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Characteristics of Humic Acids in Drained Floodplain Soils in Temperate Climates: A Spectroscopic Study

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Abstract: This study aims to assess the characteristics of humic acids (HAs) in floodplain soils. HAs were isolated from the Fluvisols located out of the embankment in the riparian forest (unflooded riparian forest) and within the embankment (inter-embankment), in the area periodically flooded. HAs from these soils were examined for quantity, structure, and humification degree using extraction methods as well as elemental analysis, UV-Vis, FTIR, EPR, and ^{1}HNMR spectroscopies. In the soils after drainage, a significant decrease in HAs has been observed compared to the periodically flooded areas. Obtained results showed that organic matter from periodically flooded soils is more humified and contains HAs with a more aromatic, lignin-like structure compared to the humus matter from unflooded Fluvisols. Humic acids from periodically flooded soil contained a lower amount of C and H compared to those isolated from unflooded soils located out of the embankment, which resulted in a less aliphatic or more aromatic character of their molecules. A higher H/C ratio of HAs from the Fluvisols after drainage exhibits more condensed aromatic ring or substituted ring structures in the molecules. Soils with organic matter with a higher humification index contained HAs with lower radical concentration values in comparison to soils with less humified organic matter. Results obtained show that in flooded areas with periodically reductive conditions, humic acids do not lose as many -OCH_3 groups as in better oxidized soils and therefore exhibit a lignin-like aromatic structure. It has been proven that the formation and dynamics of HA transformation may vary due to the water regime in soils.

Keywords: humic substances; spectroscopic analysis; E4/E6 ratio; FTIR analysis; ^{1}H NMR spectra; anthropogenic impact on soil

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1. Introduction

Soil organic matter (SOM) is particularly important since it forms one of the largest reservoirs of organic C in ecosystems [1]. Therefore, any change in the turnover rate of soil C, size, and quality may potentially alter the atmospheric CO_2 concentration and affect the global climate. Pedogenic processes, plant cover, and soil water regime affect organic matter decomposition rates in soils and the global biogeochemical cycle [2–5]. Most of the organic carbon, over 70%, occurs as highly reactive and recalcitrant humic substances [6,7]. Humic acids (HA) and fulvic acids (FA) are fractions recognized as the most reactive within soil components, which maintain soil fertility and affect the physical, chemical, and biological status of the soil [8–13]. Lately, growing attention has been paid to humin, which is considered a significant SOM component and plays an important role in carbon sequestration or binding pollutants [14–16]. However, it is difficult to discuss the role of humic substances in the environment without knowing their chemical structure. Transformation of organic matter under the influence of external factors, e.g., land use, changes in pH, changes in soil water regime, fertilization, etc., results in a change in the molecular characteristics of HS, affecting soil properties and the whole ecosystem [17].

Humification of organic matter usually involves variability in the elemental composition of HS molecules, as manifested by changes in the composition of C, H, N, and O, as well as changes in functional groups that are extremely valuable (mainly carboxylic and phenolic). More humified organic matter is characterized by an increase in the aromaticity of the molecules, an increase in carbon and decrease in hydrogen content, as well as an increase in the content of carboxyl and methoxy groups compared to less humified matter [18]. Advanced instrumental techniques such as FTIR, ^{13}C CP MAS NMR/^{1}H NMR, or EPR spectroscopy can help in describing the detailed chemical properties of humic substances as well as following the direction of humification [19–21]. Decreasing in SOM, particularly HAs (a very stable humus fraction), contributes to an increase in compactness of the soil, diminishing soil aeration, and the appearance of reductive chemical conditions [22], as well as reducing the stability of soil aggregates.

In river valleys, soils enriched with organic matter occur very often [23]. Floodplain organic carbon accumulation as well as soil development depend on coastal flooding and sedimentation [24]. The deposited sediments show clear variation both vertically and spatially [25,26]. This variability is visible both within the alluvia of the same river and between the sediments of different watercourses. Conditions of periodic high moisture result in a large accumulation of organic matter in the surface horizons. At the same time, human activities that use agrotechnical treatments to transform riverside forest areas into agricultural land have caused the surface organic layer to mix with the mineral substrate, causing a significant deepening of arable horizons and consequently changing the resources of organic matter. The physicochemical properties of soils formed under the influence of the above-mentioned factors may determine the formation of various organo-mineral complexes.

The channelization of the Odra River, the second largest river in Poland, performed in the 19th century and the construction of flood embankments at the beginning of the 20th century initiated the large-scale regulation of the Odra valley and resulted in far-reaching changes in the natural conditions in the valley [27–29]. A significant part of the riparian forests was separated from the river by hydrotechnical facilities (flood embankments), which effectively prevented their flooding and initiated the changes in vegetation known as transformation into oak-hornbeam communities. Principles of flood protection in the so-called inter-embankment zone involved the removal of trees in this zone, which resulted in favoring its use first as meadows and then for agricultural purposes. On the other hand, the commissioning of the hydroelectric power plant in Brzeg Dolny (1958) resulted in a lowering of the Odras’ bottom level and caused the lowering of the groundwater table in alluvial soils in the areas downstream the dam [30,31]. Lowering of the groundwater causes drying of the soil and, consequently, brings about changes in its properties, like changes in sediment volume or liquid limit [32]. These changes persist even after the soil has been rewetted and the effects of the drying persist. The lowering of the groundwater table enables deeper penetration of the soil by plant roots, microorganisms, and soil fauna [33], but at the same time affects the direction of organic matter humification and often causes an increase in decomposition rate. However, there are not many studies describing the humification rate and quality of organic matter under conditions of flood elimination and change of soil water regime in alluvial soils of temperate climates.

Organic C stocks in floodplain soils have been well described in the literature [23,24,34–36], but still little is known about the quality of organic matter, including the molecular characteristics of humic substances, in these areas. River valleys are a mosaic of terrestrial and aquatic environments. Most of the natural habitats found in these areas are associated with regular flooding, and the frequency and length of these floods are the most important ecological factors determining their existence [25,37,38]. Fluctuations in the water level of the river combined with river flooding affect the water regime of alluvial soils, including the level and mobility of groundwater, enabling naturally formed river valleys to retain relatively high soil moisture even in periods of drought [39]. Fluvial processes occur on all continents and in all climatic zones [40]. Alluvial terraces are characterized by strongly developed relief with oxbow lakes, periodic river channels, and gutter depressions, which, if they are subject to terrestrialization, are still slightly lower and therefore preferentially hold water in them. Vast areas of alluvial soils on the flooding terraces were covered with riparian forests [41,42], whose high fertility was conducive to human activities aimed at transforming riverside forest areas into agricultural land. At the same time, numerous floods causing significant material and social losses prompted various attempts to tame the river [43,44].

