DEVELOPMENT OF HUMICS-BASED DETOXICANTS OF COMPLEX EFFECT

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Abstract: This research demonstrated development and properties of detoxicants of integrated effect based on humic derivatives. Set of samples of humic-based derivatives including carbonylated, hydrophobizated, oxygenated, cryodestructed and biosolubilized have been synthesized. It has been demonstrated that all the produced detoxicants possessed plant growth promoting activity and detoxifying potential in relation to heavy metals.

Keywords: humic substances, heavy metals, de-toxicants, detoxifying ability

Introduction

Chemical contamination makes significant harm to natural biocenosis, and sometimes causes their whole destruction. So, toxic metals (mercury, cadmium, lead) can be bound with different cell enzymes, disturbing normal functioning of the whole organism. Chlorine-organic hydrocarbons exhibit high mutagenic activity. Poly-nucleic aromatic hydrocarbons possess the expressed cancerous properties. Pesticides are of special danger: along with toxic effect they cause genome and structural mutations. To rehabilitate normal vital activity of biogeocenosis it is necessary to provide detoxification of the contaminated objects, the success of that is mainly dependent on the efficacy of detoxification agents applied. Consequently, an urgent problem is to create ecologically safe detoxification agents of complex effect with high rehabilitation potential in respect to the damaged biogeocenosis.

For the development of de-toxicants with such properties it is rather prospective to use humic substances (HS) and their derivatives. It is so, because humic substances comprise the properties of (1) reclaiming agents – favourably influence on physical and chemical properties of soil by their structuring, increasing moisture capacity, improving gas exchange, etc., (2) sorbents - bind hydrophobic organic compounds by a physical sorption mechanism, and ions of metals – by a ion exchange mechanism, (3) antidotes - enter into chemical reactions with a wide range as well as organic and inorganic compounds. Humic substances are natural organic compounds comprising 50 to 90 % of the organic matter of peat, lignites, sapropels, as well as of the non-living organic matter of soil and water ecosystems [1, 3, 20, 23]. Soil enriched with humic substances can endure significantly higher technogenic loads. Toxic effect of heavy metals and organic compounds decrease in them for biota, the penetration of eco-toxicants into the plants is reducing, the level of ground waters contamination is lower [9, 12, 21]. As a rule, protective effect of humic substances is explained by the formation of non-toxic and inaccessible for live organisms complexes with eco-toxicants [5, 16, 17, 19]. It is approved by data obtained on the reduction of SAH (surface active hydrocarbons) and heavy metal accumulation in water organisms in the presence of humic substances [14, 17, 22, 24]. At the same time it is known, that humic substances can speed up the processes of abiotic and biotic decomposition of eco-toxicants. So, humic substances increase the solubility of high-hydrophobic chlorine-organic pesticides in water [2], speed up photolysis of SAH and catalyze hydrolysis of sym-tryasines [25]. Thanks to redox - mediatory properties, humic substances are able to play a role of terminal acceptor of electrons, speeding up the processes of anaerobic decomposition of organic contamination substances [4, 15]. Adaptogenic activity of humic substances is of special interest, because it is exhibited in the increase of live organism resistance to stress loads, in particular to chemical stress [21].

The properties mentioned above allow considering humic substances as natural ecologically safe detoxicants of complex effect. In order to increase the specificity of humic de-toxicants it is prospective to produce the derivatives on their basis. The goal of this work is to demonstrate a new strategy whereby the directed design of humic detoxicants is used to bring about a desired remedial action. To reach this goal, the following objectives were formulated: (1) to produce the humic derivatives using the methods of fractionation, chemical and microbiological modification and complex-formation; (2) to characterize the structure and physical-chemical properties of the produced humic derivatives; (3) to evaluate biological activity of the obtained humic derivatives; (4) to evaluate detoxifying properties of humic derivatives with respect to primary ecotoxicants using laboratory express-tests and vegetation experiments.

The scope of work includes: isolation of the native humic materials from main types of raw materials and synthesis of their derivatives (more than 20 samples as a total) under laboratory conditions; characterization of the structure of the natural and modified humic materials using elemental analysis, functional group analysis, 1H-, 13C NMR-, FTIR-, Mössbauer spectroscopy; X-ray diffraction SEM and TEM analysis, evaluation of the biological activity of the produced natural and modified humic materials with respect to higher plants; and performance assessment of the detoxifying ability of the produced humic derivatives with respect to heavy metals under laboratory conditions.

Materials used and methods

Protocols of native humic substances isolation

Coal humic acids (HA) were isolated from two Kyrgyz oxidized brown coal deposits (Kara-Keche and Kyzyl-Kiya). To obtain HA samples, the potassium humates were dissolved in distilled water, centrifuged from the mineral components, and acidified until pH 1. The dark precipitate was washed out with distilled water, dialyzed against distilled water using dialysis and dried in vacuo at 60º.

Protocol for carbonylation of humic acids (CarbHP)

Carbonylated humic derivatives (CarbHP) were obtained by Hatterman-Koch reaction in the medium of the sulphuric acid. A weight of the HA (10 g) was placed in the three-neck flask equipped with mixer and reverse fridge. 50 mL of the mixture of $AlCl_3$ and CuCl as catalysts were added in the reaction system at vigorous stirring. 50 mL of concentrated H_2SO_4 was dropped in the reaction system. Gaseous CO and HCl were passed through reaction mixture. The retort was deeply lowered in the bath, filled by ice in the first hours of the process at 5°C, and then after 5 hours at 20°C. Total duration of the reaction is 12 hours. After the completion of the reaction the products mixture was diluted by water in the ratio 1:2, sediment was separated by the centrifugation, washed out from ions SO_4^{2-} and Cl^- and dried out.

