
SOIL CHEMISTRY

Humic Substances: Hypotheses and Reality (a Review)

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Received June 2, 2021; revised July 19, 2021; accepted July 22, 2021

Abstract—The term humic substances (HS) refers to a group of heterogeneous, dark-colored polydisperse substances found in soils, peats, natural waters, and sediments. It is hypothesized that HSs are formed as a result of degradation and transformation of biomolecules of organic residues and free radical condensation reactions (a process called humification). Humic substances are classified as a special category of natural compounds that are dissimilar to the biomolecules of plant and microbial tissues, and are resistant to biodegradation. On the basis of their solubility in alkalis, HSs are divided into humic acids (HAs, soluble, precipitated at pH < 2), fulvic acids (FAs, soluble at all pH values), and humin (insoluble residue). The review provides a critical analysis of the HS terminology and nomenclature, the method of extraction of HS from natural objects, and the hypotheses describing their formation. The ambiguity and duality of the concept of HSs (specific compounds and the sum of operational fractions) are demonstrated, as well as an arbitrary character of the division of organic matter (OM) into the dark-colored compounds of poorly defined structure (HSs) and the substances with a known structure (non-HSs). The interpretation of HSs as mass products of a secondary synthesis requires revision. The possibility of extracellular free radical reactions in soils leaves no doubt; however, a quantitative contribution of the corresponding products to the natural OM has not been established yet. The traditional alkaline extraction should be considered as a method for isolation of hydrophilic polar substances, while the precipitation with acid as their concentration for further analysis. The historical names of humic fractions (HAs, FAs, and humin) should be retained as well-established generic terms and names of the preparations obtained in a certain way without attaching unique features and specificity to all components of these fractions. The C_{HA}/C_{FA} (or C_{HA}/C_{org}) ratios are simple and convenient indicators of the types of humus reflecting bioclimatic conditions of its formation.

Keywords: natural organic matter, non-living organic matter, soil organic matter, substances of alkaline extracts, humus formation, humus, humification, humic acids, fulvic acids, humin

DOI: 10.1134/S1064229321120164

INTRODUCTION

Soils are the major reservoir of C_{org} in terrestrial ecosystems. Globally, the soil humus stores approximately 1500 Gt C in the upper 1-m layer, which is threefold larger as compared to the aboveground biomass (559 Gt C) [44]. Soils serve as a long-term sink of atmospheric CO_2 , the process of humus accumulation largely formed and still determines the Earth climate. The substances of humus are responsible for the fertility of soils and their biospheric functions. Humus is the basis for terrestrial life — it is “the product of living matter and the source of it” (A.D. Thaer, cited from [3]).

Substances of humus have been studied for more than 200 years, but there is still no consensus on their nature, origin, and mechanisms of accumulation. The debate on humic substances (HSs) as specific and abundant components of humus is continued. The

discussion is of a cyclic character. The basically diverging views characteristic of the first two decades of this century [49, 58, 64, 81, 83, 92, 97, 108], were also typical of the first three decades of the 20th century. “Some researchers have taken the position that humus [humic] substances is a group of peculiar compounds ...formed via complex processes of transformation of organic residues ... and insistently continued to study their properties, origin, and mechanisms of formation. Others believed that humus is a mixture of organic compounds which are the products of decomposition of plant and animal residues, whereas the humic substances were regarded as artificial products formed during their extraction from soil with alkaline solutions” [15, 16]. In the 1930s, the first point of view was supported by Tyurin and Springer [15, 16] while the second one, by Waksman [3].

According to the concept of HSs, these specific dark-colored compounds are formed by decomposi-

tion of biopolymers of organic residues and by free radical condensation reactions (secondary synthesis reactions) and account for 80–90% of humus. The HSs differ in their structure from the substances of known classes (biomolecules), have a high molecular weight, and are resistant to biodegradation. The HSs are divided into three groups according to their solubility in alkalis, namely, humic acids (HAs, soluble and precipitate at $\text{pH} < 2$), fulvic acids (FAs, soluble at any pH values), and humin (insoluble residue). The HSs paradigm was widely developed in the second half of the 20th century [15, 16, 21, 22, 32, 36, 157, 158]. Its promotion was also facilitated by the fact that the leader of “opposition”, microbiologist S. Waksman, switched from humus to the research into antibiotics (the Nobel Prize in Physiology and Medicine, 1952, for the discovery of streptomycin). The views of Waksman and his forerunners, who were skeptical about the specific nature of the HSs (for example, Schreiner and Shorey, 1908–1930; Trusov, 1915, 1916), had largely lost their importance and were put aside. The current revival of these ideas [97, 108, 170] is largely associated with the introduction of high-resolution mass-spectrometry methods (pyrolysis-field ionization mass spectrometry (Py-FIMS), nanoscale secondary ion mass spectroscopy (Nano-SIMS), ion cyclotron resonance mass spectrometry (ICR-MS) and the use of nondestructive methods—nuclear magnetic resonance (NMR) spectrometry and X-ray absorption near edge structure spectroscopy (XANES) in soil organic matter (OM) research. These methods have provided information on structural fragments [92], composition, gross formulas [56, 89], and spatial organization of the substances comprising humus, including the in situ variants [105, 107]. The interpretation of the corresponding data restored the concept that humus is represented by a mixture of microbial and plant biomolecules at different stages of decomposition [49, 92, 169] rather than by specific “chemically unique” [58] compounds formed outside the living organisms via degradation and condensation reactions [15, 31, 157, 158]. It is considered that the accumulation of organic substances in soils is provided by physicochemical, biological, and ecological conditions that limit the rate of decomposition [142] rather than by complexity of molecular structure. The interaction with soil mineral components plays an important role in long-time preservation of C_{org} [103, 169].

In 2015, a paper in *Nature* actually denied the existence of HSs as a special class of natural compounds [108]. The authors of this paper proposed the “soil continuum model” (SCM), stating that the soil OM is a continuum of biomolecules at various stages of decomposition leading to a decrease in molecular weights and an increase in solubility. The authors of the SCM negate a *de novo* formation of high molecular weight and stable “HSs” and any reactions of extracellular secondary synthesis, relating a characteristic dark color of soil extracts to either alkaline treatment or to

the presence of pigments (including melanins) and products of abiotic reactions [97, 108]. The entire humic terminology is also criticized as well as the alkaline extraction as the method for isolation and analysis of natural OM that creates artifacts [97, 108]. This paper evoked a wide response and brought forth to a new wave of the historical debate on humus. As a result, several reviews were published, supporting HSs [58, 64, 81, 83], alkaline extraction as a method for studying natural OM [121], and secondary synthesis reactions generating the new structures initially absent in the living organisms [55].

The problem of HSs can be divided into three main issues:

- (1) Terminological—what is to be referred to as HSs, whether it is adequate to separate humus into HSs and non-HSs, and whether the term HSs should be retained for characterizing natural OM;
- (2) Fundamental—what are the substances that make up soil humus and humic material of other environments; whether an extracellular secondary synthesis lead to the formation of compounds initially absent in the tissues of living organisms, and what is their contribution to the natural OM;
- (3) Practical—which properties of natural OM can be adequately studied using the traditional alkaline extraction.

The review aims to provide a critical analysis of the above mentioned issues related to HSs research avoiding a detailed description of the overall set of corresponding data, which has been more or less comprehensively reviewed in recent international [45, 55, 58, 64, 81, 83, 121, 140] and Russian [12, 29, 30] literature. In this review, we consider the problems related to the HS terminology and nomenclature (historical retrospective included), the problems related to the secondary synthesis reactions, to the concept of HSs stability and their molecular weight organization, because these issues underlie the separation of HSs into a special class of geomolecules. The discussion mainly deals with the alkali-soluble substances since they are most frequently grouped under the term HSs [121].

THE CONCEPT OF HUMIC SUBSTANCES AND HYPOTHESES OF HUMIFICATION

Before discussing the controversial issues related to HSs, it is purposeful to initially dwell on the concept of HS in its traditional form [15, 16, 22, 21, 31, 157, 158].

According to the humic nomenclature (Fig. 1), the soil organic matter is divided into organic residues and humus; the latter is defined as dark-colored finely dispersed and amorphous OM or the total of the organic compounds in soil exclusive of those in living organisms and their tissues that preserve the anatomic structure [21, 158]. Humus is further divided into nonspecific compounds (non-HSs), i.e., the substances belonging to the known chemical classes (lignin, pro-

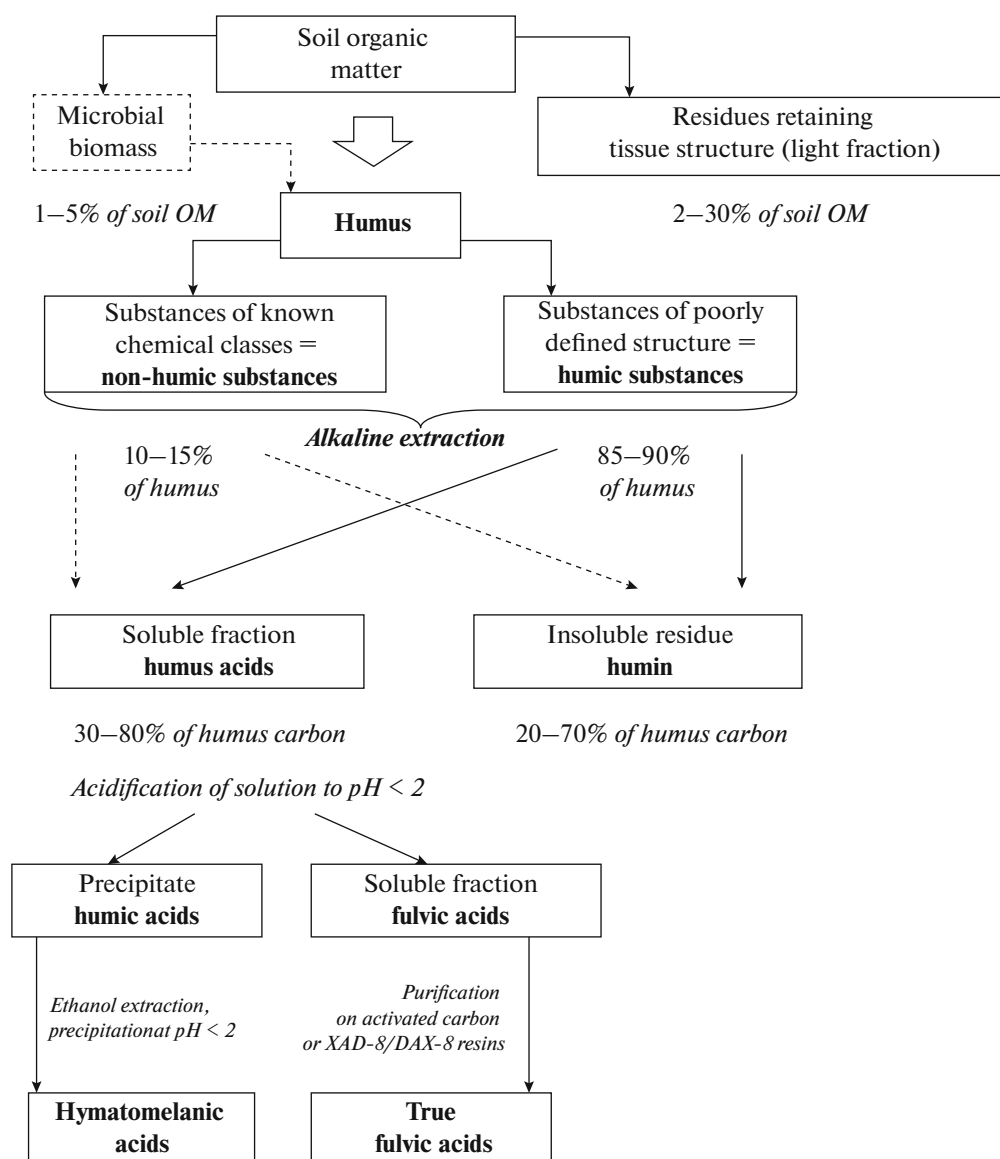


Fig. 1. Traditional scheme of the nomenclature of soil OM with approximate contents of the components in soils. It shows the discrepancy between separation of HSs into a special category of substances that have no analogues in the living organisms and the alkaline extraction, which is applied to humus as a whole. Both the biomolecules (substances of known chemical classes) and the products of their transformation pass into alkaline extract. The share of the substances with a known structure in humus can be considerably larger than 10–15% [15] taking into account the data on a considerable contribution of individual hydrophobic substances to humin [80, 82].

teins, lipids, and so on) and the compounds of poorly determined structure, HSs [15, 21, 22, 31, 157, 158]. According to solubility, the HSs are divided into HAs, FAs, and humin [21, 81, 157]. Treatment of the fresh HA precipitate with ethanol gives hymatomelanic acid, while “generic FAs” can be obtained from the FA fraction using the adsorption on either activated carbon [26] or XAD-8 [163] and DAX-8 [128] resins. Aquatic HS contain only HAs and FAs (www.humic-substances.org).

