also plant residues, bitumens, mineral inclusion and mainly high content of water can be identified in the structure. It has been already recognized that the most attractive way of non-energetic exploitation of lignites is their use as a source of humic substances [10].

2 LITERATURE REVIEW

2.1 Humic Substances

Humic substances are the most widely distributed natural products on the earth´s surface, occurring in soils, lakes, rivers, and the sea [11]. In spite of their extensive distribution, much remains to be learned about their origin, chemical structure and reactions.

Comprehensive historical review of chemical investigation of humic substances is referred in the book of Kononova [12]. But even before, one have to go back to ancient Romans who first used the term "humus" as equivalent of entire soil. Later the term was used to denominate soil organic matter (SOM) and compost or for different parts of this organic matter, as well as for complexes created by chemical agent treatments to a wide palette of organic substances. In 1760´s Wallerius defined humus in terms of decomposed organic matter [12]. In 1830´s Sprengel introduced the first relevant study of the origin and chemical nature of HS [13]. His comprehensive study on the acidic nature of humic acids is thought to be his most important benefit to humus chemistry. Research on the chemical properties of HS was extended by the Swedish researcher Berzelius, whose main contribution was the isolation of two light-yellow colored HS from mineral water and slimy mud rich in iron oxides [13].

In 1994 Stevenson [13] stated that soil organic matter includes a broad spectrum of organic constituents, many of which have their counterparts in biological tissues. However, to simplify this very complex system, two groups of organic compounds can be distinguished:

Nonhumic substances are consisting of compounds belonging to the well-known classes of organic chemistry such as amino acids, carbohydrates, lipids, lignin, nucleic acids, and other low-molecular-weight organic substances. In general, these compounds are easily attacked by microorganisms in the soil and are converted relatively quickly to other compounds.

Humic substances are unspecified, transformed, dark colored, heterogeneous, amorphous and high molecular weight materials which are relatively recalcitrant to microorganisms attack. They can be generally characterized as being rich in oxygen-containing functional groups notably COOH but also phenolic and/or enolic OH, alcoholic OH, and C = O of quinines.

However, it is not easy to separate the humic and nonhumic substances, because some nonhumic substances (usually lipids or carbohydrates) may be covalently bonded to the humic matter. Humus probably contains most, if not all, of the compounds of biological origin [13].

The classical definitions of HS are operational only and are based on solubility properties in the aqueous solutions used as soil extractants. HS are usually divided into three main fraction (1) humic acids (HA), which is soluble in alkaline solution but is precipitated by acidification of alkaline extract; (2) fulvic acids (FA), which is that humic fraction which remains in the aqueous acidified solution, i.e. it is soluble in both acid and base; and (3) the humic fraction that cannot be extracted by dilute base and acid, which is referred to as humin. The boundaries between these fractions have not been yet clarified in chemical terms and recent

results indicate that it is perhaps impossible [14]. The scheme of classification of soil organic components is described in Figure 1.

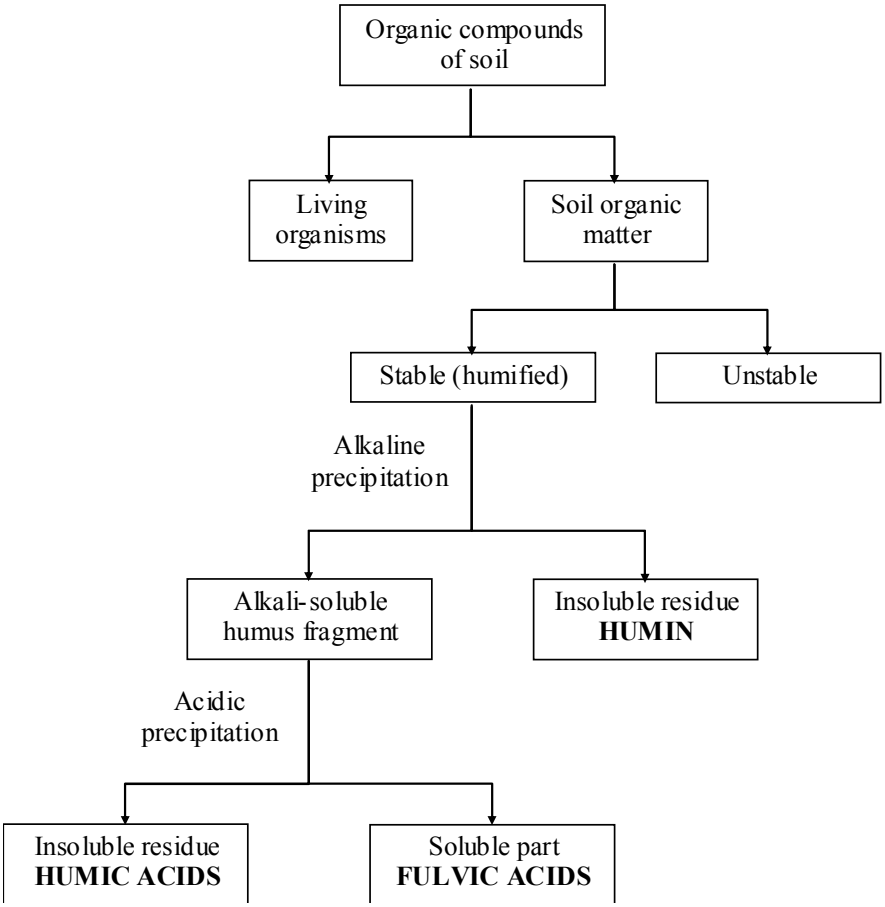

Figure 1 *Scheme of division of humic substances in dependence on their solubility* [15].

2.1.1 Chemical structure of HS

From the chemical point of view, HS consist of a mixture of substituted aliphatic as well as aromatic molecules. It is noteworthy that, in spite of general assumptions, there is not convincing evidence from chemical degradation studies for condensed aromatic components in humic structures [16], although, the recent studies on char coal and related compounds can indicate their occurrence [17, 18].

From the point of view of elementary analysis, HS consist of C, O, H and sometimes small amounts of N and occasionally also P and S. The C content ranges from 53.8 to 58.7 atomic % in case of soil HA, for coal HS is reported slightly higher. FA have lower C content (usual range from 40.7 to 50.6 atomic %), but higher oxygen (39.7−49.8 atomic %) content. A variety of functional groups, including carboxyl COOH, phenolic OH, enolic OH, quinone, hydroxyquinone, lactone, ether, and alcoholic OH, have been detected in HS [13].

Many model structures of HA and FA were suggested, but they should be considered only as models taking into account average composition. Their structures can be found for example in Stevenson [13]. In fact, in the real humic mixture, such structures may not be necessarily presents; however proposed model compounds can serve for specific purposes such as for example study of interactions with metal ions or organic polutants. Nevertheless, the most recent HA model structure takes into account the system complexity and heterogeneity, mutual interactions and implies the flexibility and versatility of HS. Model is presented in Figure 2.

During the long history of humus chemistry, several hypothetic physical structures, including different schematic models without chemical structural formulas, have been proposed [13]. The most frequently adopted view was that humic-like constituents in solution were polymers which will coil at high concentrations, low acidity and high ionic strength but become linear at neutral acidity, low ionic strength and low concentration [19]. This random coil model had been a leading concept in humus chemistry for decades. As a matter of fact, the terms polymer, polymeric and polymerization do not have their original meaning in the context of humic matter-like constituents because for example the term polymer is accurate only when the structural units are linked to each other in a regular manner and by the same kind of linkages.

This random-coiled model for humic matter-like macromolecules had been strongly criticized, e.g. by Wershaw [20], because mathematical equations used to define the model were originally derived for high-molecular-mass linear polymers. Instead, Wershaw et al. [20, 21] had presented an alternative schematic membrane model for the secondary structure of humic matter. In this membrane model, humic materials were pictured as composed of partially degraded molecular components from natural organisms (mainly from plants) which were held together in ordered, membrane-like or micelle-like, aggregated structures by weak interactions such as hydrogen bonding, π-bonding, van der Waals and hydrophobic forces. These aggregated structures with hydrophobic interiors and hydrophilic exteriors constitute a separate phase in soil-water and sediment-water systems, thus better fitting the theory presented for small organic molecules in aqueous solvents [22].

Piccolo et al. [23] introduced an extended theory where he stated that, instead of being stable polymers, humic constituents at neutral and alkaline acidities (pH) are supramolecular associations of relatively small heterogeneous molecules held together by weak dispersive forces such as van der Waals, π–π and CH–π interactions. This conclusion was based on the large-scale experimental data that after addition of modifiers such as natural organic acids, e.g. acetic acid, to the original humic-solute mixture, the macroscopic dimension of this supramolecular association was disrupted in smaller sized associations with reduced chemical complexity. This disruption by organic acid additions was attributed to the formation of new inter- and intramolecular hydrogen bonds which are thermodynamically more stable than the hydrophobic interactions stabilizing humic conformations at neutral acidities (pH) [24].

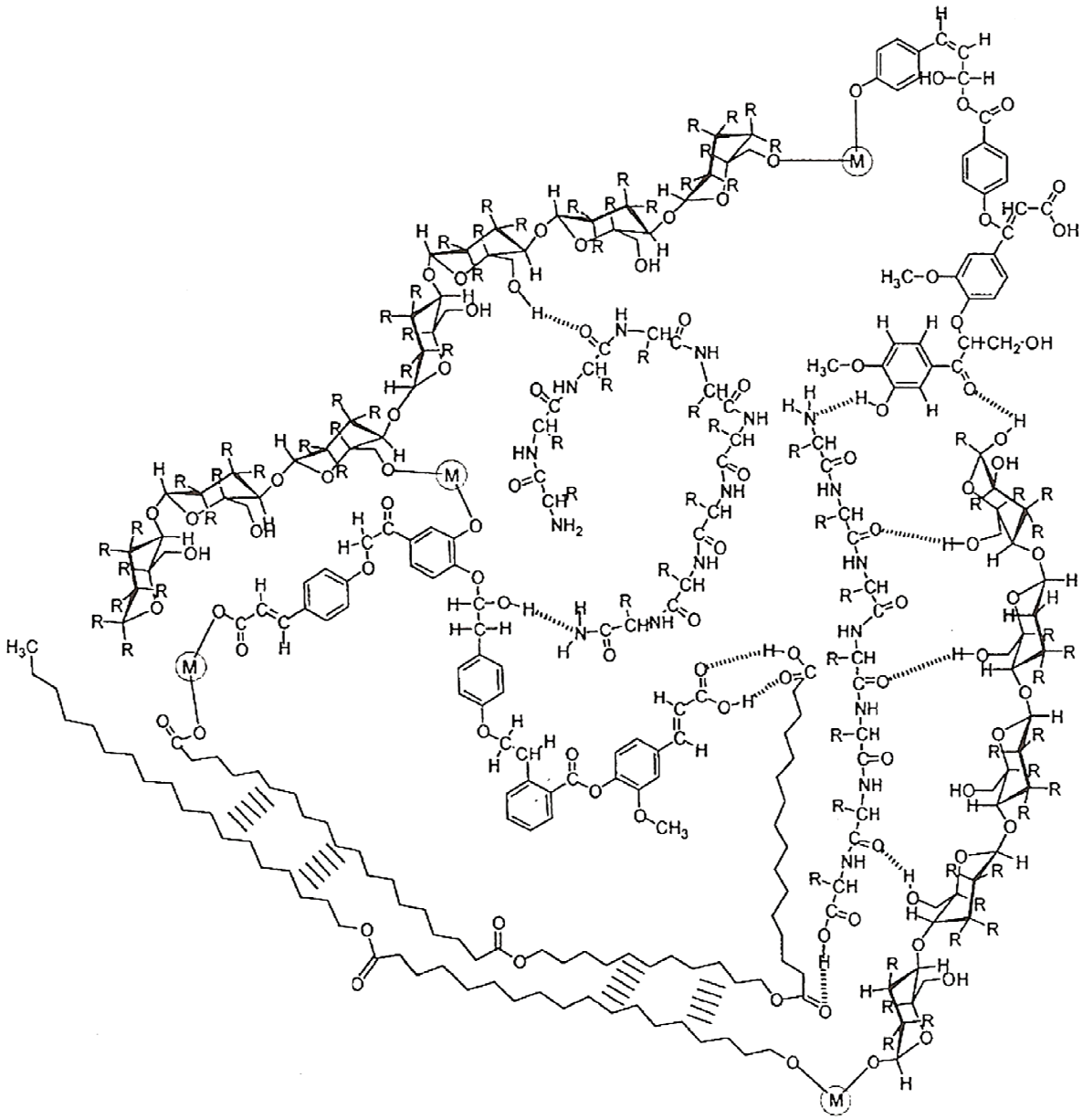

Figure 2 *Recent model structure of humic acid according to Simpson et al. (2002)* [25].

Results obtained later by fluorescence spectroscopy, nuclear magnetic resonance (NMR) [26], thermal analysis [27], mass spectrometry [28] and high resolution ultrasonic spectrometry (HRUS) [14, 29, 30] supported such conclusions. Further, other researchers adopted this view [24] although they do not refuse the presence of high molecular moieties which are the rest of parental plant tissues which are protected by humic molecules from biological degradation [31].

2.1.1.1 Theories of the molecular formation of HS

Several theories appeared for the formation of humic substances, the most accepted are the lignin theory, the polyphenol theory or the sugar-amine condensation theory. A review of such theories can be found for example in the work of Davies and Ghabbour [32].

First classical theory, the *lignin theory* was popularized by Waksman in 1932 [13]. According to this theory HS were derived from lignin which is incompletely utilized by microorganisms and the residuum becomes part of the soil humus. Modifications of lignin include loss of methoxyl (OCH_3) groups with the generation of *o*−hydroxyphenols and oxidation of aliphatic side chains to form carboxyl (COOH) groups. Assuming that HS represent a system of polymers, the initial product would be components of humin, further oxidation and fragmentation would yield first humic acids and then fulvic acids. Pathway of this theory is illustrated in Figure 3.

Flaig in his *polyphenol theory* [13] claims that lignin still plays an important role in humus synthesis, but in a different way. The starting material consists of low molecular weight organic compounds from which large molecules are formed through condensation and polymerization. In this case, phenolic aldehydes and acids released from lignin during microbiological attack undergo enzymatic conversion to quinones, which subsequently polymerize in the presence or absence of amino compounds to form humic-like macromolecules. Or, there is a second process, which is somewhat similar to the previous except that the polyphenols are synthesized by microorganisms from non-lignin carbon sources (e.g. cellulose). The polyphenols are then enzymatically oxidized to quinones and converted to humic substances. (Pathway 2 and 3 in Figure 3)

The notion that humus is formed from sugars dates back to the early days of humus chemistry [13]. According to this concept reducing sugars and amino acids, formed as byproduct of microbial metabolism, undergo non-enzymatic polymerization to form brown nitrogenous polymers of the type produced during dehydration of certain food products. This *sugar-amine condensation theory* was initially proposed in 1911 by Maillard and it can be simply illustrated as pathway 1 in Figure 3.

In 2001, Piccolo [33] introduced the *supramolecular theory* in which he proposed that humification in soil can be considered as a two-step process, i.e., biodegradation of dead-cells components following by aggregation of the degradation products. Piccolo in his supramolecular model assumes, that there is no formation of new covalent bonds during the process of humification that leads to the production of humus. Humification is the progressive self-association of the mainly hydrophobic molecules that resist the biodegradation. These suprastructures are thermodynamically separated by the water medium and adsorbed on the surfaces of soil minerals and other preexisting humic aggregates. The exclusion from water means exclusion from microbial degradation and the long-term persistence of humic matter in soil.

2.1.2 Humic acids

Humic acids represent the fraction of HS that is not soluble in water under acidic conditions (pH < 2) but is soluble at higher pH values.

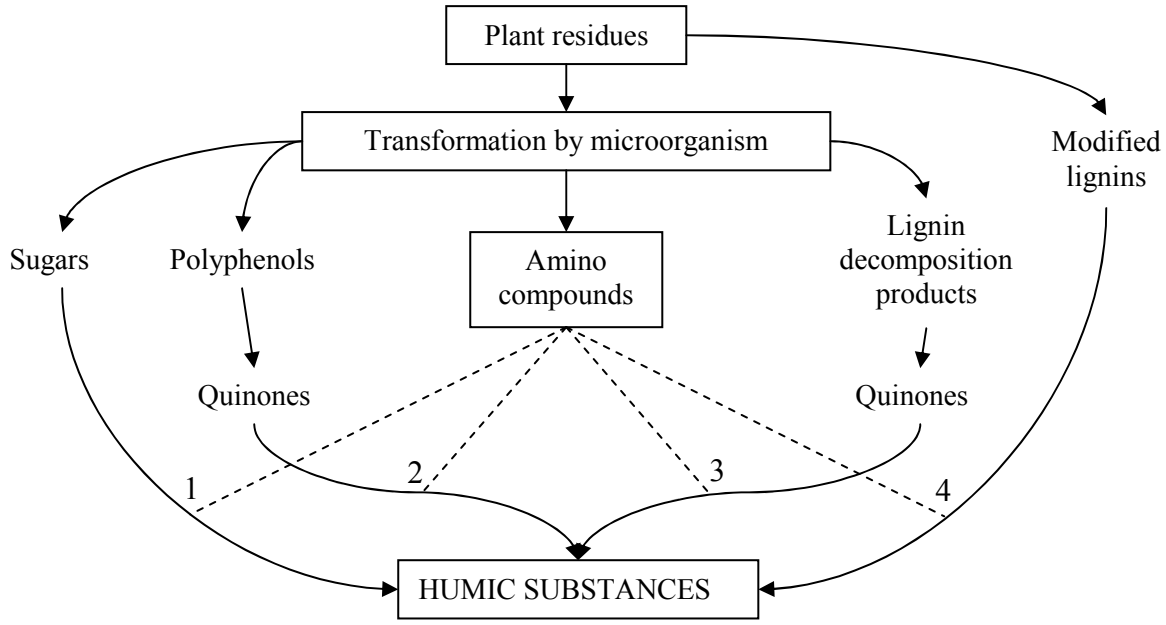

Figure 3 *Several most accepted mechanisms of humic substance formation. Amino compounds synthesized by microorganisms are seen to react with modified lignins (pathway 4), quinones (pathways 2 and 3), and reducing sugars (pathway 1) to form complex dark-coloured polymers.*

2.1.3 Colloidal properties of HS: aggregation and CMC

HS are a major class of naturally occurring organic colloidal particles, which not only demonstrate colloidal phenomena by themselves, but display a range of important colloidal interactions in the presence of other substances. Specifically, HS not only interact with other naturally occurring soil components such as clays and metals ions, but also with man-made materials such as herbicides and pesticides used in agriculture [34].

The mechanism of interaction between dissolved HA and nonionic organic compounds (especially nonpolar ones) continues to be subject to some controversy. It is, however, clear that these interactions are largely predicated on the detergent character of HA. It is generally recognized that these materials are surface active and can solubilize a wide variety of hydrophobic species [35]. A view that is presently widely accepted holds that this is due to a micelle-like organization in HA polymers in aqueous solution. The concept was introduced in a fundamental form by Rochus and Sipos in 1978 [36] and was subsequently elaborated and refined by Wershaw [20, 37, 38] who established the present mode of thinking on the subject. The essence of the theory is that the HA amphiphile consists of an elongated hydrophobic portion with one or more anionic (carboxylate) groups attached at the end. These entities aggregate in the manner of synthetic surfactants, forming micellar or membrane-like structures [20].

Aqueous solutions of synthetic surfactants have a characteristic concentration known as the critical micelle concentration (CMC), at which the monomers spontaneously aggregate to form micellar assemblies. The same has been reported for the concentrated HA solutions,

which have estimated CMC values as high as 10 g L^{-1} [39, 40]. In other experiment [41] several HS were analyzed by DOSY-NMR and the apparent CMC was established to be greater than 4 g L^{-1}. For dilute HA solutions, however, Engebretson et al. [42, 43] found evidence for micelle-like organizations which does not feature a CMC. In this model, the amphiphilic HA molecules are considered to "aggregate" both intra- and intermolecularly. The former is made possible by the chain length and flexibility of the humic polymer, which allow them to fold and coil in a manner that directs hydrophilic (e.g. carboxy and hydroxy) groups outward and keeps more hydrophobic (e.g. hydrocarbon) moieties isolated in the center. This process, which could in principle occur also 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. To indicate both similarities and differences with normal surfactant micelles, these HA structures have been referred to as *pseudomicelles*. A portion of a generalized structure, which may be visualized as a "knot" in a HA polymer "string" is shown in Fig. 4 [35]. This was upheld by other experiments [30, 44] where different methods for observation of aggregation behavior and surface activity of diluted HA were utilized in broad spectrum of concentration (from 10^{-4} to 10 g L^{-1}). Obtained results proved the presence of premicelle organizations in highly diluted HA solutions which question the existence of “true” CMC and the nature of humic micelle-like structures occurring in solutions [42, 43].

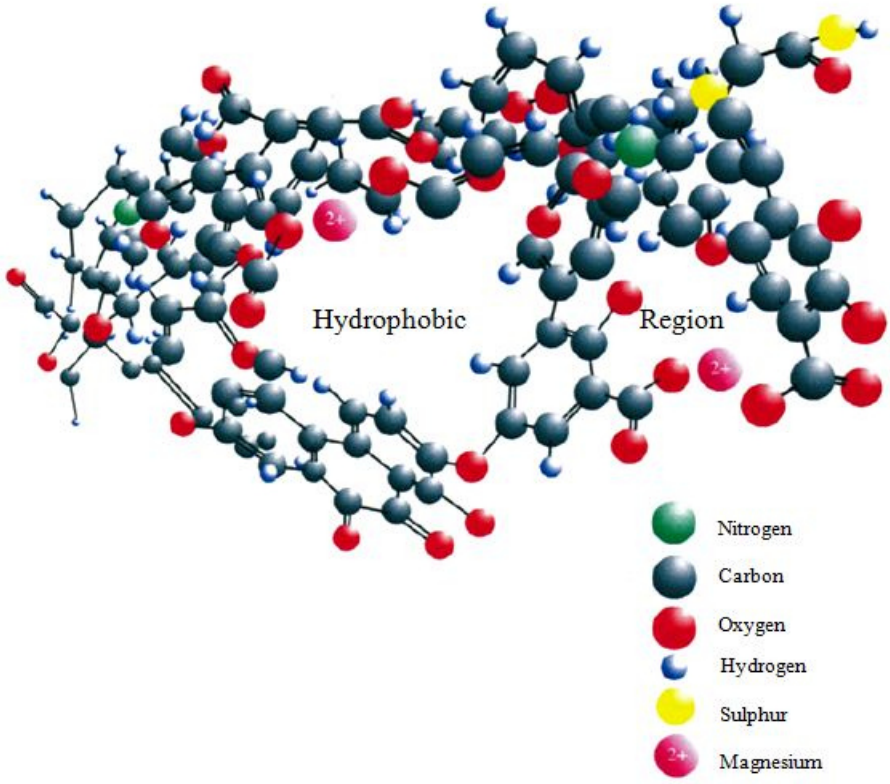

Figure 4 *Portion of the proposed "type" structure of humic acid* [35]

It has been shown that metal ions, especially polyvalent ones, have a major influence on humic aggregation and the formation of pseudomicellar structures. This is thought to be due to the ability of the cations to both neutralize the negative charges on HA (reducing repulsion) and engage in bridging interactions [45]. The propensity of HA to complex with metal ions and sequester organic molecules suggests that they may be used to remove contaminants from polluted water. This has been recognized in various studies and is the subject of a patent by Zanin and Boetti [46] who reported the use of extracted HA for the removal of heavy metals, chlorinated organics, phosphorus and nitrogen from waste water [35].