This study aims to assess the properties of the HAs molecules using spectroscopic instrumental techniques as well as to describe and determine the humification rate and indexes in floodplain soils affected by drainage. The obtained results may contribute to the development of good practices to ensure high quality organic matter and the stability of ecosystems based on floodplain soils.

2. Materials and Methods

2.1. Site Location, Soil Characteristics and Sampling

The research was carried out in the low valley of the Odra River, SW Poland, in its middle course downstream from Wrocław (Figure 1). The Pleistocene terraces of the Odra river, shaped by the fluvioglacial water of the Weichselian glaciation, are mainly built of sands and gravels, while the Holocene terraces often contain fine-textured interbedding. The surface layers of floodplains are typically built of loamy or silty soil materials [33,45,46]. As a result of riverbed regulation and the construction of levees, in particular the weirs in Brzeg Dolny and Malczyce, a decrease in groundwater level has been documented. Studies conducted in the period 1970–1990 showed a groundwater level decrease of 44 cm (compared to the previous state) at a distance of 550 m from the Odra River and of 65 cm at a distance of 120 m. As a result, for a considerable part of the year, the groundwater level is recorded beyond the conventional lower limit profiles of the soil [47–49].

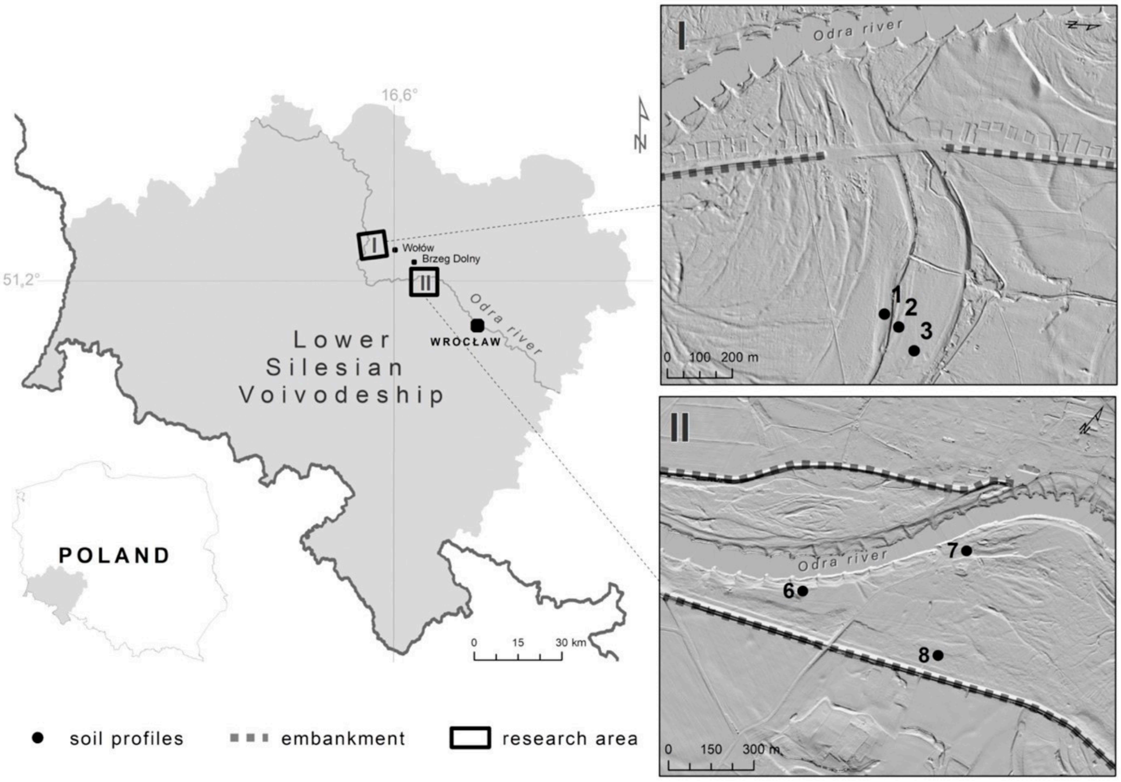

Figure 1. Site location and sampling points (1, 2, 3...8).

The study included soil profiles within two transects, which were located on the lowest terraces (former floodplain). According to the WRB 2015 [50], they are classified as Fluvic Cambisols (Rp1a–Rp3a and Mw7a–Mw8a soil profiles) and Fluvic Phaeozems (Mw6a soil profile). The first transect was located out of the embankment in the riparian forest and the oak-hornbeam forest (unflooded riparian forest within different distances from the river: 900 m, Rp1a; 950 m, Rp2a; 1050 m, Rp3a). Another transect was located within the embankment (inter-embankment), in the area periodically flooded and used as grassland (within different distances from the riverbed: 120 m for Mw6a, 150 m for Mw7a, and 200 m for Mw8a). Samples were taken from July to September, so groundwater was not recorded in any of the profiles to a depth of 150 cm. Soil morphology was described according to FAO guidelines [51]. The bulk soil sample was a sample of three singleout, taken from 0–0.1 m depth.

The basic parameters of the investigated soil horizons are presented in Table 1. The results presented are the mean values of three replicates.

Table 1. Basic properties of the Fluvisols studied (depth of sampling 0–0.1 m).

Object	**Distance from River (m)**	**pH 1M KCl**	**TOC $g\cdot kg^{-1}$**	**Nt $g\cdot kg^{-1}$**	**C/N**	**CEC $cmol(+)\cdot kg^{-1}$**	**Silt Content (%)**	**Clay Content (%)**
Rp1a	900	4.5	39.7	2.9	14	24.33	52.7	0
Rp2a	950	4.9	26.0	3.7	7	25.41	67.2	20.7
Rp3a	1050	4.9	39.9	3.1	13	17.68	41.4	9.4
Mw6a	120	4.7	45.8	3.9	12	18.20	62.0	25
Mw7a	150	4.8	56.9	2.8	20	16.28	62.0	14
Mw8a	200	4.6	54.2	2.8	19	16.98	60.0	16

Explanations: Rp—riparian forest, Mw—grassland; TOC—total organic carbon, Nt—total content of nitrogen, CEC—cation exchange capacity.