Any commonly accepted definition of HSs is absent. According to Orlov et al. [25], HSs are formed

during decomposition and transformation of plant and microbial residues and have no analogues in the living organisms; they are dark in color, polydisperse, of high molecular weight and high biothermodynamic stability. Other authors propose similar definitions of HSs as a general category of relatively high molecular weight and recalcitrant compounds, being yellow to black in color and differing in their structure from compounds of known chemical classes (Table 1). According to traditional views, HSs account for 80–90% of C_{org} in humus (the remaining part are the substances of known classes) and largely determine fertil-

Table 1. Terminology used in the chemistry of humus

Term	Definition	Author(s)	Reference
Natural organic matter, NOM	A complex mixture of thousands of organic compounds found in water, soils and sediments that was naturally formed from the residues of plants, microorganisms and animal matter at various stages of the decaying process. In soils, it appears as soil organic matter (SOM) and in surface water mostly as dissolved (DOM) or particulate organic matter (POM)	Knicker et al., 2018 (p. 1209)	[101]
Soil organic matter, SOM	All organic compounds present in soils in a free state or as organomineral compounds except for the substances within living organisms; SOM comprises both the organic residues (plant and animal remains that in part retain their initial tissue structure) and individual organic compounds of a specific or a non-specific nature; SOM distinctly falls into two large groups: organic residues and humus	Orlov, 1992 (pp. 185, 186, and 188)	[24]
	The whole of the organic materials in soils, including litter, light fraction, microbial biomass, water-soluble organics, and stabilized organic matter (humus)	Stevenson, 1994 (p. 6)	[158]
	All the organic compounds of a soil: (1) living biomass (intact plant and animal tissues and microorganisms); (2) dead roots and other recognizable plant residues or litter; (3) a largely amorphous and colloidal mixture of complex organic substances no longer unidentifiable as tissues	Brady and Weil, 2008 (p. 510)	[48]
	The sum of all natural and thermally altered biologically derived organic materials found in the soil or on the soil surface irrespective of its source, whether it is living or dead, or stage of decomposition, but excluding the aboveground portion of living plants	Baldock and Broos, 2011	[43]
	Multicomponent, heterogeneous, and polyfunctional continuum of individual particles and ensembles of biomolecules of partially or completely transformed residues of biota, which differ in size, weight, chemical structure, age, nutritive quality, and availability to microorganisms; have different nature and stability of internal and external chemical bonds; and are characterized by a three-dimensional configuration and spatial inhomogeneity of their arrangement in the conglomerate of mineral particles	Semenov and Kogut, 2015	[29]
	Should include live, dead, and decaying materials in soil	Kleber and Lehmann, 2019	[96]
	Heterogeneous mixture of all organic components found in soil which can be subdivided into two groups with different morphological and chemical characteristics: (1) unaltered materials that include fresh organic debris and nontransformed components of older debris; (2) transformed products, or humus, that bear no morphological resemblances to the structures from which they were derived. These transformed are referred to as humified products. They consist of both humic and nonhumic substances	Hayes and Swift, 2020	[83]

Table 1. (Contd.)

Term	Definition	Author(s)	Reference
Humus	The same as humus	Stevenson, 1994 (p. 6 and 33); Piccolo, 1996 (p. 225); Huang and Hardie, 2009 (p. 43)	[132, 158] [88]
	Organic matter of the soil as a whole (note that undecayed plant tissues are excluded from soil organic matter)	Waksman, 1937	[3]
	Part of the organic substances of soil that lost the anatomic structure of the initial plant residues, has undergone humification processes, and forms humus horizons uniformly coloring dark their mineral mass	Aleksandrova, 1980 (p. 34)	[1]
	All the organic compounds found in soil excluding that in the living organisms or in associations that retain the anatomical structure (tissues of living organisms). Humus is composed of specific humic substances, nonspecific organic compounds, and intermediate products of degradation and humification in a free form or as organomineral compounds	Orlov, 1992 (p. 188)	[24]
	The term has a restricted meaning and refers to humic substances plus resynthesis products of microorganisms that have become stabilized, and are thus an integral part of the soil	Stevenson, 1994 (p. 32)	[158]
	Total of the organic compounds in soil except for undecayed plant and animal tissues or their partial decomposition products (i.e. light fraction), and the soil biomass	Stevenson, 1994 (p. 33)	[158]
	A largely amorphous and colloidal mixture of complex organic substances no longer identifiable as tissues	Brady and Weil, 2008 (p. 510)	[48]
	Organic materials <53 µm remaining after removal of particulate organic matter and dissolved organic matter	Baldock and Broos, 2011	[43]
Nonhumic substances	Subsystem of soil organic matter which is formed from organic materials and compounds of plant, animal, and microbial origins that have undergone humification and non-humification stages of stabilization with the complete decomposition time of the constituents over 10 years	Kogut et al., 2021	[14]
	Substances of a known structure, individual compounds Compounds belonging to known classes of biochemistry (amino acids, carbohydrates, fats, waxes, organic acids)	Orlov, 1992; Stevenson, 1994 (p. 33)	[24] [158]
Humic substances	A complex of organic substances of brown, gray brown, or yellow color extracted by the alkaline solutions, neutral salts, or organic solvents	Kononova, 1963	[15]
	A general category of naturally occurring, biogenic, heterogeneous organic substances that can generally be characterized as being yellow to black in color, of high molecular weight and refractory	Aiken et al., 1985 (p. 4)	[36]

Table 1. (Contd.)

Term	Definition	Author(s)	Reference
	More or less dark-colored nitrogen-containing compounds of high molecular weight, characteristic products of soil formation; represented by humus acids (most characteristic substances), prohumic substances (of the type of “young” humic-like products formed in laboratory experiments in culture media), and humin	Orlov, 1992 (p. 189)	[24]
	Total of the organic compounds formed during decomposition and transformation of plant and microbial residues, that have no analogues in the living organisms. They are dark in color, polydisperse, of high molecular weight and high biothermodynamic stability	Orlov et al., 1996	[25]
	A series of relatively high-molecular weight, brown to black-colored substances formed by secondary synthesis reactions: the term is used as a generic name to describe the colored material or its fractions obtained on the basis of solubility characteristics; these materials are distinctive to the soil (or sediment) environment in that they are dissimilar to the biopolymers of microorganisms and higher plants (including lignin)	Stevenson, 1994 (pp. 32–33)	[158]
	Humic substances are comprised of huge molecules with variable, rather than specific, structures and composition. They are generally dark-colored, amorphous substances with molecular weights varying from 20000 to 300000 g/mol. Because of their complexity they are highly resistant to microbial attack.	Brady and Weil, 2008 (pp. 511–513)	[48]
	Dark-colored substances resistant to biodestruction and formed as a result of Dark-colored, biologically refractory, heterogeneous organic compounds produced as by product of microbial metabolism; they differ from the biomolecules present in humus because of their long-term persistence and their molecular architecture. Emerge from a slow process of biological decomposition, oxidation and condensation as characteristic organic mixtures having two fundamental properties: (1) supramolecular association (self-organized assemblies of low molecular mass compounds; (2) biomolecular provenance—identifiable biopolymeric fragments that form an integral part of a labile molecular architecture and that govern both conformational behavior and chemical reactivity	Sposito, 2008 (p. 70)	[155]
	Biogenic, chemically active, and stable in nature heterogeneous continuum of biomolecules that forms polymeric supramolecular ensembles via random chemical transformations and nonvalent interactions of manifold precursor molecules	Semenov et al., 2013	[30]
	A series of relatively high-molecular weight substances yellow to black-colored substances formed by secondary synthesis reactions in soils	Soil Science Society of America, 2019	https://www.soils.org/
	Specific components formed during the humification process which can be isolated and fractionated in a variety of ways... Amorphous brown-colored humic components differentiated on the basis of solubility properties into humic acids (HAs), fulvic acids (FAs), and humins.	Hayes and Swift, 2020 (p. 4)	[83]

Table 1. (Contd.)

Term	Definition	Author(s)	Reference
Humus acids	A series of highly acidic, relatively high molecular weight, and yellow to black colored substances formed by biochemical and chemical reactions during the decay and transformation of plant and microbial remains (a process named humification)	IHSS definition of December 30, 2020; see [58]	[58]
	A complex and heterogeneous mixture of polydisperse materials formed in soils, sediments, and natural waters by biochemical and chemical reactions during the decay and transformation of plant and microbial remains (a process called humification)	IHSS definition of June 01, 2021	[90]
	A special class of organic compounds formed during humification of organic residues	Aleksandrova, 1980 (p. 35)	[1]
	A special class of organic compounds, the main and specific products of humification (p. 208); they are represented by nitrogen-containing high molecular weight hydroxycarboxylic acids with intensive brown or reddish-brown color (p. 190); heterogeneous and polydisperse (p. 232); the polydispersity is true (due to molecules of different sizes) and secondary (formation of supramolecular associations by hydrogen bonding or intermolecular interaction; p. 233). Humus acids are extractable from soil with alkaline solutions and are divided according to solubility into humic acids, humatmelanic acids, and fulvic acids (p. 190)	Orlov, 1992	[24]
Humic acids	A group of humic substances soluble in alkalis that is separated of the components of alkaline extract by acidification to pH 1–2; the most typical components of humus	Orlov, 1992 (p. 190)	[24]
Humatmelanic acids	Fraction of humic substances that is insoluble in water under acid conditions (below pH 2), but becomes soluble at greater pH values	Aiken, 1985 (p. 5)	[36]
	The dark-colored organic material that can be extracted from soil by dilute alkali and other reagents and that is insoluble in dilute acid	Stevenson, 1994 (p. 33)	[158]
	The substances that pass into solution after ethanol treatment of the fresh precipitate of humic acids; give cherry red solution (p. 190)	Orlov, 1992 (p. 190)	[24]
Fulvic acids	Alcohol-soluble portion of humic acid; soluble in alkali, precipitated by acid, soluble in alcohol	Stevenson, 1994 (p. 33)	[158]
	This term is used in two senses: on the one hand, as the sum of acid-soluble organic substances that remain in solution after precipitation of humic acids and on the other hand, “proper fulvic acids”, i.e., the humic acids soluble in water, alkaline, and acid solutions, that can be isolated by adsorption on activated carbon	Orlov, 1992 (p. 190)	[24]
	Fraction of humic substances that is soluble under all pH conditions	Aiken, 1985 (p. 5)	[36]
	Fulvic acid fraction—fraction of soil organic matter that is soluble in both alkali and acid. Generic fulvic acid—pigmented material in the fulvic acid fraction	Stevenson, 1994 (p. 32)	[158]

Table 1. (Contd.)

Term	Definition	Author(s)	Reference
Humins, non-hydrolyzable residue	A heterogeneous group of [soil] organic compounds dissimilar to other groups of HS by insolubility in alkalis. Presumably, contains the humus acids intimately bound to mineral soil matrix, decarboxylated humic substances, nonhumic substances, and alkali-insoluble organic compounds	Orlov, 1992 (p. 189)	[24]
	Fraction of humic substances that is not soluble in water at any pH value	Aiken, 1985 (p. 5)	[36]
	Alkali insoluble component of humus	Stevenson, 1994 (p. 197)	[158]
	Mixture of largely identifiable biological molecules derived from plant materials composed predominantly of resistant non-polar moieties in intimate associations with, and protecting some biodegradable biomolecules, all in intimate associations with the soil mineral colloids	Hayes et al., 2010	[84]

ity of soils and their biospheric functions [15, 20, 157]. The stability of soil HSs have been explained by their complex condensed structure [15, 22, 31, 36] as well as by the interaction with soil mineral components [1, 22, 158]. It was assumed that accumulation of HS within humus determines a long-term residence time (hundreds and thousands of years) of C_{org} in soils [21, 158].