2.1.4 Lignite as a source of HS

Lignite in the Czech terminology includes a variety of brown coal, which exhibits the lowest degree of coalification, is mostly of xylitic character with large or small fragments of wood and tree trunks with preserved growth rings (the elemental analysis of particular products of the process of coalification is reported in Table 1. From the petrological viewpoints, it is a brown coal hemitype. Its calorific value is lower than 17 MJ/kg. World proved recoverable reserves of lignite are estimated at about 170 billion tonnes. Their predominant part is located on the territory of Australia (22 %), the USA (19 %), China (1 %), Serbia (9 %) and Russia (6 %). Lignite is used in energy generation and for heating. It represents the lowest quality mineral fuel, consumption of which gradually decreases [47].

Figure 5 *Lignite* [48]

The largest deposits of lignite in Czech Republic occur along the northern margin of the Vienna Basin, which extends from Austria into southern Moravia. South Moravian lignite is of a xylodetrital character with numerous tree trunks. It is rich in water (45–49 %), ash content is between 23 and 26 %, average content of sulphur is 1.5–2.2 % and its calorific value is 8–10 MJ/kg. Other deposits of low-quality lignite in Czech Republic occur in the South Bohemian Basin and the Zittau (Žitava) Basin (Figure 6).

Lignite as a material system can be defined as a transition organic-mineral substrate in the process of conversion of the phytomass into the high degree of dehydrogenated/dehydrated and deoxidized coal. It is morphologically and molecularly polydisperse system, which consists of complex of cyclano/aromatic compounds with important functional groups, big

volume of water incorporated partly in the free space of lignite particles (e.g. pores) microcracks, original interstitium of the plexus of original material, partly physically bonded on the oxidized carbon structures, special mineral structures based on the compounds of silicon, aluminium, iron and other elements and macroscopic components of the incident origin and occurrence.

The physical model of a lignite structure includes fibrous, lamellar and spatially-symmetrical or asymmetrical components in different rank of coalification, micro and macro-dispersion of the admixtures and free intermolecular volume involving capillaries, microcracks and vacuoles. Lignite therefore represents a complicated macromolecular complex consisting of polyelectrolytes, polysaccharides, polyaromates, carbon chains modified by sulphate and nitrate groups consisted of oxygenous parts connected with the main chains [49, 50].

Table 1 Elemental analysis of solid fuels line up according to increasing degree of coalification [49].

Solid fuel	C [%]	H [%]	O [%]	N [%]
Wood	50.0	6.0	43.8	0.2
Peat	57.0	6.0	35.0	2.0
Lignite	65.5	5.5	28.0	1.0
Brown coal	73.0	6.0	19.8	1.2
Black coal (gaseous)	85.8	5.5	7.0	1.7
Anthracite	94.0	2.4	1.9	1.2

Lignites are almost entirely used as fuel. Moreover, because of low calorific value, lignites were recognized to be low-cost raw materials suitable for non-fuel utilization. In this case, mainly specific surface properties of lignite particles were exploited. The important and promising field of exploitation is in agriculture for increasing of soil fertility and nutrients availability [51]. In environmental protection industry lignites could serve as materials for treating or preventing ecological accidents, e.g. in remediation technologies [6]. Due to their high cation exchange capacity attributed mainly to high content of HS, lignites can effectively serve as organic components in organo-mineral fertilizers [52]. This lead to increase of the availability of mineral components and macronutrients (e.g. phosphorus) and simultaneously to decrease of the release of phytotoxic substances (e.g. soluble Al and Mn) in the soils by the plants. Subsequently, addition of lignites to fertilizers can increase the yield and improve the quality of growing plants and crops.

Coals of variable degree of coalification possess variable amount of extractable humic acids. Use of oxidation procedures represents an ideal inverse diagenetic process resulting in a higher content of extractable humic matter [53]. 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) and have similar chemical-physical properties as humic

substances which were precursors of coal formation [54-56]. Several works has been dealing with characterization of RHA [15, 56, 57].

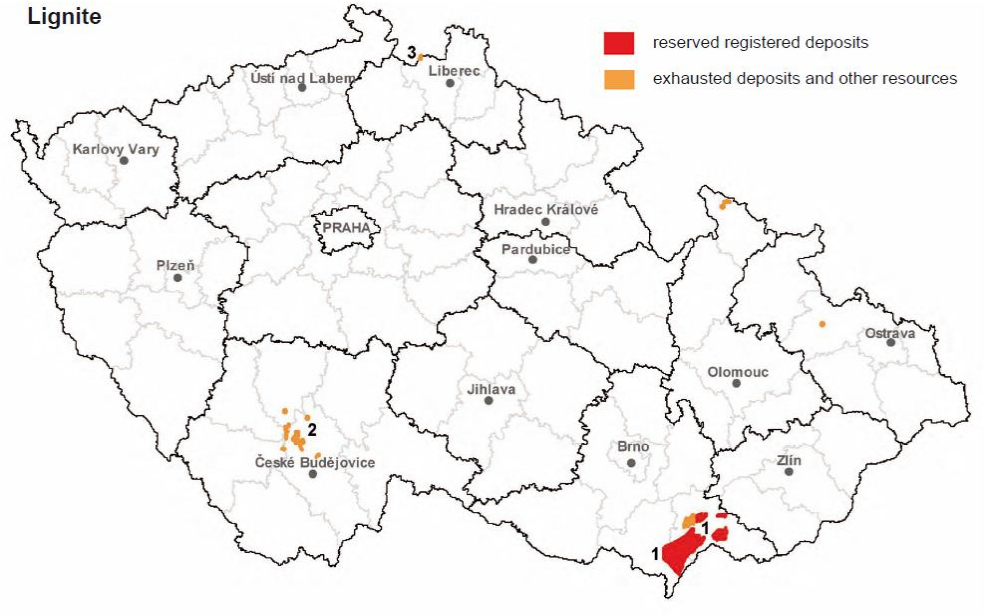

Figure 6 *Deposits and other resources of lignite in the Czech Republic: (1) Vienna Basin, (2) South Bohemian Basin and (3) Zittau (Žitava) Basin* [47].

2.1.5 Biological activity of HS

Increasing evidences have indicated that humic substances can induce plant growth [58]. This effect has often been attributed to the formation of complexes between HS and nutrients [59]. However, it was demonstrated by many authors that HS act as protein carriers of ions trough permeable cell membrane [58, 60], they activate respiration, the Krebs cycle, photosynthesis and production of adenosine triphosphate [58, 61, 62]. The mechanism by which HS stimulate plant biological activity is not still well clarified, however, it is already clear that origin, molecular size, chemical characteristics, pH and concentration play a crucial role. It was demonstrated that functional carboxylic and phenolic C groups seem to have an important role in determining their biological activity [58, 63]. Many authors have demonstrated that low-molecular-size (LMS) fractions of HS are biologically more active than the high ones [64, 65]. The LMS may enter the plant and affect plant metabolism by either enzyme activation or inhibition and inducing or repressing protein synthesis and functional changes in root architecture [66-68]. More recent works suggested the hormonal activity of HS [69, 70]. Pinton et al. [71] showed that low molecular weight water extractable humic fraction affects nitrate uptake and plasma membrane (PM) H^+-ATPase activity in maize roots. This findings was supported by Canellas et al. [70], who showed that HS isolated from earthworm compost induced maize H^+-ATPase activity, while Zandonadi et al. [72] investigated the effect of HAs

on lateral root development concerted with PM H^+-ATPase activity. This implies that predominantly LMS fraction of HS can serve as an environmental source of auxin-like activity [69, 70]. (Auxins are hormones which are involved in plant cell elongation, apical dominance and rooting [72].)

2.1.6 Environmental significance of HS

Humic acids and related substances are among the most widely distributed organic materials on the Earth. The amount of C on the Earth as humic acids (60×10^{11} t) exceeds that which occurs in living organisms (7×10^{11} t) [13]. Humic substances are fundamental in geochemistry and in the environment for the following reasons [13]:

They may be involved in the transportation and subsequent concentration of mineral substances, such as bog ores and nodules of marine strata. Also they may be responsible for the enrichment of uranium and other metals in various bioliths, including coal.

HS can serve as carriers of organic xenobiotics (as well as trace elements) in natural waters. HS *per se* are not believed to be physiologically harmful, but they are aesthetically unacceptable because they impart a reddish-black color to potable waters and recreational lakes. HS play an important role in reducing the toxicities of certain heavy metals (e.g. Cu^{2+} and Al^{3+}) to aquatic organisms, including fish.

HS act as oxidizers or reducing agents, depending on environmental conditions. They may affect photochemical processes in natural waters, including photoalteration of xenobiotics. HS have been shown to reduce Hg(II) to volatile Hg^0 under natural pH conditions, thereby providing a potential pathways for the mobilization of Hg in the environment.

The sorption capacity of the soil for a variety of organic and inorganic gases is strongly influenced by humus. The ability of the soil to function as “sink” for N and S oxides in the atmosphere may be due in part to reactions involving organic colloids.

Humic-like materials in waste waters treated by biological secondary treatment processes create problems of considerable importance in many water works. For example, they can react with chlorine (added during chlorination) to produce the carcinogen chloroform and other undesirable halogenated organics. A major problem in the treatment of waste waters for reuse involves removal of HS and other organics; techniques such as coagulation and carbon adsorption have been proposed for this purpose.

HS provide numerous benefits to crop production. They help break up clay and compacted soils, assist in transferring micronutrients from the soil to the plant, increase seed germination rate and penetration, enhance water retention, and stimulate development of micro flora populations in soils. HS also slow down water evaporation from soils. Their benefits have been proven both experimentally and in the field [73].

As it was mentioned herein, environmental significance of HS is in their strong association with organic and inorganic compounds in soil and water, acting as both storage and transport

agents for these species. Effects of HS on the solubility and mobility of organic contaminants have been the subject of numerous studies. The apparent aqueous solubility of chlordane, DDT, PCBs and chlorodioxins has been observed to increase in the presence of HS [74-76]. This is because the binding of a particular PAH compound by HS depends not only on the hydrophobicity of the PAH solute but also on the size of the solute molecule and its ability to fit into hydrophobic cavities in HS. An increase in pH and ionic strength was found to decrease the binding of PAHs by HS [77]. Hydrophobic organic contaminants (PAHs, benzene, carbon tetrachloride and DDT) can also be adsorbed by dissolved HS [78-82]. The mobility of PAHs, PCBs, and chlordane in groundwater systems was observed to have increased in the presence of HS [8, 9, 75, 83, 84]. Backhus and Gschwend [83] concluded that the presence of HS (<40 mg C/L) will double the mobile load of hydrophobic pollutants such as benzo[a]pyrene or perylene, but will have little effect on the mobility of less hydrophobic pollutants. The enhanced solubility of petroleum-derived compounds in HA solutions is the basis for a new groundwater remediation technology [85]. Additionally, the surfactant activity of HS was found to reduce sorption of organic contaminants on spiked soils, thereby enabling desorption-remediation of PAH [9, 86-88], dioxins [89], and heavy metals [90, 91].

2.2 Soil

There are many different opinions as to what constitutes soil, and there is no commonly agreed definition [92]. Soil is the superficial covering of most of the land area of the Earth and varies in thickness from a few millimeters to many meters. One definition is that soil is weathered rock material at the Earth´s surface, which may or may not contain organic matter and often also contains air and water. A more all-embracing is the definition that soil is a natural body composed of minerals, organic compounds, living organisms, air and water in interactive combinations produced by physical, chemical and biological processes. This would be a genetic soil definition. The primary components of soil are inorganic material, mostly produced by the weathering of bedrock or other parent material, various forms of organic matter, gas and water required by plants and soil organisms, and soluble nutrients used by plants. These constituents differ from the parent material in their morphology, physical, chemical and mineralogical properties, and their biological characteristics. [92]

2.2.1 Soil health and soil quality

Soil health is defined as the continued capacity of soil to function as a vital living system, by recognizing that it contains biological elements that are key to ecosystem function within land-use boundaries. These functions are able to sustain biological productivity of soil, maintain the quality of surrounding air and water environments, as well as promote plant, animal, and human health [93, 94]. The concept of soil quality emerged in the literature in the early 1990s, and the first official application of the term was approved by the Soil Science Society of America Ad Hoc Committee on Soil Quality (S-581) and discussed by Karlen et al., 1997 [95]. Definition of soil quality was as follows: “the capacity of a reference soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation”. [96] Subsequently the two terms are used interchangeably although it is important to distinguish that, soil quality is related to soil function, whereas soil health presents the soil as a finite non-renewable and dynamic living resource [93, 94, 96, 97].

Soil quality cannot be measured directly, but soil properties that are sensitive to changes in management can be used as indicators [98]. *Soil health indicators* are needed that help smallholder farmers understand the chain of cause and effect that links farm decisions to ultimate productivity and health of plants and animals. The quality of soil is rather dynamic and can affect the sustainability and productivity of land use. It is the end product of soil degradative or conserving processes and is controlled by chemical, physical, and biological components of a soil and their interactions [99]. Indicators, however, will vary according to the location, and the level of sophistication at which measurements are likely to be made [100]. Identification of *biological indicators* of soil quality is reported as critically important by several authors [101, 102] because soil quality is strongly influenced by microbiological mediated processes (nutrient cycling, nutrient capacity, aggregate stability). Biological indicators of soil quality that are commonly measured include soil organic matter, respiration, microbial biomass (total bacteria and fungi,) and mineralizable nitrogen. Soil organic matter plays a key role in soil function, determining soil quality, water holding capacity and

susceptibility of soil to degradation [103]. In addition, soil organic matter may serve as a source or sink to atmospheric CO_2 and an increase in the soil C content is indicated by a higher microbial biomass and elevated respiration [103, 104]. It is also the principal reserve of nutrients such as N in the soil and some tropical soils may contain large quantities of mineral N in the top 2m depth [103]. *Chemical indicators*: In order to achieve high crop yields smallholder farmers have to provide soil nutrients in large quantities [105]. Therefore it is possible to alter the pool of available nutrients by adding inorganic fertilizers, incorporating cover crops, and using other organic materials in form of manures and composts [106]. Results of chemical tests are soil quality indicators, which provide information on the capacity of soil to supply mineral nutrients, which is dependent on the soil pH. Soil pH is an estimate of the activity of hydrogen ions in the soil solution. It is also an indicator of plant available nutrients. High activity is not desirable and the soil may require liming with base cations Ca or Mg in order to bring the solution back to neutral. *Physical Indicators* - Soil physical properties are estimated from the soil's texture, bulk density (a measure of compaction), porosity, water-holding capacity [107]. The presence or absence of hard pans usually presents barriers to rooting depth. These properties are all improved through additions of organic matter to soils. Therefore, the suitability of soil for sustaining plant growth and biological activity is a function of its physical properties (porosity, water holding capacity, structure, and tilth).

2.2.2 Soil remediation

Maturity of modern society is no longer determined by levels of production and consumption, but primarily by ability to take care of the environment. Generally, it is desirable to create harmony between man and nature in order to increase care and quality of the environment, i.e. minimize inputs of hazardous substances and remove previous contaminations.

Soil and ground water pollution became a major environmental issue for the developed world later than, for example, air pollution or waste treatment [108]. In the 1980s the developed world became much more aware of the significance of soil contamination as an environmental issue. In many countries, research institutes and companies began to develop technologies to solve the problem of contaminated soil. Most of these were based on treatment of excavated soil. Thermal desorption and soil washing were typical technologies resulting from this phase of development. These technologies, which are often also heavy consumers of energy have become known as "intensive" treatment technologies [109]. However, these technologies can be associated with high energy consumption and sometimes lead to new environmental problems [108]. So, both from an environmental and an economic point of view, there is an emerging international demand for low input, low energy remedial technologies. These technologies have become known as "extensive" technologies. Being low input, extensive technologies may often be long-term treatments, and so their application implies a more holistic and longer-term management of the risks from contaminated land than is the case for rapid intensive response [109].

Remediation technologies

Summarized, the process of removing pollutants from the environment is called *remediation*. *Soil remediation* deals with the removal of pollution or contaminants from soil. Remediation is generally subject to an array of regulatory requirements, and also can be based on assessments of human health and ecological risks where no legislated standards exist or where standards are advisory. Before remedial action takes place there is necessary to properly estimate range of contamination, availability of contaminant and contaminant itself. Remediation technologies can be categorized into *ex-situ* and *in-situ* methods. Ex-situ methods involve excavation of affected soils and subsequent treatment at the surface; in-situ methods treat the contamination without removing the soils. Both these methods primarily use physical and chemical principals and in case of in-situ remediation also biological methods [110]. The main advantage of ex-situ treatment is that it generally requires shorter time periods than in-situ treatment, and there is more certainty about the uniformity of treatment because of the ability to homogenize, screen, and continuously mix the soil. Ex-situ treatment, however, requires excavation of soils, leading to increased costs and engineering for equipment, and material handling/worker exposure conditions. The main advantage of in-situ treatment is that it allows soil to be treated without being excavated and transported, resulting in potentially significant cost savings. However, in-situ treatment generally requires longer time periods, and there is less certainty about the uniformity of treatment because of the variability in soil and aquifer characteristics and because the efficacy of the process is more difficult to verify.

All remediation technologies can be more or less used both ex-situ and in-situ. Most widely used technologies include solidification/stabilization, encapsulation, ion exchange, soil washing, vitrification/thermal treatment, electrokinetic separation, adsorption, soil vapor extraction, and chemical treatments, such as neutralization, precipitation, etc. *Solidification/stabilization* is a remediation/treatment technology used when contaminants are physically bound or enclosed within a stabilized mass (solidification); or chemical reactions are induced between the stabilizing agent and contaminants to reduce their mobility (stabilization). *Ion exchange* removes ions from the aqueous phase by exchange with counter ions on the exchange medium. *Soil washing* - contaminants are sorbed onto fine soil particles and separated from bulk soil in an aqueous-based system on the basis of particle size. The wash water may be augmented with a basic leaching agent, surfactant, pH adjustment, or chelating agent to help remove organics and heavy metals. *Vitrification*, is a thermal method that employ heat up to 1200 °C to melt and convert waste materials into glass or other glass and crystalline products. The high temperatures destroy any organic constituents with very few byproducts. Materials, such as heavy metals and radionuclides, are actually incorporated into the glass structure which is, generally, a relatively strong, durable material that is resistant to leaching. The *electrokinetic* remediation uses electrochemical and electrokinetic processes to desorb, and then remove, metals and polar organics. *Soil vapor extraction* employs vacuum which is applied to the soil to induce the controlled flow of air and remove volatile and some semivolatile contaminants from the soil [110].

Bioremediation

Bioremediation is defined as the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities [111]. By definition, bioremediation is the use of living organisms, primarily microorganisms, to degrade the environmental contaminants into less toxic forms. It uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment. The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. Contaminant compounds are transformed by living organisms through reactions that take place as a part of their metabolic processes. Biodegradation of a compound is often a result of the actions of multiple organisms. When microorganisms are imported to a contaminated site to enhance degradation, the process is known as *bioaugmentation*. For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products. Like other technologies, bioremediation has its limitations. Some contaminants, such as chlorinated organic or high aromatic hydrocarbons, are resistant to microbial attack. They are degraded either slowly or not at all, hence it is not easy to predict the rates of clean-up for a bioremediation exercise; there are no rules to predict if a contaminant can be degraded. Bioremediation techniques are typically more economical than traditional methods such as incineration, and some pollutants can be treated on site, thus reducing exposure risks for clean-up personnel, or potentially wider exposure as a result of transportation accidents. Since bioremediation is based on natural attenuation the public considers it more acceptable than other technologies. [112]

Phytoremediation

Although the application of microbe biotechnology has been successful with petroleum-based constituents, microbial digestion has met limited success for widespread residual organic and metals pollutants. Vegetation-based remediation shows potential for accumulating, immobilizing, and transforming a low level of persistent contaminants. In natural ecosystems, plants act as filters and metabolize substances generated by nature. Phytoremediation is an emerging technology that uses plants to remove contaminants from soil and water [113-115]. The term “phytoremediation” is relatively new, coined in 1991. Its potential for encouraging the biodegradation of organic contaminants requires further research, although it may be a promising area for the future. It can be found five types of phytoremediation techniques, classified based on the contaminant fate: phytoextraction, phytotransformation, phytostabilization, phytodegradation, rhizofiltration, even if a combination of these can be found in nature. [112]

Humic acids as remediation agent

Due to the fact that HA are defined as a surface active substances, based on their significant effects on surface tension and with their tendency to complex metal ions and sequester organic molecules (see chapter 2.1.6), it could be suggested that they may be used to remove contaminants from polluted water. This has been recognized in various studies [116-119] and is the subject of a patent by Zanin and Boetti, who reported the use of extracted HA for the

removal of heavy metals, chlorinated organics, phosphorus, and nitrogen from waste water [46]. The main difficulty in using HA for this purpose, however, is that their isolation from natural matrices (usually soil) is laborious, time consuming, and costly. One exception to this is leonardite humic acid (LHA), which is available in bulk and requires little or no further treatment. LHA is a material found in association with leonardite, lignite distributed in vast deposits across North America and it presently enjoys wide use as an agricultural soil conditioner [35]. HA prepared in this work are produced from lignite as well. Therefore, one can observe some similarities with LHA.