2.2. Content of Total Organic Carbon and C/N Ratio

Total organic carbon (TOC) was measured using a Ströhlein CS-mat 5500 analyzer (Strohlein GmbH & Co., Kaarst, Germany; currently Bruker AXS Inc., Madison, WI, USA); samples did not contain carbonates; thus, no pre-treatment was applied. The total nitrogen content (Nt) was measured by the Kjeldahl method using a Büchi semi-automated analyzer.

2.3. Chemical Fractionation of the Humic Material

The extraction and chemical fractionation of the humic substances were accomplished through the method suggested by the International Society of Humic Substances (IHSS), described in Swift [52] with some modification. The first step in the procedure is decalcitation of the soil sample to obtain a low-molecular-weight fulvic fraction (CAC), followed by exhausting alkali extraction using 0.1 M NaOH until the supernatant is a light color. This fraction, called total extractable carbon (TEC), consists of humic acids and fulvic acids.

Quantitative determination of humic fractions was performed using H_2SO_4 instead of HCl [53] for decalcitation (CAC). The carbon of fulvic acids (CFA) fraction was calculated from the difference between total extractable carbon (TEC) measured in alkali (NaOH) extract and carbon of humic acids (CHA) fraction using sulfuric acid (H_2SO_4) to precipitate HA at about pH = 2 of the alkaline extract and separation of solid HA from the FA by centrifugation.

Carbon of nonhydrolyzable humic substances (CR)–commonly named humin fraction was calculated according to the formula:

$$\mathrm{CR} = \mathrm{TOC} - (\mathrm{CHA} + \mathrm{CFA}) \tag{1}$$

To evaluate the humification degree some indexes were calculated [53–55]:

$$\text{degree of humification HD} = \mathrm{CHA} + \mathrm{CFA} \tag{2}$$

$$\text{humification rate HR} = (\text{CHA} + \text{CFA})/\text{TOC} \tag{3}$$

$$\text{humification index HI} = (\text{CHA}/\text{TOC}) \times 100 \tag{4}$$

$$\text{humic acid to fulvic acid ratio CHA}/\text{CFA}$$

2.4. Extraction and Purification Procedure of Humic Acids

Humic acids were extracted from the soil of the humus horizon (A) using the procedure described by Swift [52], which was found satisfactory by the International Humic Substances Society for most soil types. 1 g of the dried soil sample sieved by 2 mm was decalcified with a few drops of 1 M HCl and then 0.1 M HCl was added to obtain a sample-solution ratio of 1:10; the suspension was shaken, left for 1 h, and separated by centrifugation. After neutralizing the soil with 1 M NaOH, 0.1 M NaOH was added (under N_2) to obtain the final 1:10 (soil: extractant). The alkaline suspension was collected by centrifugation. The supernatant was acidified with 6 M HCl to pH 1. HA gel was separated by centrifugation and purified with a 0.1 M HCl/0.3 M HF solution, left overnight, and centrifuged; this procedure was repeated three times. The precipitate was transferred to a Visking dialysis tube (Spectra/Por 7 MWCO 10,000, Spectrum Europe B.V., Breda, The Netherlands) and dialyzed against distilled water until a Cl^- test was negative. After that, the HA were freeze-dried.

Elemental analysis of HA was performed with a Perkin-Elmer 2000 instrument (C, H, and N). Oxygen was calculated from the mass balance. The ash content was determined by combustion in a muffle furnace at 550 C. The degree of internal oxidation (ω) was calculated according to the formula [56]:

$$\omega = (2\text{O} + 3\text{N-H})/\text{C} \tag{5}$$

O, N, H, and C represent concentrations of elements in HA molecules expressed as atomic percent in ash-free samples. Furthermore, the atomic ratios H/C and O/C were calculated to express the relationship of the elements in the composition of HA.

2.5. Spectroscopic Analysis of HA

2.5.1. E_4/E_6

Vis spectroscopy analysis of HA was performed using UV-Vis Cary 60 (Agilent Technologies, Santa Clara, CA, USA). About 1 mg of HA was dissolved in 100 mL of 0.1 mol/dm^3 NaOH and a clear solution. Vis absorbance was measured in 1 cm quartz quvettes at wavelengths of 465 and 665 nm. The ratio of both absorbances was calculated as the E_4/E_6 ratio.

2.5.2. FTIR

Fourier-transformed infrared (FTIR) spectroscopy of HA was used to analyze the nature and structure of HA molecules. FTIR spectroscopy provides information on the presence of functional groups in both aliphatic and aromatic components. FTIR spectra of HA were recorded with a Bruker Vertex 70 FT-IR spectrometer on KBr pellets (an approximately 1 mg sample in 400 mg of KBr). The collected spectra were presented in the figure as transmittance (T). The same spectra, but in absorbance (A) mode (A = −log(T)), were used for quantitative analysis. Spectra were divided into characteristic peak regions and integrated using OriginPro 2016. Ten areas were chosen with a maximum wavenumber of (cm^{-1}): 1040, 1076, 1229, 1076, 2441, 1451, 1540, 1653, 1229, 1451, 1540, 1653, 1725, 2851, 2922, and 3421.

2.5.3. ^{1}H NMR

^{1}H NMR spectra were taken for HA samples diluted in NaOH solutions dissolved in D_2O. These studies were done to compare proton content in different structures (aromatic/aliphatic methoxy, alcohol groups) of humic acids. Automatic measurements were performed using the Bruker Avance III 500 MHz Spectrometer with the liquid probe BBI, 5 mm, 1H/2H/31P-109Ag, Z-grad, ATM. Standard measurement parameters were used: 16 scans, pulse sequence zg30, d1 = 1 s, acquisition time 2.62 s, fid resolution 0.38 Hz, rotation frequency of sample 20 Hz.

For quantitative analysis, integration of NMR spectra was performed using TopSpin 4.1 software.