According to the concept of HSs, these compounds are abundant not only in soils, but also in composts, peats, brown coals, natural waters, and bottom sediments [20, 27, 36]. However, the chemical composition of dead tissues and the conditions of their transformation in these environments are different. The main starting material for the HSs in terrestrial ecosystems are lignified plant residues. This determines a high degree of aromaticity of the HAs and FAs of terrestrial origin; their formation and degradation processes are mainly aerobic [1, 3, 15, 22, 158]. The HSs of bottom sediments are formed under oxygen deficiency from the residues of aquatic organisms lacking lignin. The marine HAs and FAs, accounting for 10–50% of the C_{org} in seawater and known as the marine HSs, are largely composed of phospholipids and polyunsaturated fatty acids [75], and are considered to be formed during oxidative crosslinking of two and more unsaturated lipids [76]. Yellow marine HS have little in common with the soil HAs and FAs. Thus, the term HSs is used in relation to quite dissimilar products of decomposition of organic residues, soluble or insoluble in alkalis.

The humus acids (HAs and FAs) of terrestrial origin is the best studied group among the entire range of HSs. These compounds are enriched in ionizable functional groups, first and foremost, carboxyl and phenolic ones; contain heterocyclic and amide nitrogen (N_{tot} , 3–6%), and are considered as the most

important and most chemically reactive representatives of HS [21, 121]. The sum of HAs and FAs in soils accounts for 40–70% of C_{org} [21, 158] and in freshwater they comprise about 50% of the dissolved organic materials (www.humic-substances.org). According to Tyurin, the group of HAs is the most typical with respect to its relative content in humus and its absolute amount in soil; HAs account for 10 to 40% of the total humus content with maximum values in chernozems [31]. The C_{HA}/C_{FA} ratio in soils varies from <0.5 to >2 and reflects the conditions of humus formation [15, 21, 32]. As for the HA structure, it has been widely accepted that these substances are nitrogen-enriched heteropolymers with an aromatic backbone and an aliphatic “periphery” [30]. A number of hypothetical structural models of HA have been proposed on the basis of structural components determined after the hydrolysis and oxidative degradation of substances in alkali extracts [21, 158].

Hypotheses of humification. The formation of HS is attributed to humification, which is an intricate process of decomposition of organic residues largely driven by the activity of biota [1, 15, 21, 31, 158]. Humification takes place in all environments [20] and under all climatic conditions except for glacial ones [4]. However, the processes that accompany the transformation of the initial components of dead tissues into dark-colored HSs are rather vague. They are an area of hypotheses largely limited by an aerobic branch of destruction [15, 21, 158].

According to the traditional views, the humification in soils include hydrolytic and oxidative degradation and transformation of biopolymers as well as the extracellular condensation reactions (secondary synthesis), generating N-containing heteropolymers. The condensation reactions are regarded as specific of

humification [15, 158]. These reactions include the interaction of phenols oxidized to phenoxy radicals and quinones with the N-containing compounds as well as sugar—amino acid condensation (Maillard reaction) [158]. In the first case, oxidized lignin, low molecular weight products of its degradation or low molecular weight metabolites of plants and microscopic fungi can enter the reaction (see polyphenol theory by Flaig and Kononova and the works on fungal humus by Martin and Haider) [140, 158]. Extracellular phenol oxidases and peroxidases of microorganisms are the key biotic catalysts in the oxidation of phenols [1, 15, 158] although the abiotic catalytic reactions involving iron and manganese ions in soil minerals are also possible [88]. According to the condensation hypothesis in its initial form, the process of humification comprises two stages, namely, decomposition of plant residues to simpler monomeric compounds and after that synthesis of more complex substances, HSs (Williams, 1914; cited from [16]). This approach (first, decomposition and then, synthesis) was further developed by Kononova [15, 16]: as is assumed, HSs are represented by a system of the macromolecules formed *de novo* via the condensation of monomers. Thus, lower molecular weight FAs are the first to be formed followed by higher molecular weight HAs.

The theories relying on degradation of biopolymers are more popular as compared with the theories of monomer condensation. A large contribution of the oxidative degradation processes to the formation of humified OM is the point of general consensus among researches supporting [21, 143, 157] and opposing [108, 142] the humic concept. Degradative theories include the biodegradation of lignin (for example, [1]) and other relatively stable plant (cutin and suberin) and microbial (e.g. melanins) macromolecules (Hatcher and Spiker, 1988; cited from [158]). According to Aleksandrova [1], humification process includes lignin demethylation, formation of carboxyl and phenolic groups (“oxidative acids formation”), and depolymerization reactions giving first HAs and then FAs. The decomposition of biopolymers results in a decrease in their molecular weights, being accompanied by the interaction of the degradation products with soil mineral components. According to the theory of Hatcher and Spiker (1988), the stable microbial and plant biopolymers selectively accumulate in soils and act as HS precursors. The hydrophobic substances accumulate in the humin fraction and their oxidation gives HAs and then FAs; the latter are regarded as the most humified fraction of HSs (cited from [158]). In general, it is considered that the degradation and condensation reactions can run concurrently; however, the humification process has one principal direction, i.e., accumulation of the compounds that are most stable under given bioclimatic conditions [21, 31].

There is no universally accepted definition of what are HSs; their structure is poorly defined and mecha-

nisms underlying their genesis are hypothetical; the HS concept is strongly associated with secondary synthesis reactions [15, 108, 158, 170] and alkaline extraction, leading to artifacts [81, 97, 121]. Nonetheless, “the decomposition of organic residues leads to formation of a specific group of dark-colored substances” ([3], p. 101). The questions arise on what are these substances as well as whether it is reasonable to distinguish them into a special category of geomolecules and to divide the components of humus and humified OM into HSs and non-HSs?

THE PROBLEMS IN TERMINOLOGY AND NOMENCLATURE

The problem of HSs begins with the problems in their terminology. According to Kleber and Lehmann [97], “to enable constructive debate, it needs to be clear what is being debated”. Indeed, “no other phase of chemistry has been so much confused as that of humus, as a result of which it frequently becomes necessary to lay considerable emphasis upon the proper definition of the terms used” [3, p. V]. The origin of the terms and the definition of their meanings are directly related to the understanding of what are the HSs.

Humus and humic matter. When using the term HSs, it is necessary to define the substrate from which they are isolated. The term “humic substances” originates from *humus* (the Latin word for land or soil). However, different researchers understand the term humus in different ways. Most frequently, humus is the total of organic compounds in soil exclusive the microbial biomass and the residues preserving their tissue structure [21, 158]. The substances constituting humus can be in a free form or as organomineral compounds [21]. Tyurin included microbial biomass into humus [31], which draws objections in terms of the nomenclature [1, 21, 29] but is justified with a practical standpoint since it is almost impossible to separate the biomass. In addition, the carbon of microbial biomass is an insignificant contributor to humus, accounting for less than 4% of C_{org} in humus (cited from [58]).

There is still no consensus on the genesis of humus. Some researchers believe that humus is a purely soil product [1] formed within the soil [29] and others, that humus is ubiquitous in nature wherever organic residues are subject to aerobic and anaerobic decomposition, be it soil, compost, peat bogs, or water bodies [3]. The latter opinion looks rather more compliant with the concept of HSs as the substances ubiquitous in the biosphere and originating from the word “humus” [20, 27, 36].

The terms humus and soil OM are frequently regarded as identical. This dates back to Waksman: having defined humus as “a complex aggregate of brown to dark-colored amorphous substances which have originated during the decomposition of plant and

animal residues by microorganisms” ([3], p. 5), he proposed to use this name to designate the organic matter of the soil as a whole ([3]). Correspondingly, the terms humus and soil OM are frequently used as synonyms [58, 88, 158]. This brought about certain confusion since the term “soil OM” is regarded as the total of the organic compounds present in a soil including the litter, undecayed organic residues, and humus [1, 24, 97, 158], occasionally including the microbial biomass [158] (Fig. 1).

Regarding the HSs as the compounds ubiquitous in the non-living OM [20, 27, 36], it is necessary to keep in mind that these substances are a characteristic part of “humified” OM which is formed during decomposition of the dead tissues and lose with time visual structural similarity to these tissues. It is necessary and purposeful to distinguish this humified part from the standpoint of its composition, functional and physico-chemical properties, which considerably differ from the properties of undecayed residues [21]. The question on how we name this part, be it “humus” *sensu lato* according to Waksman (the humus of soils, peats, and water bodies) or “humic matter” of soils, peats, etc., if consider humus as a purely soil product, is still an open issue of terminology.

Humification. There is much confusion associated with the term “humification” as well as with the remaining HS terminology. Initially, the term humification belongs to the process of transformation of organic residues into humus [3, 82]. Correspondingly, all substances of humus, both belonging to known classes and HSs, were regarded as humified ones [82]. However, the following objections emerged: it is unclear why proteins, enzymes, and polysaccharides (all belonging to the substances of a known structure) become humified only because they are present in humus (Oades and Ladd, 1977; cited from [97]). In addition, once humification is the formation of humus, it is not clear what is the difference between the processes of humification and humus formation [12, 28]. Two terms designate one and the same phenomenon. On the other hand, formation of only specific substances—HSs in general [20, 90], HAs plus FAs [24], or HAs alone [24]—is regarded as humification. Once again, it is unclear what are the differences between the transformation of organic residues into HAs and FAs and the transformation of these residues into the substances of humus in general. Presumably, this gave rise to a narrow interpretation of humification as the secondary synthesis reactions, also referred to as “humification reactions” [108, 170]. In a classical meaning [15], this is the synthesis of high molecular weight and stable compounds from low molecular weight degradation products. This interpretation of the term leads to denial of the HS existence as quantitatively significant products of this process [97, 108].

Presumably, it is reasonable to define humification as the process of transformation of the tissues of dead

organisms into colloidal dark-colored material (humic matter) in different natural environments.

Humic acids and humin. In terms of the debate on humus, the question arises on why the concept of HSs and their classification are so tightly associated with alkaline extraction. Note that the terms HAs, FAs, and humin are primary, whereas the term HSs is secondary. The research into HSs dates back to Achard (1786), who treated peat with alkali and got a dark-colored solution. The alkaline solution was later named as humus acid (Döbereiner, 1822) and the dark precipitate obtained upon addition of a mineral acid as humic acid (Sprengel, 1826; cited from [3]). These substances were referred to as acids because of their negative charge and the ability to react with bases to form salts, “humates”. The dark-colored substances insoluble in alkalis were named humin (Berzelius, 1839; cited from [3]). Berzelius also succeeded in isolating light-yellow substances from the mineral waters and bog silt rich in iron (1833) as well as from a decaying elm tree (1839). These substances, soluble in water and alcohol and precipitated by copper acetate were called crenic and apocrenic acids. Following the studies by Tyurin (1940), crenic and apocrenic acids were replaced by the term FAs (from *fulvus* for yellow color in Latin) proposed by Auden (1912–1919) to designate the water-soluble substances of peat. The term hymatome-lanic acid as introduced by Hoppe-Seyler (1889, cited from [3]) is still retained for the fraction isolated by alcohol from fresh HA precipitate [26, 157, 158].

The very method used to isolate HSs demonstrates that they are heterogeneous preparations rather than specific compounds. However, the researches of the first half of the 19th century believed that the isolated “acids” were individual substances and gave them their individual names according to either their source or the method used for their isolation (for example, mucic acid, fumic acid, and ulmic acid [3, 16]). The names of these “acids” were used in the singular. Humus formation was regarded exclusively in terms of chemistry as oxidation and dehydration of individual plant substances because microbiology as a science was absent at that time [3, 16]. Along with the isolation of natural HSs, it was attempted to obtain these substances artificially, mainly from sugars via their treatment with concentrated acids and alkalis (see for example, Germann, 1837; Braconnot, 1819; Mulder, 1839; cited from [3]). As early as that time, the fact that chemical treatment gave dark-colored substances was the cause that induced the continuing discussion on whether the natural HSs are an artifact of alkaline extraction or not [97, 121].