2.2.3 Biochar

One of the most popular soil organic amendments of recent times is biochar. It is carbonaceous porous material obtained by pyrolysis of biomasses. It shows great potential in improving soil fertility [120-122]. The effect of biochar on soil biological, chemical and physical properties is complex but, in general, it establishes a carbon sink and maintains soil fertility for over the long time of soil cultivation. This was proved by many authors and agriculturists on many types of used soils. [123-125] Among others the most advantageous application of biochar is in remediation of contaminated soils [126, 127]. In the experiment of Abdelhafez et al. 2013 [128], the restoration of shooting range soils with high lead contamination by application of biochar was successfully provided. The results showed that the addition of biochar significantly increased the soil water holding capacity, availability of nutrients (N, P and K), cation exchange capacity, and stimulated the microbial growth (bacteria and fungi) in soil. Many studies have indicated that the carbon in biochar remains stable for millennia, what resulted in reducing of greenhouse gas emission [129]. In summary, biochar maintain soil fertility and establish a carbon sink, enhances the microbial and chemical transformation and cause a significant increase in crop production.

2.2.4 Soil water repellency

Soil water repellency (SWR) is a physical property of soil that limits water infiltration, as well as a chemical property in it that consists of organic compound. In a hydrophobic soil, water will not readily penetrate and infiltrate into the soil, but will blockade and remain on the surface [130, 131]. Surface attraction of repellency for water originates from the attractive forces between water and solid surfaces. If the attraction is greater between water and soil particles than between individual water molecules, the water will spread out and be absorbed. However, when the attraction between water molecules is greater than that between the water and soil surface, the water will be repelled by the soil particle rather than infiltrate [130, 131]. The main factors affecting the soil water repellency are soil texture and organic matter [130]. Hydrophobic substances are naturally occurring and derived from organic compounds of most living and decomposing plant species or microorganisms. The most commonly associated species with water repellency are those containing significant amount of resins, waxes and aromatic soils [130-132]. Organic compounds most suspected to cause SWR among others are aliphatic hydrocarbons and amphiphilic compounds such as long-chained fatty acids. Fatty acids form very insoluble soaps with calcium, magnesium and other bi and trivalent metals, when dry, they become extremely water-repellent [133].

3 THE GOAL OF THE WORK

As can be seen in the above-mentioned paragraphs, the potential of humic substances applied in environmental protection technologies is enormous. Therefore, the main goal of this work is the optimization of procedures leading to the production of modified lignite humic acids, which have improved properties and modified chemical characteristics applicable in environmental technologies. In light of above discussion, this challenge seems to be very ambitious and also very optimistic. In fact, this study follows previous pilot researches carried out at Faculty of Chemistry of Brno University of Technology, focused on production of modified humic acids [15, 57]. In mentioned works, two ways of modification of parent lignite were chosen, i) chemical and ii) physical. In both cases, humic acids were extracted by standard procedures [10, 134-136]. It was demonstrated that both types of modifications provided possibilities to produce humic acids, which have biological activity comparable with commercially available biostimulators [137]. In fact, the highest biological activity showed humic acids produced using way "ii)". In our previous work [44], using the same set of humic acids, the effect of products on the surface tension was studied. It was shown that the way ii) is slightly more efficient to produce HA with higher surface activity than was "i)". The way "i)" was studied more deeply in the recent work of David et al. [138] who used a comprehensive approach in which a multitude of techniques was used to characterize humic products; however applied statistical approach revealed only weak correlations between primary and secondary characteristics of HA. Nevertheless the promising potential of humic acids as a sorbent and biostimulant were confirmed and discussed.

In this work, the production of a set of "modified" humic acids (MHA) is planned also using approach "ii)". This approach mimics the processes observed in rhizosphere where "small" organic molecules (typically acids) are produced by root system of higher plants and possibly cause the reaggregation of HA present in rhizosphere [33]. As a result, apparently large molecules of humic acid are disaggregated into small subunits or even molecules, which allow and support the transport of nutrients and simultaneously, they are able to penetrate through the root cell walls and participate in cell biological processes. Therefore, MHA produced in this work will be characterized mainly for their biological and surface activity. In other words, the aim of this research is to find out the optimal physical pre-treatment of raw lignite with respect to maximal surface activity and biological activity. Furthermore, we would like to continue the search for the relation between the type of lignite modification agent and the HA structure and properties in order to design an approach leading to production of humic acids with desired properties.

4 EXPERIMENTAL PART

Several methods presented in this part were already described in detail in our previous work [44].The publication is attached to present study as Appendix I.

4.1 Sample preparation

4.1.1 Sample origin

As a source of studied humic acids was used South Moravian lignite collected from the Mír mine in the area of Mikulčice, near Hodonín (Vienna basin, Czech Republic - Figure 6, chapter 2.1.4).

4.1.2 Extraction of humic acids

Ten modified humic acids (MHA) were prepared by pre-treatment of parental lignite. To obtain MHA samples, milled and sieved (at 0.2-0.3 mm) lignite was first pre-treated by various organic acids. Their list is given in Table 2. 50 g of raw lignite were soaked for 30 min under the constant stirring in a suspension with 500 mL of appropriate organic acid solution. Pre-treated lignite was then thoroughly washed by deionized water on the sintered glass filter till agent-free. Humic acids were extracted from lignite by standard alkaline extraction with 0.5 M NaOH and 0.1 M $Na_4P_2O_7$ (1:10) [44, 139, 140]. For comparison, humic acid without previous modification of parental lignite was also extracted. Part of MHA samples was then dissolved in deionized water, titrated by 0.1 M KOH to pH 7 and freeze-dried to produce water soluble potassium humates. [44]

Table 2 List of studied samples

Sample	Modifier
MHA0	None
MHA1	formic acid (20%)
MHA2	acetic acid (20%)
MHA3	propionic acid (20%)
MHA4	butyric acid (20%)
MHA5	maleic acid (20%)
MHA6	benzoic acid (0.3%)
MHA7	fumaric acid (0.5%)
MHA8	oxalic acid (10%)
MHA9	phenylacetic acid (1.5%)
MHA10	picric acid (1.4%)

4.2 Physico-chemical characterization

4.2.1 Elemental Analysis

Elemental analysis represents the fundamental characterization of humic acids. For elemental analysis the PE 2400 CHNS/O Elemental Analyzer performed by Strojírenský zkušební ústav, s. p., Brno, Czech Republic was used.

4.2.2 FTIR spectroscopy

FTIR spectroscopy was used in order to assess the molecular changes in individual MHA induced by modification of parental lignite. For analysis the Nicolet Impact 400 spectrometer was used. Conventional KBr pellet technique was applied [44, 141]. For analysis, potassium humates of studied samples were used. From 0.5 to 1 mg of previously dried (110 °C for 4 hours), cooled and stored humic samples in desiccator were mixed in agate mortar with 200 mg of KBr. Obtained powder was squeezed to form the pallet and put into the spectrometer to be analyzed. [44]

4.2.3 HPSEC analysis

To assess the aggregation behaviour and molecular size distribution of individual HAs samples, high pressure size exclusion chromatography (HPSEC) was carried out. [140, 142, 143] The Agilent system with two detectors in series was used; UV detector at 280 nm (calibrated by sodium polystyrensulphonates (PSS)) and RI refractive index (RI) detector calibration with polysaccharides (PSC)). For the size exclusion separations, a Phenomenex Biosep S2000 (600 x 7.5 mm) column preceded by a guard column and by a 0.2 μm stainless-steel inlet filter was used. The HPSEC eluent was a 50 mM $NaH_2PO_4 \cdot H_2O$ solution adjusted to pH 7 with 1 M NaOH. The flow rate was set 0.6 mL min^{-1}. For the column calibration, standards of known molecular weight were used [44]. Potassium humates of studied samples were dissolved in the HPSEC eluent to achieve concentration 0.6 mg mL^{-1}, filtered through quartz filters (Glass Microfibre Filterm Whatman International, LTD) and subjected to HPSEC analysis. The weight-averaged molecular weight (M_w) was calculated using PE-TC-SEC 4.01 software [44]:

$$M_W = \frac{\sum_{i=1}^{N} (h_i \cdot M_i)}{\sum_{i=1}^{N} h_i}, \qquad (1)$$

where M_i and h_i are molecular weight and the height of each i-th fraction in chromatogram, respectively. [44]

4.2.4 Surface tension measurement

To assess the surface activity of studied set of modified humic acids, the surface tension measurement employing Sigma 700 tensiometer (KSV Instruments Ltd.) using 19 mm diameter platinum-irridium (Pt-Ir) ring was used. The measurement was carried out as follows: eleven samples with concentrations from 0.001 to 8 g L^{-1} of each MHA were

prepared by diluting humic solutions one day before the measurement. Each solution was placed into the shallow glass measuring dish and carefully stirred for 5 min (Figure 7).

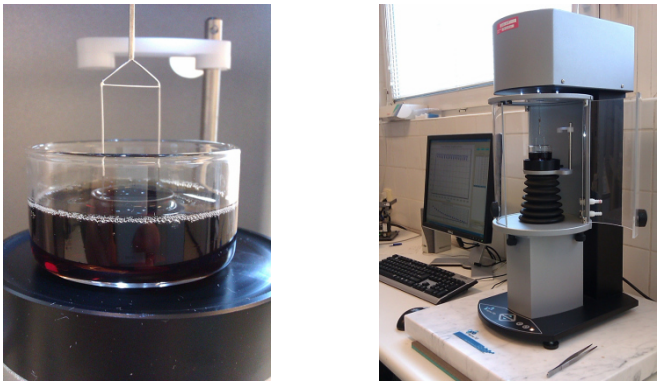

Figure 7 *Measurement of surface tension with 19-mm diameter Pt-Ir ring.*

Then, stirring was switched off and after 10 min of sample repose the surface tension was measured repetitive by Pt-Ir ring. Within 10 min long analysis 20 values of surface tension were recorded. For the subsequent calculations, the mean value was applied. During 10 min of measurement, no significant changes in ST were observed (data not shown). After the measurement, the glass dish with sample solution was placed into the desiccators with water to keep moisture conditions and after 24 hours the whole measurement was repeated directly without stirring. All measurements were carried out at 25±2 °C. To investigate the molecular structure of humic acid adsorbed in water/air interface, data obtained from ST measurement were fitted by Szyszkowski equation [144-146] using Origin 7.5 [44]:

$$\gamma_0 - \gamma = a \log(1 + bc), \tag{2}$$

where the *a* and *b* are empirical parameters of Szyszkowski equations. For the maximal surface saturation by adsorbed molecules was derived following equation:

$$\Gamma_{\max} = a / 2{,}303 RT, \tag{3}$$

where Γ_{max} is the maximal Gibbs surface excess quantity.

4.2.5 Thermochemolysis and GC-MS analysis

To determine the content of free and bound fatty acids and compare the primary structure of individual humic acids extracted from variously pre-treated lignite, the method of chemical degradation as thermochemolysis was used. Obtain products were analysed by gas chromatography-mass spectrometry.

Thermochemolysis

The thermochemolysis method was based on the off-line procedure previously used on lignite humic acids [147]. MHA samples (1 g) were milled and thoroughly mixed in a ratio of 1:1

with a 25% w/w solution of TMAH in methanol. After 1 h of impregnation, the sample was transferred to a 60 x 3 cm i.d. Pyrex tube and heated at 400 °C (30 min isothermal). Thermochemolysis products were swept using helium (flow rate 100 ml/min) to a trap containing chloroform cooled to 0 °C. When thermochemolysis was complete, the chloroform solution was drawn off and filtered through Na_2SO_4 to scavenge any associated water. The sample was evaporated under a gentle stream of nitrogen to dryness and re-dissolved in chloroform spiked with a known amount of eicosane (n-C20 alkane, n-C20) as internal standard and transferred to a 100 µl low-volume glass insert in 1.5 ml glass screw-cap vial for analysis using gas chromatography–mass spectrometry (GC–MS).

Gas chromatography–mass spectrometry (GC–MS)

After thermochemolysis, samples were analyzed using a Trace GC Thermo Finnigan gas chromatograph equipped with a split injector (250 °C) and a Supelco Equity 5 fused silica column (30 m x 0.25 mm, 0.25 µm film thickness), and He as carrier gas, connected to a Thermo Finnigan Automass quadrupole mass spectrometer. The mass spectrometer was operated in the electron ionization mode at 70 eV, with ion separation in a quadrupole filter. The oven temperature was programmed from 60 °C to 300 °C at 5 °C.min^{-1} and held there for 20 min. Products were identified on the basis of their retention times, their mass spectra (comparison with standards) and literature data [147].

4.3 Plant grow experiment

To determine biological activity of extracted HK the experiment with the maize growing hydroponically in HA solutions was carried out. For the experiment, the corns of the common maize *Zea mays* CEKLAD 235 species (Oseva Bzenec Ltd., Czech Republic) were used. The species was chosen for its wide spread of use for kernel and silage purpose, high percent of germinative activity and high durability. The experiment was carried out according to previous work of [72, 138, 148] and is illustrated in Figure 8-10.

4.3.1 Seed germination experiment

To remove the fungicidal treatment, the corns were first treated with 0.05 mol L^{-1} solution of sodium hypochlorite (NaClO) for 5 min. In order to absorb water for following easier germination, the corns were carefully washed and soaked for 5 hours in distilled water. The germination phase was conducted in wet paper laboratory towels, where the corns were rolled in approx 3 cm gaps. The paper rolls with seeds were put in a glass beaker with certain amount of distilled water and let to germinate for 2 days in the dark at 28±2 °C employing the BT-120 Biological Thermostat (Laboratorní přístroje Praha Ltd., Prague, Czech Republic) [138].

4.3.2 Root growth experiment

Selected germs (2 to 4 cm) were planted hydroponically. For good nutrient properties and minimum risk of synergistic activity along with humates [72], the plants grew in the solution of potassium humate sample in predicted optimum concentration 40 mg L^{-1} dissolved in 1 L of 2 mmol L^{-1} solution of $CaCl_2$ [148]. Thirty germs were put onto the foamed poly(styrene) floating beds lying in the container with 1 L of tested solution. For each sample one container. For the comparison the commercial humate preparations Lignohumate B and Lignohumate AM (Amagro s.r.o., Czech Republic) of 40 mg L^{-1} dissolved in 1 L of 2 mmol L^{-1} solution of $CaCl_2$ was tested. The growth was carried out in the biological thermostat (BT-120 Biological Thermostat) for 5 days at 25±2 °C, every 12 hours at daylight and every 12 hours at dark. All containers were constantly aerated using aquarium pump. [138]

4.3.3 Plant growth assessment

Total mass increment assessment

All 30 sprouts and later the grown seedlings for each sample solution were weighted and the difference was calculated as a *total mass increment*.

Root length increment assessment

The roots of five selected seedlings were measured before and after the growing experiment and the difference was calculated as a *root length increment*.

Image analysis

The roots of previously selected five seedlings were scanned against black paper background using common desktop office scanner. The obtained images (300×300 dpi, 2424×3426 pix

and 24 bits pix^{-1}) were loaded by Harmonic and Fractal image Analyzer software (HarFa) (http://www.fch.vutbr.cz/lectures/imagesci) [149], trimmed to desired square images (300×300 dpi; 2048×2048 pix×24 bits pix^{-1}), saved as bitmaps and subjected to 2D Wavelet Analysis with black and white thresholding set at the value of 160. The *K*[BW] value represents the number of pixels on the black and white border, therefore the value stands for the measure of *root division* [138, 149, 150].

4.3.4 Sugar and protein assessment

After the plant grow experiment and image analysis, whole seedlings were dried in a laboratory dryer for 3 days at 60 °C. Total dry material was subjected to sugar and protein assessment. The sugar content was determined polarimetrically using the customary Ewers polarimetric method [151]. The proteins were determined by the Kjeldahl method [152] for the determination of total nitrogen. An automatic Kjeldahl analyzer Kjel-Tec™ 2100 (FOSS Inc., Hillerød, Denmark) was employed. [138] The measurement was performed in cooperation with Mendel University Brno.

Figure 8 *Seed germination experiment.*

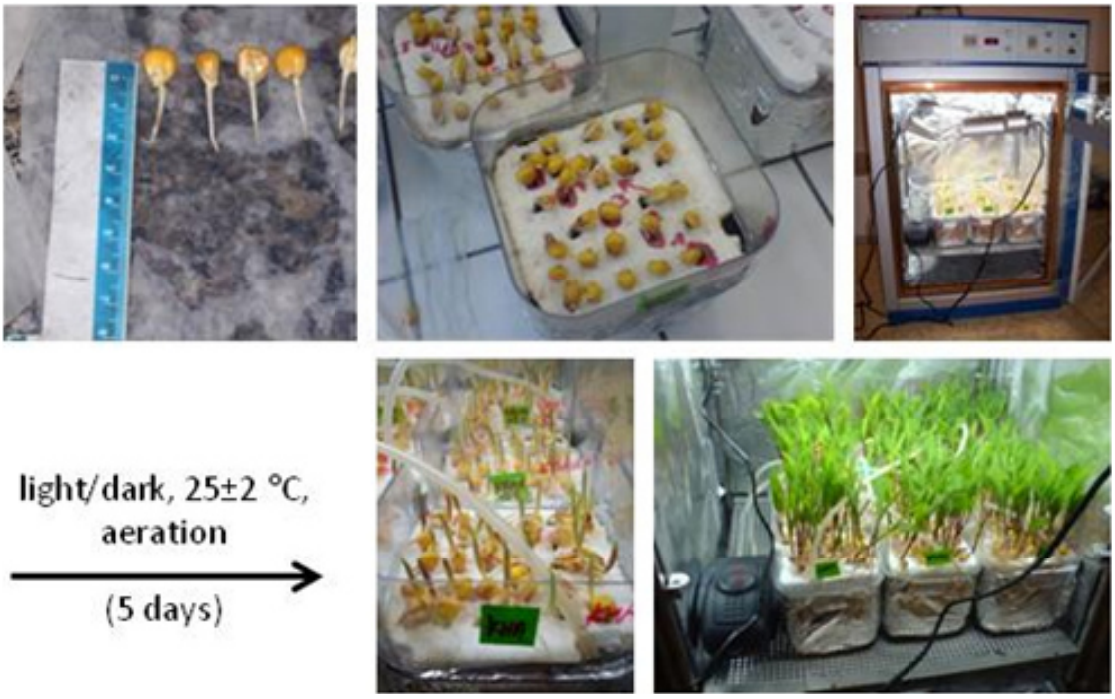

Figure 9 *Root growth experiment.*

Figure 10 *Plant growth assessments.*

4.4 Influence on soil

To assess the soil remediation potential of studied humic acid samples, the MHA were mixed with arable soil. Common soil experiments such as soil respiration and soil hydrophobicity were performed. For comparison experiments without addition of humic acid were performed. The soil used for the experiment came from the "university testing field" of Faculty of Agriculture / Landscape Management, University of Applied Science in Dresden. The soil experiments were carried out at the same place. The type of the soil was Albeluvisol and its characterisation is described in Table 3.

Table 3 Characterisation of the soil used for soil experiments carried out in this research.

Horizon	Depth	Stone (%)	Sand (%)	Silt (%)	Clay (%)	C (%)	pH H_2O	pH $CaCl_2$
Ap	0-26	< 2	60	32	8	2.1	6.3	5.4
Al	26-43	< 2	53	37	10	1.5	6.4	5.8
Bv	43-94	< 2	50	36	14	0.9	6.5	5.8
Bv-Bt	> 94	< 2	52	30	18	0.8	6.7	5.7

4.4.1 Soil respiration

Soil respiration is one of the main soil quality indicators and its measurement represents a common tool to evaluate soil biological processes. It reflects the primary path by which CO_2 returns to the atmosphere [153-155]. In such experiments, biological activity is usually assessed by the measurement of CO_2 evolution, O_2 consumption, microbial activity, enzyme activity, or other parameters. In present work, the soil respiration experiment was carried out with the aim to prove the positive remediation effect of lignite humic acids on soil microbiological activity.

Soil water-retention capacity

Water retention capacity is an important property of soil indicating the amount of bounded C in soil and soil texture, respectively, and is necessary parameter for incubation experiment. For determination of the water-retention capacity of the soil used for incubation experiment performed in this research, the ceramic plates at pF 1.8 according to DIN/ISO 11274 were used [156] (Figure 11).

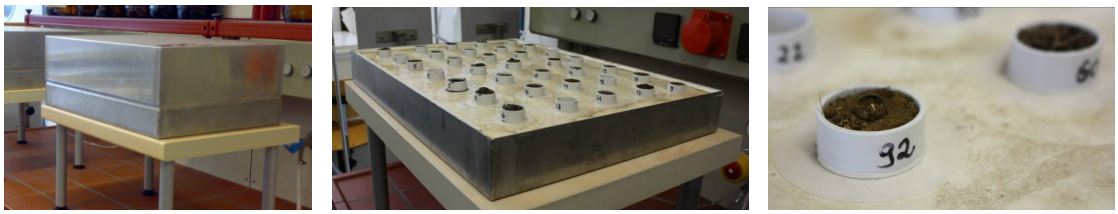

Figure 11 *Water retention capacity experiment.*

Soil incubation experiment

Incubation experiment was carried out with RESPICOND device from Nordgren Innovations (Sweden) (Figure 12). Twenty grams of soil (air-dried and sieved to <2 mm) was placed in a 250-mL vessel with a small container of 10 mL of 0.6 M KOH solution suspended above. Electrical conductivity of this solution was measured to monitor the CO_2 evolution during the experiment in varying intervals from 30 to 60 minutes. Before starting the experiments, the air-dried and sieved soil samples were re-moistened by addition of water solution of studied modified humic acid samples at appropriate concentrations until 76 % of their field water retention capacity (pF 1.8). As a reference the soils were re-moistened with water solution of chemical surfactants (SDS, Triton X-100) and pure water. The soil respiration measurements were carried out in three replicates, which are presented in this study as averaged values. Measurement of evolved CO_2 began immediately within 2 h after re-moistening and ended after 50 days of incubation at a constant temperature 20 °C [153]. Data obtained from soil incubation experiment were fitted by a two-compartment (double-exponential) model function (using Origin 7.5) [157]:

$$C_t = C_r \exp(-k_r t_r) + C_s \exp(-k_s t_s), \quad (4)$$

where C_t is total carbon in the soil at time t, C_r is the rapidly mineralizable C pool, C_s is the slowly mineralizable C pool, k_r and k_s represent the mineralization constants and t_r and t_s are respective time constants [157]

Figure 12 *RESPICOND-device for soil incubation experiment with 96 positions.*

4.4.2 Soil water repellency

To assess the changes of wettability / hydrophobicity of the soils treated by studied humic acid samples before and after incubation experiment, the soil water repellency experiment (SWR) was carried out. [158, 159]

Contact angle by Wilhelmy plate method

The SWR for all studied samples was determined by measuring of contact angle by Wilhelmy plate method. One measurement was provided before and one after incubation experiment on the soils treated with studied MHA samples. The principal of this method was described in the textbook of Butt et al. [160]. A thin plate of glass, platinum, or filter paper is vertically placed halfway into the liquid. Close to the three-phase contact line the liquid surface is oriented almost vertically (provided the contact angle is 0°). Thus the surface tension can exert a downward force. One measures the force required to prevent the plate from being drawn into the liquid. After subtracting the gravitational force this force is $2l\gamma$, where l is the length of the plate. The adaption of Wilhelmy plate method to soil science was described in detail in the paper of Diehl [158] and Diehl et. al [159].

