

Original Research

Correlation Biological Activity and Physicochemical Properties of Humic Acids in Aqueous Solutions

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Abstract

Objective. The biological activity of humic acids (HA), considering them as open non-equilibrium systems, in which properties are determined by the state or level of organization of the system.

Methods. The dependence of physicochemical properties of HA on their concentration (optical and sorption properties, ζ -potential and electrical conductivity, average size of optical homogeneities) were analyzed by several methods: optical microscopy; low-angle laser light scattering (LALLS) method on a Zetasizer Nano ZS analyzer; absorption on a Shimadzu UV-3600 spectrophotometer; chemical analysis on an ICP-QMS Agilent 7500CE spectrometer. Biological activity of HA was studied on the crustacean *Daphnia magna* and the monocotyledonous plants *Allium cepa* L.

Results. It was established that the nature of the HA effect on biological objects is non-linear and has an extremum at 1 mg•L⁻¹. It was shown that the mineralization of solutions affects the biological activity of HA. So in the presence of the addition of mineral salts, for *D. magna* the effect of HA is insensible, while for *A. cepa* L. it changes its direction. In the absence of mineral salts at HA concentrations < 5-10 mg•L⁻¹, a sharp drop in the survival of *D. magna* and suppression of root growth of *A. cepa* L. was observed, and in saline solution – stimulation of root growth. It has been established that the concentration range of 5-10 mg•L⁻¹ was also critical for concentration changes in a number of physicochemical parameters of aqueous solutions of HA, which makes it possible to consider it a phase transition characterized by new properties.

Findings. It was shown that changes in various properties of aqueous solutions of HA, including biological activity, were characterized by non-linear concentration dependencies with the presence of intermittent changes indicating qualitative rearrangements in the system.

Keywords: humic substances, humic acids, bioassays, biological activities, physicochemical parameters

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Introduction

Humic substances (HS) are ubiquitous natural products of the decomposition of organic material, as well as a necessary and mandatory part of all processes in the biosphere. They perform a number of important functions: accumulative, transport, regulatory, protective and physiological [1-4]. In this paper, attention is paid to the physiological function, i.e., the ability of HS to cause certain reactions when they interact with living organisms. Numerous studies allow us to state the presence of a positive response of living cells to the HS in the environment. Thus interacting with living organisms, HS in small quantities stimulate plant growth [5, 6], and are able to protect living cells from the toxic effects of natural and anthropogenic compounds [7]. HS not only regulates the processes of nutrition and plant development [8], but they themselves can serve as a source of [9-11]. That is, nature in its genius has come up with a completely unique product that combines the properties of both organic and inorganic fertilizers, being like a universal growth factor.

At the same time, there are quite numerous data in the literature on the negative effect of HS on the state of living organisms. For example, HS can also inhibit plant growth [12], cause expression of heat shock proteins [13, 14], modulate biotransformation enzymes because of oxidative stress [15-17], inhibit photosynthetic oxygen release in plants [18-21], alter ionic homeostasis in freshwater animals [22-25], as well as have hormone-like effects on water crustaceans [26, 27], amphibians [28], fish [29, 30] and other animals [31, 32].

The pharmaceutical industry has seen an increased interest in the use of HS [33, 34] as potent biomaterials for therapeutic and drug delivery systems [35, 36]. HS and their derivatives were used as auxiliary substances with oral drugs [37], treatment drugs of eczema [38] and other drugs [39]. However, in these studies we did not consider the possible effect of HA. Investigated toxic effects in the use of drugs in combination with HA, for example natural phytocomplex with potential procognitive activity [40], peat applied to organic-mineral fertilizer production [41], and other drugs [42, 43], but these studies had not fully explored the possible mechanisms.

The relevance of the work associated with modifications of water purification systems comes from surface-active substances, including HA [44]. Different properties of HAs should also be taken into account in studies of decolorization of azo dye [45], soil cleaning and water purification with electrophysical [46] and electrochemical [47] methods, and water purification with different adsorbents [48].