At the end of the 19th century, the development of microbiology and biochemistry brought about the data on the role of microorganisms in the decomposition of organic residues and demonstrated the inhomogeneity of the substances extracted by alkalis. It was shown that xylan and oxidized lignin derivatives

accounted for an important part of the group of substances dissolved in alkalis and precipitated by acids (Hoppe-Seyler, 1889; cited from [3]). Over 40 individual compounds were isolated from humic “acids” (Schreiner and Shorey, 1909–1913); it was postulated that “the idea that numerous plant constituents are in some mysterious way transformed into a single group of closely related bodies, called “humic acids” was entirely wrong” ([3], p. 46). Thus, the current views on the substances of alkaline extracts as biomolecules at different stages of decomposition [92, 97] are far from being new, dating back to over a century ago. Summarizing the research into humus starting from 1786 (the study by Achard), Waksman proposed to “abandon without reservation the whole nomenclature of ... numerous acids that designate not definite chemical compounds but merely certain preparations which have been obtained by specific procedures” ([3], p. 62). This recommendation failed to cancel humic terminology; however, the names of “acids” began to be used in plural as group terms. According to Tyurin, the term “humic acids” should imply “a group of high molecular weight compounds with rather different composition but possessing common properties and ... a common type of structure” [31, p. 116]. The concept of HAs and FAs as groups of related specific compounds was retained as the central in the humic theory. To be sure when ascribing compounds to the class of soil humic acids, Orlov [22] proposed the combination of five characteristics: (1) elemental composition (46–61% of C in HAs, 36–45% of C in FAs, and 3–6% of N); (2) obligatory presence of benzene polycarboxylic acids, containing 3–6% of “heterocyclic” nitrogen; (3) the presence of “nonhydrolyzable” nitrogen (25–55% of the total nitrogen), part of which is represented by the nitrogen of heterocycles; (4) characteristic patterns of electron absorption spectra and the extinction

coefficients $\left(E \frac{0.001\%}{465 \text{ nm}} \right)$ of approximately 0.01–0.2;

and (5) characteristic set of bands in the IR spectra. However, the common character of the averaged properties can be interpreted in another way, namely, as that the properties of the substances constituting the humic fractions reflect the changes in the initial OM with time during humification [121]. They also reflect the specific features of the substances constituting humic material. For example, the elemental composition close to the soil HA fraction (55–58% of C and 3–6% of N) is also characteristic of humus as a whole [68]; the nitrogen of heterocycles may well be part of microbial metabolites (melanins [65, 120]) and melanoids [88]; the visible spectra similar to HAs are also characteristic of polyphenols, for example, gallic acid [59] and may also result from an alkaline treatment of a mixture of phenolic acids (Zavarzina and Demin, unpublished data). Thus, these diagnostic features of HAs do not prove the existence of HAs and FAs as groups of related compounds. Nonetheless, HAs are the

most characteristic and, perhaps, the only group among the compounds constituting humus that is unambiguously ascribed to HSs.

As for the FAs, it has been repeatedly proposed that the acid-soluble fraction obtained after HA precipitation is a set of individual substances and a product of partial hydrolysis of diverse compounds belonging to the HA fraction [15, 23, 31]: “according to several authors, these assumed acids belong to the group of intermediate degradation products rather than to humic substances” [31]. It was proposed to replace the ratio of C_{HA}/C_{FA} , widely used in Russian soil research practice to characterize the types of humus [26, 31] with the C_{HA}/C_{org} ratio [23]. Nonetheless, the term FAs was retained and is used in two ways: for the acid-soluble substances according to Tyurin and “true FAs” according to Forsyth (1947). Forsyth designed a method for purification of the acid-soluble fraction from non-specific impurities (hydrocarbons, uronic acids, and nitrogen-containing compounds) by adsorption on activated carbon. This method has been used for a long time in the soil research [26] however, currently, the FA fraction is purified on DAX-8 resins [90, 128]. The DAX method is also applied to isolate HAs and FAs from aquatic OM [88].

Finally, the debate about humin should be also mentioned, namely, whether it is a HS or not [15, 21, 83, 158]. According to Kononova “the accumulating [experimental] material ... indicates a real presence in soil of only two groups of substances: humic and crenic (and apocrenic) acids” [16, p. 94]. Indeed, in early papers only HAs and FAs were ascribed to HSs ([16], Scheffer and Ulrich, 1960; Stevenson and Butler, 1969; cited from [158]); it was believed that humin was represented by HAs and FAs tightly bound to the mineral constituents and thus poorly extractable [15, 31]. As was found out later, humin is a heterogeneous fraction largely composed of hydrophobic substances unextractable by aqueous solvents [22, 136, 158]. After an exhaustive alkaline extraction of the HA and FA fractions from soil using 6 M urea and dissolution of the residue with DMSO, it was demonstrated using ^{13}C -NMR spectroscopy that the nature of humin is mostly aliphatic and the sources of humin are lipids, waxes, resins, and the components of plant cuticles [83]. Since the HSs by definition are the substances not belonging to known chemical classes, it was proposed to exclude humin from the category of HS [83]. Note here that the humin fraction accounts for 30–70% of humus in soil mineral horizons [21, 136], plays an important role in binding of xenobiotics [136], and the degree of transformation of the hydrophobic substances in humin requires further research. Nonetheless, when speaking about HSs, only hydrophilic polar substances extractable by alkalis are frequently kept in mind [108, 121]. This does not contribute to the certainty of HS definition.

Isolation of operational fractions aiming to decrease the heterogeneity of humus constituents has become a routine practice in soil chemistry although this approach is recognized rather conventional [15, 16]. For example, the ability of the substances constituting HA fraction to precipitate depends not only on the pH of the solution, but also on its ionic strength and the degree of HA saturation by metal ions (ash content) [23]. Noteworthy that the fractionation procedure refers to humus [158] or the humified OM of composts, peats, etc. and not exclusively to HSs [25, 81] because in the latter case these specific compounds must be preliminarily isolated from their source material (Fig. 1). Thus, HAs, FAs, and humin are no more than operational parts of a heterogeneous system of substances constituting the humified OM. The sum of HA, FA, and humin fractions includes all components of humus (or humified OM) both of defined and undefined chemical structure. Alkaline extraction should be regarded therefore as the method for extraction of hydrophilic polar substances of humus, the precipitation with acid as the method of their concentration for further studies rather than the way of selective isolation of specific products of humification. The alkaline extraction leads to transformation of the extracted substances (find the details below).

Humic substances. Until the 1980s, the term “humus substances” was used in the Russian literature for describing the components of humus as a whole [1, 15, 16, 22, 26, 31], while the term “proper humus substances” was used to denote the specific substances different from those with a known structure [1, 15, 31, 26]. Most likely, the latter was replaced with the term “humic substances” [21, 24] after publication of the Stevenson’s textbook [157].

The term HSs in its traditional interpretation (with discrimination of HS from compounds with a known structure) most probably dates back to the 1930s. Page proposed to avoid using the term humus because of the widely different meanings attached to this word (see [3]). He suggested the term “humic matter” to describe the “dark-colored high molecular colloidal organic matter” representing a characteristic constituent of soil [3, p. 70]. Presumably, this put the origin to the term “HSs” as compounds comprising this matter [31, 157]. The term “non-humic matter” was used to designate the “colorless organic substances, largely soluble and formed during decomposition of cellulose, lignin, or other components of plant and animal residues as well as from the further decomposition of “humic matter” itself” [3, p. 58]. This approach has been preserved till the present time.

It should be recognized that the subdivision of humified OM into dark-colored substances of an unknown structure (HSs) and the substances belonging to known classes of chemistry (non-HSs) is hardly justified in terms of the functioning of organic matter in soils and other environments since its components

comprise a complex dynamic entity. Waksman (1936) proposed to use the terms “humus substances” or “humic matter” “to describe the humic complexes as a whole” (note that according to Waksman, humus is present not only in soils [3]). However, this approach was criticized (Springer, 1934, 1935; Tyurin, 1937; Lane, 1940; Kononova, 1943, 1946 [15, 16]). According to Tyurin, the presence of dark-colored [humic] substances “distinguishes the non-living OM of natural formations from the intact matter of plants, animals, and microorganisms”; “these substances are formed extracellularly, the process goes towards selection of the most stable compounds”; and “there is none of the incomprehensible mystery, that Waksman talks about, in this phenomenon” [31, p. 146]. Thus, the grouping of dark-colored substances of undetermined structure into a separate chemical category and their designation as HSs became commonly accepted in the second half of the 20th century [22, 157]. In 1981, the International Humic Substances Society (IHSS) was founded in Denver, Colorado to bring together scientists with interests in HSs (the current motto of IHSS is “natural organic matter research”).

At present time the term HS is used as a generic name of fractions obtained on the basis of solubility characteristics (HA, FA, humin) along with the definition of HSs as specific products of humification (Fig. 2). This brings about the discrepancy between the theory and practice: on the one hand, HSs are dark-colored substances that have no analogues in living organisms [25, 81, 82], “chemically unique compounds” [58], and on the other hand, the sum of operational fractions [58, 81, 158]. Evidently, the extracts and the residue after the extraction contain biomolecules of known classes; their removal by “purification” is mainly used for the FA fraction. Complete separation of HS from non-HS is hardly possible (moreover, it is hardly reasonable). The attribution of HA, FA, and humin fractions to specific HSs [15, 21, 22, 157] is relied on the concepts of rather low (10–15%) contribution of the substances of known classes to humus [15]. Noteworthy that in the second edition of the textbook by Stevenson, the HAs, FAs, and humin were referred to as the fractions of humus, while the HS definition “as a series of relatively high molecular weight, yellow to black-colored substances formed by secondary synthesis reactions” exists somewhat separately [158, Table 2.3, p. 33]. Correspondingly, the term HSs turns into a certain theoretical abstraction.

Problems in identification of HSs in humus. Perhaps, the most problematic section in the humic theory is the identification of HSs and their separation from non-HS. It should be admitted that such division is possible only on paper. For example, what are the criteria for distinguishing HSs from non-HSs? Various definitions of HSs (Table 1) are rather descriptions based on the current state of knowledge about the structure and properties of the substances in extracts. The definition of HSs as high molecular weight com-

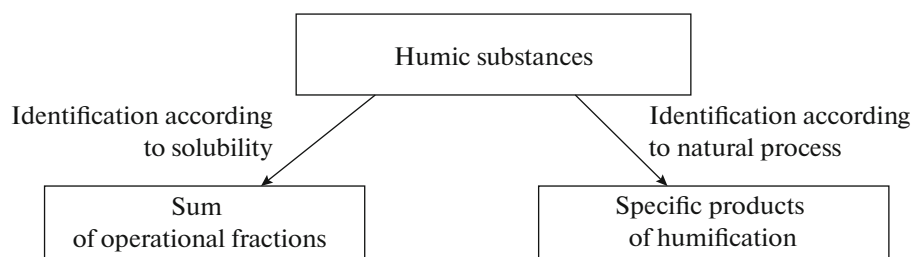


Fig. 2. Duality and ambiguity of the concept of humic substances: on the one hand, the sum of operational fractions constituting together all substances of humus and, on the other hand, specific compounds that have no analogues in the living organisms.

pounds [90, 158] preceded the theory of HAs as supra-molecular associations of small molecules [130, 159, 174, 177]. The recalcitrance of HSs is also questionable. As has been shown HAs are biochemically unstable when unbound to the mineral matrix and are efficiently decomposed in fungal cultures [7, 19, 70, 71, 185]. The description of HAs and FAs as dark-colored molecules [15, 21, 36] is disputable because not all substances in humic material possess dark color. It is more adequate to describe the HSs as a dark-colored mixture of molecules [55] than a mixture of dark-colored molecules. Problems also emerge when defining HSs as the products of transformation of organic residues (including the products of extracellular resynthesis) that differ at the functional and structural levels from their precursor compounds [15, 21, 31, 158]. Strict chemical criteria for these differences are absent. What particular characteristics or a stage of biotransformation of the initial molecules determines the transition from non-HSs to HSs [110]? Should the biomolecules covalently bound to HSs be regarded as their integral part [159], or should we try to separate them, e.g. with the help of chemical extraction methods destructing ester bonds (see ‘humeomics’ of Piccolo [117])? How can the products of secondary synthesis (extracellular condensation) be distinguished and separated from the heterostructures initially present in plant and microbial tissues? The tissues of living organisms contain both polymeric complexes (e.g. of lignin with proteins and polysaccharides, of melanins with proteins, and of tannins with proteins [3, 49]) and oligomeric soluble complexes of phenolic compounds with sugars and/or amino acids, referred to as conjugates in plant physiology [10]. The complexes of these types are abundant in plant tissues and were found in lichens [9, 155]. In the latter case, they are easily washed out by water from living thalli into the environment. The above listed uncertainties are endemic for the area of HS research and contribute to “the problem of HAs” [110]. Unfortunately, it is impossible to identify the HSs as certain new substances based on the existing definitions.