5 OVERVIEW OF RESULTS AND DISCUSSION

5.1 Physico-chemical properties of modified lignite HAs

5.1.1 Elemental analysis

The results of elemental analysis of individual samples are listed in Table 4. While C, N, H content were measured directly, O content was calculated as a difference according to equation O[%] = 100 – C[%] – N[%] – H[%]. It must be taken into account that percentages of oxygen include also a small amount of other elements such as sulphur and phosphorus. Nevertheless, according to the literature data, such amount rarely exceeds 2 %. [13].

Table 4 List of analyzed samples and their elemental analysis in % (w/w).

Sample	Modifier	C	H	N	O	Moist.	C/O	C/H	C/N
MHA0	none	58.0 ±0.03	4.3 ±0.05	1.4 ±0.01	36.3	7.0	1.6	13.4	41.4
MHA1	formic a.	58.6 ±0.05	5.3 ±0.15	1.3 ±0.01	34.8	7.3	1.7	11.1	45.1
MHA2	acetic a.	54.0 ±0.03	5.1 ±0.07	1.3 ±0.01	39.6	11.3	1.4	10.6	41.5
MHA3	propionic a.	59.7 ±0.13	3.8 ±0.05	1.4 ±0.01	35.1	2.2	1.7	15.7	42.6
MHA4	butyric a.	58.2 ±0.05	4.0 ±0.06	1.4 ±0.01	36.4	5.9	1.6	14.5	41.6
MHA5	maleic a.	58.4 ±0.06	4.6 ±0.02	1.4 ±0.01	35.6	5.6	1.6	12.6	41.7
MHA6	benzoic a.	58.0 ±0.01	4.6 ±0.06	1.4 ±0.02	36.0	5.3	1.6	12.5	41.4
MHA7	fumaric a.	57.8 ±0.06	4.8 ±0.06	1.4 ±0.01	35.9	5.9	1.6	11.9	41.3
MHA8	oxalic a.	58.1 ±0.02	4.7 ±0.02	1.4 ±0.01	35.8	5.0	1.6	12.4	41.5
MHA9	phenylac. a.	57.1 ±0.02	4.2 ±0.05	1.4 ±0.01	37.3	6.4	1.5	13.7	40.8
MHA10	picric a.	57.1 ±0.09	4.1 ±0.06	1.7 ±0.01	37.2	6.3	1.5	13.9	33.6

a.=acid; Moist.=Moisture

Modification of lignite caused both increase and decrease of the content of all elements. Content of C, H and N varied within 54.0-59.7 %, 3.8-5.3 % and 1.3-1.7 %. O content varied within 34.8-39.6 %. The lowest content of C was obtained for HA prepared by modification with acetic acid. On the contrary, the highest C content was recorded for HA modified by propionic acid. The lowest H content was recorded for HA treated with propionic acid; the highest H content was recorded for HA treated with formic acid. The lowest N content was recorded for HA modified by formic acid and the highest N content was observed in HA treated with picric acid. To study the differences among samples, the ratio C/H, that is indicative for aromaticity/alifaticity degree of the HA, was calculated. High C/H ratio indicated that during the modification of parental lignite, preferably aromatic rather than aliphatic structures were released and consequently extracted. The highest C/H ratio was recorded for HA treated with propionic acid, whereas the lowest ratio was recorded for formic acid. Next determined ratio, the C/O, reflects the degree of carbon oxidation extracted after lignite pre-treatment. The lowest value of C/O ratio was recorded for MHA2 sample, what indicates that the HA treated with acetic acid contained largest amount of oxygenous functional groups in the molecular structure, in comparison with other samples (Table 4). In

overall results from elemental analysis, the most remarkable changes in molecular structure of all studied samples induced by physical pre-treatment of lignite were observed in case of formic acid, acetic acid and propionic acid. Other samples showed average values.

5.1.2 FTIR spectroscopy

In order to assess the molecular changes in individual HAs samples induced by modification of parental lignite, FTIR spectroscopy was applied. The patterns of spectra of all studied samples were similar, the differences were observed only in intensities of individual peaks. The obtained spectra were also similar to those obtained previously by many authors [13, 161, 162] Exemplary spectra of not-treated HA, HA treated with acetic acid and propionic acid are shown in Figure 13. The interpretation of FTIR spectra of humic acids have already been described by many authors [13, 44, 163]. Briefly, the first broad peaks observed in the range 3450-3300 cm^{-1} and 2950–2900 cm^{-1} described hydrogen bonded –OH and aliphatic C–H stretching. Sharp peaks recorded in the range 1590-1517 cm^{-1} and 1380 cm^{-1} described C=O stretching of COO–, and 1640-1600 cm^{-1} described C=O stretching of COO–, ketonic C=O and aromatic C=C conjugated with COO–. Two smaller peaks were observed in the region 1270 cm^{-1} and 1220 cm^{-1} belonging to C–OH vibrations of phenolic –OH and possibly also to aromatic C=C conjugated with COO–. The last weak peak was observed at around 1050 cm^{-1} what corresponds with C–O stretching of polysaccharides and/or Si–O vibrations of mineral impurities.

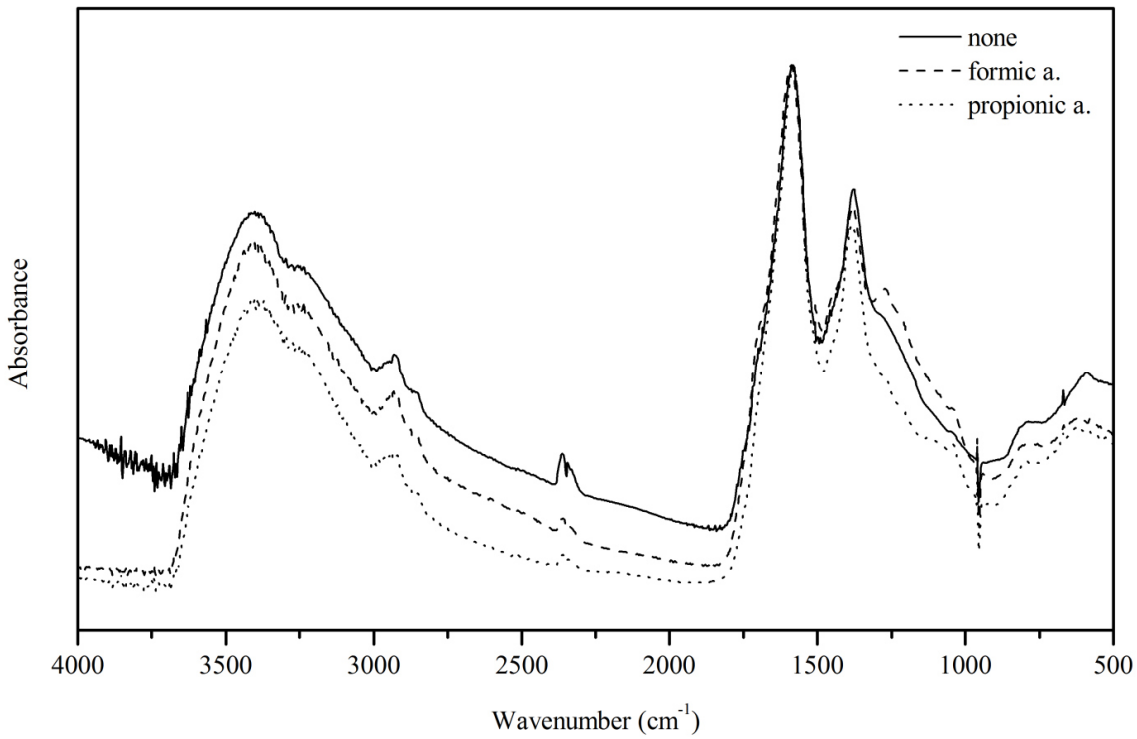

Figure 13 *Comparison of FTIR spectra of not modified MHA0 and modified MHA1 and MHA3 (treated with 20% formic acid and 20% propionic acid, respectively)*

To distinguish the differences among studied HAs samples in detail, the ratio of relative intensities of specific absorption bands was calculated. Similarly to the results obtained from elemental analysis, the ratio of relative intensities 1610/2930, describing aromaticity /aliphaticity degree, was performed. The list of these values for all samples is shown in Table 5 and indicates an increase in aromatic moieties after the lignite pre-treatment of all HA samples. The highest ratio was observed for propionic acid, while the lowest value was observed for fumaric and formic acid.

Table 5 List of proportion of relative intensity of selected absorption bands.

Sample	Modifier	arom./aliph. 1610/2930
MHA0	none	1.9
MHA1	formic a.	2.2
MHA2	acetic a.	2.6
MHA3	propionic a.	2.8
MHA4	butyric a.	2.5
MHA5	maleic a.	2.5
MHA6	benzoic a.	2.4
MHA7	fumaric a.	2.0
MHA8	oxalic a.	2.3
MHA9	phenylac. a.	2.4
MHA10	picric a.	2.7

5.1.3 Molecular size distribution

In order to assess the changes in physical structure and self-assembling behaviour of HA induced by modification of raw lignite, the molecular-size distribution was analysed. High performance size exclusion chromatography (HPSEC) was carried out, because it is recognized as a highly precise method for evaluation of the relative molecular-size distribution of dissolved HAs [140, 164-166].

The HPSEC records were obtained using two detectors; UV detector set at 280 nm, which could detect only chromophores (i.e. predominantly unsaturated moieties), and refractive index (RI) detector, monitoring elution of whole sample mass. Both detectors recorded similar shape of chromatograms consisting of two peaks. Obtained chromatograms were almost identical for all studied samples and similar to those reported in literature for HAs isolated from soils, sediments and natural waters [3, 140, 167]. The exemplary chromatograms recorded for both UV and RI detectors, are reported in Figure 14. The first peak was eluted at retention time 23 min and indicated the elution of the largest components (aggregates) of HAs samples. Conte et al. [168] demonstrated that this fraction with the largest molecular size is rich in aromatic, aliphatic and alkyl components. Aliphatic structures contained preferentially longer alkyl chains, which strongly associated due to stronger hydrophobic effect and formed supramolecular structures, which eluted at short elution times typical for apparently large molecular-size components. Shorter alkyl chains than associated in lower molecular-size

fractions eluted with increasing elution times, represented by the second broad peak with maximum at approximately 32 min. The elution of aromatic structures decreased with reducing molecular size and increasing elution time [168]. While the least intensive first peak, and thus the lowest amount of large aggregates excluded, was recorded for HA treated with formic acid, the most intense first peak was recorded for HA treated with propionic acid. This observation was completed by the results from weight-average molecular weight (M_w) calculations, while both detectors indicated an increase in M_w, except formic acid, comparing to non-treated HA. The M_w was calculated based on respective calibration from both detectors and gained values for all samples are listed in Table 6. While the lowest M_w was obtained for formic acid, the highest values showed HA treated with phenylacetic acid > propionic acid > acetic acid (Table 6). This could indicate the fact, that during treatment, only very small organic acid molecules (i.e. formic acid) could diffuse more easily through loosely-bound HA aggregates in lignite and could cause the rearrangement of larger compartments into the smaller aggregates [140, 143], while the other organic acids act in a different way. Kučerík et al. [10] showed that addition of formic acid into the HAs solution, caused the protonation of the functional group with subsequent formation of intermolecular H-bonds, leading to larger M_w in comparison with propionic acid. However, the propionic acid still showed lower M_w value than parental HAs [10]. It indicates that behaviour of already extracted HAs differs in comparison with HAs still present in lignites. This might be caused by interactions of humic acids with other lignite compartments, which might be both weak interaction and covalent bonds. The latter can be disrupted during extraction as suggested by other studies [169]. This, however, also implies that, at least for lignites, the results do not support the supramolecular theory suggested by Piccolo [33] and support the alternative explanation considering humic acids as "molecular artefacts" [169] produced and largely influenced by used extraction technique.

To study more in detail the differences in distribution of molecular size after the modification of parental lignite among individual HA samples, the area under the peaks was divided into six intervals of M_w, which were divided by total-peak area and multiplied by factor 100 to obtain percentage contents of particular molecular fractions. The molecular weights, which were equal or over 150 kDa, were covered up in one group. Based on UV detector, the most remarkable part of humic aggregates (24-46 %) occurred in the lowest defined interval of M_w 0-25 kDa. The most obvious increase was occurred after addition of formic acid into the HS system (up to 5.2%). On the contrary, addition of propionic acid into the HS system caused apparent decrease in the formation of low molecular-size aggregates, down to 16.8%. From 12.6 % (formic acid) up to 31.0 % (propionic acid) of HA aggregates occurred in the interval of M_w 100-150 kDa (UV det.) with only 10.6 % occurred in non-treated HA. The calculations from RI detector are comparable and confirmative to that obtained from UV detector. The results are summarized in the Table 7 and Table 8 and illustrated in the Figure 15 and Figure 16, for UV and RI detector, respectively. Again, the results are in contrast to HPSEC studies reported earlier [10, 33] and underline the importance of extraction procedure.

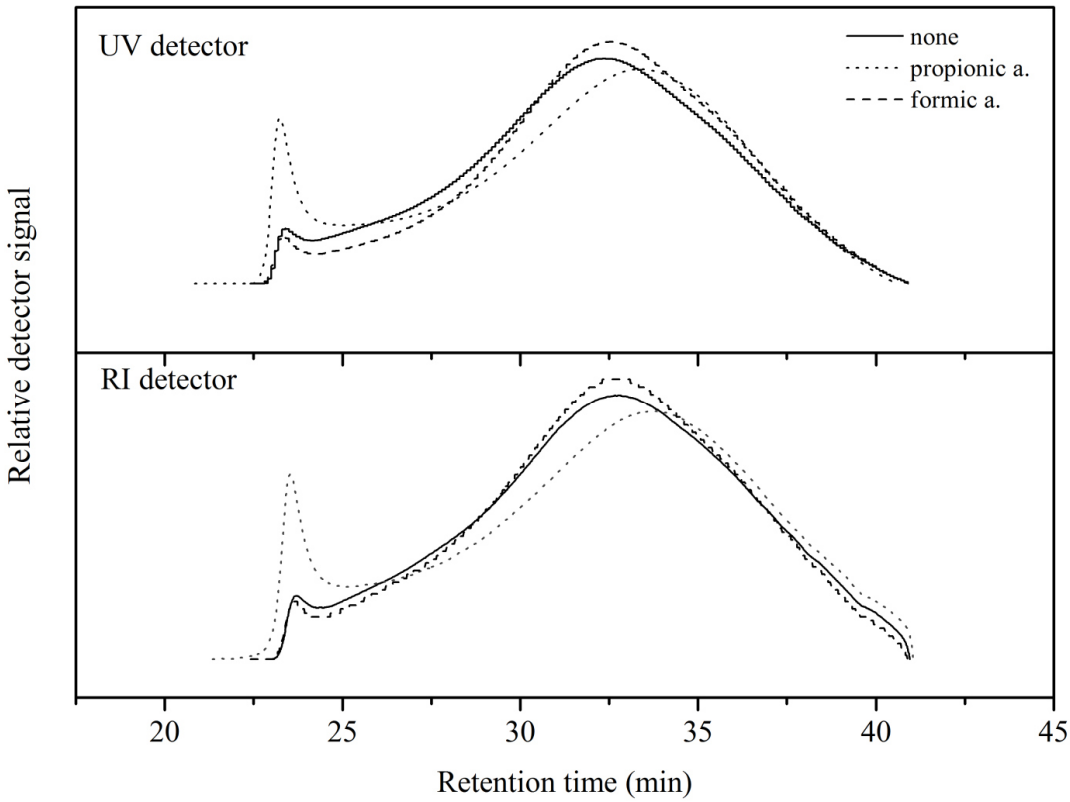

Figure 14 *Comparison of HPSEC chromatograms obtained from UV (280 nm) and RI detectors of not modified HA and HA treated with formic acid and propionic acid.*

Table 6 List of weight-average molecular weight in g mol^{-1} obtained from HPSEC analysis (UV and RI detector) of all studied MHA samples.

Sample	Modifier	M_w (g mol^{-1})	
		UV	RI
MHA0	none	10060	29330
MHA1	formic a.	8373	27780
MHA2	acetic a.	13480	38590
MHA3	propionic a.	14650	38640
MHA4	butyric a.	10110	29520
MHA5	maleic a.	11810	32900
MHA6	benzoic a.	13010	30140
MHA7	fumaric a.	11730	33700
MHA8	oxalic a.	13210	35830
MHA9	phenylac. a.	16940	46900
MHA10	picric a.	10750	31160

Table 7 Molecular size distribution calculated from data detected by UV (280 nm) detector.

Sample	Modifier	Fraction of molecules (%) in the molecular mass interval (kDa) based on VWD-UV (280 nm)					
		0-25	25-50	50-75	75-100	100-150	>150
MHA0	none	40.8	19.5	14.7	14.5	10.6	0
MHA1	formic a.	46.0	16.7	11.9	13.0	12.5	0
MHA2	acetic a.	30.3	16.2	13.8	16.8	23.0	0
MHA3	propionic a.	24.0	13.2	12.9	18.9	31.0	0
MHA4	butyric a.	39.4	17.6	13.2	14.6	15.2	0.1
MHA5	maleic a.	34.1	16.9	13.7	16.3	19.1	0
MHA6	benzoic a.	31.9	16.8	14.0	17.4	20.0	0
MHA7	fumaric a.	35.2	16.8	13.4	16.1	18.5	0.1
MHA8	oxalic a.	30.3	16.0	13.9	17.8	22.1	0
MHA9	phenylac. a.	24.7	14.9	13.4	18.1	28.7	0.3
MHA10	picric a.	38.7	16.6	12.4	14.6	17.7	0

Table 8 Molecular size distribution calculated from data detected by RI detector.

Sample	Modifier	Fraction of molecules (%) in the molecular mass interval (kDa) based on RID					
		0-25	25-50	50-75	75-100	100-150	>150
MHA0	none	21.1	15.1	10.8	8.6	13.7	30.7
MHA1	formic a.	23.3	16.1	10.8	8.4	12.8	28.6
MHA2	acetic a.	15.5	12.0	8.7	7.1	12.1	44.6
MHA3	propionic a.	14.4	9.4	6.9	5.9	10.9	52.5
MHA4	butyric a.	20.4	14.7	10.2	8.1	12.7	33.9
MHA5	maleic a.	18.5	13.5	9.2	7.4	12.0	39.4
MHA6	benzoic a.	18.3	13.5	9.7	8.0	13.0	37.5
MHA7	fumaric a.	18.5	14.0	9.6	7.6	12.3	38.0
MHA8	oxalic a.	16.7	12.1	8.3	6.8	11.7	44.4
MHA9	phenylac. a.	12.4	10.3	7.6	6.4	11.4	51.9
MHA10	picric a.	20.7	14.9	9.5	7.2	11.2	36.5

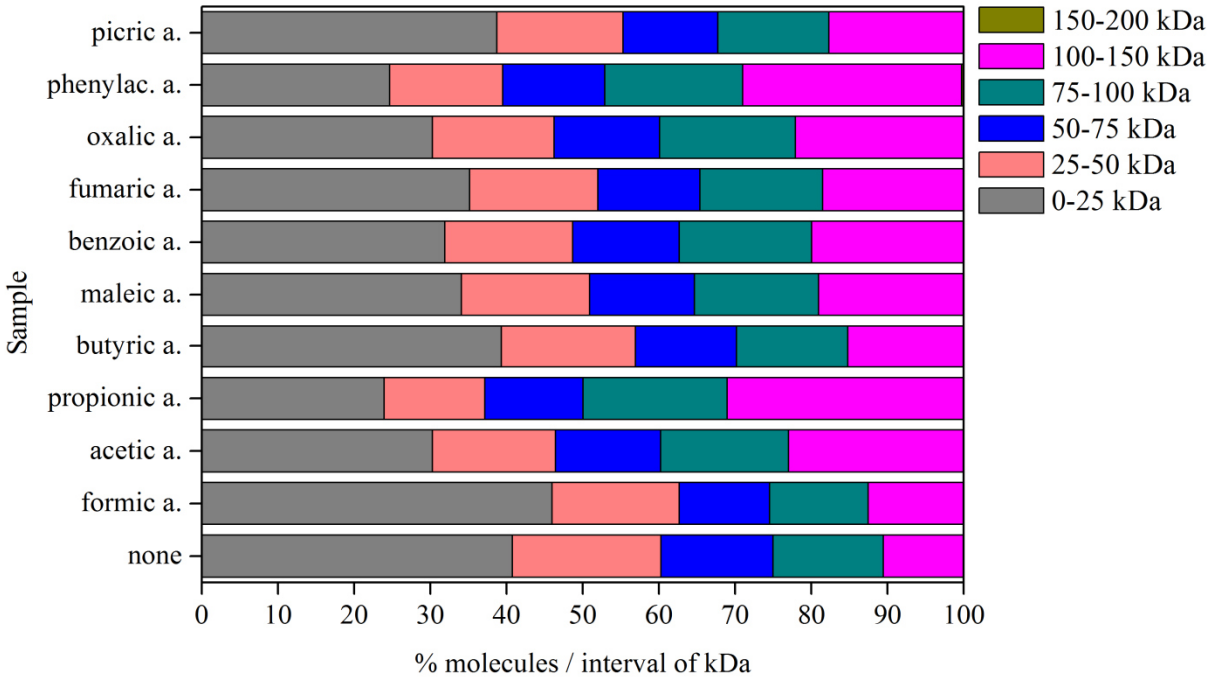

Figure 15 *Molecular size distribution calculated from data detected by UV detector.*