Protocol for oxygenating of humic acids (Oxy-HP)

10 g of HA was suspended in 500 mL of 3.5% $KMnO_4$ in 1% KOH. The reaction mixture was placed in the three-neck flask equipped with the mixer and reflux condenser. Oxidation was carried out at 50°C in the water bath within 15 min and then was kept at 22°C up to a brown colour. Liquid product was separated from MnO_2 by filtration. Oxy-HP was extracted with methylethylketone (MEK). Filtrate was saturated by Na_2SO_4. Extract was washed out from MEK and dried.

Protocol for hydrophobization of humic acids (HymA- hymatomelanic acids)

Hydrophobic fraction of HA was isolated from the parent HA by extraction with alcohol. 5 g of HA was put in the Erlenmeyer flask with 100 mL of 96% ethanol and kept for 24 hours at the continuous shaking. Then 50 mL of ethanol was added and shaked within 30 min and extract was centrifuged at 6000 rtm. Sediment was transferred into flask and processed with 50-100 mL of ethanol in dependence on content of HymA and kept at the continious shaking. The extract was separated on the centrifuge. This operation was reiterated 5-7 times till orange or brown colour of solution. Combined extracts were purified from impurities with ethyl alcohol using rotary evaporator at 40-45°C. The concentrated extract was air-dried at 30°C.

Protocol for biotransformation of humic acids (BioHP)

Natural microbial populations from a cultivated soil (Bishkek, Kyrgyzstan), a biohumus from *Eisenia foetida*, a wood rot from *Ulmus Pamila* were used as inocula. Colony forming units (CFU) were counted after 7 days at 25°C on MPA for bacteria and on Czapek agar for microscopic fungi (standard deviation of CFU counts: 10%). Numbers of bacteria were determined microscopially after fluorescent staining of cells according to Bloem.

The basal solution (per litre of distilled water) KH_2PO_4 – 0.5 g, K_2HPO_4 – 0.5 g, $MgSO_4$ – 0.4 g, NaCI – 0.1 g, $CaCl_2$ – 0.01 g, $(NH_4)_2SO_4$ – 0.5 g, glucose – 15 g, was used in full strength ($(NH_4)_2SO_4$) or without $(NH_4)_2SO_4$ to receive a nitrogen-deficient nutrient solution. The salt solution, sterilized by autoclaving (160°C, 30 min), 5 mL of inoculum, and 1 g of HS were added into flasks to final concentration of 1.0 mg/mL. pH was 6.8. The flasks were incubated in the dark at 28°C for 12 months. All experiments were performed in duplicate. After incubation biosolubilized HA were separated by centrifugation, washed out with H_2O, desalted using dialysis and dried in vacuo at 60º. Noninoculated sterile HS served as a control.

Protocol for cryodestruction of humic acids (CryoHD)

Sample of parent HA was freezed at -16°C with following defrosting at 30°C. This procedure was repeated fivefold.

Elemental analysis

Elemental analyses (C, H, N) were performed on a Carlo Erba Strumentazione elemental analyzer. Ash contents were determined manually. Oxygen contents were calculated as a difference. The H/C and O/C atomic ratios were derived from the contents of the elements calculated on ash- and water-free basis.

Determination of total acidity

5-10 mL aliquot of HA solution containing 5-20 mg HA was transferred into a vial (~ 22 mL) and 10 mL of 0.03 M $Ba(OH)_2$ were added. The vial was tightly sealed, shaken well and left for equilibration for 24 hours at room temperature. Aliquots of transparent solution above the precipitate of Ba humates were transferred to titration cell and titrated with HCl standard solution (~0.1 M) using phenolphtaleine as an indicator. Total acidity (TA, mmol/g) was calculated according to the formula

$$TA = \frac{(V_0 - V_{HA}) \cdot c_{HCl}}{m}$$

where V_0 and V_{HA} are the volumes of HCl (mL) consumed for blank and sample titrations respectively, C_{HCl} is the titrant concentration (mmol/mL) and m is the mass (g) of HA in the aliquot.

Saturated $Ba(OH)_2$ solution was prepared from BaO by dissolving it in CO_2-free deionized water (boiled during 1 hour) in a sealed volumetric flask under intensive shaking. The solution was left for 3-4 days until complete precipitation of $BaCO_3$ occurred. Work solutions were prepared immediately prior to the analysis by diluting an aliquot of transparent supernatant and standardized against HCl.

Determination of carboxylic acidity and contents of strong acidic groups (COOH and SO_3H)

Calcium acetate method was used to determine carboxylic acidity or the content of strong acid groups (in case of sulphonated derivatives) in the humic samples. 5-10 mL aliquot of HA solution containing 5-20 mg HA was transferred into a vial (~ 22 mL) and 10 mL of 0.6 M $Ca(CH_3COO)_2$ were added. The vial was tightly sealed, shaken well and left for equilibration for 24 hours at room temperature. Aliquots of transparent solution above the precipitate of Ca humates were transferred into titration cell and titrated with NaOH standard solution (~0.05 M) using autotitrator. Carboxyl acidity (CA, mmol/g) was calculated according to the formula

$$D = \left(1 - \frac{R_d - R_{d+t}}{R_d} \Big/ \frac{R_o - R_t}{R_o}\right)$$

where V_0 and V_{HA} are the volumes of NaOH (mL) consumed for blank and sample titrations respectively, C_{NaOH} is the titrant concentration (mmol/mL) and m is the mass (g) of HA in the aliquot.