2.5.4. EPR

EPR (Electron Paramagnetic Resonance) spectra were applied for quantitative and qualitative analysis of radicals, which are built into humic substance structures [52,55,57]. All EPR spectra of humic acids were recorded at room temperature using a Bruker Elexsys E500 spectrometer equipped with standard Bruker instrumentation (frequency and teslameter counters). For quantitative measurements, the resonator of a double cavity was applied; the radical species concentration standards (IHSS peat and Leonardite HA as well as Bruker alanine pill) were placed in the first cavity while the analyzed sample was placed in its second cavity. After tuning, the spectra were recorded separately for each of the two cavities without changing any of the measurement parameters. X-band spectra were performed at a microwave power of 20 mW and a modulation amplitude of 1 G. The double integrations were conducted using the WinEPR 2.22 rev.12 program (developed by Bruker).

2.6. Statistical Analysis

Results of the humic substances properties were compared between the mean values of groups with different water regimes (meadow flooded soils vs. riparian forest unflooded soils) using analysis of variance (ANOVA). Tukey's honest significant difference (HSD) test was used to find pairs of means that were significantly different from each other at $p < 0.05$ after ANOVA revealed statistically significant differences. Before running the ANOVA, the Shapiro-Wilk normality test was performed. The raw data on humic fraction content as well as molecular properties of the humic acids were log-transformed based on the results of Shapiro–Wilk's test that revealed the lack of their normal distribution. All the data were processed using the software package Statistica version 13.3 from TIBCO Software Inc., Karifuku Niya Pavilion, CA, USA.

3. Results and Discussion

3.1. Total Organic Carbon and Nitrogen

The studied Fluvisols were characterized by various total organic carbon (TOC) contents depending on the land use and distance from the river (Table 2). Fluvisols under the non-flooded riparian forest contained significantly lower TOC compared to the meadow soils (26.0 to 39.9 g kg^{-1} and from 45.8 to 56.9 g kg^{-1}, respectively). Lowering the groundwater level causes aerobic conditions in the soil. This favors an increase in organic matter decomposition, as confirmed by the lower C/N ratio compared to the floodplain soils. Similar results were described by Banach-Szott et al. [58] and Kercheva et al. [59], who found the highest TOC for the regularly flooded Fluvisols.

3.2. Content of Humic Substances

The process of organic matter transformation after drainage led to a change in the fractional composition of humus in the investigated soils. However, in soils from both transects, humin-residual carbon (CR) was the main fraction of total organic carbon (Table 2, Figure 2). CR values decreased with an increase in the distance from the river (61.4 to 44.5% of TOC for the soils under riparian forest (Rp sample code) and 60.8 to 47% of TOC for the grassland soils (Mw sample code). Interesting phenomena have been observed for the Rp1a soil with 0% clay content and the highest content of CR fraction (Table 1 and Figure 2). This seems to confirm the theory that the CR/humin fraction, or at least much of this fraction, is probably derived from cuticular components mostly of aliphatic character, which emphasize a much slower rate of decomposition than the other plant constituents [14]. Greater distance from the river banks and particularly drainage conditions favor deeper transformation of organic residues, which is reflected in the lower content and share of CR fraction in TOC in unflooded Fluvisols (13.98–24.39 g kg^{-1}) compared to the soils within the embankment (25.46–32.33 g kg^{-1}). The decrease in humin fraction is correlated with an increase in total extractable carbon (TEC fraction, isolated with alkali extracts and consisting of humic and fulvic acids) (Table 1 and Figure 2), which suggests that this fraction may play a significant role in the humification path [60]. In the soils after drainage, a significant decrease of humic acids has been observed compared to the flooded areas (4.77–7.51 g kg^{-1} and 8.54–14.97 g kg^{-1}, respectively) and a share of this fraction in the total organic carbon content as well (Table 2, Figure 2). This resulted in a decrease in the humic acid/fulvic acid (CHA/CFA) ratio and changes in humification parameters (Table 2). This ratio is commonly used to determine the direction of the humification process. Fertile soils such as chernozems are characterized by humic substances with CHA/CFA ranges of about 2.0–2.5, which means that organic matter transformation leads to the formation of two times more humic acids than fulvic acids [10,11]. The main reason for the lower CHA/CFA ratio in the riparian soils was presumably the more oxidized conditions in the unflooded soils, which enhanced mineralization of the organic matter [36].

Table 2. Content of carbon fractions in the Fluvisols (depth of sampling 0–10 cm).

Factor	**Distance from River (m)**	**TOC**	**CAC**	**TEC**	**CHA**	**CFA**	**CR**
		g·kg^{-1}					
Mw6a	120	45.8	1.58	16.37	8.54	7.83	27.85
Mw7a	150	56.9	1.84	22.73	12.00	10.73	32.33
Mw8a	200	54.2	2.56	26.17	14.97	11.20	25.46
Rp1a	900	39.7	3.45	11.86	5.37	6.49	24.39
Rp2a	950	26.0	1.76	10.26	4.77	5.50	13.98
Rp3a	1050	39.9	4.28	17.87	7.51	10.36	17.75
	LSD	3.099	0.485	3.142	2.742	1.240	1.314
Land use	Grassland	52.3	1.993	21.757	11.837	9.92	28.547
	SD	6.99	0.47	4.67	3.17	1.72	3.15
	Riparian forest	35.4	3.163	13.330	5.883	7.45	18.707
	SD	5.26	1.17	3.66	1.35	2.32	4.74
	LSD	2.017	0.316	2.045	1.785	0.807	0.855

Explanations: LSD—lowest significant difference, SD—standard deviation at $p < 0.05$; Rp—riparian forest, Mw—grassland; TOC—total organic carbon, CAC—carbon of low-molecular fulvic fraction, TEC—total extractable carbon, CHA—carbon of humic acids, CFA—carbon of fulvic acids, CR—carbon of humin fraction.

3.3. E4/E6 Ratio Results

Another parameter determined for humic acids extracted from the investigated soil was the E4/E6 ratio. The ratio that is calculated to express the degree of humification on the basis of the absorbance, called the color ratio, provides information on the degree of aromaticity and is commonly used in the characterization of organic matter [61,62]. This ratio ranged from 3.16 to 3.90 (Table 3) for the humic acids studied, achieving values typical for this organic matter fraction (5.0) [63]. The E4/E6 relationship was significantly lower for the meadow soils (3.35) than the forest ones (3.79). In general, the lower parameter values are typical for relatively large humic substances with high molecular masses and degrees of humification [64].