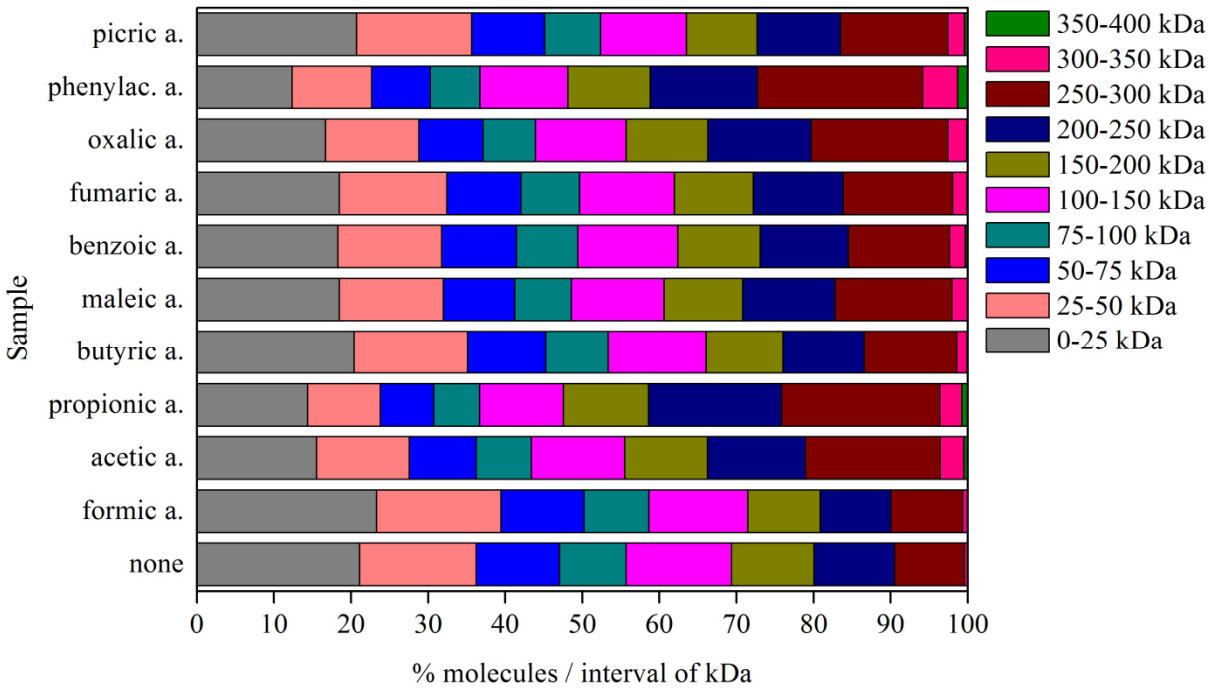

Figure 16 *Molecular size distribution calculated from data detected by RI detector.*

5.2 Fatty acids content

It has already been published by many authors that long-chained fatty acids (FAs) are one of the groups of compounds responsible for water repellency of the soil [133, 170-172]. Based on their natural hydrophobic tail, Holmberg (2001) [173] classified FAs as natural surfactants. In the light of the goal of present work, it was convenient to analyze the content of FAs in individual HA samples, with the main aim, to study the changes in primary structure induced by modification of parental lignite. FAs usually occur in the HS in a "free" form and tightly trapped with the humic acid organic network [147, 174]. The content of FAs in studied HAs samples was analysed by thermochemolysis, method of chemical degradation, using tetramethylammonium hydroxide (TMAH). This method allows both, transesterification of esters and methylation of free carboxylic groups, but does not distinguish between individual forms of FAs occurring in HS [147]. FAs as methyl esters (FAMEs), released after thermochemolysis with TMAH, presented in studied HAs, mostly ranged from the C16 to C32 dominated by the C28 (moltanic acid) and C30 (melissic acid) members, which are the result of the alteration of biopolymers such as suberins or waxes of higher plants that underwent depolymerisation (leading to fatty acids) [175]. Figure 17 shows the distribution of analysed FAMEs in studied samples. The most FAMEs-rich sample was HA treated with propionic acid. This indicates that propionic acid is most effective pre-treatment for extraction of fatty acids, while the other procedures, for unknown reason, decrease the yield significantly.

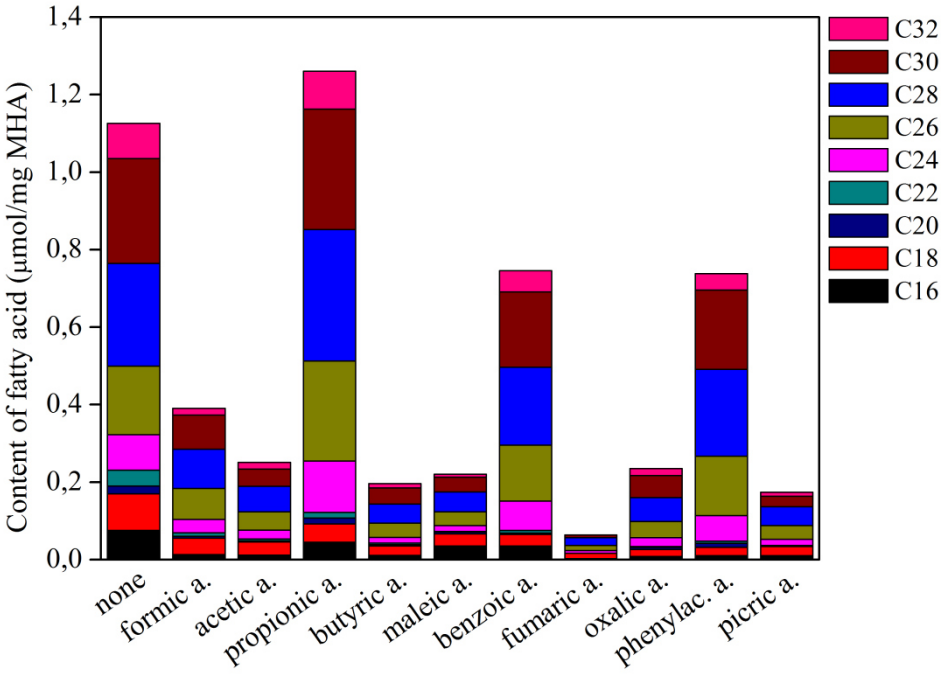

Figure 17 *Content of fatty acids in studied HAs samples.*

5.3 Surface activity

In order to use HAs in environmental applications, such as soil remediation technologies, one essential condition, i.e. surface activity, must be fulfilled. The surface activity of HA have already been studied extensively by many authors [35, 37, 45, 176-179].

Line of eleven HAs produced in this work were analysed for their surface activity, with the aim to disclose the influence of changes induced by individual modifications of parental lignite on their surface activity in aqueous solutions. The surface tension was measured as a function of humate´s water solution concentration and time. In fact, with increasing concentration the surface tension progressively decreased. In contrast to our previous research [44], where one 12 hours long measurement was performed, in this work we measured ST of "fresh surface" produced by an intensive solution shaking and after 24 hours of surface "aging". Figure 18a shows the decreasing tendency of ST at both $t = 0$ h and $t = 24$ h. and Figure 18b shows their differences. The pattern showed in Figures 18 was recorded for all studied samples. This behaviour confirms the above mentioned results from previous studies, that aggregation of HAs molecules takes place from very low concentrations [30]. The most significant difference (12 mN m^{-1}) in ST before and after 24-hours repose was observed in HA treated with butyric acid at concentration 2 g L^{-1}. In overall results and in comparison with not-modified HA, the most significant decrease in ST was observed in following order: butyric acid > picric acid > phenylacetic acid. The lowest decrease was observed in case of propionic acid in all measured concentrations. Generally saying, modifications of raw lignite carried out in this research induced changes in HAs structure, which resulted in an increase of its surface activity in aqueous solutions.

Data obtained from ST measurement were subtracted from the surface tension of the solvent (water) and the results were plotted versus respective concentrations. Plotted data were fitted by Szyszkowski equation [145], which was generally derived for water solutions of fatty acids and aliphatic alcohols. With the presumption, that the main amphiphilic-like molecules occurred in HAs structures are predominantly aliphatic moieties, such as lipids and alcohols, which are very likely to be excluded from the bulk of water solution to the surface, the Szyszkowski equation could be used for fitting. This approach was applied and repeated according to the work of Čtvrtníčková et. al [44]. Obtained parameters from Szyszkowski eq. are listed in Table 9. Parameter *a* reflects the nature of surface active substances and has a constant value for the surface active moieties of one molecular type, homologous series. At the time 0 h the parameter *a* ranged between 4.1 and 14.7 mN m^{-1} (maximal standard deviation was ± 67 %) and after 24 hours of equilibration between 2.5 and 6.3 mN m^{-1} (maximal standard deviation was ± 31 %). While the values at $t = 0$ h were higher and variable, the values after the equilibration showed much more similar values. The studied HAs samples can be divided into four groups according to obtained similar values of parameter *a* relating to more or less the same type of molecules absorbed at the surface. To the first group belongs HAs treated with butyric and maleic acid with the *a*-parameter between 2.5-2.7 mN m^{-1}, to the second group not-treated HA, acetic and benzoic acid, to the third group belongs formic, oxalic and picric acid and the fourth group includes propionic,

fumaric and phenylacetic acid (Table 9). Even if the SD is high (data not shown), the results show a trend indicating existence of specific groups of molecules at the water/air interface. Parameter *b* is different for different molecules and characterizes the efficiency of surface activity of the absorbed molecules to decrease surface tension of water [145, 146]. The variability of *b*-parameter indicated different way of rearrangement, depending on type of modification agent (Table 9). While in comparison with not-modified HA (46.1 $dm^3\ g^{-1}$), five studied modifiers showed higher efficiency of surface activity with calculated values ranging between 48.1 (picric acid) and 2058.8 $dm^3\ g^{-1}$ (butyric acid), other five modifiers showed lower efficiency ranging from the lowest values in order phenylacetic acid < propionic acid < fumaric acid < oxalic acid < formic acid (Table 9). For assessment of the surface saturation, the parameter describing the maximal surface saturation (Γ_{max}) was calculated. After 24 hours of equilibrating, decreasing tendency was observed for all studied samples (Table 9), while the highest value was recorded for phenylacetic and propionic acid.

Table 9 Parameters *a* and *b* and Γ_{max} (maximal surface saturation) obtained from the Szyszkowski equation [145] at *t* = 0h and *t* = 24h.

Sample	Modifier	*t* = 0 h		*t* = 24 h		*t* = 0 h	*t* = 24 h
		a ($mN\ m^{-1}$)	*b* ($dm^3\ g^{-1}$)	*a* ($mN\ m^{-1}$)	*b* ($dm^3\ g^{-1}$)	Γ_{max} ($\times 10^{-3}$ $mol\ m^{-2}$)	
MHA0	none	7.1	1.6	3.9	46.1	1.2	0.7
MHA1	formic a.	8.1	1.2	4.3	35.8	1.4	0.8
MHA2	acetic a.	4.1	16.4	3.7	82.7	0.7	0.6
MHA3	propionic a.	14.7	0.3	5.6	6.4	2.6	1.0
MHA4	butyric a.	9.0	1.0	2.5	2058.8	1.6	0.4
MHA5	maleic a.	10.9	0.5	2.7	300.8	1.9	0.5
MHA6	benzoic a.	11.0	0.6	3.9	70.2	1.9	0.7
MHA7	fumaric a.	11.6	0.5	5.1	18.3	2.0	0.9
MHA8	oxalic a.	10.3	0.4	4.0	32.1	1.8	0.7
MHA9	phenylac. a.	10.1	0.5	6.3	6.3	1.8	1.1
MHA10	picric a.	5.5	3.8	4.5	48.1	1.0	0.8

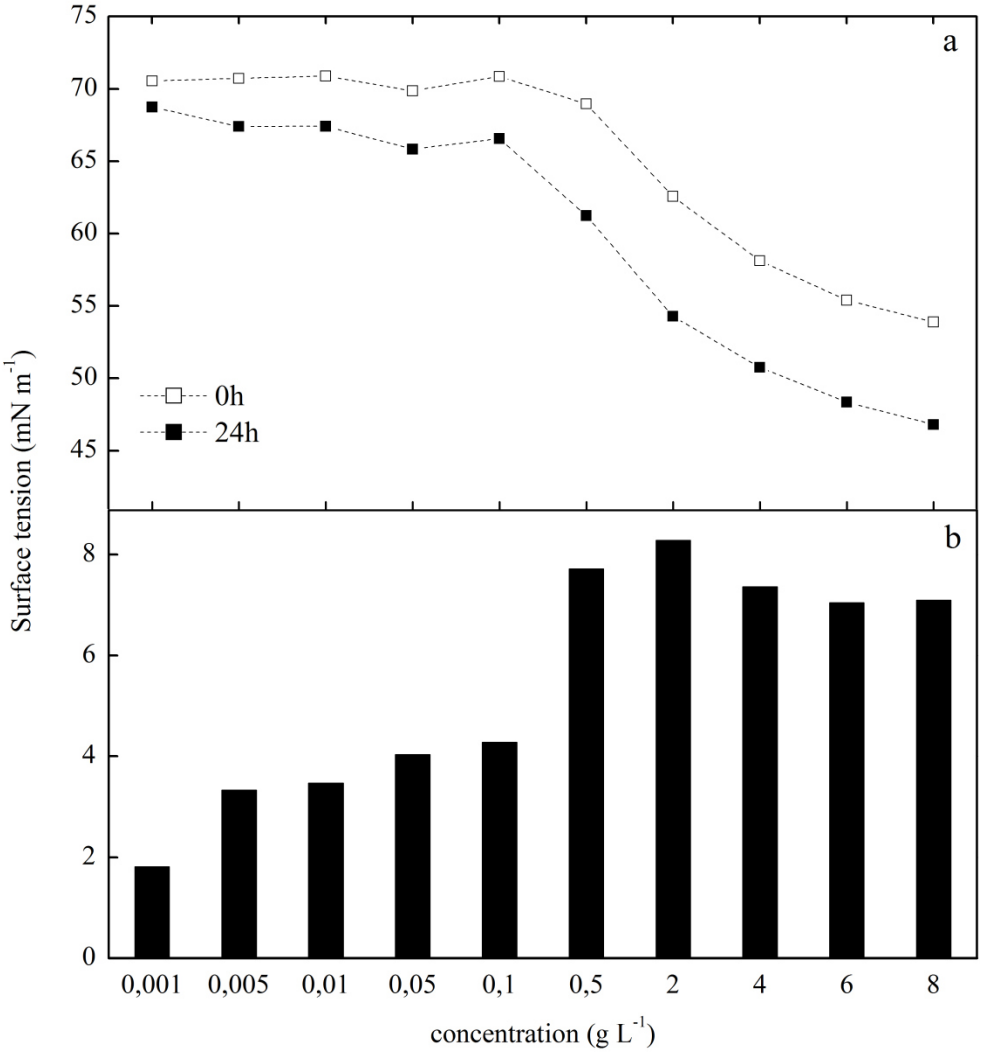

Figure 18 *Surface tension of MHA water solution as a function of concentration at t = 0h and t = 24h (a). The difference between surface tension at t = 24h and at t = 0h (b).*

5.4 Biological activity towards higher plants

The aim of this part was to assess the biological activity of studied humic acid samples. The most important parameter in such investigation was the effect on growth of plants root represented by maize corn.

The assessment of biological activity included measuring of root length increment and weighting of mass increment of maize plants after the plant grow experiment. The roots length and weight of five selected seedlings were measured before and after the growing experiments and the difference was calculated as a root length increment. Since the experiments were conducted twice, this gave the data for ten seedlings, which were averaged and the standard deviations were calculated in MS Excel software. Figure 20 shows average root length increment for selected plants growing in solutions of individual HA samples (results for MHA7 not shown) and for control solution without any addition of humic acid. As a reference were tested commercial humate preparations Lignohumate B and Lignohumate AM and as a control was measured solution of $CaCl_2$. It was evident that addition of humic acids had positive effect on the root growth increment. The most significant increase in root length was obtained for HA treated with oxalic acid, non-treated HA and HA treated with propionic acid. On the contrary, the smallest root length increment was calculated for control solution and for HA treated with butyric acid. To assess the total mass increment during the growing experiment, all 30 sprouts and later the grown seedlings for each sample solution were weighted and subtracted. Figure 21 shows average total mass increment for all maize plants growing in solutions of individual HA samples and for control solution without addition of humic acid. The highest difference was obtained for HA treated with butyric acid and the lowest difference was calculated for HA treated with acetic acid (Figure 21).

The plant root system consists of the primary roots (already assessed by the measuring of length increment) and secondary roots, the lateral roots. The lateral roots extend horizontally from the primary root and, while they spread through rhizosphere, they facilitate the water and nutrient uptake for the growth and development of the plant. Therefore, their assessment was, together with length and weight of roots, one of the further main objectives of this part. For the quick and more accurate assessment of root division of lateral roots of maize plants after plant grow experiment was used HarFa image analyzer software, calculated as a root division parameter. Image analysis represents useful tool in many field of science. The most popular is in biological and environmental science [150, 180]. Selected five roots for each HA-solution were scanned and obtained images (Figure 19) were subjected to thresholding from the gray scale to black and white images. Obtained values after tresholding - fractal measure *K*[BW] - showed the number of pixels on the black and white border what corresponded to the root division. With increasing value of *K*[BW] pixels, the longer is the border between root and background and bigger is the growth increment. Figure 22 shows average root division of five selected maize for each studied HA sample. The highest root division was observed for HA treated with butyric acid. Not-treated HA showed higher value, but with significantly big standard deviation (data not shown).

Next parameter of HAs-biological activity was their effect on the starch and protein content in maize plants after the plant grow experiment. The starch and protein content assessments were evaluated for 30 whole (but dried) plants and the contents are shown in Figure 23. Generally, the content of starch varied between 26.6 and 36.9 % (wt). The most remarkable was lower content of starch in the plant growing in the solution of HA treated with butyric acid. The protein content varied between 10.6 and 12.7 % (wt) with highest content recorded for picric acid. All the results obtained from plant grow experiment are summarized in Table 10 and demonstrate that all studied samples showed biological activity.

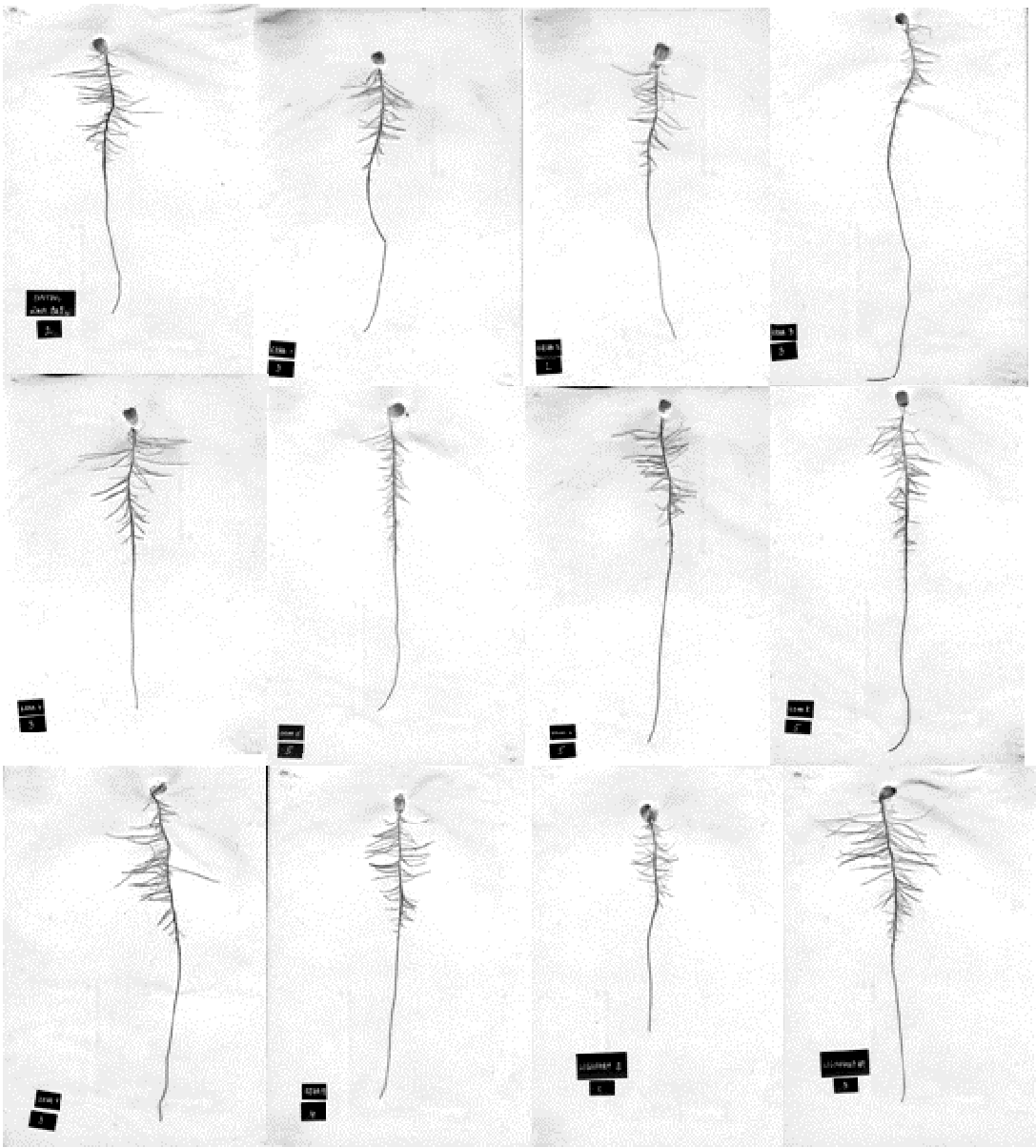

Figure 19 *Diversity of selected root scans of Zea mays grown in MHAs solutions. Pictures are placed upwardly from MHA0 to Lignohumate AM according to Table 10.*

Table 10 Biological activity results from plant grow experiments for studied MHA samples.