We have to recognize that the term HSs in its classical understanding is complex and ambiguous; correspondingly, this concept must be revised. Apparently,

a certain step in this direction has been made following a paper by Lehmann and Kleber [10] that denies the existence of HSs as mass products of secondary synthesis and the subsequent attempts to preserve this concept [55, 58, 81, 83, 121]. Currently, the IHSS defines HSs as “*complex and heterogeneous mixtures of polydispersed materials formed in soils, sediments, and natural waters by biochemical and chemical reactions during the decay and transformation of plant and microbial remains (a process called humification)*” (www.humic-substances.org).

Current trends in terminology and the reasons to preserve humic terms. The uncertainty of the HS concept in its traditional interpretation, unfeasibility of HS identification within humus, association of the HS notion with alkaline extraction, and the absence of correlation between the HS operational fractions and functional OM pools in soils [12] have led to the situation when researchers prefer to use the terms other than HSs when studying OM, in particular, non-living OM [85], soil OM (SOM) [97, 140], and natural OM (NOM) [101, 118, 121]. It is proposed to refer to HSs as the substances of alkaline extracts [108] or to use the term MUC (molecularly uncharacterized component of non-living organic matter) instead of HS to describe dark-colored natural material of unknown structure [43, 85].

However, these terms not only fail to resolve the problem in terminology but rather aggravate it. For example, the term SOM comprises all soil OM, including the biomass, organic residues, and humus [97, 158]. Correspondingly, the use of this term automatically involves into the consideration not only the OM transformed by biota (humus) but also live and partially decayed materials. Altogether, according to Lehmann and Kleber, gives a continuum of substances at different stages of decomposition [108]. However, it remains unclear what particular part of the SOM is studied when using this term. The term NOM creates the problem with the OM of an anthropogenic origin, which is part of humus, and the term non-living OM, the problems with microbial biomass, which is inseparable.

The humus and humified OM distinctly differ from detritus (poorly decomposed organic residues) in their morphological, chemical, and functional characteris-

tics [24]. That is why, it is necessary to add a brief designation of the constituent substances. In practice, the term HSs when used to describe the “peculiar” [15, 31], “specific” [21, 31], and “chemically unique” [58] substances formed via biotransformation of organic residues and extracellular synthesis reactions [15, 158] creates difficulties with their identification in the already formed humus because these substances inherit the structural units of the initial components of plant tissues. Thus, the separation of these substances from those with a known structure is no less arbitrary as their partition into HAs, FAs, and humin. Both the substances of known classes and the products of their transformation form a dynamic entity: “humus exists in a dynamic rather than a static state because it is constantly formed from plant and animal residues and further is constantly decomposed by microorganisms” [3, p. 22]. The degradation products of biopolymers of plant and animal tissues integrate into microbial biomass (which is the secondary synthesis in a strict sense), can undergo oxidative coupling and copolymerization [55], and form supramolecular associations. They form an integrated system of a colloidal nature, which would be appropriate to refer to as “humus substances” or “humic matter” in a broad sense according to the proposal by Waksman [3].

As for classification, Waksman put it down as follows: “The accumulated knowledge about the genesis and chemistry of humus permits a more logical [as compared with solubility of substances] system of classification based entirely upon the processes and conditions of humus formation: (A) humus types formed by decomposition of plant and animal residues under aerobic or only partially anaerobic conditions in composts and in soil; (B) humus types formed by decomposition of plant and animal residues under anaerobic conditions; and (C) humus types formed in water basins” [3, p. 8]. This classification is also applicable to “humic matter”. On the other hand, the classification based on solubility and the terms HAs, FAs, and humin has been in use for such a long time that the attempts to abandon them were unsuccessful both after the period since the Waksman suggestion and after the paper in *Nature* (2015). According to the Web of Science data, the number of publications on HSs that use the humic terminology does not decrease [58]. Stevenson [158] stated that the cancellation of existing terms could lead to even a greater confusion; the replacement of the well-established terms with cumbersome descriptions (such as “the fraction soluble in alkalis but insoluble in acids” for HAs) is hardly reasonable. The historical names of humic fractions (HAs, FAs, and humin) should be preserved as brief group terms and the names of the preparations produced in a certain way without attaching the meaning of specific substances to these fractions. Another reason justifying the retention of the traditional terminology are the ratios C_{HA}/C_{org} (or C_{HA}/C_{FA}) which serve as a simple and convenient characteristic of the types

of humus, reflecting the bioclimatic conditions of its formation.

CHEMICAL NATURE OF SUBSTANCES COMPRISING HUMUS AND SECONDARY SYNTHESIS AS PART OF HUMIFICATION PROCESS

According to Tyurin, the most significant question with a fundamental importance for the overall problem of a chemical nature of humus “...is whether humus is a complex entity of different compounds known from the chemistry of plant and animal substances or humus contains certain specific characteristic compounds that are absent in the list of substances of plant and animal origin” [32, p. 116]. This question is also discussed at present time [49, 58, 81, 92, 108].

The answer to this question is evident and was given as early as in 1927 by Schreiner and Dowson: in their view, “chemically, humus consists of certain constituents of the original plant material resistant to further degradation; of substances undergoing decomposition; of complexes resulting from decomposition, either by processes of hydrolysis or by oxidation and reduction; and of various compounds synthesized by microorganisms” (cited from [3, p. 21–22]). This description rather accurately describes composition of humus without using the term HS: (1) initial substances; (2) transformed biomolecules, “the substances undergoing decomposition and the complexes resulting from hydrolysis, oxidation, or reduction”; and (3) the products of microbial synthesis, “various compounds synthesized by microorganisms”. Intuitively, the latter include the products of extracellular synthesis. Thus, even the opponents of the humic paradigm (Schreiner) recognized the possibility of extracellular secondary synthesis reactions although not distinguishing the products of the biotransformation and resynthesis into a separate category of specific substances and referring to them as HSs [158].

Transformed biomolecules as the constituents of HS.

The opinion that HSs is a mixture of microbial and plant biomolecules rather than compounds synthesized *de novo* was repeatedly uttered as early as the beginning of the last century. Current rebirth of these ideas is largely based on the data of NMR spectroscopy interpreted as an absence of any additional signals except for those characteristic of the mixtures of known biopolymers [49, 77, 92, 111]. However, the fact that the signals in NMR spectra of heterogeneous mixtures overlap suggests that the chemical environment of atoms is the same but not the structure in general [58]. The presence of signals characteristic of biomolecules in the spectra of HA is not surprising since the humic material inherits the structures of initial compounds. Any spectral methods have their own limitations; in particular, two-dimensional ^1H -NMR [92] detects only protonated C-atoms [51].

Semi-quantitative solid-phase ^{13}C -NMR spectroscopy, which detects all carbon atoms irrespective of solubility and molecular weight, and dipolar dephasing (DD ^{13}C -NMR), decreasing the overlapping of signals from individual carbon atoms, have made it possible to discover considerable differences between the peat HA and FA spectra, chernozem HS spectra (IHSS standards) and the spectra of biomolecules (lignin, cellulose, and proteins) as well as spectra of plant and microbial tissues [51]. Characteristic of the ^{13}C -NMR spectra of plant and microbial tissues is the prevalence of the signals of carbohydrates and proteins, respectively; however, the spectra of HA considerably differ from nonhumified materials by the prevalence of the signals of unprotonated “aromatics” (which is not visible in the ^1H -NMR spectra) [92]. The aromatic carbon atoms not bound to hydrogen or oxygen atoms account for 28–33% in HAs versus <10% in the studied biomolecules [51]. In HAs, part of them is represented by condensed structures. The HA spectra differ from biomolecules and the material of biomass by intensive signals of the carbon atoms bound with oxygen, namely, COOH groups, C=O ketones, and C–O alkyl groups. In addition, HA spectra contain the signals that correspond to the aromatic carbon bound to three carbon atoms (region of <125 ppm), and alkyl carbon, bound to three carbon atoms and one oxygen atom (approximately 85 ppm). These signals are interpreted as the result of newly formed C–C bonds (cross-links) between aromatic and aliphatic structures during biotransformation of lignin and other biopolymers in soils and as an evidence of secondary synthesis reactions [51]. Thus, the ^{13}C -NMR data suggest accumulation of aromatic oxygen-substituted structures during humification [51, 64]; their content is rather low in the initial plant material, and they are absent in the initial microbial tissues.

Nonetheless, the analysis of extremely heterogeneous humus and humus extracts with spectral methods, not particularly designed for this purpose, sometimes leads to quite unexpected conclusions. For example, that soil humus has an aliphatic nature or mainly microbial rather than plant origin (see reviews [104, 111] and references therein). In particular, solid-phase ^{13}C DP/MAS NMR has shown that solid-phase ^{13}C CP/MAS NMR underestimates up to 65% of C_{org} mainly of aromatic and carboxylic origin [153]. In addition, ^{13}C DP/MAS NMR demonstrates that the content of aromatic carbon in HSs varies from 33 to 55% [113]. Thus, the conclusion on the aliphatic nature of HSs is incorrect. In the widely cited paper [113], Simpson et al. [149] compared the NMR spectra of microbial biomass and soil OM and made a conclusion that the microbial biomass of some soils constitutes >50% of the extractable OM fractions, approximately 45% of humin fraction, and >80% of soil nitrogen. These values are evidently overestimated [40].

The enrichment in oxidized aromatic fragments is a characteristic feature of the HA and FA of terrestrial origin [21, 158]. The main source of these structures in the soils of the modern biosphere is lignin and other polyphenols, for example, hydrolyzable and condensed tannins [15, 21]. Numerous studies have shown a high contribution of the aromatic structures of lignin to soil HSs [60, 72, 115, 162, 188]. Vanillic and syringic acids, the corresponding aldehydes and ketones, as well as cinnamic (ferulic and *p*-coumaric) acids are identifiable among the products of humus and HA oxidation by copper oxide in an alkaline medium [60]. These monomers are known as lignin biomarkers [39, 86] and are widely used for assaying lignin structures and their transformation in soils [13, 72, 162, 188]. Unlike the plant residues, humus and its alkaline extracts are enriched in acids as compared with aldehydes and ketones [60], which suggests an oxidative transformation of the initial lignin structures in soils. This complies with the NMR data of HAs [51, 64].

In the context of the components of tissues of living organisms, which contribute to the dark-colored humic matter it is worse to mention the fungal pigments melanins [11]. There is an opinion that substances constituting the HA fraction are actually the intact melanins [11]. Indeed, these pigments display a considerable similarity to soil HAs in elemental composition, molecular weights, and optical properties. However, the fungal melanins have been shown to mineralize more rapidly as compared with HAs; correspondingly, HAs are not intact melanins [187]. The contribution of these pigments to HSs is unknown.

According to the SCM model [108], the process of OM decomposition is directed towards an increase in the solubility and a decrease in the molecular weights of the decomposing substances, which facilitates the sorption of decomposition products by mineral phases. Indeed, oxidation processes contribute to an increase in water solubility of biopolymers, such as lignin and cellulose. However, the increase in solubility may be regarded as an intermediate rather than a final stage [55]. For example, the OM degradation processes in the absence of a mineral component (in composts) lead to a decrease in the content of water-soluble hydrophilic components in the course of composting [53]. During humification, the alkyl components of lipids and aromatic compounds accumulate, the content of polysaccharides and amino acids decreases [189], and the relative hydrophobicity of the product increases, i.e., its solubility decreases.

Overall a large volume of data on the structural and molecular composition of humic matter in different environments has been accumulated during the last 30 years. However, despite their increasing complexity the spectral methods fail to provide basically new information on the components of humus. For example, the study of the HA and FA by ion cyclotron resonance mass spectrometry (ICR-MS) gives thousands

of brutto-formulas plotted on Van Krevelen diagrams, which represent the composition of substances in the hydrogen to carbon and oxygen to carbon coordinates [94]. The analysis of the diagrams gives regions belonging to HS precursors: lignin, tannins, terpenoids, lipids, polysaccharides, proteins, and so on [151]. Nano-SIMS and NEXAFS have revealed a cluster in situ organization of the humus components [105, 107]. Nonetheless, complex spectral methods for analyzing HSs are unable to unambiguously answer the question on whether the secondary synthesis products are present in humus and what is their contribution. These problems are resolvable only in terms of biochemistry: “instead of confining all attention to humus which has already been formed, it would be more logical to begin with fresh plant residues and follow the course of change which takes place during the process of their transformation into humus [3, p. 91].