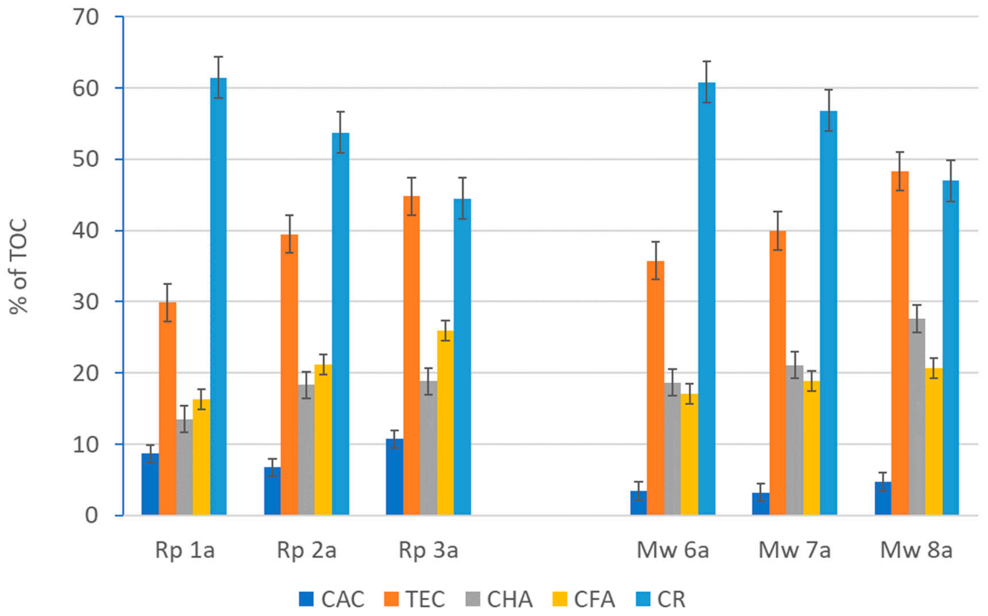

Figure 2. Carbon fractions in the fluvisols (in % of total organic carbon) (depth of sampling 0–10 cm). Error bars represent standard error at $p < 0.05$. Explanations: Rp—riparian forest, Mw—grassland. CAC—carbon of low-molecular fulvic fraction, TEC—total extractable carbon, CHA—carbon of humic acids, CFA—carbon of fulvic acids, CR—carbon of humin fraction.

Table 3. Humification indexes of the Fluvisols (depth of sampling 0–0.1 m).

Factor	Distance from River (m)	HD	HR	HI	CHA/CFA	E4/E6
Mw6a	120	16.37	0.41	21.51	1.09	3.25
Mw7a	150	22.73	0.86	44.97	1.11	3.16
Mw8a	200	26.17	0.66	37.51	1.34	3.65
Rp1a	900	11.86	0.26	11.72	0.83	3.90
Rp2a	950	10.27	0.18	8.38	0.87	3.82
Rp3a	1050	17.87	0.33	13.84	0.72	3.66
	LSD	3.91	0.06	5.60	n.s.	n.s.
	Grassland	21.76	0.64	34.66	1.18	3.35
Land use	SD	4.81	0.20	11.08	0.14	0.33
	Riparian forest	13.33	0.26	11.32	0.81	3.79
	SD	3.66	0.22	2.52	0.07	0.26
	LSD	2.55	0.04	3.64	0.09	0.43

Explanation: n.s.—not significant, LSD—lowest significant difference, SD—standsrd deviation at $p < 0.05$; Rp—riparian forest, Mw—grassland; HD—humification degree, HR—humification ratio, HI—humification index, CHA/CFA—humic acids to fulvic acids ratio, E4/E6—absorbance ratio measured at 465 (E4) and 665 nm (E6).

In our studies, changes in the quantity of humic acids under different water regime influences may originate from two processes: the production of HA precursors and the transformation of native HAs during modification of the soil redox conditions [65].

3.4. Elemental Composition of and Atomic Ratio of HAs

The content of carbon in the HA molecules exhibits a change in the soil-water regime and is connected with an increasing distance from the riverbed (Table 4). Humic acids from periodically flooded soil (120–200 m from the riverbed) contain a lower amount of C (50.00–51.88%) compared to those isolated from unflooded soils located out of the embankment (50.95–52.86%).

Table 4. Elemental composition (in weight percent), atomic ratio and degree of internal oxidation of humic acids from the fluvisols (depth of sampling 0–0.1 m).

Object	C (SD)	H (SD)	N (SD)	S (SD)	O	H/C	O/C	ω
			%					
Mw6a	51.88^{a} (0.16)	5.57^{a} (0.37)	4.99^{a} (0.06)	0.85^{a} (0.01)	36.71	1.29	0.53	0.0208
Mw7a	51.48^{ac} (0.07)	5.62^{a} (0.36)	4.97^{a} (0.04)	0.86^{a} (0.01)	37.06	1.31	0.54	0.0177
Mw8a	50.00^{b} (0.26)	5.58^{a} (0.36)	5.37^{b} (0.05)	0.92^{a} (0.03)	38.13	1.34	0.57	0.0817
Rp1a	50.95^{c} (0.01)	5.80^{a} (0.25)	5.06^{a} (0.05)	0.66^{b} (0.03)	37.54	1.37	0.55	−0.0046
Rp2a	51.90^{a} (0.13)	5.75^{a} (0.36)	4.90^{a} (0.06)	0.67^{b} (0.02)	36.77	1.33	0.53	−0.0247
Rp3a	52.86^{d} (0.08)	6.00^{a} (0.40)	5.39^{b} (0.04)	0.65^{b} (0.02)	38.04	1.36	0.54	−0.0205

Explanaition: ^{a,b,c,d} means followed by the same letter are not significantly different at $p < 0.05$; Rp—riparian forest, Mw—grassland; (SD)—standard deviation, ω—degree of internal oxidation.