The generally accepted approach to assessing biological activity and the manifestation of, for example, a detoxifying effect by identifying the relationship with the source of origin of HS, as well as the chemical structure of commercial preparations, is not sufficiently correct. First, regardless of the source of origin, all HS

have a number of common properties: the ability to hydrophilic-hydrophobic interactions and complexation, anti-radical, antioxidant and radioprotective activity, enzymatic activity, as well as membranotropic properties [49-52]. Secondly, the existing approach does not explain the inconsistency of the experimental data obtained. In explaining the dual effects of HS on living organisms, it is necessary to proceed from the fact that HS are not just an accidental product of a specific biosynthetic cycle of utilization of biomass residues. They are a necessary link in the evolution of the biosphere, the most important factor in the stability of life processes. The functioning of HS should be of a targeted nature and be aimed at ensuring the existence of comfortable conditions for living organisms in the biosphere with minimal energy expenditure. In order to understand whereby HS provides the performance of the previously mentioned functions in order to continue their further directional use, it is necessary to have a clear idea of what HS are and their properties. For a long time, in accordance with the concept of D.S. Orlov [1], HS was considered a complex mixture of specific nitrogen-containing high molecular weight heteropolymers. Relatively recently, A. Piccolo [53] put forward the theory of the dominant contribution of low molecular weight components in the composition of HS, which exists in a solution in the form of micelles. We then formed a new concept of molecular organization of HS based on the main principles of supramolecular chemistry, according to which HS are ensembles of organic molecules of relatively small size, built according to the "guest-host" principle and interconnected mainly by weak rather than covalent bonds into a stable, degradation-resistant complex. This is based on the work of Gilli P. and Gilli N. [54], Pignatello J. and Ni J. [55, 56], where at the first time we recognized the contribution of exceptionally strong hydrogen bonds to the intermolecular forces holding HS molecules together. Finally, according to Wells M. and Stretz H. [57], supramolecular associations of HS consist of metachemical hydrogels formed by near-covalent strong hydrogen bonds that are encapsulated within dispersible physical hydrogel scaffolds produced by non-covalent weak intermolecular forces (van der Waals, weak hydrogen bonding, ionic effects) in a hierarchical 'supramolecular within supramolecular' self-assembling architecture.

According to I.V. Perminova [58], HS can be considered as open non-equilibrium systems with complex multi-level space-time self-organization. A high level of fluctuations and the occurrence of critical states, as well as bifurcations should characterize such systems during the transition between these states [59]. It is not correct to determine the properties of such systems by the sum of the components' properties or their averaged values. The properties of a system are determined by its state, which depends on the composition of the system (up to trace impurities), on external factors (temperature, pressure, electromagnetic and sound waves, and other physical

effects) and on time as a factor of non-equilibrium systems [60]. That is why the obtained experimental data of various properties of HS, which always characterize a specific state of an individually developing system, often differ greatly, which is reflected in the usual discrepancy for HS and even the contradiction of literary data.

In this regard, we propose to change the approach to the study of HS, namely, not from the point of view of their composition, which in turn is determined by the history of origin, as most researchers do, but to consider the change in their state in a particular system. The change in the system state can be tracked by the control parameter changes. In this study, HS concentration is used as such a parameter. In this way, the purpose of this work was to establish the patterns of influence of different concentrations of humic substances on living organisms and to analyze the obtained results from the point of view of open non-equilibrium systems.

Classical bioassays were chosen for assessing the effect of HS on living organisms, namely, daphnia (*Daphnia magna*) [61] – the crustacean object of the invertebrate freshwater fauna, and the representative of monocotyledonous plants – bulb onion (*Allium cepa* L.) [62].

Therefore, the aim of the work was to determine the critical concentrations of humic acids in an aqueous solution relative to changes in physicochemical indicators and indicators for living organisms.

Materials and Methods

The scheme of the experimental design is shown in Fig. 1.