Fourier Transform Infra Red Spectroscopy

Fourier transform infrared (FTIR) spectra were obtained by pressing the HA sample into KBr pellet and analyzing with a FTIR spectrometer IR-200 (ThermoNicolet, USA) on spectral range of 400-4000 cm-1 (4 cm-1 resolution, 64 scans per spectrum).

Quantitative 13C NMR Spectroscopy

13C solution-state NMR spectra of HA samples were measured on solutions of humic materials in 0.3 M NaOD/D_2O at concentration of 100 g/L. Measurements were made on a Bruker Aspect 3000 spectrometer operating at 100MHz 13C observation frequency using inverse gate decoupling. MeOH/D2O (d = 49.0 ppm) was used as an external standard. All the spectra were recorded at 8-s delay time. These conditions were shown to provide quantitative determination of carbon distribution among the main structural fragments of HA. To quantify the observed spectra, the assignments were made after Kovalevskii (Kovalevskii, 1998) and were as follows (in ppm): 5-50, aliphatic H and C-substituted C (CAlk); 50-108, aliphatic O-substituted C (CAlk-O); 108-145, aromatic H and C-substituted C (CAr-H,C); 145-165, aromatic O-substituted C-atoms (CAr-O); 165-187, - C of COOH/R groups (CCOO); 187-220 – ketonic/quinoic groups (CC=O).

Quantitative 1H NMR Spectroscopy

1H NMR spectra were acquired with Bruker DMX 500 NMR spectroscopy operating at 500 MHz proton frequency. The spectra were recorded at 303K using the 1-st increment presat-NOESY (90-deg), acquisition time = 4.7 s, relaxation delay = 15 sec.

All the spectra were acquired with 5-mm broadband probe, the samples were dissolved in 700 ml 0.1N NaOD/D_2O.

Size Exclusion Chromatography (SEC)

SEC-Analysis was performed as described in Perminova et al. (1998). SEC system Abimed (Gilson, France) included HPLC pump, autosampler, and UV detector. The column 25 mm × 20 cm packed with Toyopearl HW-55S gel (Toso Haas, Japan). The 0.028 M phosphate buffer (pH 6.8) was used as a mobile phase. The flow rate was 1 ml min-1. The absorbance of eluate was detected at 254 nm. The void volume and total permeation volume of the column were determined using blue dextran (2000 kDa) and acetone (48 Da), respectively. Sodium salts of polystyrenesulfonic acids of peak molecular weight of 14.00, 20.70, 45.10, and 80.84 kDa (Polymer Standard Service, Germany) were used as markers for molecular weight calculations. On the basis of the obtained data, the number-, weight, and z-average molecular weight and polydispersity (Mn, Mw, Mz, and Mw/Mn, respectively) were calculated as described in our previous publications (Kudryavtsev et al., 2000).

Protocol of bioassay with seedlings

Biological activity of the preparations was estimated by bioassay technique with seedlings. Seedlings of wheat *Triticum aestivum L.* were used as a target object, and a length of the longest root was used as a response. Ten wheat seeds were placed in Petri dishes with solutions containing 10 mL of 5, 15, 30, 50, 100 mg/L of humic derivative obtained during hydroxylation of humics.

The stock solutions of humic materials were prepared by dissolution of a certain amount of the sample in a small volume of 1M NaOH. Values of pH of all the solutions were adjusted to 5.5-5.9 using 0.1M HCl. Seeds were grown for 72 hours at 25°C in the dark.

Protocol of the quantitative assessment of detoxifying ability of the humic copolymers

For quantitative assessment of detoxification ability of HA preparations the detoxification coefficients (D) were calculated using approach described in (Perminova et al., 2001):

$$D = \left(1 - \frac{R_d - R_{d+t}}{R_d} \Big/ \frac{R_o - R_t}{R_o}\right)$$

where: R_0 – response of control; R_d - response in presence of HA; R_t - response in presence of toxicant (copper or mercury); R_{d+t} - responce in presence of toxicant (copper or mercury) and HA. On the basis of the D values, the toxicological constants of copper binding to HA normalized to the organic carbon content in HA preparation (K_{oc}^{tox}) were calculated as described previously (ibid.). The values of K_{oc}^{tox} were obtained by approximating the following expression:

$$D = \frac{K_{OC}^{tox} \times C_{HA}}{1 + K_{OC}^{tox} \times C_{HA}}$$

where CHA is a concentration of HA. The obtained values of K_{oc}^{tox} values were further used for comparison of detoxifying ability of different HA derivatives in relation to copper.

All preparations obtained using various modification techniques are listed in Table 1 below.