A lower amount of hydrogen (although not statistically confirmed) at the same time results in a less aliphatic or more aromatic character of the HA molecules, which is also expressed in a lower H/C ratio. Higher H/C exhibits more condensed aromatic ring or substituted ring structures in the HAs molecules [66]. Periodically flooded Fluvisols covered by the grassland community create a better condition to cumulate organic matter with slow but regular transformation of organic matter compared to the drier soils located out of embankment (Rp1a–Rp3a). These results are in good agreement with the ones obtained from the Vis analysis. The E4/E6 ratios of HAs from periodically flooded soils were characterized by significantly lower values in comparison to those from unflooded soils (Table 3), which is connected with the higher molecular weight of HAs from Mw soils [67]. Negative values of the degree of internal oxidation obtained for the HAs from unflooded Fluvisols (Table 4) confirm the above observation. Young humic acids from organic matter of lower humification degree are often characterized by a lower molecular weight expressed in lower values of the parameter (negative values mean "young" unmatured HAs), as reported by some authors [67,68]. The Van Krevelen diagram presented in Figure 3 exhibits the H/C and O/C atomic ratios of HAs; thus, information on changes in their structure under the influence of different humification conditions can be obtained.

More humified organic matter from the periodically flooded Fluvisols, as indicated by the higher values of humification parameters (Table 2), is at the same time characterized by a more aromatic character of HAs molecules. Humification leads to condensation of unsaturated aliphatic chains and a decrease in hydrogen contents [18,52], as confirmed in our studies.

3.5. FTIR and EPR Analysis

FT-IR spectra presented in Figure 4 exhibit bands typical for humic acids extracted from soil [65–67]. Characteristic stretching of COOH at 1722 cm^{-1} [17] for all investigated samples is week. Although the absorbance mode integrals did not show significant changes between the two types of samples (flooded and unflooded), small differences could be found. Peaks ascribed as aromatic (1620–1630 cm^{-1}), C=C, were slightly more marked for HAs from periodically flooded soils, what is in a good agreement with results presented in Van Krevelen's diagram as well as well the degree of internal oxidation and E4/E6. HA originated from periodically flooded (Mw) soils exhibited slightly higher intensities of aliphatic C-H stretching vibrations (3000–2820 cm^{-1}). Similar results were found by Rosa et al. [69] who described the presence of aliphatic chains in the organic matter from Fluvisols.

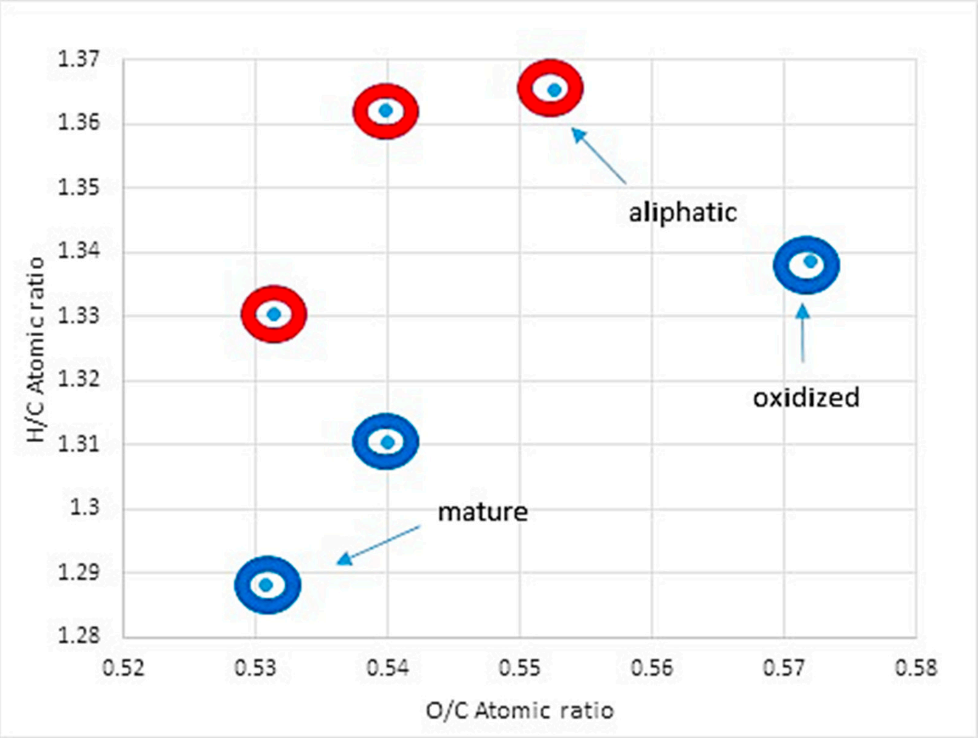

Figure 3. Van Krevelen's diagram of humic acids (HAs) from the flooded and unflooded Fluvisols.

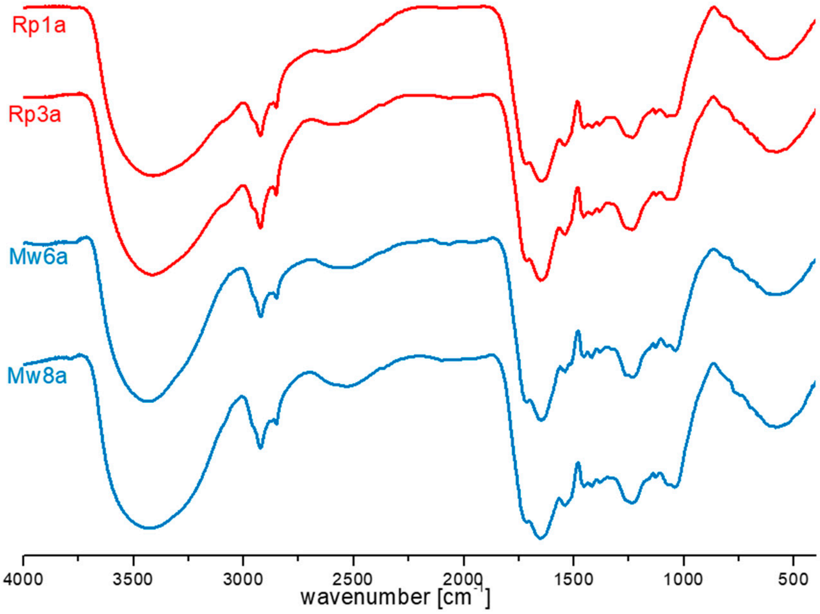

Figure 4. FT-R spectra (transmittance mode) of the humic acids from the flooded (Mw) and unflooded (Rp) Fluvisols.