Sample	Modifier	Conc.	Root length increment (cm)	Mass increment (g)	Root division K[BW] (pix)	Starch content (%wt)	Protein content (%wt)
control ($CaCl_2$)	-	2 mmol L^{-1}	14	30	34936	37	11
MHA0	none	40 mg L^{-1}	20	34	73317	33	11
MHA1	formic a.	40 mg L^{-1}	17	31	33705	35	11
MHA2	acetic a.	40 mg L^{-1}	18	27	36855	33	11
MHA3	propionic a.	40 mg L^{-1}	19	32	27374	33	11
MHA4	butyric a.	40 mg L^{-1}	16	36	48800	27	12
MHA5	maleic a.	40 mg L^{-1}	17	33	34415	34	11
MHA6	benzoic a.	40 mg L^{-1}	19	32	37065	33	12
MHA8	oxalic a.	40 mg L^{-1}	21	33	28817	34	12
MHA9	phenylac. a.	40 mg L^{-1}	18	29	36070	33	12
MHA10	picric a.	40 mg L^{-1}	16	32	37712	34	13
lignohum B	-	40 mg L^{-1}	16	36	28061	34	12
lignohum AM	-	40 mg L^{-1}	17	28	42962	33	13

Conc. = Concentration

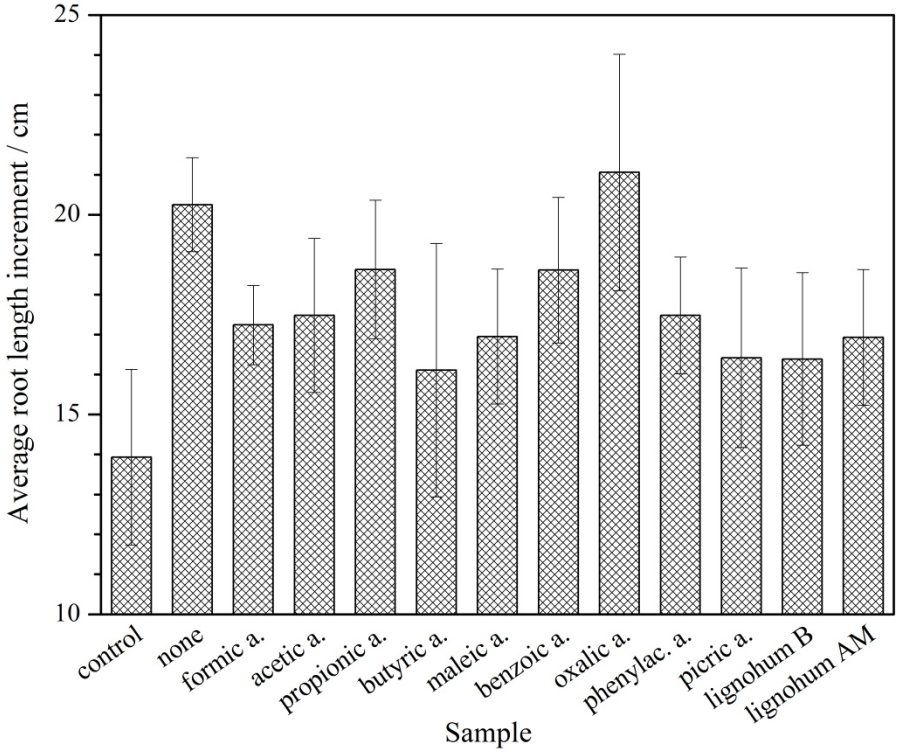

Figure 20 *Averaged root length increment of selected Zea Mays plants.*

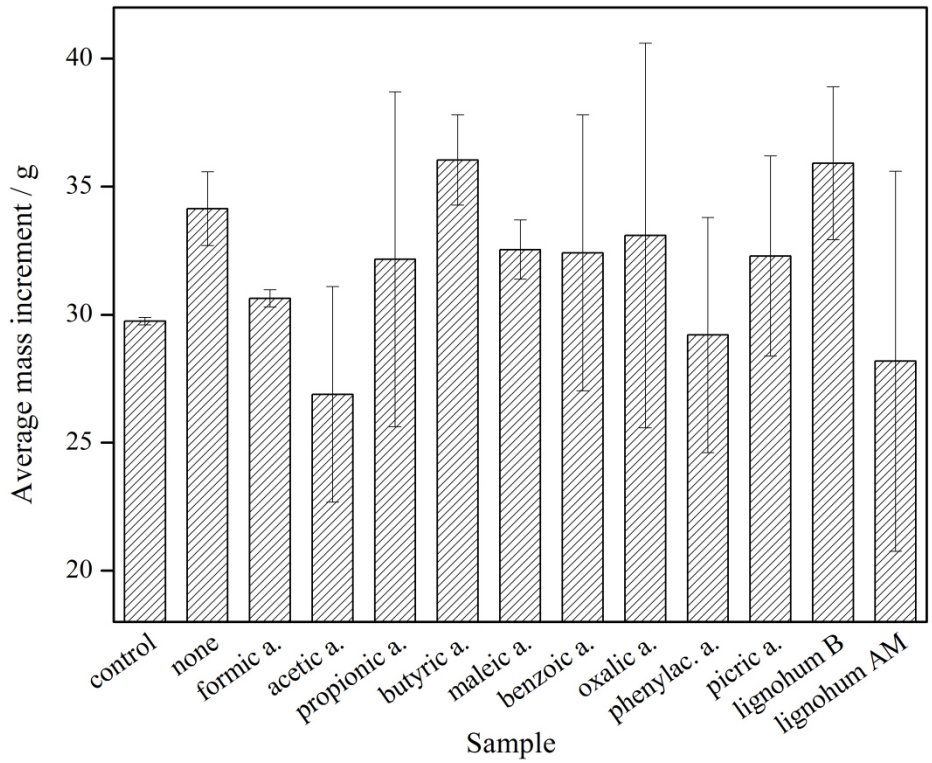

Figure 21 *Average total mass increment of all Zea Mays plants.*

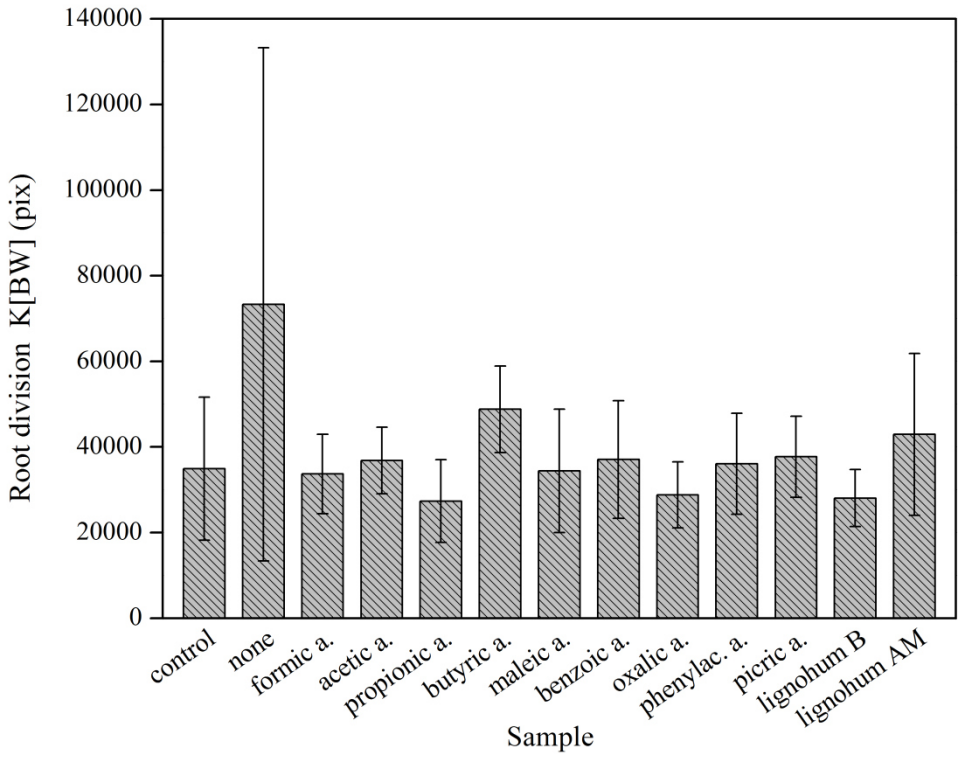

Figure 22 *Average root division of selected Zea Mays plants.*

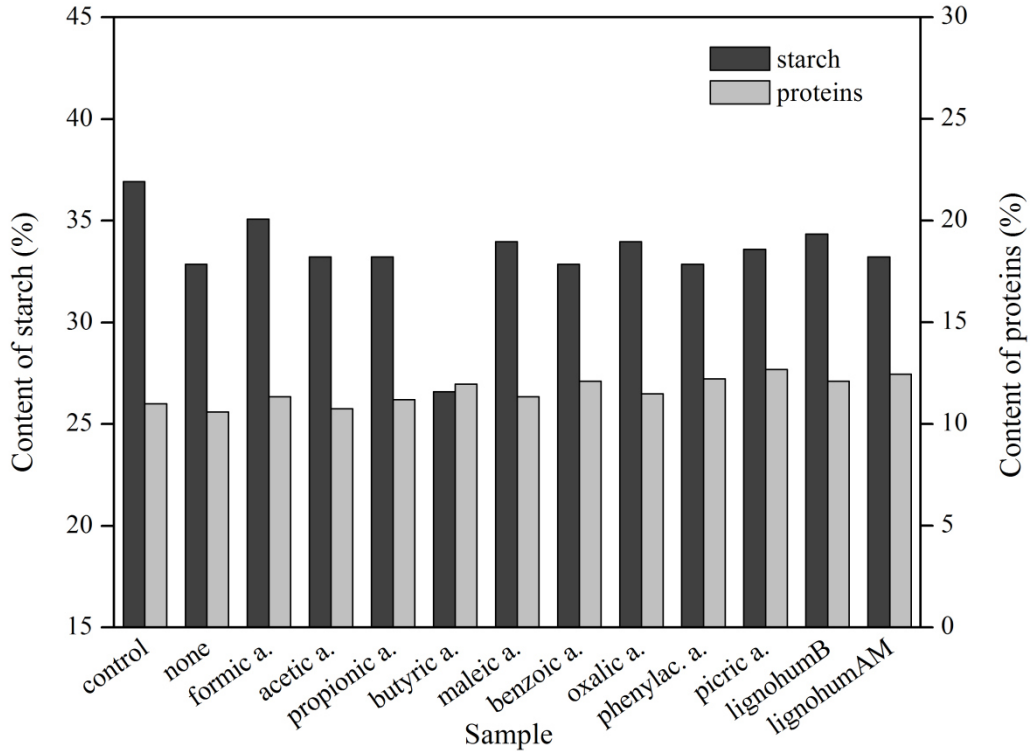

Figure 23 *Starch (dark grey) and protein (light grey) content in wt% analysed in the dry mass of Zea mays plants after the plant grow experiment for studied HA samples.*

5.5 Remediation potential

Currently, application of carbonaceous amendments for soil remediation is very popular and increasingly utilized by the agriculturists for the reconstruction and recovery of soil organic matter. These procedures are applied mainly in areas, where the soils are poorly wettable, contaminated after industry exploitation with e.g. heavy metals or just depleted from frequent cultivation. In all previously mentioned cases, introduction of organic amendment into the soil was applied and had positive effect [128, 181, 182]. However, there is a risk that when extraneous organic matter is incorporated into the soil, it can cause negative effects on soil properties such as e.g. water repellency or soil texture. One of the confirmative examples is uncontrolled disposal of olive mill waste water, when both positive and negative effects on soil quality have been reported [182]. In other study [183], it has been demonstrated that application of various organic amendments such as mulch, manures and composts to saline soils increased water infiltration, water-holding capacity and aggregate stability. Furthermore, the application of organic matter increases soil microbial biomass and some soil enzymatic activities [184]. The aim of this part was to test the use of humic acids as an organic amendment/remediation agent of soil damaged by long-term agricultural activity.

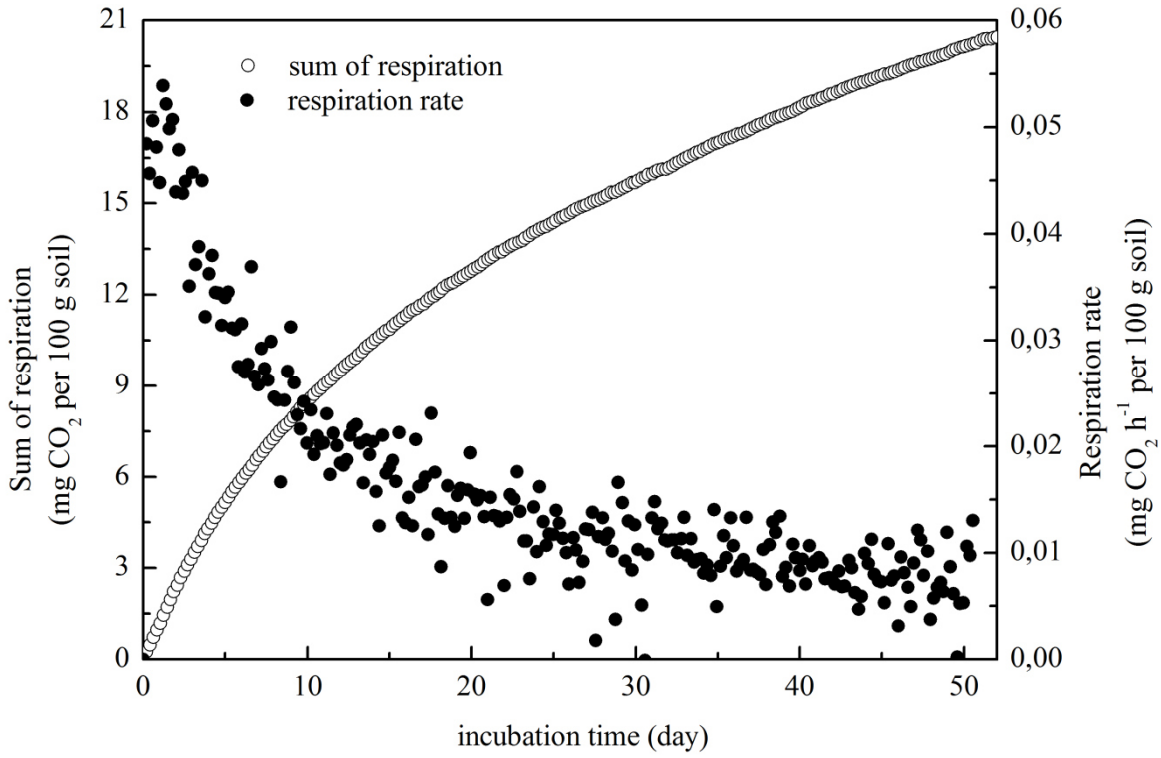

Figure 24 *Soil respiration and its derivative - respiration rate.*

To assess the effect of studied lignite HAs samples as an organic amendment in the soil, the respiration experiment, as a common tool for evaluation of biological processes performing in the soils, was carried out. The biological activity of HAs in soil was assessed by the

measurement of CO_2 evolution. CO_2 is a natural product of activity of soil organisms and thus its evolution is a positive indication of soil (microbial) activity [153, 185]. The laboratory soil respiration experiment performed in this study was carried out under constant optimal moisture condition (76% of pF 1.8) and constant temperature (20 °C) for 50 days. Figure 24 shows the mean value of cumulative evolved CO_2 and the rate of CO_2 evolution from five representative HAs samples (not-treated HA, HA treated with formic acid, acetic acid, propionic acid and phenylacetic acid). As a reference the pure soil without any treatment (Control) and soil treated with common chemical detergents such as SDS and Triton X-100, respectively, were analysed. With increasing incubation time, the respiration rate gradually decreased. The highest respiration rate (0.06 mg CO_2 h^{-1}/ 100 g soil) was recorded at the beginning of the incubation experiment immediately after re-moistening of the air-dried samples. During the first 15 days of the incubation experiment, the tendency of the CO_2 emission rate was rapidly decreasing (Figure 24). The reasons of rapid emission of CO_2 could be attributed to the anomaly called "Birch effect" [186]; when soils become dry, e.g. during summer because of lack of rain and are then rewetted e.g. by precipitation or irrigation, there is a burst of decomposition, mineralization and release of inorganic nitrogen and CO_2 [187], [188],[186]. After the first 15 days, slower and almost constant rate of CO_2 emission was recorded and this tendency persists until the end of the experiment. After 50 days, the emission decreased to 0.01 mg CO_2 h^{-1}/100 g soil. This pattern was observed for all studied samples.

Table 11 shows the amount of released CO_2 after 50 days of respiration experiment for all analysed HAs samples. The greatest release of CO_2 was observed from the Control sample, the pure soil without any treatment (24.0 mg CO_2 /100 g soil), while the lowest release was observed from soil mixed up with not-treated HA (18.4 mg CO_2 /100 g soil). Other significant decrease was observed in case of HA treated with acetic acid (18.3 mg CO_2 /100 g soil) but that could be attributed to present lower C content after the extraction process (see chapter 5.1.1). Generally, all analysed samples showed decreasing tendency of CO_2 emission after 50 days of incubation, compared to pure soil, what could indicate both, nontoxicity of studied HA while in contact with soil and successive stimulation of the soil connected with gradual release of C, respectively.

In order to describe the mineralization of total soil organic carbon during the incubation experiment, data obtained from incubation experiment were fitted by a model two-compartment (double-exponential) function $C_t = C_r \exp(-k_r t_r) + C_s \exp(-k_s t_s)$ [157], where C_t describes the total organic C consisting of two fractions, the rapidly mineralizable C pool (C_r) and the slowly mineralizable C pool (C_s). Table 11 shows the calculations of both organic C pools and their respective time constants (t_r, t_s). [157, 189] After the modification of parental lignite, the content of rapidly mineralizable C decreased, while the content of slowly mineralizable C increased. This was determined for all studied HA samples, except HA treated with propionic acid, which showed contradictory process. These findings were confirmed by additional calculation of C_r / C_s ratio (Table 11). The highest C_r was confirmed for propionic acid (27%) and Control sample (24%), while the lowest C_r was calculated for SDS (16%) and not-treated HA (18%).

Soil incubation experiment carried out in this research agreed with previously mentioned studies [129] that after addition of appropriate soil organic amendment into the soil the content of labile C decreases while the content of stabile C increases. This and previously mentioned behaviour of studied HAs remarkably remains the behaviour of biochar.

As it was already mentioned in previous paragraphs, application of extraneous organic amendments into the soil represents a big risk connected with potentially negative impact on essential properties of the soil, such as e.g. wettability. One of the examples was described in the work of Peikert et al. [182] who studied soils polluted by uncontrolled disposal of olive oil mill wastewater. All polluted soils exhibited stronger water repellency and contained higher amounts of non-aromatic compounds like fatty acids and sugars than their controls [182].

Table 11 Released CO_2 / 100 g soil after 50 days of incubation experiment. Parameters C_r, C_s and t_r, t_s obtained from fitting the model function [157]

Sample	Modifier	mg CO_2 / 100 g after 50 days	C_r (% of C_t)	t_r (days)	C_s (% of C_t)	t_s (days)	C_r / C_s
MHA0	none	18.4	18	5	82	54	0.2
MHA1	formic a.	20.6	19	6	81	53	0.2
MHA2	acetic a.	18.3	20	6	80	61	0.3
MHA3	propionic a.	21.9	27	10	73	78	0.4
MHA9	phenylacetic a.	22.3	19	6	81	54	0.2
SDS	-	19.9	16	7	84	120	0.2
Triton	-	21.9	20	6	80	50	0.3
Control	-	24.0	24	5	76	44	0.3

Table 12 Contact angles of soils measured before and after incubation experiment.

Sample	Modifier	Contact angle (°)			
		concentration 0.1 g L^{-1} (*bellow CMC)		concentration 8 g L^{-1} (*above CMC)	
		before incubation	after incubation	before incubation	after incubation
MHA0	none	49	51	53	51
MHA1	formic a.	47	47	55	47
MHA2	acetic a.	50	45	54	51
MHA3	propionic a.	82	47	69	50
MHA9	phenylacetic a.	49	52	50	49
Control	-	47	48	47	48
SDS*	-	48	50	48	50
Triton*	-	47	51	47	51

When the studied HAs samples were mixed up with soil to perform the soil incubation experiment, the soil water repellency (SWR), as a parameter of soil wettability, was determined. To study the effect of present HA on the soil wettability, the SWR was measured both, before and after the soil incubation experiment. The SWR was determined by measuring of contact angle by Wilhelmy plate method [158-160]. Table 12 summarizes and Figure 25 illustrates the results of contact angles recorded for selected studied HAs samples at two analysed concentrations, 0.1 g L^{-1} and 8 g L^{-1}, respectively. As a control, pure soil without any treatment was investigated. For comparison, common synthetic surfactants, such as SDS and Triton X-100, were analysed at concentrations bellow and above CMC, respectively. Before incubation experiment, with increasing concentration increasing tendency of contact angle were observed for all studied HAs. The extremely high values were found for HA treated with propionic acid, at both concentrations. On the contrary, after the incubation experiment, all studied samples showed stabilized values. In summary, before incubation experiment, only HA treated with propionic acid induced increase in soil water repellency, all other studied samples showed averaged stabilized values, in comparison to pure control soil. After the incubation, no significant variations in the soil-hydrophobocity were observed.

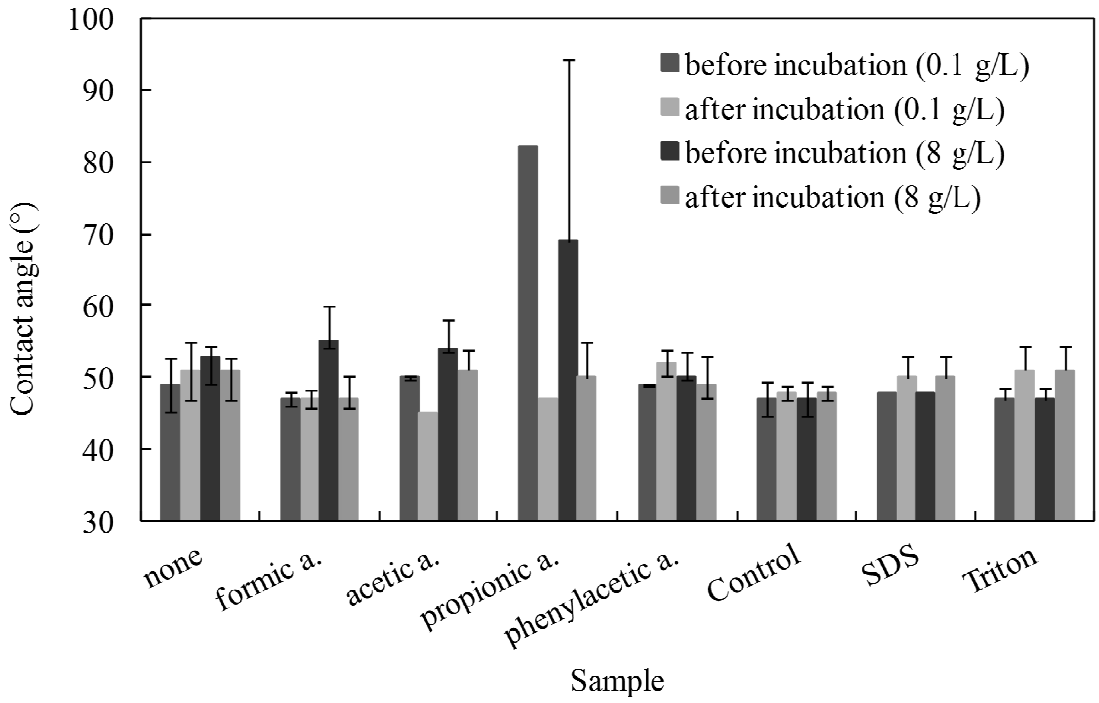

Figure 25 *Contact angles before and after the incubation of the soils treated with selected HAs samples at concentration 0.1 and 8 g L^{-1}, synthetic surfactants (SDS, Triton X-100) bellow and above CMC, and pure soil (Control).*

5.6 Statistical analysis

In this part, the attempt is paid to find the connection between chemical characteristics of studied HAs samples, their physicochemical properties and effects on soils and maize. To study their relationship, the Pearson´s correlation coefficient (*r*) (MS Excell) was applied. The results are summarized in Table 13.