Table 1

The sources and names of the humic derivatives

Sample designation	Description of the source humic material
HA1	Humic acids from oxidized brown coal of Kyrgyz Kyzyl-Kiya deposit
HymA1	Hymatomelanic acids, alcohol extraction from HA1
CarbHD1	Carbonylated humic derivatives from HA1
CryoHD1	Cryodestructed humic derivatives from HA1
BHD1-S3 (BHD1-S6, BHD1-S9)	HA biosolubilized by soil natural microbial populations in 3, 6, 9 months respectively
BHD1-W3	HA1 biosolubilized by wood-degrading natural microbial populations in 3 months
HA2	HA isolated from oxidized brown coal, Kyrgyz Kara-Keche deposit
HymA2	Hymatomelanic acids, alcohol extraction from HA2
CarbHD2	Carbonylated humic derivatives from HA2
CryoHD2	Cryodestructed humic derivatives from HA2
BHD2-S3 (BS6-HD2, BHD2-S9, BHD2-S12)	HA2 biosolubilized by soil natural microbial populations in 3, 6, 9, 12 months respectively
HA2 (Fr3)	HA2, SEC-fraction 3
BHD2-S3 (Fr3)	BHD2-S3, SEC-fraction 3
BHD2-W3, BHD2-W6, BHD2-W9	HA1 biosolubilized by wood natural microbial populations in 3, 6, 9 months respectively
CryoBHD2-S3	Cryodestructed BHD2-S3
BHD1-B12	HA1 biosolubilized by biohumus natural microbial populations in 12 months

Results and discussions

Elemental and functional group composition of parent and modified HA are summarized in Tables 2 and 3.

Table 2

Elemental composition of humic derivatives

Index	Mass %					Atomic correlation		ώ
	C	H	N	S	O	H/C	O/C	
HA1	65.99	3.86	1.08	0.43	28.71	0.70	0.36	-0.05
HymA1	63.15	4.41	0.67	0.25	31.61	0.84	0.38	-0.06
CarbHD1	64.59	3.60	0.90	0.24	30.73	0.50	0.26	-0.02
CryoHD1	64.30	4.05	0.80	0.23	30.58	0.77	0.36	-0.05

BHD1-S3	63.30	5.55	0.81	0.11	30.35	1.04	0.35	-0.35
BHD1-S6	64.60	5.83	1.24	n.d.	28.42	1.07	0.32	-
BHD1-S9	59.54	5.64	1.99	n.d.	32.83	1.12	0.41	-
BHD1-W3	65.20	4.98	0.82	n.d.	29.02	0.92	0.33	-
HA2	65.51	3.91	1.08	0.41	29.22	0.71	0.33	-0.05
HymA2	62.74	4.06	0.73	0.27	31.82	0.88	0.38	-0.08
CarbHD2	63.38	4.11	0.90	0.35	31.44	0.68	0.51	-0.04
CryoHD2	63.20	4.18	0.98	0.34	31.30	0.80	0.37	-0.05
CryoBHD2-S3	62.26	4.33	1.40	0.40	31.61	0.83	0.38	-0.07
BSHD2	58.40	4.61	0.86	n.d.	36.22	0.91	0.56	-0.01
HA2 (Fr3)	62.30	3.82	1.06	n.d.	32.64	0.73	0.39	0.05
BHD2-S3 (Fr3)	58.50	4.42	1.22	n.d.	35.91	0.90	0.50	0.01
BHD2-S3	62.02	4.55	1.06	n.d.	32.50	0.87	0.36	-
BHD2-S6	64.24	4.61	1.24	n.d.	30.05	0.85	0.34	-
BHD2-S12	59.54	5.28	1.95	0.40	32.83	1.06	0.41	-0.23
BHD2-W3	62.05	5.02	0.95	n.d.	32.17	0.98	0.40	-0.01
BHD2-W6	60.22	5.00	1.88	0.40	32.50	0.99	0.40	-0.19

As can be seen from the obtained data (Table 2), HA isolated from coal Kara-Keche deposit as well as Kyzyl-Kya had the lowest H/C ratio. Such values indicate of high content of non-saturated structural moieties in the corresponding molecular structure. Analysis formylated and biotransformed HS are characterized with more highest H/C ratio. Such values indicate of high content of saturated structural moieties of humic substances macromolecules.

Table 3

Functional group composition of humic derivatives

Sample	**Functional groups**					
	COOH		**OH**		**CO**	
	mM/g	**%**	**mM/g**	**%**	**mM/g**	**%**
HA1	**4.03**	**18.14**	**2.67**	**4.54**	**1.39**	**3.90**
CryoHP1	4.08	18.36	2.72	4.62	1.61	4.51
BCryoHP1-S3	4.16	18.17	2.99	5.08	1.54	4.31
BHP1-S3	4.09	18.41	3.01	5.12	1.57	4.40
BHP1-W3	4.03	18.14	2.73	4.64	1.48	4.14
BHP1-B3	4.04	18.18	2.81	4.78	1.57	4.40
BHP1-S6	3.97	17.87	3.13	5.32	1.52	4.26
BHP1-S9	3.79	17.06	3.55	6.04	1.36	3.81
BCryoHP1-W9	3.86	17.37	3.53	6.00	1.34	3.76
BCryoHP1-B9	3.81	17.15	3.62	6.15	1.35	3.78
BHP1-W12	4.35	19.58	3.62	6.15	1.40	3.92
BHP1-B12	4.29	19.31	3.71	6.31	1.43	4.60
HA2	**5.00**	**22.5**	**2.40**	**4.08**	**1.44**	**4.03**
CryoHP2	5.34	24.03	2.76	4.69	1.60	4.48
BHP2-S3	5.34	24.03	2.65	4.50	1.48	4.14
BHP2-W3	5.47	24.62	2.47	4.12	1.47	4.12
BCryoHP2-S6	4.83	21.74	3.11	5.29	1.25	3.50
BHP2-S6	5.05	22.72	2.94	5.00	1.29	3.62
BCryoHP2-W6	5.00	22.50	2.59	4.41	1.30	3.64
BCryoHP2-B6	4.99	22.45	2.80	4.76	1.31	3.66
BCryoHP2-S9	4.57	20.57	3.61	6.14	1.42	4.17
BHP2-S9	4.59	20.66	3.45	5.87	1.43	4.00
BCryoHP2-W9	4.64	20.88	3.50	5.95	1.38	3.86
BCryoHP2-B9	4.75	21.37	3.32	5.64	1.35	3.78