Products of secondary synthesis in humus. This section of humus chemistry is actually the most debatable. Whereas it was previously thought that the bulk of humus substances is formed with the involvement of free radical condensation reactions [3, 15, 157], there is a trend at the present time to completely deny the secondary synthesis [108, 170]. Supramolecular theories [130, 176], rejecting formation of secondary polymeric compounds under natural conditions, have become generally accepted. According to Piccolo et al. [130], the process of humification consists in accumulation of small molecules (to 2000 Da) that further form supramolecular associations via noncovalent interactions (van der Waals forces, hydrophobic, and hydrogen bonds). Hydrophobic molecules form an envelope that protects hydrophilic molecules from destruction [133]. The theory by Piccolo further develops the hypothesis by Wershaw [176] of a micellar structure of HSs.

Thus, the backbone of the discussion on humus is the criticism of the “HS paradigm”, which is interpreted by Kleber and Lehmann [97] (following Wershaw, 177) as the concept that “soil humus is composed of the end products of synthetic reactions that alter the structure of plant degradation products in a way that provides these newly synthesized materials with unique properties that are distinct from non-humified organic matter.” According to Kleber and Lehman [97], “the fundamental contention in this debate is the question of whether there is indeed a naturally occurring process of secondary synthesis in the biosphere that operates independent of and in addition to standard processes of decay and, in doing so, reassembles plant degradation products into new, molecularly and functionally distinct compounds at a quantitatively relevant scale.” Earlier, Burdon reviewed the hypothetical structures of HA and FA and concluded that there is “no compelling reason for putting forward as HSs structures complex molecules that do not occur in plants or in microorganisms or are not ratio-

nal products of degradation of these” [49]. The discussion in general became reduced to the criticism of condensation reactions (humification reactions) producing polymeric and stable HSs [108] and providing C_{org} stabilization in the soils [170].

One of the arguments against extracellular synthesis of complex humic structures is the evolutionary press: microorganisms, which spend their energy and resources on production of recalcitrant materials, would not survive because of interspecies competition [49]. If complex HAs are undesired products of spontaneous resynthesis, a biochemical mistake, the contribution of such mistakes to the natural OM must be small [49]. These arguments were also used later in the relevant criticism [96, 97, 108]. However, there is no question of evolutionary press since microorganisms produce extracellular phenoloxidas and peroxidases, oxidizing phenolic substrates, for their own metabolic purposes, while free radical condensation is a spontaneous process not requiring energy spending. The biocatalysts providing formation of free radicals, for example, laccase, tyrosinase, and peroxidase, are abundant in soils [50, 150] as well as abiogenic catalysts—such as Fe^{3+} and Mn^{4+} ions in soil oxides and hydroxides [88].

Much more essential is the question what is meant by secondary synthesis—exclusively the condensation of low molecular weight decomposition products (according to Kononova) or these reactions include copolymerization as well (for example, attachment of amino acids to lignin).

Biochemical free radical processes during degradation of polymers. This part of the humic theory is the least disputable. It is convenient to consider the radical formation during oxidation of lignin. Lignin is an ubiquitous wood biopolymer, accounting for 10–30% of wood, and the main source of reactive phenolic compounds in the soils of the modern biosphere. Fungi are the main degraders of lignin (see reviews [28, 178, 182]). The white rot fungi and the related litter-decomposing basidiomycetes [124] depolymerize lignin to low molecular weight fragments by breaking the C–C bonds of both the aromatic ring and phenylpropane chains [28, 95]. The nonspecific lignolytic peroxidases with a high redox potential: lignin peroxidase (EC 1.11.11.14, LP; found only in wood-decomposing fungi), Mn-dependent peroxidase (EC 1.11.1.33, MnP), versatile peroxidase (EC 1.11.1.16, VP), and the phenoloxidase laccase (EC 1.10.3.2) [178, 182] are believed to play a key role in this process. These enzymes catalyze oxidization of their substrates by molecular oxygen (laccase), peroxide (VP and LP), or Mn^{3+} (MnP and VP) to give quinones, semiquinones, aryl radicals, and phenoxy radicals. These unstable species can undergo spontaneous condensation reactions, while the reactive oxygen species (ROS) formed in quinone/hydroquinone cycles initiate substrate degradation [69]. As a result, the lignin is mineralized or

depolymerized [87, 155] down to monomers [158]. The radicals, carbonyl compounds, and resonance structures generated by enzymes during lignin degradation [138] undergo condensation and copolymerization reactions binding other molecules with nucleophilic groups (amino acids, carbohydrates, etc.). Laccases play important role in condensation reactions. One of their functions is considered to be associated with polymerization of low molecular weight lignin degradation products which may be toxic to the fungus [164]). This gives dark-colored FA- and HA-like products of varying molecular weights, which has been demonstrated for the lignin degradation in fungal cultures [2, 179, 185]. High molecular weight HA-like compounds can be also formed during the partial lignin degradation by cellulolytic fungi, which lack lignolytic peroxidases. The basidiomycetes causing brown rot of wood are also able to partially oxidize lignin with the help of ROS formed in the Fenton reaction; in this case, lignin retains its high molecular weight structure and acquires brown color [41]. Ascomycetes and soil micromycetes causing soft rot decay can partially depolymerize lignin by laccases [28, 185]. The lignin oxidation by laccase is accompanied by the emergence of dark coloration [123]. The cellulolytic fungi cause demethylation of lignin and the enrichment of aromatic rings in carboxyl groups. As has been shown, the same nonspecific fungal enzymes that are involved in lignin transformation convert the substances of HA fraction into FAs (and vice versa) [19, 70, 71, 155, 185]. The listed biochemical processes, including degradation and copolymerization, form the background for degradation theories of humification [1, 3, 62] and imply the formation of dark-colored heterostructures of varying molecular weight differing in their properties from the initial molecules. For example, oxidized lignin and cellulose become more soluble in alkalis (native lignin and cellulose are poorly soluble).

Condensation of low molecular weight substances. The low molecular weight phenolic compounds formed during lignin degradation as well as phenolic metabolites of fungi can be involved in the condensation reactions producing dark-colored heterostructures, which is the basis of condensation theories of humification [15, 62]. However, the scale of this process was repeatedly questioned with regard to low concentrations of low molecular weight precursors in soil solutions [24]. What is the extent of such polymerization reactions under natural conditions? Can macromolecules be formed in this way?

Most experimental confirmations of secondary synthesis have been obtained using model closed systems and/or high concentrations of the initial substances (1–10 mg/mL), namely, the reactions in homogeneous systems in the presence of phenoloxidases and peroxidases [15, 37, 47], the reactions in heterogeneous catalytic systems in the presence of abiogenic catalysts [88] or immobilized tyrosinase [116] or laccase [182, 184]. Formation of dark-colored

humic-like molecules has been shown in the cultures of myxobacteria [16]. Some microscopic fungi also produce phenolic compounds. Their oxidation in the presence of amino acids gives dark-colored substances precipitated with mineral acids (Haider et al., 1965; Haider and Martin, 1967; Martin and Haider, 1969; cited from [140]). Formation of heteropolymers is explained by the reactions of phenolic substances with nucleophiles, for example, the free amino groups of lysine and the thiol groups of cysteine followed by deamination and decarboxylation [15, 74].

Laboratory experiments have demonstrated that the presence of a biocatalyst and the interface boundary concentrating both the substrates and products enhances synthesis of dark-colored macromolecular HA-like substances. The time required for microscopic fungi to form HA-like condensates is reduced in the presence of clay minerals; the amount of HA condensates increases (Martin et al., 1972; cited from [140]) as compared with a homogeneous system; the products with a higher molecular weight were formed from phenolic and amino acids in the presence of clay minerals and immobilized fungal laccase [182, 184]; re-polymerization of low molecular weight products of HA degradation on fungal mycelium was demonstrated in submerged fungal cultures [185]. In general, a large set of experimental data suggests the feasibility of extracellular synthesis of HA-like structures, including polymeric compounds, as well as the important role of biocatalysts and the interface boundary in this process. However, the possibility that such structures are formed at low concentrations of substrates (0.1–0.01 mg/mL) and under the dynamic conditions, characteristic of soils, is questionable and needs experimental confirmation or refuting. We have shown that polymeric substances do not form when a mixture of phenolic acids (0.01 mM each) is passed through a column with a clay mineral with immobilized laccase; however, the presence of a biocatalyst enhances the binding of certain phenolic acids with the mineral [182]. The question thus arises on whether the formation of polymers is so important for C_{org} stabilization?

One more essential question is the contribution of condensation reactions to humification in the soils of the modern biosphere, where the transformation of plant biopolymers is the leading process. Presumably, the condensation reactions with involvement of low molecular weight phenolic compounds are important for the primary humus formation in the absence of lignin, for example, at the initial stages of colonization of mineral substrates by a microbiota. Konrad Haider and James P. Martin (1965–1978) [140] significantly contributed to the research into such reactions and formation of “fungal” humus. Later on, phenolic compounds, their water-soluble conjugates with sugars and amino acids [9], as well as the humification catalysts laccases and tyrosinases were discovered in lichens, the organisms that dominate the soil cover in

tundra and oligotrophic forests [46]. This implies potentially important role of phenolic compounds and phenoloxidases of lower plants in humus formation. It is important that the time segment for such reactions may be of approximately 2 billion years prior to appearance of lignin in the Devonian [186]. In the history of the oxygen biosphere (3 billion years), the humus formation in the presence of lignin took only the last 400 million years, which is the period when the modern soils with humus horizons as the root habitable layer were formed. Biochemical free radical condensation reactions should be in focus of further research in order to resolve their role in organic carbon stabilization at different stages of the evolution of terrestrial ecosystems [140, 143].

In the context of the biochemical processes of HS formation in the early biosphere, it is worth to mention the potential role of bacterial laccases. Bacteria have two forms of enzyme—three-domain laccase (also characteristic of fungi, with an acidic pH optimum in the oxidation of phenolic substrates) and two-domain laccase (evolutionarily more ancient form; see references in [106, 163]). The two-domain laccase has an alkaline optimum in oxidizing phenolic substrates, is thermostable, and resistant to inhibitors (sodium azide). As has been shown recently, two-domain laccases of streptomycetes enhance polymerization of low molecular weight and high molecular weight HA fractions, which leads to formation of new covalent bonds in the humic ensemble [109]. In addition, two-domain laccases were shown to take part in co-polymerization of phenolic acids and HAs, thereby contributing to an increase in the relative content of high molecular weight fractions in HAs [166]. The humification processes under alkaline conditions require further studies.

In general, a large set of experimental data demonstrates that the rejection of secondary synthesis in soils is rather groundless. These reactions can play an important role in the molecular weight organization of humic material as well as in sequestration of organic carbon and nitrogen in soils.

Stability of HSs. This postulate is among the main ones in the HS theory: “It is perfectly clear that of the manifold reactions of organic matter transformation beyond living organisms, only the reactions that lead to formation of relatively stable compounds—and these are humic substances—are important” [31]. This in many respects explains the interest to secondary synthesis. As was believed, these reactions give complex polymeric condensed heterostructures resistant to biodegradation [15]. The particular stability ascribed to humic compounds (and HAs are their most prominent representatives) served for a long time as one of the reasons for distinguishing HAs as a special category of geomolecules [22, 158].

However, the falsehood of this concept can now be regarded as proved. A wide range of data suggest that a long-term persistence of C_{org} in soils is largely a result

of OM isolation from the activity of microbiota (the driving force of destruction and synthesis) rather than molecular recalcitrance of the substances constituting humic fractions, as has been earlier believed [15, 158]. The complexity of molecular structure is important only at the early stages of decomposition in soil organic horizons [104, 170]. Lignin and lipids display a relative molecular stability, whereas carbohydrates and proteins belong to a labile pool [102]. However, lignin, as well as other materials of plant origin, can be effectively decomposed in the presence of easily available substrates e.g. glucose (Haider and Martin, 1981; cited from [39, 135, 140]). A relatively low molecular stability is also characteristic of the HAs during their incubation in fungal cultures. The HAs not bound to mineral matrix are efficiently decolorized and depolymerized by both lignolytic [70, 182] and cellulolytic [8, 19, 71] fungi.