Humic Substances

The commercial product of the sodium salt of humic acid (HA) (Sigma-Aldrich, USA) without further purification was used in this study. The concentrations of HA were $1 \text{ mg}\cdot\text{L}^{-1}$, $5 \text{ mg}\cdot\text{L}^{-1}$ and $15 \text{ mg}\cdot\text{L}^{-1}$.

The aqueous solutions were prepared by dissolving the precise dosage of the product (analytical scales

ATL-80d4 Acculab, Russia) in deionized water (MilliQ-system, UK).

Chemical analysis of deionized water was performed by inductively coupled plasma-mass spectrometry on the ICP-QMS Agilent 7500CE spectrometer (Agilent Technologies, USA). Calibration solutions with a high range of element concentrations (from $0.1 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ to $100 \text{ }\mu\text{g}\cdot\text{L}^{-1}$) were used for device calibration. The solutions were prepared based on the international standard 2.74473.0100 “ICP Multi Element Standard Solution XXI CertiPUR,” which contains the following elements: Ag, Al, As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Tl, U, V, Zn, and Hg. The concentration of all above-listed 24 elements in the deionized water did not exceed the upper detection limit (detection limit range 0.1-10 ppm). Deionized water had no differences in physical characteristic or in trace element composition, except the humic content. This excluded the multifactor influence in the system for all comparison groups.

Effect of Concentration of Humic Substances on Their Physicochemical Properties

The size and ζ -potential of particles of sodium humate (NaH) in aqueous solution, and also the electric conductance of solutions were determined by the dynamic and electrophoretic light scattering methods, accordingly, on an analyser Zetasizer Nano ZS (Malvern Instruments, UK) with He-Ne laser (wavelength of 633 nm, maximum power of 4 mW), while using Version 6.20 Software. Each measurement was repeated at least 5 times, and the obtained results were averaged for further analysis. The relative error did not exceed 10%. The absorption spectra of aqueous solutions were recorded on a Shimadzu UV-3600 spectrophotometer (Shimadzu, Japan). The pH values were measured using a pH-meter pH-150 (Acculab, Russia). The sorption properties of HA were studied on the example of their adsorption by kaolinite in a static mode [63]. The number of functional groups in the HA molecule was determined by potentiometric titration [64].

Daphnia magna Culture Assays

D. magna is a standard sensitive species of crustaceans used in bioassay of water [61]. Laboratory culture of *D. magna* was established from gravid females originally acquired from Laboratory of biotesting and bioindication (Dumanskii Institute of Colloid and Water Chemistry, Ukraine). The culture was maintained in synthetic laboratory water [61]. *D. magna* were kept under constant light/dark (16:8 hours) and temperature (20-22°C) conditions and were fed a slurry of yeast, with water renewal every two days. All bioassay procedures were performed according to standard methods [61, 62].

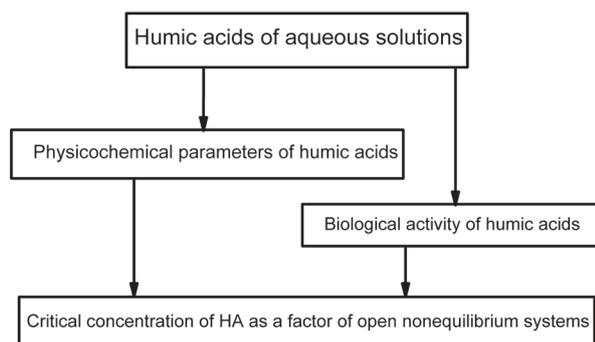


Fig. 1. Scheme of the experimental design.

Table 1. Survival results of *D. magna* at different concentrations of HA in model solution (MS) and in deionized water (DW). Experimental water samples: No. 1 – MS + 1 mg·L⁻¹ HA; No. 2 – MS + 5 mg·L⁻¹ HA; No. 3 – MS + 15 mg·L⁻¹ HA; No. 1* – DW +1 mg·L⁻¹ HA; No. 2* – DW +5 mg·L⁻¹ HA; No. 3* – DW + 15 mg·L⁻¹ HA. * p<0.05 the reliability of the differences between the tested samples in relation to MS and DW accordingly.