Functional group analysis showed (Table 3) that a remarkable increase in the content of ArOH is observed for biosolubilized humic derivatives; it should be noted for all that derivatives studied, the content of ArOH increases along

with an increase in the inoculation time. For the cryodestructed derivatives an increase in the content of phenolic groups was observed as compared to the corresponding humic acids. For the synthesized humic derivatives enriched in carbonyl fragments a slight increase in the content of carboxylic groups was revealed as compared to the corresponding parent humic material.

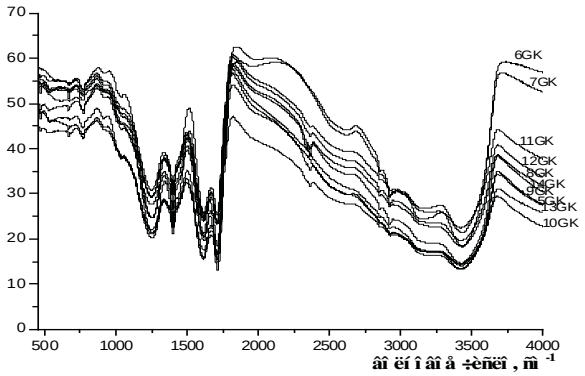

Figure 1. FTIR spectra of humic derivatives

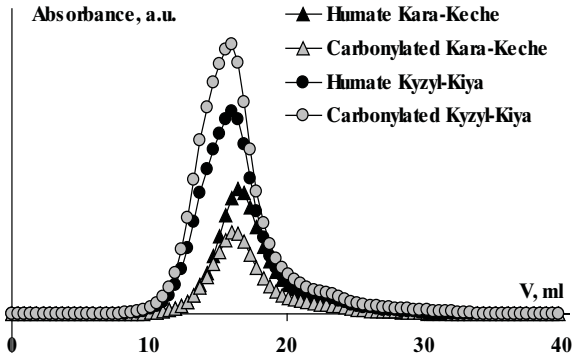

Figure 2. SEC-profiles of the parent humic material and carbonylated derivatives

FTIR-spectra of the parent and humic derivatives (Figure 1) turned out to be very similar and their shapes were typical for spectra of coal HA. The FTIR spectra of enriched humic derivatives exhibit the bands typical for parent humic material with negligible differences associated with the modification of parent material.

SEC analysis (Figure 2) showed that enrichment with carbonyl groups caused an increase in molecular weights of the derivatives, whereas oxy-, as well cryo-derivatives did not change molecular weight of the parent humic material. Humic derivatives enriched in carbonyl fragments exhibited the peak typical for the humics and also indicated the increase in peak molecular weight comparing to the parent humic material. The same tendency is also observed for biosolubilized derivatives in 9-12 months of inoculation.

^{13}H-NMR and ^{13}C-NMR spectra of the investigated humic substances are presented in Figures 3-4. As it can be seen from the figures, the spectra are typical for those of coal humic acids, characterized by large peaks corresponding to aromatic (100-165 ppm) and carboxylic/ester (165-187 ppm) signals and reducted hydrocarbon (48-108 ppm) signals. Spectral partial integrals, corresponding with 9 structural fragments of HS, are presented in the Table 4.

Table 4

^{13}C-NMR spectral integrals of studied samples (%)

Structural fragments	C_{ALK}	CH_3O	CH_2-O,N	CH-O,N	OC-O,N	C_{AR}	C_{AR}-O,N	CO-O,N	C=O
Intervals, ppm	0-48	48-58	58-64	64-90	90-108	108-145	145-165	165-187	187-220
HA1	7.7	1.1	1	5.2	3.9	48.5	8.7	18.3	5.6
HymA1	11.9	1.7	0.8	1	1.5	51.7	7.8	18.8	4.8
HA2	9.5	1.5	1.5	4.2	5.5	44.1	12.1	14.7	6.9
HymA2	17	1.8	0.2	1.5	2	48.6	6.48	18.2	4.2
BHD1-W12	8.2	1.6	0.8	2.4	2.7	48.5	10.8	18.8	6.2
BHD2-W12	7.1	0.7	0.1	1.9	0.9	51.1	11.6	18.6	7.9
BHD1-S12	7.5	1.3	0.4	0.5	1.8	55.2	9.2	19.5	4.6
BHD2-S12	10.7	1.2	0.7	3.4	4.9	54.9	5.7	16.4	2.2
BHD1-B12	8.9	1.2	0.4	1.6	3.7	54.9	6.7	18.8	3.8
BHD2-B12	10.2	1.2	0.4	2	2.8	51.3	8.2	18.1	5.8