In the context of stability of humic molecules, it is worth noting the problem of pyrogenic carbon. The presence of condensed aromatic structures is characteristic of soil HAs [51]. It has been suggested that they originate from pyrogenic sources (black carbon, BC) [141]. It was also suggested [68, 141, 142] that BC is the main contributor to aromatic carbon in soil, determines dark color of chernozems, and their enrichment with aromatic fragments [e.g. 137]. An indirect confirmation here is the ^{13}C CP/MAS NMR data on the chemical structure of OM in soil profiles: the contribution of nonphenolic aromatic carbon to soil OM increases with depth and, vice versa, the contribution of phenolic, decreases [189]. However, examination of chernozem samples collected from the experimental plots of the Central Chernozem Nature Reserve [167] has shown that the content of pyrogenic carbon in the 0–30-cm soil layer assayed according to benzene carboxylic acids accounts for 3–4% of C_{org} for the unmown steppe and 5–7% for long-term black fallow. Thus, the high contribution of BC-like substances to the total content of aromatic compounds is not characteristic of all types of chernozem soils. In addition, the thermal and chemical methods used to assay BC in soil are rather arbitrary [17]. The BC markers, for example, benzene polycarboxylic acids [68], may have other origin than a pyrogenic one [64]: as is shown, BC-like substances as well as alicyclic acids are formed during the lignin degradation initiated by hydroxyl radicals (Fenton reaction) [171]. As for a long-term preservation in soils, it has been shown that 42–66% of the pyrogenic carbon in the near-surface soil layer can be destroyed as early as the next wildfire [57].

Thus, the molecular stability is not absolute and any structural molecular description of the most recalcitrant soil OM components is still unavailable [102]. In the long run, all substances constituting humus are decomposed to CO_2 and H_2O [91]. Oxygen deficiency (for example, in hydromorphic soils) does not prevent OM mineralization [114]. On the other hand, the aver-

age residence time measured for different identified individual substances in humus is shorter as compared with the OM of humus as a whole [39, 142]. This may suggest that the OM stability is determined not by the structure of individual compounds but rather by covalent and noncovalent interactions in the complex system of non-living OM, adsorption interactions with the mineral components, and physical isolation within soil aggregates [96, 103, 104, 142, 170]. The burial in sediments (brown coals) also contributes to long-term OM conservation.

Molecular weight distribution of HSs. It has long time been considered that, the HAs are relatively high molecular weight compounds (5–100 kDa) [15, 158], which can form supramolecular associations [21, 122]; however, it is generally accepted now that HAs are supramolecular ensembles of relatively small molecules (2–6 kDa) [130, 159, 175]. In the context of the HS molecular weight organization, it is worth noting that the researchers dealing with humus always relied on the current trends of classical chemistry when interpreting their results.

HSs as a system of polymers. The concept of HSs as a system of polymers [15, 16] followed the studies by Staudinger (the 1953 Nobel Prize in Chemistry), who formulated in 1922 the concept of polymers, i.e., large molecules composed of many repeating subunits linked together by covalent bonds. To describe such molecules, Staudinger introduced the concept of macromolecule [35]. The macromolecular theory was soon applied to the chemistry of humus. The schemes for HS synthesis from monomers, formed during the breakdown of biopolymers, were proposed [16]. The system of biopolymers had to degrade almost completely to “trigger” the reactions of HS synthesis. However, such factors as limited diffusion of monomers to the reaction site and low concentration of soil solutions, were omitted. The macromolecular theory was dominant in the chemistry of humus until the 1990s [25, 158]. The conformational behavior of HA and FA macromolecules in solutions was described in terms of protein behavior; here, it is appropriate to recall the random coil model [161]. According to this model, the HS molecules acquire an elongated conformation under alkaline conditions because of the electrostatic repulsion between negatively charged functional groups and become more compact with a decrease in pH and an increase in the ionic strength of the solution [161].

Although the condensation theory has its shortcomings, the presence of high molecular weight substances in humic matter is evident at least on the basis of the initial composition of organic residues and the biochemical processes that accompany destruction. In the 1970s–1990s, D.S. Orlov formulated the concept of HSs as a series of molecules displaying (1) true polydispersity because of different molecular weights of their components and (2) secondary polydispersity

due to formation of supramolecular associations. Chromatography data also demonstrated the presence of true macromolecular components in HAs. Toyo-Pearl gel filtration chromatography of 77 samples of humic materials of different origins gave average-number molecular weights (M_n) of approximately 2.9–9.7 kDa and average-weight molecular weights (M_w) of 4.7–30.4 kDa [127]. High performance liquid chromatography of 33 soil HA samples gave similar results (M_n 1.3 ± 0.5 kDa and M_w 13.2 ± 1.2 kDa [63]). The M_n values of 130 aqueous HA and FA samples were assessed as approximately 800–1700 Da [125]. Note that the HS molecular weights determined experimentally are rather arbitrary because they depend on the type of the used matrices and eluting buffers [55]. For example, Toyo Pearl gel [127] has the affinity for hydrophobic/high molecular weight HA components, which sorb on the gel leading to underestimated M_n and M_w . Another problem is the absence of molecular weight markers for HAs and FAs. Chromatography columns are calibrated using globular proteins or polystyrene sulfonic acids. The HS conformation that determines their size characteristics may well be considerably different as compared with the model polymers. The presence of true macromolecular components in HAs also confirmed by biochemical studies of HA depolymerization in fungal cultures in the presence of the enzymes affecting covalent bonds in phenolic substrates [19, 70, 185]. Diffusion-ordered ^1H NMR spectroscopy (DOSY) was used to clarify the nature of high molecular weight HA components [147, 148]. The components with the chemical shifts characteristic of lignins, polysaccharides, and peptides and the diffusivity matching molecular weight of approximately 2500, 1000, 200–600 Da, respectively, were observed. These data confirm the general conclusion that alkaline extracts contain a series of components at different stages of decomposition with a wide range of molecular weights and structures.

HSs as supramolecular structures. In the 1990s, Lehn formulated the main concepts of supramolecular chemistry [18] (the 1987 Nobel Prize in Chemistry jointly with Pedersen and Cram); after this, the theory of a supramolecular structure of the HSs became popular. It should be noted that the supramolecular theories of natural OM structure had been discussed before. In particular, as early as 1972, the model of HSs as an ensemble (loose network) of acids and phenols, which entrap other compounds, was proposed [144]. In 1975, Orlov et al. [22, 122] described HSs as a polydisperse mixture of substances and their supramolecular associations. The micellar theory of HS structure by Wershaw appeared in the 1990s [176] and was further developed by Piccolo [130]. Starting from the studies by Piccolo, the supramolecular theory firmly settled in both soil science and aquatic chemistry. According to Piccolo, HAs are regarded as supramolecular associations of small molecules (to 2 kDa)

stabilized by weak dispersion interactions (van der Waals forces, π – π , and CH– π) and hydrogen bonds. Conclusions on supramolecular nature of HAs were based on the HA molecular weight distributions assessed by gel chromatography in the presence of the organic acids causing HA disaggregation [131]. The experimental conditions did not take into account electrostatic and other non-exclusion effects [126] and the conclusions on low molecular weight of HA were relied on the mass spectrometry data, unable to determine the compounds with a molecular weight exceeding 2 kDa. Nonetheless, this model was further developed [146, 159] and is currently almost commonly recognized.

Finally, the advent of the universal theory of hydrogen bonds (Gilli and Gilli [67]), the strength of which varies from weak (<1 kcal/mol) to the level close to that of covalent bonds (30–45 kcal/mol) led Wells in 2015–2019 to a new conceptual model of supramolecular aggregation of natural OM [61, 175]. This model takes into account not only weak (according to Piccolo) hydrogen bonds, but also strong (near-covalent) ones in natural OM aggregations [174, 175]. According to the model [175], the primary OM associations with a size of <1 μ m, stable to perturbations (turbulence, change in pH, etc.) and referred to as metachemical hydrogel, are formed by crosslinking of individual molecules by strong hydrogen bonding. This include the so-called charge-assisted H-bonds, formed when a proton is distributed between two dissociated aromatic carboxyl or phenolic groups; resonance-assisted H-bonds between uncharged groups; and polarization-assisted H-bonds between undissociated phenolic groups. Metachemical gel is not degradable by either alkaline extraction or alkaline desorption of OM from DAX resins. The associations of <1 μ m are preserved in HS solutions and natural OM even at pH 13 [99]. Under favorable conditions, metachemical gel particles form loose three-dimensional networks with a size of >1 μ m at the expense of weak hydrogen bonding; these aggregates are referred to as physical hydrogel. Perturbations cause reversible dissociation of physical hydrogel, which spontaneously reform over turbulent/quiescent cycles. These two types of associations are distinguished as the basic types of aggregates in the hierarchical self-assembling supramolecular architecture of natural OM. The authors believe that formation of the metachemical hydrogel, that is, the primary dense supramolecular structures inside loose physical supramolecular structures, is the particular mechanism responsible for the biochemical recalcitrance, characteristic of natural OM.

It should be noted that the supramolecular theories set aside the models of supramolecular structures formed from organic ligand molecules and metal ions as bridges, the existence of which is beyond doubts [158]. In addition, these models have been mainly developed for aqueous solutions. Correspondingly, it is relevant to apply them to undissolved OM. This approach was commenced by studies of Wershaw [176] with repre-

sensation of soil OM as membranes on a mineral surface, followed by a zonal model of OM self-assembly on mineral surfaces [98], as well as a humic matrix of soil gels [33, 34].

ALKALINE EXTRACTION

One more traditional point for criticism of the HS concept [3, 97, 108] is the method used for HS extraction with alkalis (0.1–0.5 M NaOH), which are used to isolate HSs from soils, peat, and brown coals as well as to desorb HA and FA fractions from XAD8/DAX8 resins when studying aquatic OM [12]. The main questionable issues are (1) the extraction selectivity towards humification products since components of fresh microbial and plant residues as well as components of biomass and metabolites (the substances of known classes) can pass into solution; (2) the chemical changes in the native OM producing artifacts; and (3) the relevance of alkaline extraction for studying properties and functions of natural OM [3, 108, 121, 122, 144].

Selectivity of extraction. The exact quantitative contribution of the substances of known chemical classes to the alkaline extracts is not known. It depends on the degree of oxidative transformation of organic residues and is regarded as insignificant (10–15% of C_{org} [15]) in the materials with a high degree of humification (the degree of humification can be estimated as a ratio $C_{HA}/C_{org} \times 100\%$) [26]. Non-humic low molecular weight substances are partially separated during precipitation of HA fraction or purification of FA fraction on DAX/XAD resins [121]. However, it is impossible to completely separate biomolecules from the products of humification. For example, the protein components of microbial tissues, the extracellular enzymes associated with soil OM and minerals, and fungal melanins pass into extracts and co-precipitate in the HA fraction. However, the transformation of plant residues in soils is accompanied by oxidation reactions leading to carboxylation and hydroxylation of biopolymers, such as lignin, thereby increasing the solubility of initial biomolecules in aqueous solvents. That is why, alkaline extraction is in a certain way selective towards the oxidized products of transformation of plant biopolymers. Intact lignocellulose is poorly soluble in alkalis as well as lipids, plant cuticles, and other hydrophobic substances, remaining in “humin” fraction [80]. As is justified above, alkaline extraction should be hardly interpreted as the method for selective isolation of specific substances (HSs) but rather as a procedure aimed at dissolution of quantitatively significant (30–80% of soil C_{org} [158]) part of OM, which includes its intact and altered (humified) components. Alkaline extraction makes it possible to study hydrophilic components of OM, their molecular properties [121], including the use of such powerful methods as FT-ICR MS. The acid precipitation of HA fraction is an efficient method for concentration of the OM soluble under alkaline conditions.