Relationship between primary and secondary structure of studied HAs was studied by the correlation between fatty acids and intervals of molecular size of HAs. The positive linear correlations found between longer-chained fatty acids (C24-C32) and intervals of large molecular size 150-200 kDa ($r = 0.63$) obtained from RI-detector, was in agreement with the work of Conte et al. [140], who demonstrated that the large molecular size fractions of HAs are rich in aliphatic structures containing preferentially longer alkyl chains. No significant correlation found for intervals of small molecular size 25-50 kDa only confirmed later suggestion. The correlation coefficients between all fatty acids and respective intervals of molecular size are shown in Table 13.

Fatty acids are recognized as natural surfactants [173], thus the relationship with the data from ST measurement and parameters *a* and *b* from Szyszkowski equation, are discussed. The correlations between ST and FAs searched after 24 hours, when the system reached equilibrium. Table 13 shows a connection between these two parameters for concentration 0.5 g L^{-1}. In fact, lower measured concentration 0.1 g L^{-1} gave no correlation, while next higher concentration gave still significant, but lower correlation than 0.5 g L^{-1}. This observation underlines the changing character of humic aggregates in interface layer at different concentrations. Furthermore, it roughly correlates with observation of other authors [29, 190, 191] that conformation of dilute humic acids changes at 1 g L^{-1}. Drastik et al. [190] concluded that it is due to changing character of present aggregates, below this concentration they are stabilized by hydrophobic interactions and the aggregates are very hydrophobic, while above, these aggregates are successively incorporated into a hydrogel-like structure formed by aggregating hydrophilic and amphiphilic molecules. This would also explain the difference between individual groups of fatty acids. On the contrary, low correlation coefficients were found between FAs and parameters *a* and *b,* which can be attributed to above mentioned changes in aggregation.

High positive linear correlations ($r = 0.65$-0.79) were found between long-chained FAs and parameter of soil hydrophobicity, the contact angle (CA). This correlation supports the assumption that long-chained fatty acids are responsible for soil water repellency [133, 172]. However, the correlations were found only before incubation experiment (Table 13), which implies some changes in soils during incubation. It implies that soil microbiological activity affected the long-chained FAs and they were relatively quickly transformed by soil microorganisms. We speculate that they were either mineralized or transformed into soil matrix and they were not involved any more in soil hydrophobicity. This hypotheises is confirmed by relatively low correlation coefficients calculated after the incubation experiment (Table 13) and by a decrease in contact angle after incubation. We prefer the transformation way due to a high negative linear correlation ($r = -0.71$) between long-chained FAs and the

rapidly mineralizable C (C_r) (Table 13). In other words, their presence negatively influences the rapid part of respiration represented by amount of mineralized C. The FAs are assumed to be resistant to mineralization for several reasons; they are trapped with the humic acid organic network either by weak, mostly hydrophobic interactions [168] as bound esters [147, 174], or form the stable and biologically resistant crystallites [192]. Neither of these explanations seems to be acceptable, thus we assume that this observation can be connected with interaction of biofilms produced by with fatty acids, while decreasing their hydrophobicity effect. It is also worth to mention that the soil hydrophobicity experiment (CA) was carried out only for five HAs samples, thus obtained correlations had lower significance, but indicate a trend of respective effects.

The relationship between primary structure of HAs and their biological activity was demonstrated by relatively high positive linear correlation ($r = 0.78$) between fatty acids and parameter of biological activity towards higher plants, the root division (K[BW]) of maize (Table 13).

One of the primary hypotheses of this work was that biological activity is connected with surfactant properties of studied HAs, however, no correlation between these two parameters was found.

Table 13 The Pearson´s correlation coefficients between FAs and parameters of primary and secondary structure of studied HAs samples and parameters of soil properties.

	Fatty acids								
	C16	C18	C20	C22	C24	C26	C28	C30	C32
25-50 kDa	0.02	0.19	-0.30	0.12	-0.48	-0.50	-0.48	-0.44	-0.38
150-200 kDa	0.43	0.25	0.52	0.36	0.63	0.61	0.63	0.63	0.63
ST (0.1 g L^{-1})*	-0.15	0.17	0.35	0.27	0.12	0.12	0.08	0.06	0.14
ST (0.5 g L^{-1})*	0.40	0.57	0.74	0.70	0.67	0.67	0.65	0.65	0.64
ST (2 g L^{-1})*	0.48	0.34	0.50	0.37	0.42	0.38	0.36	0.36	0.44
a-parameter	-0.11	-0.07	0.29	0.04	0.46	0.50	0.49	0.45	0.35
b-parameter	0.18	-0.03	-0.19	-0.19	-0.35	-0.35	-0.35	-0.34	-0.32
CA before inc.	0.27	-0.01	0.36	-0.01	0.79	0.77	0.69	0.62	0.65
CA after inc.	0.34	0.24	0.63	0.41	0.31	0.38	0.48	0.54	0.36
C_r	-0.04	0.25	-0.21	0.24	-0.71	-0.71	-0.62	-0.53	-0.50
C_s	0.35	0.61	-0.03	0.53	-0.43	-0.52	-0.46	-0.37	-0.16
K[BW]	0.62	0.75	0.51	0.78	0.13	0.12	0.19	0.25	0.33

*t = 24h

6 CONCLUSION

In this work, a set of ten "modified" humic acids (MHA) was produced using physico-chemical modification of parental lignite. The multiple methods were used to characterize the samples and determine the biological and surface activity of MHAs and its relationship.

The results from elemental analysis and FTIR spectroscopy showed an increase in aromatic moieties after the lignite pre-treatment. The result from HPSEC analysis indicated an increase in weight-average molecular weight (M_w) in all studied samples, only except formic acid. These results were not in agreement with our hypothesis that small organic molecules could cause the rearrangement of larger compartments into the smaller aggregates. This was confirmed only for very small organic acids (i.e. formic acid); the other organic acids acted in a different way. However, even if the smaller aggregates were produced, they could be simply washed out during the extraction procedure of HA. Molecular size distribution showed that in produced MHAs were present mainly the fractions of molecules/aggregates 0-25 kDa and >150 kDa.

Fatty acids presented in studied HAs ranged from the C16 to C32, the dominant fractions were the C28 (moltanic acid) and C30 (melissic acid) members. The distribution of presented FAs varied with different modifiers, while the richest sample was HA treated with propionic acid.

Surface tension measurement showed high surface activity of all studied MHAs samples comparable with chemical surfactants, while the most effective was HA treated with butyric acid. HA treated with propionic acid showed the lowest efficiency.

The results obtained from plant grow experiment demonstrated that all studied MHAs samples showed biological activity and they are able to participate in plant growth processes.

Soil incubation experiment and water repellency experiment proved that the samples can be used for soil remediations with no risk of toxicity or initiation of soil processes leading to an increase in soil hydrophobicity.

The result from statistical analysis showed that the biological activity of HAs was influenced by their physico-chemical properties. However, the surface properties did not show any correlations with physico-chemical properties of studied HAs, which was in conflict with out hypothesis. It was also demonstrated that there was no relationship between biological activity and surface tension of studied HAs.

In overall results and from the point of view of biological and surface activity, the most efficient modifier was 20% formic acid and the less efficient 20% propionic acid.

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8 LIST OF ABBREVIATIONS

BA	biological activity
CA	contact angle
CMC	critical micelle concentration
FA	fatty acid
FAMEs	fatty acids methyl esters
FTIR	Fourier transform infrared (spectroscopy)
HA	humic acid
HPSEC	high performance size-exclusion chromatography
HS	humic substance
HU	humin
MHA	modified humic acid
PAH	polycyclic aromatic hydrocarbons
PCB	polychlorinated biphenyls
PSC	polysaccharides
PSS	polystyrensulphonates
RHA	regenerated humic acid
RID	refractive index detector
SDS	sodium dodecyl sulphate
ST	surface tension
SWR	soil water repellency
TMAH	tetramethylamonium hydroxide
UV	ultraviolet
VIS	visible

9 LIST OF APPENDICES

Appendix I

Čtvrtníčková, A., Drastík, M., David, J., and Kučerík, J., *Surface and solution behavior of surfactants produced from lignite humic acids*. Fresenius Environmental Bulletin, 2011. 20(7a): p. 1764-1771.

Appendix I

SURFACE AND SOLUTION BEHAVIOR OF SURFACTANTS PRODUCED FROM LIGNITE HUMIC ACIDS

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ABSTRACT

Analytical and physical-chemical techniques, such as elemental analysis, FTIR, surface tension (ST) measurement, high performance size exclusion chromatography (HPSEC) and high resolution ultrasonic spectroscopy (HRUS) were combined in order to assess the surface activity of lignite sodium humic acids salts (humates). Humates were obtained from parental and modified South Moravian lignite pretreated either by oxidation or by small organic acids. It was shown that unlike common surfactants, humates dissolved in water do not exhibit the critical micelle concentration and form micelle-like aggregates also in diluted solutions. As revealed by HRUS, the aggregation and/or reconformation of humic aggregates in solution takes up to ten minutes; in contrast, the ST measurements showed long-term stabilization associated with a slow decrease in ST of the solution. Correlation with results of HPSEC indicated that the surface activity of lignite humates is caused predominantly by aggregates with apparent molecular weight between 50–75 kDa (detected by refractivity index detector). Therefore, the long-term processes of ST equilibration were attributed to the slow re-orientation of molecules in humate aggregates adsorbed in the air/water interface in which hydrophobic parts of humates were moved towards air and hydrophilic parts towards to the water bulk. The efficacy of prepared surfactants to decrease the surface tension of aqueous solution was compared with activity of common surface active molecules using Szyszkowski equation and it was found out that humic products can compete with some commercially available surfactants used in remediation technologies.

KEYWORDS: Lignite humic acids, natural surfactants, ultrasonic resonator, size exclusion chromatography, surface tension

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1. INTRODUCTION

Industrial accidents as well as anthropogenic activities are the main reasons for soil pollution. As a result the development of soil remediation technologies suitable for the transformation and detoxification of pollutants is of great interest [1]. Most of the soil pollutants are nonaqueous phase liquids (e.g., tetrachlorethene) which are due to their low aqueous solubility and slow rate of dissolution considered as persistent groundwater contaminants [2]. For remediation technologies many synthetic surfactants such as Tween 80, Triton X 100 or sodium dodecyl sulphate are successfully used. These usually belong to the group of petroleum derived chemicals that are proposed to have cleaning or solubilization properties. Because of their synthetic origin, surfactants can be resistant to biodegradation, persist in the environment, and affect biota [3]. Remediation technologies which use so called natural or bio-based surfactants have been subjected to a growing interest as well. In this case, applied surfactants originate from natural sources, e.g., fatty acid esters of sugars and fatty acid esters or amides of amino acids [4].

Humic acids (HA), main organic constituents of soils, natural waters organic matter and low rank coals have been recognized as natural surfactants as well [1, 5, 6]. HA are complicated mixtures of organic molecules originating from death plant tissues and animal bodies. In the chemical structure of HA hydrophilic segments composed of ionic and/or nonionic groups as well as hydrophobic aliphatic and aromatic moieties can be identified. In this way they partially resemble classical amphiphilic surfactants. The potential of HA to solubilize some organic compounds has already been demonstrated [7, 8].

One of the most important parameters for pollutant solubilization is the critical micelle concentration of the particular remediation surfactant (CMC). In the case of common surfactants, under CMC they exist as monomers which are partly adsorbed at the interfaces (e.g., water/air) causing a decrease of the surface tension of the solution [9]. At higher concentrations surfactant monomers associate into the aggregates called micelles. The concentration at which the micelle formation occurs is defined as CMC. In the past, the estimated value of CMC of concentrated HA solutions has been reported as high as 10 g L^{-1} [10].

In contrast, other experiments proved the presence of pre-micelle organizations in highly diluted HA solution which questions the existence of "true" CMC and the nature of humic micelle-like structures occurring in solutions [11, 12]. Recently, using the high resolution ultrasonic spectroscopy (HRUS) the nature of humic assemblies was described as planar structures stabilized predominantly by hydrophobic interactions at low concentrations and by H-bonds at higher concentrations [13]. Humic substances represent a complicated mixture, thus, in order to develop most efficient humic acid based natural surfactant, it is important to combine and compare data obtained from different techniques in order to understand the character of behavior of humic substances in aqueous solutions and their surfaces. For this purpose, in this work several salts of lignite humic acids (humates) were prepared. The first part of the samples consists of humic acids extracted from previously oxidized lignites. In this way, the treatment is relatively strong, structure of lignite is irreversibly changed and humic substances with higher number of polar groups can be prepared. In contrast, the second part of the humic sample set is prepared by a relatively non-invasive treatment which is supposed to reversibly change the physical structure of humic acids. As a result humic acids with altered physical structure can be produced. At last, humic acids from non-treated "parental" lignite are investigated.

The objective of this work is to investigate the relationship between surface activity of prepared salts of lignite humic acids (humates), their solution behavior and chemical composition. For this purpose, the modification of lignite prior to the extraction results in the production of materials with a wider range of chemical composition and properties as well as different influence on the surface tension in aqueous solutions.

2. MATERIALS AND METHODS

2.1. Sample preparation

Seven samples of modified "regenerated" humic acids (RHA), i.e. RHA1–RHA7 and one reference "not regenerated" HA were extracted from the lignite mined in "Mír" mine in the area of Mikulčice located nearby Hodonín (Czech Republic). 7 fractions of lignite, previously sieved at 0.2–0.3 mm were soaked with two oxidizers (HNO_3, H_2O_2) and two "amphiphilic-like" agents (acetic and citric acids, respectively), followed by the alkali extraction. The process of lignite modification was carried out according to the following procedure: 20 g of raw lignite was placed into a beaker, 200 mL of an appropriate modifier was added and the suspension was stirred for 30 minutes at laboratory temperature. Further, the pre-treated lignite was thoroughly washed with distilled water until free from modifiers. Pre-treated lignite as well as the non-pre-treated part were mixed with 400 mL of extraction agent consisting of 0.5 M NaOH and 0.1 M $Na_4P_2O_7$ (1:10). The extraction was carried out under gentle heating (≈40°C) and stirring for 60 minutes. After centrifugation (15 min, 4000 rpm), the supernatant was treated with concentrated HCl to reach pH 1–2 in order to precipitate the RHA. Then approximately 500 mL of 8 % (v/v) HF was added and shaken overnight in a plastic vessel to remove residual ashes. After that the samples were centrifuged, rinsed with distilled water, centrifuged again and then dialyzed (Spectra/Por® dialysis tubes, 1000 Da cut-off) against distilled water until chloride-free (five to seven days). The final process was freeze-drying (Labconco FreeZone) of dialyzed humic products. The list of samples is given in Table 1. Obtained RHA were then divided into two parts: one part was titrated by 0.1 M NaOH to produce water-soluble sodium humates (NaRHA) and the second part remained in the protonated form. NaRHA samples were then used for characterization by high performance size exclusion chromatography (HPSEC), surface tension (ST) and high resolution ultrasonic spectroscopy (HRUS). The protonated forms of RHA and HA were used for Elemental Analysis (EA) and FTIR spectroscopy. For EA the PE 2400 CHNS/O Elemental Analyzer performed by Strojírenský zkušební ústav, s. p., Brno, Czech Republic was used (Table 1).

2.2. FTIR spectroscopy

In order to evaluate humic acids molecular changes induced by the modification of parental lignite, FTIR spectroscopy was used (Nicolet Impact 400). Conventional KBr pellet technique was applied. From 0.5 to 1 mg of previously dried (110 °C for 4 hours), cooled and in desiccator stored humic samples were mixed in agate mortar with 200 mg of KBr. Obtained powder was squeezed to form a pellet and put into the spectrometer to be analyzed. Number of scans of FTIR analysis was 256, resolution 4.

2.3. HPSEC analysis

The Agilent system was used for HPSEC analysis. Two detectors in series were used: UV detector at 280 nm (calibrated by sodium polystyrensulphonates (PSS)) and refractive index detector (RI, calibration with polysaccharides (PSC)). An automatic injector, with a 100 μL sample loop, was used to load HPSEC solutions. A Phenomenex Biosep S2000 (600 x 7.5 mm) column was used for the size exclusion separations. The column was preceded by a guard column and by a 0.2 μm stainless-steel inlet filter. Flow rate was set 0.6 mL min^{-1}, the HPSEC eluent was a 50 mM $NaH_2PO_4 \cdot H_2O$ solution adjusted to pH 7 with 1 M NaOH. The salt concentration was chosen to have a constant ionic strength of 50 mM in order to minimize ionic exclusion or hydrophobic interactions with the column [14]. Standards of known molecular weight, such as PSC of 186, 100, 23.7 and 12.2 kDa (Polymer Sciences Laboratories, UK) and PSS of 169, 123, 30.9 and 6.78 kDa (Polymer Standard Service, Germany) were used for the column calibration. NaRHA samples were dissolved in the HPSEC eluent to achieve concentration 0.6 mg mL^{-1}, filtered through quartz filters (Glass Microfibre Filterm Whatman International, LTD) and subjected to HPSEC

analysis. Weight-averaged molecular weight (M_w) was calculated using following equation (PE-TC-SEC 4.01 software)

$$M_W = \frac{\sum_{i=1}^{N}\left(h_i {M_i}^2\right)}{\sum_{i=1}^{N} h_i M_i}, \quad (1)$$

where M_i and h_i are molecular weight and the height of each $i^{\text{-th}}$ fraction in chromatogram, respectively.

2.4. Surface tension

Surface tension (ST) measurements were conducted employing Sigma 700 tensiometer (KSV Instruments Ltd.) using a 19-mm-diameter platinum-iridium (Pt-Ir) ring. Thirteen samples with concentrations from 0.001 to 10 g L^{-1} (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2, 3, 4, 5, 8, 10 g L^{-1}) of individual NaRHA were prepared by diluting humic solutions one day before the measurement. Each solution was placed in a shallow glass measuring dish and carefully stirred for 5 min. Then, stirring was switched off and after 10 min the surface tension was measured repetitive by Pt-Ir ring. The total time of measurement was 12 hours. All measurements were carried out at the temperature of 25±2 °C. Obtained data were fitted by Szyszkowski equation (Eq. 2) [15, 16] using Origin 7.5

$$\gamma_0 - \gamma = a \log(1 + bc), \quad (2)$$

where a and b are empirical parameters of Szyszkowski equations depending on the structure of dissolved matter; parameter a reflects the nature of surface active substances and has a constant value for the surface active moieties of one molecular type, parameter b is different for different molecules and characterizes the efficiency of the absorbed molecules to decrease surface tension and also surface activity. γ_0 is the surface tension of the solvent (water at 25 °C: γ_0 = 72.1 mN m^{-1}), γ is the surface tension of the solution and c is the concentration of the solute.

2.5. HRUS analysis

For monitoring of ultrasonic velocity, HRUS 102 device (Ultrasonic-Scientific, Dublin, Ireland) was employed. It consists of two independent quartz cells tempered by a water bath; cell 1 serves as a sample cell and cell 2 as a reference. All experiments were carried out at 25.00±0.02 °C, under constant stirring (600 rpm) and at eight different initial ultrasound frequencies (2370, 5108, 5478, 7850, 8219, 11950, 12196 and 14692 kHz). For the measurement of ultrasonic velocity, the resolution of the spectrometer was set down to 10^{-5} %. Sodium humates samples were dissolved in deionized water in order to prepare the stock solution 10 g L^{-1}. 10 µL of the NaRHA solution was added stepwise every 10 min into cell 1 (i.e., when constant values were achieved); cell 2 was treated in the same way with the same amount of water to control the influence of temperature fluctuation on ultrasonic velocity. The concentration increment in the ultrasonic velocity A was calculated according to the equation published in [17]:

$$A \cong \left(U - U_0\right) / \left(U_0 m \rho_0\right), \quad (3)$$

where U and U_0 are the values of ultrasonic velocity in solution and solvent, respectively, m is the weight concentration of the solute and ρ_0 is the density of the solvent.

2.6. Statistical analysis

For correlation of the values obtained in this study the linear regression using the least squares method was used (Microsoft Excel).

3. RESULTS AND DISCUSSION

3.1. Molecular structure of humic acids

The molecular characteristics of the obtained samples such as elemental analysis, selected parameters obtained from FTIR measurements, parameters a and b obtained from Szyszkowski equation and M_w obtained from HPSEC are reported in Table 1. First, the differences in the chemical composition of the individual samples were indicated by their elemental analysis. Content of C, H, N and O in all samples varied within 41.5-45.4 %, 32.2-38.2 %, 0.92-2.13 % and 19.3-21.0 % (atomic), respectively. The most remarkable is the variation of N content, namely in the case of RHA3 treated by 20% nitric acid. The larger amount can be attributed to the N-substitution or the processes of nitration occurred during the modification with nitric acid. In contrast, RHA1 which was also prepared by modification with nitric acid showed a smaller amount of N in comparison with other samples. It seems that 5% nitric acid is strong enough to oxidize the parental lignite but too weak to introduce a significant amount of nitrogen into the extractable humic molecular structure. The C/H ratio for all samples indicates that during the modification of parental lignite preferentially aliphatic moieties have been oxidized which is in accordance with previous observations [18]. In fact, aromaticity of the samples decreases with increasing concentration of individual modifiers. The lowest value of C/O ratio among all of the samples showed RHA3 treated with 20% nitric acid which indicates larger content of oxygenous functional groups in the molecular structure in comparison with other samples. It is probably the consequence of the strongest oxidative attack of the parental lignite followed by the formation of predominantly phenolic OH groups [19]. On the other hand, other samples showed practically the same value which was already explained in ref. [18] – the oxidation changed the molecular feature of humic acids and simultaneously the yield of extractable humic matter increased. Therefore, the averaged distribution of oxygen within molecules of regenerated humic acids was more or less constant but a larger amount of humic matter was obtained.