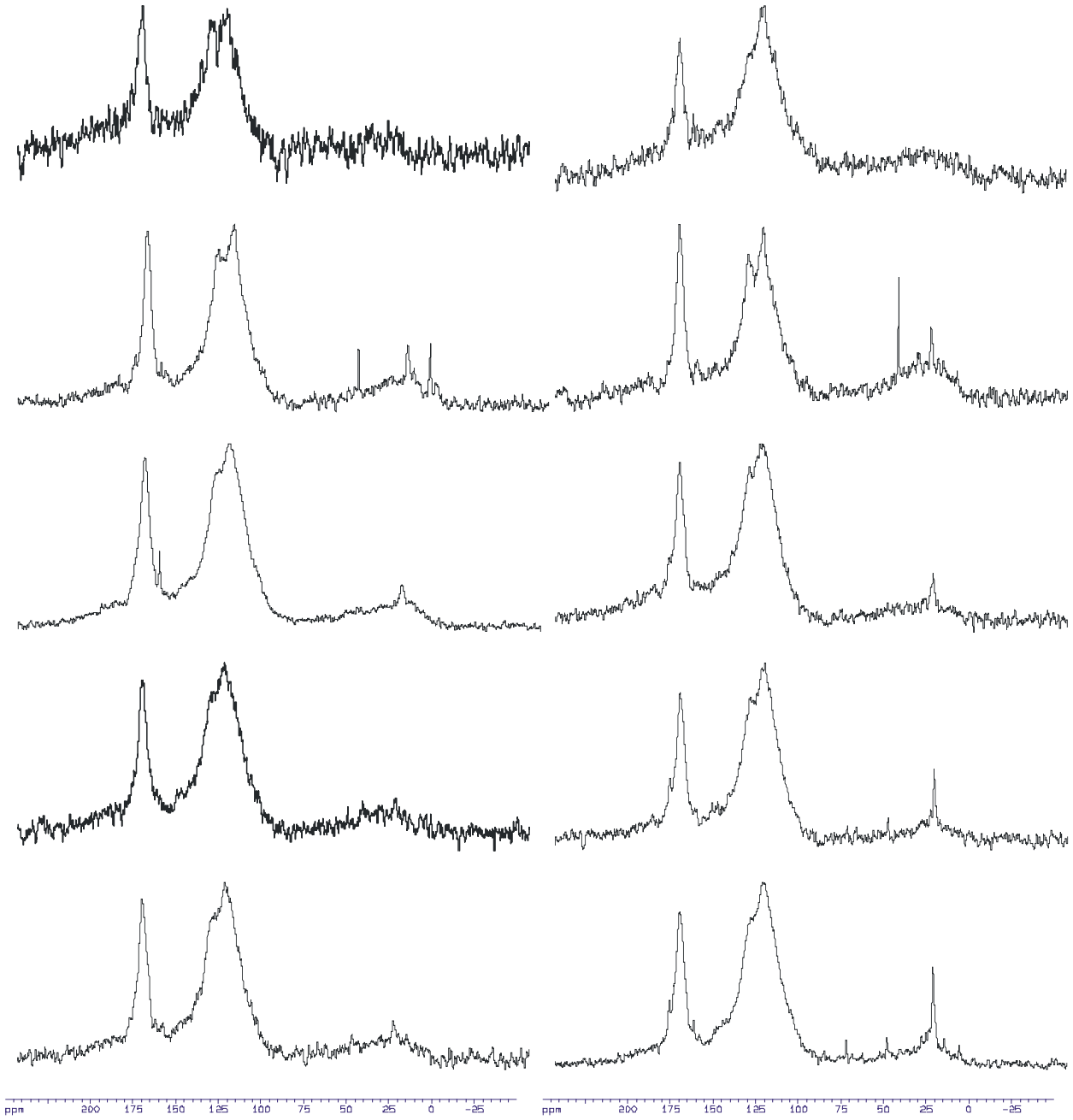

Figure 3. ^{13}C-NMR spectra of humic derivatives: HA1; HA2; Hym1; HymA2; BHD1-W12-1; BHD1-W12-2; BHD1-S12-1; BHD1-S12-2; BHD1-S12-1; BHD1-S12-2

As it was expected, the ^{13}C NMR-spectra of HymAs in comparison with the HAs demonstrated increasing in the intensities of the hydrophobic (alkyl and non-substituted by heteroatoms aromatic) groups signals. The Table 3 also shows increasing of aromatic spectral signals intensity (108-145 ppm) and decreasing of spectral integrals in 58-90 ppm area, corresponding to hydrocarbon and peptide functional groups of HS, as a result of the HS long time biotreatment.

Comparison of the ^{1}H and ^{13}C NMR spectroscopy results shows that NMR-signals of alkyl-H and alkyl-C structural fragments increase from the humic acids to the hymatomelanic acids (Tables 4-5).

Aromatic carbon signals increase as a result of microbiological long treatment of the HAs. At the same time, aromatic hydrogen signals decrease from the HAs to the HymAs and to the microbiological treated HAs. It can be interpreted as a result of carbon substituted aromatic rings contribution relatively increased. The significant rise of alpha-groups proton signals shown in the Table 3 confirms this supposition.