Valuable information on the type and quantity of semiquinone radicals was provided by the EPR measurements. The obtained results on the diversity of identified radical spin concentrations among studied flooded and riparian soils are presented in Table 5.

Quantitative studies of semiquinone radicals indicate lower values of radical concentration for HAs from flooded Fluvisols compared to those from unflooded soils (Table 5). Statistical analysis—calculated correlation coefficients (Table 6) confirmed that soils with organic matter of higher humification index (HI) (Mw soils) contained HAs of lower radical concentration in comparison to the soils with lower humification index (HI) (Rp soils).

There are several known reasons for the changes in the radical concentration in humic acids. Particularly critical is the influence of the pH, whose changes not only modify the base-acid equilibria but, what is more important, also affect the redox reaction equilibria in the structural units of organic matter [70]. The change of the pH results in the shift of the equilibria: hydroquinone, semiquinone (radicals A, B, and C in Figure 5), quinone, and similarly, phenol, the phenoxyl radical. As presented in Figure 5, the presence of a specific form of quinone depends not only on pH but also on the redox conditions [57,71]. From a very simple polyphenol such as pyrogallol, at least three radicals could be formed depending on the pH and redox conditions, as presented in Figure 5. More complex structures like HA could lead to a much bigger variety of radicals with similar but not the same structures. The most reduced form of the discussed equilibria, hydroquinone, is not paramagnetic, so a decrease in radical concentration is observed when reduced conditions for HA are observed. Additionally, radicals that are still present in the humic acids are protonated (smaller g parameter) [72,73]. While deprotonated radicals (C) are formed at higher pH, an increase in the value of the g-parameter is also observed [74,75]. All forms presented in Figure 5 exist in equilibria that could be shifted [74]. In the studied case for HA of flooded areas, a very small but repeatable decrease of the g parameter is observed, paralleled by a decrease of the radical concentration, which could be an indicator of reduced conditions in the soils of flooded areas.

Table 5. Radicals concentration and values of g parameter (obtained from EPR spectra) of the analysed HAs.

	Spin Concentration per Gram × 10^{17}	**g Parameter**
Rp1a	2.35	2.0034
Rp2a	1.73	2.0034
Rp3a	1.34	2.0034
Average Rp	1.81 (SD 0.5)	--
Mw6a	1.37	2.0033
Mw7a	1.22	2.0033
Mw8a	1.47	2.0032
Average Mw	1.35 (SD 0.13)	

Explanations: Rp—riparian forest, Mw—grassland; (SD)—standard deviation.

Table 6. Correlation coefficients between properties of humic substances isolated from flooded and unflooded soils.

	TEC	**CHA**	**CFA**	**CR**	**HD**	**CHA/ CFA**	**Spin Concentration**	**Alcoholmethoxy**	**Aromatic**	**Attached to Benzene**
Distance from the river	−0.407	−0.622	0.008	−0.342	−0.407	−0.987 *	0.521	−0.972 *	−0.983 *	−0.447
TOC	0.827 *	0.774	0.752	0.8322	0.827 *	0.417	−0.351	0.322	0.410	−0.253
CAC	−0.358	−0.475	−0.105	−0.801	−0.358	−0.835 *	0.336	−0.691	−0.853 *	−0.406
TEC	-	0.954 *	0.881 *	0.384	0.999 *	0.394	−0.592	0.377	0.385	−0.514
CHA		-	0.698	0.367	0.954 *	0.615	−0.555	0.6243	0.578	−0.348
CFA			-	0.336	0.881 *	−0.028	−0.537	−0.085	0.008	−0.675
HI				−0.005	0.501	0.674	−0.822 *	0.579	0.745	−0.031
CHA/CFA					0.394	-	−0.436	0.963 *	0.982 *	0.404
Methylen						0.131	−0.089	0.181	0.197	0.889 *
Alcoholmethoxy							−0.373	-	0.919 *	0.411

Explanations: * significant at $p < 0.05$; TOC—total organic carbon, CAC—carbon of low-molecular fulvic fraction, TEC—total extractable carbon, CHA—carbon of humic acids, CFA—carbon of fulvic acids, CR—carbon of humin fraction, HI- humification index.

Another reason for radical concentration changes is interaction with metal ions. Some metal ions, such as Ca(II) and Zn(II), are known to increase radical concentration [70,73]. Paramagnetic metal ions like Cu(II), Mn(II), Gd(III), and Fe(II)/(III) cause a decrease in radical concentration in humic substances [74,75]. This is the result of the antiferromagnetic interaction between the unpaired electrons of the metal ion and the radical. This phenomenon could additionally explain the decrease in radical concentration in HA from Mw because of the higher content of Cu(II) of anthropogenic origin in the soils located inside the embankment (flooded Fluvisols) compared to the soils from riparian forests. The area of the research is located in the middle Odra valley, which carries a bag of pollutants from PCC Rokita, a manufacturer of specialized chemical products and industrial formulations. That was reported in the previous paper by Kawałko and Karczewska [76].

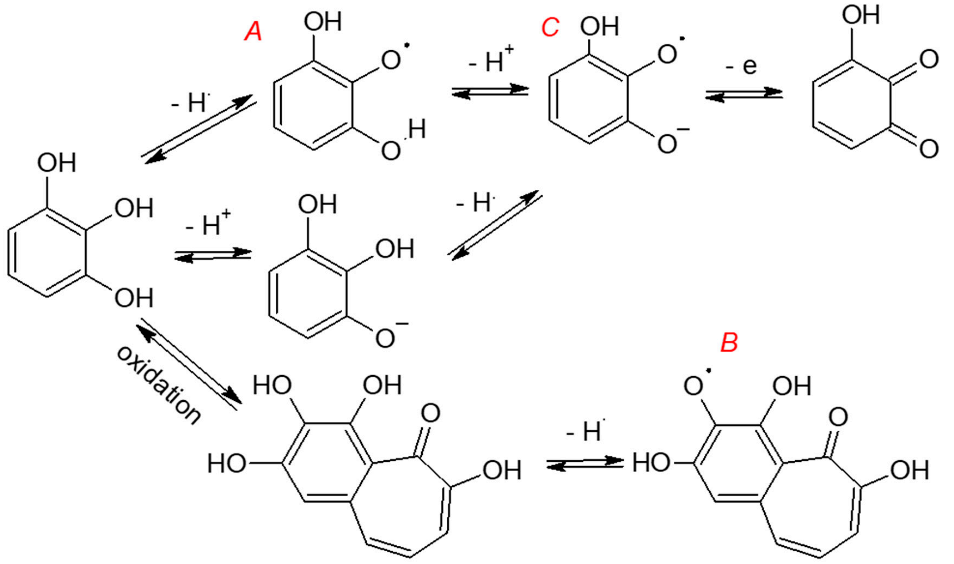

Figure 5. Formation of semiquinone radicals, illustrated for dehydrogenation of pyrogallol. A,B,C—radicals.