FTIR spectroscopy data supported the results obtained from elemental analysis. Vibration spectra of all studied

samples (RHAs) were similar to the spectra of humic acids published and described by many authors and the results seem to be almost identical. A typical record of HA extracted from non-treated lignite is reported in Figure 1.

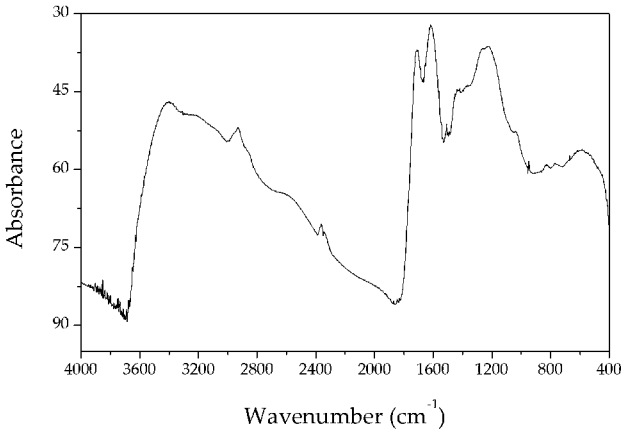

FIGURE 1 - FTIR record of parental humic acids.

The interpretation of FTIR spectra of HA can be found elsewhere (e.g. [18, 19]). Briefly, the first broad peaks were observed in the range 3450–3300 cm^{-1} (hydrogen bonded –OH) and in region 2950–2900 cm^{-1} (aliphatic C–H stretching). Sharp peaks can be seen around 1710 cm^{-1} (C=O of COOH), another in region 1640–1600 cm^{-1} (C=O stretching of COO–, ketonic C=O and aromatic C=C conjugated with COO–) and other at around 1280–1180 cm^{-1} (aromatic C, C–O stretch). To distinguish the differences between HA and RHA samples the ratio of relative intensities of specific absorption bands 2930/1514 and 1514/1400 was used. The first ratio (2930/1514) describes the relations between aliphatic (C–H stretching vibration) and aromatic (C=C aromatic vibration) character and confirms increase of aromaticity of the samples during the modification of parental lignite. The biggest change was noticed in the case of RHA4 treated by hydrogen peroxide which indicates that during the oxidation of raw lignite the ratio aliphatic/aromatic was shifted towards aromatic structures because the aliphatic ones were chemically affected. It seems that the original aromatic structures were also oxidized but not as extensively as aliphatic ones.

The ratio 1514/1400 describes the correlation of aromatic C=C vs. combination of phenolic O–H deformation vibration and C–O stretching vibrations. In the work of Tandler [22] it was shown the mutual connection between those bands because they represent the main parts of the HA structure. In comparison with non-treated HA, RHA4 and RHA2 have the smallest value of the ratio. This indicates a possible carbon oxidation associated with formation of phenols and/or alcohols.

3.2. Size distribution of humic aggregates

As indicated in the previous paragraphs, the modification changed the chemical structure of individual humic samples. As a result, a change in their physical (supramolecular) structure could be expected. In order to assess the distribution of molecular dimensions of humic aggregates reflecting the ability of humic molecules to form aggregates of variable size, the HPSEC was employed [14]. The chromatograms of humates obtained using two detectors are reported in Figure 2. UV detector showed a record with two peaks eluted at 20 min and 28 min, while RI detector showed a record with an intensive peak eluted at 35 min. In fact, UV detector set up at 280 nm could detect only chromophores and groups absorbing at that particular wavelength which is in contrast to the RI detector which allows monitoring of elution of the whole sample mass. Both M_w values based on the UV and RI detection showed that molecular sizes of humic aggregates during the modification were changed; results are summarized in Table 1. In fact, the results obtained from HPSEC reflect the affinity of molecules to form aggregates stabilized by weak interactions [23]. It has been stated that at pH 7, used in this work, mainly hydrophobic interactions contribute to the stabilization of formed aggregates.

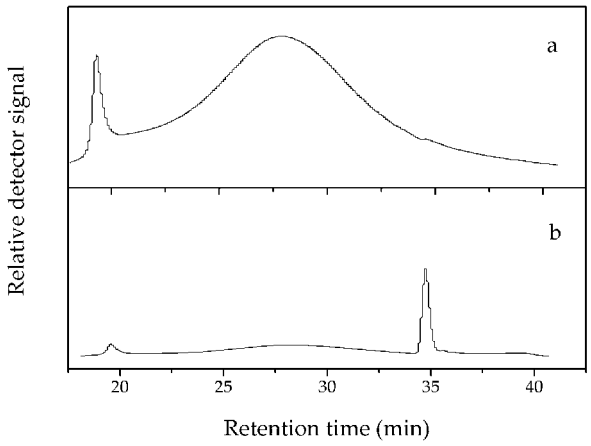

FIGURE 2 - HPSEC records of sample NaRHA3, a) UV detection, b) RI detection.

The averaged molecular sizes of humic substances covered similar ranges, however, significant differences exist in their distributions. The area under the peaks was divided into six intervals of M_w, divided by total peaks area and multiplied by factor 100 to obtain percentage contents of particular molecular fractions (Table 2). In some cases the results obtained by UV and RI detectors differed remarkably. The significant part (23–35 %) of humic samples occurs in the low molecular weight interval 0–25 kDa. The largest fraction was recorded for sample HA which was extracted from non-treated lignite. Samples RHA3 and RHA5 treated with 20% nitric acid and 5% hydrogen peroxide, respectively, gave smaller values. Other four intervals showed similar results except for the interval 100–150 kDa. In this particular interval about 20–40 % of humic molecular fractions were eluted and sample RHA3 showed the largest content in comparison with other measured samples. Sample RHA1, prepared by the treatment of parental lignite by 5% nitric acid rather deviates from this

TABLE 1 - Elemental analysis of HA and RHAs is given in atomic %. The proportion of relative intensity of selected absorption bands. Weight-average molecular weight in g mol^{-1} for UV (280 nm) and RI detector obtained from HPSEC analysis. Parameters *a* and *b* obtained from Szyszkowski equation (Eq. 2).

sample	modifier	Elemental analysis [at. %]						FTIR ratios		M_w (g mol^{-1})		*a*	*b*
		C	H	N	O	C/O	C/H	2930/1514	1514/1422	(UV)	(RI)	(mN m^{-1})	(dm^3 g^{-1})
HA	–	41.6	38.2	0.94	19.3	2.16	1.09	1.08	0.87	9878	6256	3.95	55±8
RHA1	5% HNO_3	45.4	32.2	1.34	21.0	2.16	1.41	1.07	0.84	12180	10483	3.33	136±0
RHA2	10% HNO_3	44.9	32.8	1.53	20.8	2.16	1.37	1.02	0.77	11755	8922	3.40	153±24
RHA3	20% HNO_3	41.5	36.6	2.13	19.8	2.10	1.13	0.95	0.91	14763	17848	3.15	301±41
RHA4	2% H_2O_2	44.5	33.3	1.41	20.8	2.14	1.34	0.83	0.75	13151	14687	3.65	68±10
RHA5	5%H_2O_2	42.5	37.1	0.92	19.5	2.18	1.15	0.96	0.79	16087	16035	3.45	646±168
RHA6	20% acetic acid	44.0	34.1	1.34	20.6	2.14	1.29	1.03	0.91	10225	11110	3.50	111±34
RHA7	20% citric acid	44.1	34.1	1.49	20.3	2.17	1.29	0.94	0.95	10718	13990	3.58	482±173

TABLE 2 - Molecular size distribution calculated from data detected by UV (280 nm) and RI detectors.

sample	fraction of molecules (%) in the molecular mass interval (kDa) based on VWD-UV (280 nm)						fraction of molecules (%) in the molecular mass interval (kDa) based on RID					
	0–25	25–50	50–75	75–100	100–150	150–200	0–25	25–50	50–75	75–100	100–150	150–200
HA	35.3	15.2	12.2	15.6	21.7	0.04	38.4	14.4	7.8	5.2	13.5	20.7
RHA1	28.3	14.6	13.1	18.0	26.0	0.05	26.8	9.1	6.7	7.9	41.2	8.2
RHA2	29.5	14.6	12.9	17.2	25.7	0.07	27.7	11.3	7.6	6.7	18.8	27.9
RHA3	24.2	12.0	10.1	12.8	36.8	3.39	18.3	7.8	5.7	5.0	13.7	39.7
RHA4	27.7	14.5	12.6	16.0	28.9	0.28	22.0	13.1	9.9	8.4	18.4	26.7
RHA5	23.2	13.6	12.1	16.3	33.9	0.97	19.0	10.6	8.2	7.5	18.5	34.3
RHA6	34.1	14.9	12.2	15.8	23.0	0.05	29.2	13.1	8.9	7.5	17.6	23.3
RHA7	32.5	15.1	12.6	15.9	23.8	0.04	24.6	12.8	9.5	8.4	19.3	24.5

range since the number of the molecules occurring in this interval is only about 8 %.

3.3. High Resolution Ultrasonic Spectroscopy

For monitoring of the aggregation of humates in the bulk solution, the HRUS measurement was employed in the similar way as reported by Kučerík et al. [24]. Generally, US velocity in HA solutions is influenced by two main factors. The first factor is the number of water molecules presented in the hydration shell which increases the US velocity. In this case, the hydration shell represents less compressible, rigid and denser fraction (layer) of water molecules in comparison with the bulk water molecules and thus provides better conditions for ultrasonic wave propagation. The second factor which can play an inverse role in ultrasonic wave propagation is the formation of compressible aggregates possessing hydrophobic interiors. With this respect, the energy of the wave is partially used for the compression of the micelle-like aggregates (drop in amplitude of the wave) because the hydrophobic core is relatively soft. As a result, the velocity of the wave was decelerated.

As already demonstrated by Buckin et al. [25], in case of common surfactants, titration of a highly concentrated solution of surfactant into water resulted in a steep increase in ultrasonic velocity as a consequence of increasing number of hydrated molecules in solution. Prior to the reaching of the CMC, the value of concentration increment in US velocity (*A*) stays practically constant which demonstrates the absence of interactions between molecules. As soon as the CMC is reached the concentration increment dramatically decreases due to the effect of micelle compression [25]. When the increment of ultrasonic velocity *A* is calculated and plotted against concentration, the concentration under CMC is characterized by a constant value of *A*, while the appearance of CMC is indicated by its abrupt decrease [24].

Humate solutions behave in a different way. Figure 3a shows that with increasing time of titration, i.e. with increasing concentration, the ultrasonic velocity increases. The small frame shows that after the addition of the stock solution the ultrasonic velocity reaches constant value within 10 minutes and next addition can be done. This is partially caused by experimental arrangement but mostly by humic molecules itself (it was also proved by experiments in which the stirring was switched off after the stock solution addition followed by a quick and careful mixing). The respective increment of ultrasonic velocity calculated according to the Equation 3 is reported in Figure 3b. All the determined and calculated dependences of increment were similar for all humates which indicates that the progressive formation of aggregates occurred at low concentration in all samples.

As described in the Materials and Methods part, humic samples were measured at eight different ultrasound frequencies in range from 2 to 14 MHz. Figure 3b shows that three different frequencies provided identical results. If the formation of micelle-like aggregate occurs (soft hydrophobic core), the ultrasonic velocity would depend on the applied frequency. Simply, the relaxation time of mi-

celles at a certain (high) frequency would be smaller than the time proportional to the ultrasonic (mechanical) wave frequency applied. As a result micelles would become less compressible, i.e. more elastic, and therefore higher values of U_{12} would be recorded. Nevertheless, this behavior was not observed which confirms the fact that the humic aggregates formed at concentration used in this paper do not form "classical" micelle-like organizations. Instead, it was suggested that in diluted solutions they form planar open structures [13].

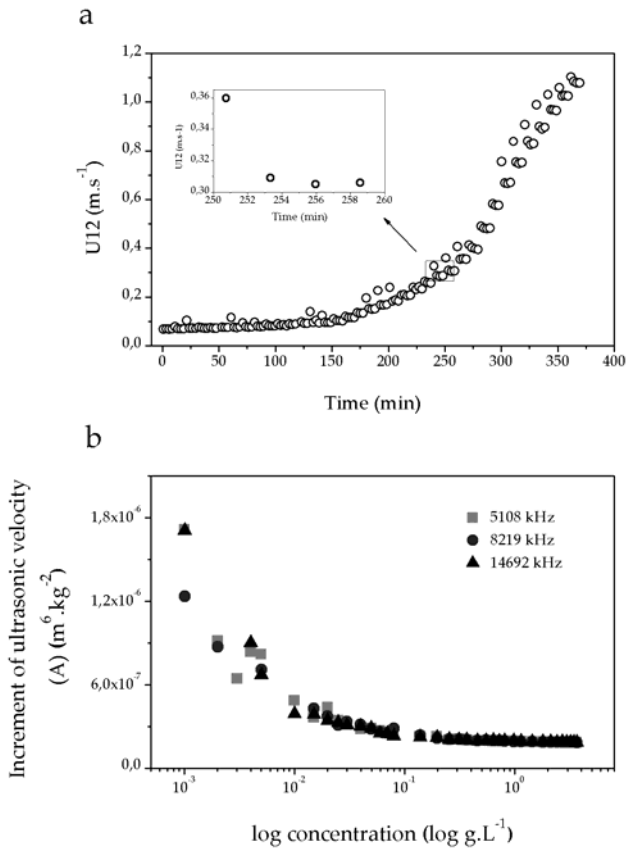

FIGURE 3 - (a) Increase of ultrasonic velocity as measured by HRUS in differential arrangement (U_1-U_2=U_{12}), stock solution was added every 10 minutes; (b) dependence of increment of ultrasonic velocity on concentration for 3 different frequencies.

3.4. Surface Tension of humate solutions

The surface tension of humic samples was measured as the function of their concentration and time. In fact, with increasing concentration of humic samples the ST progressively decreased and the constant value was reached after approximately 10 hours (Figure 4a). Obtained data of ST were subtracted from the surface tension of the solvent (water), results were plotted versus respective concentrations and fitted by the Szyszkowski equation (Eq. 2). Obtained parameters are listed in Table 1. It can be seen that all samples had a value of parameter *a* between 3 and 4 which means, that all samples included similar type of molecules adsorbed at the surface. The parameter *b,* which reflects the efficiency of surfactant to decrease the surface tension, showed a significantly high value for RHA5 (646 $dm^3\ g^{-1}$) in comparison with lower HA (55 $dm^3\ g^{-1}$). Therefore, it can be seen that the modification of parental lignite led to the production of humic acids with variable surface activity. Comparison of parameters *b* with fatty acids indicate that the efficacy of prepared humic acid to decrease the surface tension is comparable with C_{10} and higher fatty acids homologues (results for fatty acids are not reported).

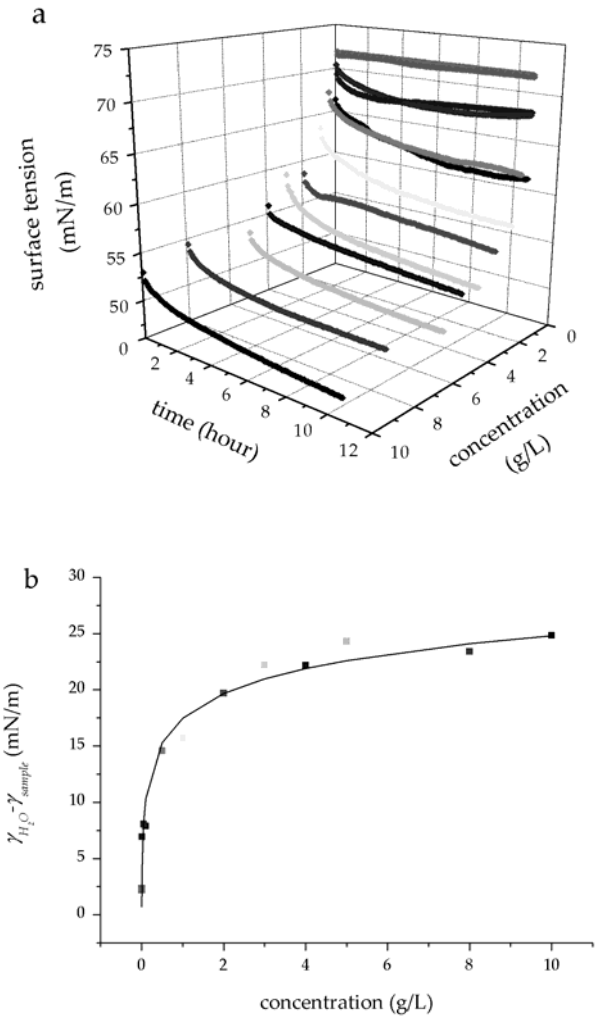

FIGURE 4 - (a) Time and concentration dependency of surface tension for sample NaRHA2; (b) the fitting by Szyszkowski equation (see Equation 2).

3.5. Correlations and consideration

In this work, the attempt was made to find the relationship between results of applied analytical techniques. The linear regression, least square method, was used for this purpose. The only significant linear correlation (intentionally ignoring one point) was found between parameter *a* determined from Szysszkowski equation and the amount of aggregates determined by HPSEC in the interval 25–50 kDa and 50–75 kDa for RI detection (Figure 5). This suggests that unlike in the solutions of common surfactants, the surface activity of humates is caused by relatively large

aggregates. As demonstrated by the experiments, aggregates are adsorbed in the interface layer (air/water) very quickly but their reconformation takes a long time (ST measurement); it is in contrast to their behavior in solution where no significant changes can be observed after several minutes (HRUS measurement).

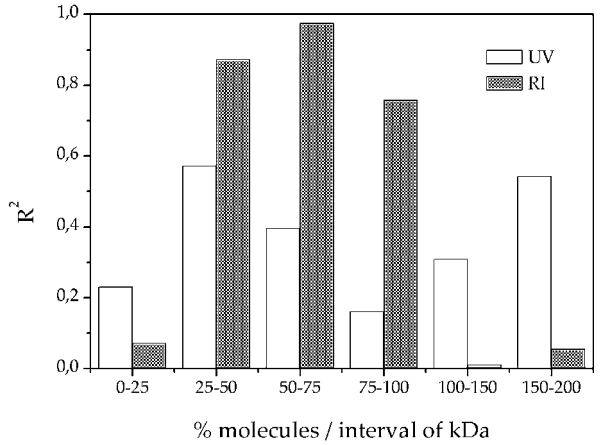

FIGURE 5 - Correlation coefficient for linear relationship between humate aggregates dimension (as detected by RI and UV detector) and parameter *a* from Szyskowski equation (from Equation 2)

The fate of humic aggregates in the interface (surface ageing) is in contrast to the behavior of pure surfactants. Prosser at al. [26] showed that in the case of *n*-dodecanoid acid an induction period occurs; it means that after a time period when no change of surface tension is observed, single molecules tend to form monolayer islands and surface tension starts decreasing. In contrast, humates did not show induction period which can be explained by the fact that when adsorbed in the interface layer, they are not surrounded by water molecules from all sides any longer and the hydrophobic effect is partially diminished. This disruption of semi-equilibrium causes the observed long-term surface tension decrease. Similarly as in living systems, hydrophobic effect plays a crucial factor in those processes. Water molecules surrounding dissolved or dispersed molecules have a strong tendency to form 3D structures stabilized by H-bonds which causes the minimization of the contact surface between water and hydrophobic compounds or their parts. In case of polar compounds the situation is different, since the affinity of polar (soluble) moieties to water is higher than mutual affinity between two water molecules. As a result, separation of hydrophobic molecules from polar water molecules occurs. In humate solutions as soon as the large heterogeneous aggregate is adsorbed in the air/solution interface an immediate tendency occurs to open its structure and to expose the hydrophobic parts towards air while hydrophilic parts tend be oriented to water. That process is kinetically driven and takes several hours as demonstrated in Fig 4a. The notion about adsorption of aggregates in the humate interfaces is of a great importance for understanding of many functions of lignite humates such as for example their biological activity, reactivity on mineral surfaces and transport properties.

4. CONCLUSIONS

In this study, it was demonstrated that lignites represent chemical raw materials useful for production of humic-based surfactants. Efficacy to decrease surface tension of water by prepared humates was demonstrated and it was found out that it is comparable with efficacy of homologues of fatty acids C_{10} and higher. Experiments indicated that the mechanism of humates to decrease the surface tension of water solution does not resemble principle known for classical surfactants. Instead, the presence of pre-micellar aggregates in diluted humate solutions was confirmed which generally indicates the difference in mechanism of solubilization of hydrophobic compounds such as for example pollutants by humate solutions. Surface measurement showed that the surface activity of lignite humates is caused by aggregates of relatively high dimension adsorbed in the air/solution interface and the surface aging takes several hours during which the aggregates slowly reach a stable conformation. It is in contrast to the state of humic aggregates dispersed in solution which can reach stable conformation in the course of several minutes. This indicates also only minimal or none exchange of aggregates from solution with those present in the surface layer. Finally, the nature of aggregates present in solution is different in comparison with classical compressible micelle assemblies containing hydrophophobic cores or bilayers present in biological systems.

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10 SUMMARY OF THE PUBLICATIONS AND ACTIVITIES

Publications

1. Kučerík, J., Čtvrtníčková A. and Siewert C., Practical application of thermogravimetry in soil science. Part 1: Thermal and biological stability of soils from contrasting regions. *Journal of Thermal Analysis and Calorimetry*, 2013. 113(3): p. 1103-1111. ISSN 1388-6150.

2. Čtvrtníčková, A., Drastík, M., David, J. and Kučerík, J., Surface and solution behavior of surfactants produced from lignite humic acids. *Fresenius Environmental Bulletin*, 2011. 20(7a): p. 1764-1771. ISSN 1018-4619.

3. Kučerík, J., Drastík, M., Zmeškal, O., Čtvrtníčková, A., Ultrasonic Spectroscopy and Fractal Analysis in the Study on Progressive Aggregation of Humic Substances in Diluted Solutions. *WSEAS Journal Transactions on Environment and Development*, 2009, 5(11), p. 705-715. ISSN 1790-5079.

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