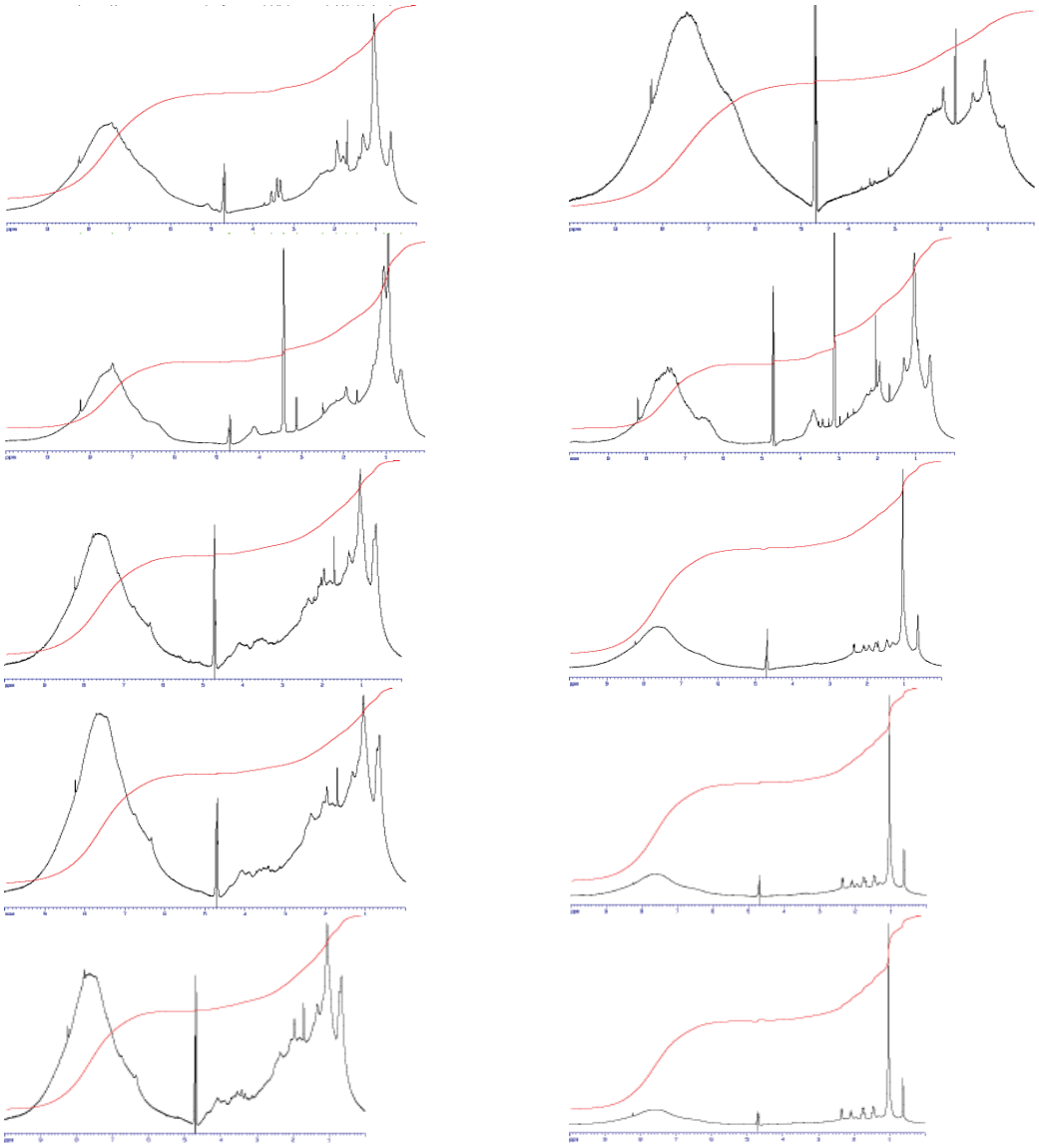

Figure 4. [1]H-NMR spectra of humic derivatives: HA1; HA2; HymA1; HymA2; BHD1-W12-1; BHD1-W12-2; BHD1-S12-1; BHD2-S12-2; BHD1-S12; BHD1-S12-2

Table 5

[1]H-NMR spectral integrals of studied samples (%)

Structural fragments	Aromatic H	O-CH-O,N	CH-O,N	alpha-CH*	Alkyl-H
Intervals, ppm	10.0-6.0	6.0-4.8	4.6-3.2	3.2-2.05	2.05-0.0
HA1	52.7	1.8	2.7	8.2	33.6
HymA1	48.2	0.0	6.3	10.7	45.5
HA2	51.4	3.1	1.8	8.2	33.6
HymA2	33.9	0.0	6.3	18.8	38.4
BHD1-W12	49.6	0.9	4.5	9.9	33.3
BHD2-W12	55.9	0.0	0.9	9.9	32.4
BHD1-S12	53.6	1.8	3.6	9.8	29.5
BHD2-S12	48.2	1.8	2.2	8.9	37.5
BHD1-B12	49.6	0.9	3.6	9.9	33.3
BHD2-B12	45.9	0.0	1.8	9.0	42.3

* *alpha-CH* – protons of aliphatic groups in alpha-position to electronegative group or to aromatic ring

Assessment of stimulating activity and potential self-toxicity of the produced humic materials and synthesized derivatives has revealed that there was no toxicity observed for all humic derivatives studied: the root length was in the range of (100±15)% or (100±17)% of control, respectively. Stimulating activity towards wheat seedlings was the highest for the parent humic material reaching 120% of control. For the both hydrophobic and carbonylated derivatives, in most cases, it was lower than for other humic preparations and laid in the range of experimental error. The results on detoxifying ability activity of the humic derivatives are given in Figure 5.