3.6. ^{1}H NMR Results

^{1}H NMR spectra of all studied HA samples show a typical pattern (Figure 6) for that kind of substance [77–80]. The ^{1}H NMR chemical shift is mainly divided into five regions [53]: 0–1.77 ppm the terminal methyl of polymethylene chain protons; 1.77–2.33 ppm methylene group protons; 2.33–2.8 ppm groups attached to benzene ring protons; 2.8–4.38 ppm protons on carbon connected to oxygen (or nitrogen) (mainly carbohydrates, organic amines, and methoxy substances); aliphatic ring H proton of alcohol methoxy groups; and 6–8.27 ppm aromatic ring protons. The proton types are also presented on the spectra (Figure 6). The region of 4.6–4.8 ppm is the water shift and was not considered in the calculations. In the current study, samples exhibit various degrees of absorption in these five chemical shift regions. The obtained results are presented in Table 7.

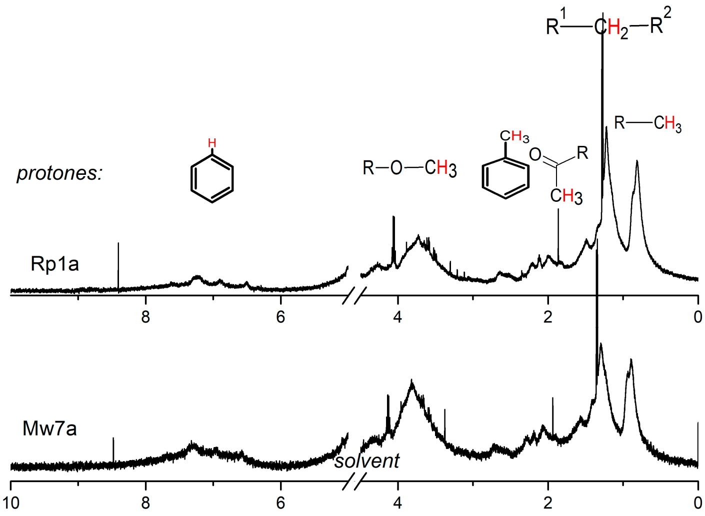

Figure 6. ^{1}H NMR spectra of the HA from riparian forest (Rp1a) and grassland soil (Mw7a).

Table 7. Integrated areas of ^{1}H NMR spectra. Integrals of methylene groups protons bands are equated to 1.

Proton Type	Methyl	Methylene	Attached to Benzene	Alcohol Methoxy	Aromatic
			ppm		
	0–1.77	1.77–2.33	2.33–2.8	2.8–4.38	6–8.27
Rp1a	1	0.19	0.06	0.47	0.03
Rp2a	1	0.12	0.02	0.37	0.06
Rp3a	1	0.16	0.03	0.35	0.01
Mw6a	1	0.18	0.07	0.66	0.28
Mw7a	1	0.24	0.07	0.67	0.23
Mw8a	1	0.10	0.02	0.71	0.21

Explanations: Rp—riparian forest, Mw—grassland.

Common characteristics of the ^{1}H NMR spectra were the highest abundance of peaks at ranges 0–1.77 ppm and 1.77–2.33, confirming the previously stated large number of aliphatic domains and carbohydrates in HA samples. Predominance of aliphatic structures in all studied samples, regardless of the soil origin (floodplains or out of embankment). HA extracted from Mw soil samples in flood-prone areas close to the riverbed exhibited an increased relative content of aromatic and methoxy protons. The methoxy groups in HA are mostly connected to aromatic structures [81], so the increased intensities of both peak areas corroborate that fact. Significantly high correlation coefficients between distance from the riverbed and content of aromatic and methoxy protons (-0.985 and -0.9722, respectively), (Table 6), confirmed the obtained results. They are in good agreement with the results of the elemental and Vis analyses as well. In floodplain areas where anaerobic conditions could be periodically observed, organic matter transformations are temporarily slowed down, thus further oxidation of -OCH_3 does not occur. Defragmentation of the lignin methoxy groups is an important stage of its natural decomposition [82]. The presented results show that in the periodically flooded areas, soil samples do not lose as many -OCH_3 groups as in the areas where anaerobic conditions are not observed.

4. Conclusions

Periodically flooded Fluvisols cumulate organic matter, with a slow transformation of organic matter towards humic substances with a predominance of aliphatic structures. However, on the basis of the obtained results, it can be concluded that humic acids from the periodically flooded soils are characterized by a less aliphatic or more aromatic character of the molecules with higher molecular weight and an increased relative content of aromatic and methoxy protons compared to the HAs from unflooded Fluvisols. A greater distance from the river results in the formation of less stable organic matter, which contributes less to carbon sequestration. It is therefore necessary to take treatments that will not contribute to changes in the water regime of alluvial soils, which may result in soil drying, increased mineralization, and, consequently, higher CO_2 emissions. Results obtained show that in flooded areas with periodically reductive conditions, humic acids do not lose as many -OCH_3 groups as in better oxidized soils and therefore exhibit a lignin-like aromatic structure. It has been proven that the formation and dynamics of HA transformation may vary due to the water regime in soils. The presented results may provide a better understanding of the processes governing the composition and structural characteristics of soil organic matter fractions in floodplain ecosystems. Future perspectives should be focused on the continuation of the research towards the determination of the impact of flooding period length on the formation and structure of the humic substances in soils in order to determine their stabilization degree in the environment.

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