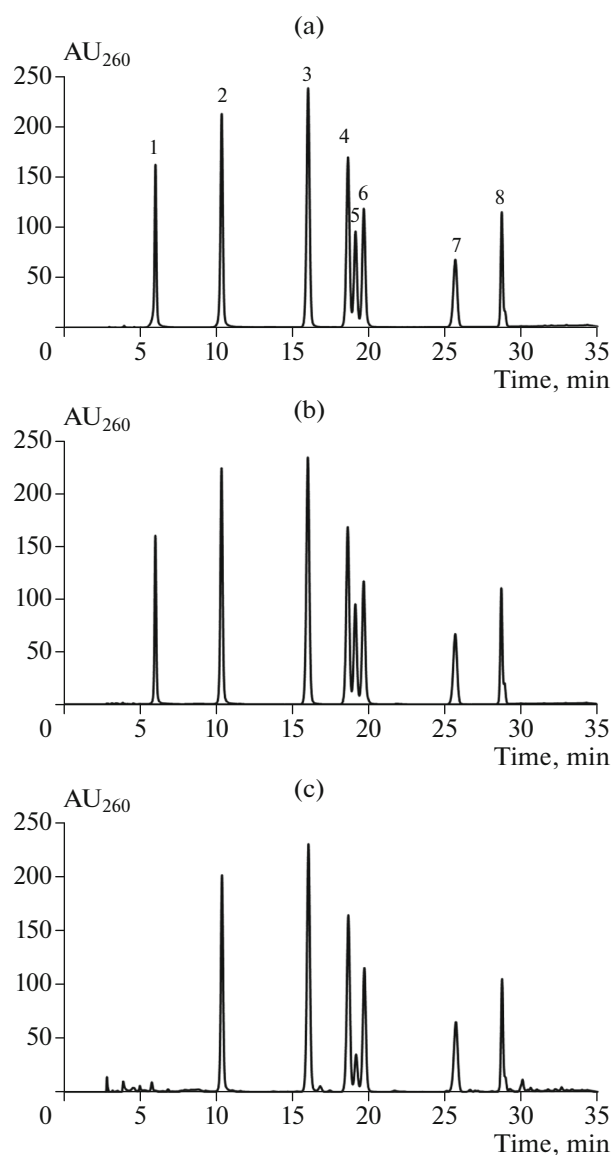


Fig. 3. The effect of alkaline pH on phenolic acids according to reverse-phase high pressure liquid chromatography data: (a) initial mixture of acids in 20 mM acetate buffer (pH 4.5); (b) the mixture of acids after 1-h treatment under alkaline conditions (NaOH pH 12) with permanent N_2 purge; and (c) the same but without N_2 purge (1, gallic acid; 2, protocatechuic acid; 3, hydroxybenzoic acid; 4, vanillic acid; 5, caffeic acid; 6, syringic acid; 7, vanillin, and 8, ferulic acid). Synergi Hydro-RP column ($150 \times 4.6 \mu\text{m}$, $4 \mu\text{m}$; Phenomenex, United States). The acids were identified as described earlier [5].

Extraction artifacts. This issue has been discussed from the very first studies of the humic matter (for details, see several recent reviews [81, 97, 121, 140]). The most active critics regard HAs and FAs as a laboratory artifact [3, 108].

The alkaline extraction of humus is accompanied by oxygen consumption. It can be as high as 700–800 mm³ O₂/0.2 g soil in 0.5 M NaOH versus 7–

37 mm³ O₂/0.2 g soil in 0.1 M Na₂P₂O₇ pH 7.0 (Bremner, 1950; cited from [158]). In the presence of oxygen, phenolic compounds (key aromatic components of the HAs and FAs of a terrestrial origin) can undergo autooxidation in alkaline medium [168]. Reactive oxygen species and ionized state of OH[–] groups enhance the oxidation [54]. As a result, both condensation and degradation reactions are possible. For example, 30-day oxygen purge of the peat HAs dissolved in 1 M NaOH converted almost half of the HAs into FAs and other low molecular weight compounds as well as led to a loss in amino acid nitrogen in HAs (Swift and Posner, 1972; cited from [121]). Oxygen purge of alkaline solutions of gallic and protocatechuic acids cause an oxidation polymerization of these polyphenols and formation of dark-colored polycondensates, similar to HAs in the pK_a of carboxylic and phenolic groups and the spectra in UV, visible, and IR regions [59, 66]. Another likely change in OM structure during the alkaline extraction is hydrolysis of ester groups, causing an increase in the number of carboxyl groups [121].

In order to minimize the chemical changes of OM in an alkaline medium, the IHSS recommends isolating HAs and FAs in the atmosphere of an inert gas, for example, nitrogen [160]. Swift and Posner (1972, cited from [121]) have shown that the HA conversion into lower molecular weight compounds during the alkaline extraction with 1 M NaOH over 30 days in N_2 atmosphere amounts to less than 15%. The conditions recommended by the IHSS for HA and FA extraction from soils—0.1 M NaOH, 24 h, and N_2 atmosphere—imply even lesser degree of OM transformation. We analyzed mixtures of phenolic acids with reverse-phase liquid chromatography before and after their alkaline treatment in the absence and presence of N_2 atmosphere and demonstrated that no transformation of polyphenols (gallic and caffeic acids) occurred during 1 h of constant purge with N_2 (Fig. 3b). Without nitrogen purge, gallic and caffeic acids “disappear” from the chromatograms almost immediately after alkali is added (Fig. 3c). It is very difficult to achieve a constant purge and the absence of oxygen when isolating large volumes of HA preparations. The use of N_2 atmosphere is not customary for the practice of humus research in Russia [26]. However, a comparison of the physicochemical properties of the HAs extracted using 0.1 M NaOH with nitrogen purge and without it from the humus horizons of two contrasting soil types—Retisol and Chernozem—demonstrates no significant degradation and condensation processes if HA are extracted in closed bottles without N_2 . It is suggested that in soils with deep oxidative transformation of OM during humification (such as chernozems) the presence of O₂ in the alkaline medium do not induce further oxidation of OM components. The nitrogen purge decreased oxidation processes during extraction of HA from Retisol [7].

Another disputable issue is a dark color of the HS solutions. As was proposed, this is caused by alkaline treatment [108]. However, decomposition of fresh plant residues is accompanied by darkening with time (for example, in composts [93]), while soil humus horizons have a natural dark coloration [38] and dark-colored substances are extractable from soils not only with alkalis [55, 64, 79, 121]. It has been shown, that the use of a diluted alkali has almost no effect on the molecular composition of the extracted natural OM. Pyrolysis-field ionization mass-spectrometry identified the same ten classes of compounds in soil OM as in the HA, FA, and humin fractions; moreover, the relative abundance of eight classes of the ten was identical for all HS fractions and the bulk soil [143]. Ion cyclotron resonance mass spectrometry demonstrated that the alkaline desorption of OM from XAD-8 resins had no significant effect on the molecular composition of dissolved OM (DOM) of natural waters: of approximately 13 000 molecular formulas found in the spectra of initial DOM, almost 90% were also present in its three fractions (HAs, FAs, and hydrophilic DOM), which is a very high similarity [121]. Thus, the HA, FA, and humin fractions satisfactory approximate the main trends in the molecular composition of natural OM.

As arguments against an “artificial” action of alkali on natural OM, De Nobili et al. [55] state that the fresh plant residues and substances of humus experience an alkaline impact (pH 8–10) under natural conditions due to action of pedofauna, e.g. when passing through the gut of invertebrates (see references in [55]). Such zoogenically transformed materials in the humus horizon of the soils with Moder or Amphimull humus types may account for 70 to 100% of the soil volume [180]. Thus, natural OM even in acid soils experiences a local impact of alkaline conditions. Although certain modification of the native OM can occur, Olk et al [121] believe that the HA and FA fractions consist of really existing natural molecules and can be used to study the molecular composition and functions of OM in soil and in aquatic systems. However, it should be noted that the structure of native colloidal humus has little in common with the HS state in alkaline extracts.

Use of alkaline extraction. Alkaline extraction (0.1–0.5 M NaOH) is the most efficient quantitatively, allowing extraction of up to 80% of humus C_{org} versus 30% of C_{org} when using neutral salts ($Na_4P_2O_7$ and NaF) [158]; correspondingly, diluted alkali solutions are preferable as compared with other extracting agents. Nonetheless, alkaline extraction selectively isolates the substances enriched with polar groups and exhibiting increased reactivity relative to the OM in general [97, 108]. That is why, the use of alkaline extraction requires that the areas of its application are justified [121]. Alkaline extraction is generally accepted method for isolation of humic preparations [139]. A high applied value of these preparations in agricul-

ture, ecology, and medicine is doubtless even for the opponents of the humic concept [96]. The content of HA fraction indicates the maturity of composts [53] and the degree of humification of soil OM [21, 23]. The C_{HA}/C_{FA} ratio may serve as a measure of soil OM hydrolyzability if FAs are not regarded as a real and independent group of soil humus [23]. Alkaline extraction makes it possible to determine molecular weights and molecular formulas of tens of thousands of individual OM components by FT-ICR MS as well as to approximately estimate their relative abundance [89, 130]. Neither bulk soil nor its density fractions are suitable for such assays. The experiments with HAs and FAs allowed construction of good prognostic models on migration and distribution of metal ions and pesticides in soil [73, 106, 165], which serve in favor of HA and FAs as representative fractions of soil OM when studying and simulating such processes [12]. On the other hand, the question arises on whether it is possible to assess the OM reactivity in soils based on the reactivity of the substances in alkaline extracts. In soils, part of the OM functional groups is “occupied” by binding with the mineral matrix, which is well reflected in the conceptual model of organomineral interactions in soils of Kleber et al [98]. For example, binding of phenolic acids by minerals occurs mainly via carboxylic groups; these groups are the main reaction centers in the pH range of 4–7, characteristic of most soils [5, 6]. Correspondingly, the metal ions complexation ability assessed for HA solutions is overestimated as compared with mineral-bound state of HA.

Summing up, many alternative approaches to the isolation of OM have been designed in addition to the alkaline extraction, with a purpose to decrease the heterogeneity and expand the range of the components accessible for structural analysis. Among these approaches is the use of aquatic extracting agents: buffers and complex-forming solutions [158], polar and nonpolar organic solvents applied before alkaline extraction (humeomics [117]), and, finally, systems of solvents, including DMSO, allowing isolation of the components of humin [78, 80, 153, 154]. As a result of successive extractions, a number of OM fractions differing in hydrophilicity can be isolated; in total, they account for a larger share of the soil C_{org} as compared with the classical alkaline extract and better reflect the diversity of properties of different soil OM components. The approaches combining densimetric fractionation and extraction [52] also contribute to a decrease in OM heterogeneity. Recent advance in the spectral methods (pyrolysis-field ionization mass-spectrometry, nano-SIMS, and solid-phase ^{13}C NMR) is promising for the in situ research into natural OM [140] but cannot completely replace extraction methods. Extraction and in situ studies must be complementary.

CONCLUSIONS

Humus and humic matter distinctly differ from detritus (poorly decomposed organic residues) in their morphological, chemical, and functional properties. In its composition, humus is the complex of initial biomolecules of plant and animal tissues, the products of their degradation and transformation, and the products of extracellular synthesis. All these substances form a dynamic system of a colloidal nature stabilized by covalent, ionic and intermolecular interactions. This suggests that the generally accepted division of this complex entity into the substances with a poorly defined structure (HSs) and the substances of known classes (non-HSs) is rather arbitrary. This division contradicts with the operational approach to HS isolation and leads to the problem of selectivity of extraction towards humification products. There is still no consensus on what is to be regarded as HSs—only polar carboxylated substances of alkaline extracts (HAs and FAs) or humin as well, which contains a considerable contribution from identifiable nonpolar compounds (lipids, cutin, etc.). The definition of HSs as dark-colored substances formed by biodegradation and free radical condensation reactions, dissimilar to the biopolymers of plant and microbial tissues should be reconsidered as it creates problems with identification of these substances in the already formed humus. There are no doubts that free radical reactions can take place in soil; however, their quantitative contribution to the formation of natural OM requires further studies.

The traditional alkaline extraction should be regarded as the method for dissolution of polar substances of humus and the HA precipitation with acid, as the method for concentration and primary purification of a quantitatively significant number of components for further analysis. This is also a commonly accepted method for production of the preparations with a high practical value. The terms HAs, FAs, and humin should be attached to the operational fractions (preparations) without regarding all their constituents as specific chemically unique substances. The C_{HA}/C_{org} ratio is a simple and convenient characteristic of humus types that reflects the bioclimatic conditions of its formation. The insight into the mechanisms underlying formation, structural organization, and functioning of the substances constituting the humic matter requires further studies.

FUNDING

This study was supported by the Russian Science Foundation (project no. 17-14-01207; sections “The Concept of Humic Substances...” and “Secondary Synthesis”), State budget (project no. 121040800154-8; section “Problems in Terminology”), and Interdisciplinary Research and Educational School of Moscow State University “The Future Planet and Global Environmental Change” (section “Alkaline Extraction”).

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Translated by G. Chirikova