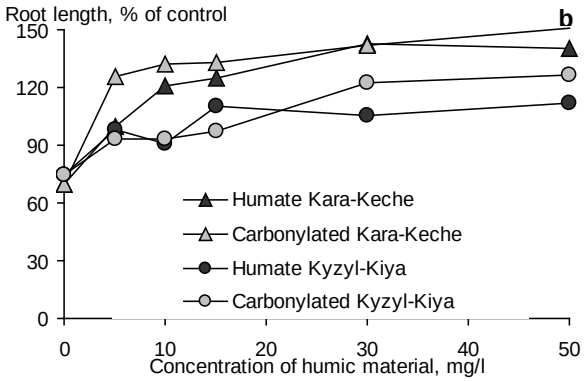

Figure 5. Dose-response relationships in the presence of 1 mg/L of copper(II) for the parent humic materials and carbonylated derivatives

Estimation of phyto-hormonal activity of the produced humic substances and their derivatives in respect to higher plants (*Rosmarinus Officinalis L.*, watercress *Coronopus*, lettuce (*Lactuca Sativa*) with auxine and gibberellic tests has been shown that parent humic substances had no hormon-like activity. But, some produced derivatives (cryodestructed, hymatomelanic, carbonylated from Kara-Keche) displayed hormone like effect. Low molecular weight fractions of cryodectructed and carbonylated HP displayed two kind of biological activity.

Detoxifying properties of the produced humic preparations

Estimation of detoxifying ability of the parent and modified humic materials towards heavy metals revealed that all the studied humic derivatives significantly decreased toxicity of copper in bioassays with seedlings, vegetative experiments and field trials. The obtained results on copper toxicity are presented in Figure 6. At the low concentrations of HA (5-30 mg/L) the tendency that in most cases the detoxification activity toward copper of the carbonylated HA was higher than that of the parent HA was observed.

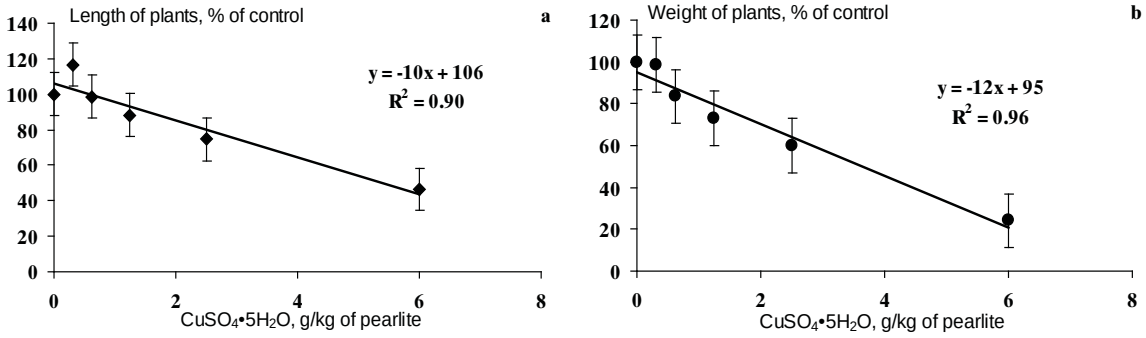

Figure 6. Dose-response relationships for copper(II) in the form of $CuSO_4 \times 5H_2O$ under conditions of lab vegetation experiments with a use of plant length (a) or weight (b) as a response. Bars represent standard deviation.

The calculated values of the toxicological binding constants of the humic materials studied with copper(II) are given in Table 6. Data presented in Table 6 confirm the above conclusion on detoxifying ability of the obtained humic derivatives. K_{OC}^{tox} values of modified humics were higher than K_{OC}^{tox} value of the parent HA. Maximum value of K_{OC}^{tox} was observed for BHP-B12 that was biosolubilized humic derivatives. Among derivatives the greatest detoxification ability toward copper was observed for biosolubilized derivatives. For the carbonylated derivatives a slight increase in detoxifying ability was registered comparing the initial humic materials.

Table 6

The toxicological constants of copper binding to the humic materials

Derivative Cipher	K_{OC}^{tox}, L/kg
Humate Kara-Keche	1.3×10^5
Humate Kyzyl-Kiya	1.0×10^5
CarbHD1	1.8×10^5
CarbHD2	1.6×10^5
BHP1-B12	3.6×10^5
BHP2-B12	3.2×10^5

Conclusion

Set of samples of humic-based de-toxicants have been synthesized. The experimental approaches undertaken to produce humic-based de-toxicants included enrichment with carbonyl groups; hydrophobic groups; oxygen groups; cryodestruction of humic substances and biosolubilization.

It has been demonstrated from both chemical characteristics and detoxifying ability point of view that all the produced de-toxicants possessed higher detoxifying potential in relation to heavy metals. All the derivatives of humics have been studied at express bioassay, laboratory vegetation experiments. It has been confirmed not only diverse detoxifying potential of those de-toxicants in relation to heavy metals but also their prolonged activity in the environment.

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