Exposure time, h	Experimental water samples							
	MS	No. 1	No. 2	No. 3	DW	No. 1*	No. 2*	No. 3*
	Survival, %							
24	100	100	100	90	80*	0*	70*	90
72	100	90	100	90	70*	0*	50*	90
96	100	90	90	90	40*	0*	40*	90

Effect of Humic Substances on *D. magna* Survival

The criterion for assessing the effect of HA on *D. magna* was the survival index of juvenile form of daphnia in two types of water solutions with different concentrations of HA:

- 1) In the model solution prepared on the basis of deionized water in the presence of mineral salts [17, 19] with the addition of HA. The model solution of mineral salts (MS) without the addition of HA served as control.
- 2) In deionized water with the addition of HA. Control was provided by deionized water (DW) without the addition of HA.

Each experiment was repeated 3 times (n = 10) for each concentration of HA (1 mg·L⁻¹, 5 mg·L⁻¹ and 15 mg·L⁻¹). The duration of the experiment was 4 days. The water temperature during the entire experiment was 22°C with the help of climatic chambers (MIR-3, Russia) with thermo regulators, the light regime corresponded to the change of day and night. All environmental conditions were maintained the same throughout the experiment.

All experiments were in accordance with ethical standards and principles adopted by EU Directives 2010/63/EU for animal testing.

Allium cepa L. Assays

Calibrated in size (1.5-2 cm diameter) «Stuttgart» (Agrofirm, Ukraine) bulb onions *A. cepa* L. from Laboratory of biotesting and bioindication (Dumanskii Institute of Colloid and Water Chemistry, Ukraine), untreated with plant growth regulators, were used for experiments. All bioassay procedures were performed according to standard methods [62].

Effect of Humic Substances on *A. cepa* L.

The assessment of the influence of HA on *A. cepa* L. was carried out by germination of reference bulbs in the investigated model solutions [62]. The criteria of influence are qualitative changes in the size

and weight of bulbs of the roots in the experimental group compared with the control after exposure for 120 hours. Each experiment was repeated 3 times (n = 10) for each concentration of HA (1 mg·L⁻¹, 5 mg·L⁻¹ and 15 mg·L⁻¹). On the fifth day the roots of the plants were weighed (analytical scales ATL-80d4 Acculab, Russia) and their lengths measured (analytical line, Acculab, Russia). Then the average length and mass of the roots were determined for each bulb, and then separately for each sample.

Statistics

The findings were processed by the statistical methods (t-student test) using software packages of Microsoft Excel. Each value on the figures represents «mean±SD» and the level of reliability was determined at p<0.05

Results and Discussion

The results of the study of the effect of HA on the survival of daphnia (juvenile form) in two types of aqueous solutions – in the presence of additives of the standard set of salts (MS) and in deionized water (DW) – are given in Table 1.

As can be seen from Table 1 in saline solutions, HA at all investigated concentrations practically does not

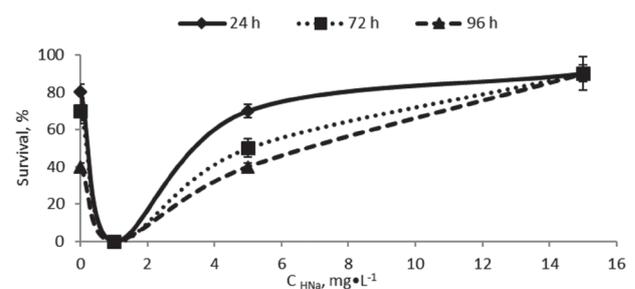


Fig. 2. Influence of HA concentration (C) on survival of *D. magna*.

affect the survival of daphnia. It should be noted that under these conditions, the survival of daphnia, both in experimental samples and in the control sample, practically does not change with an increase in the duration of the experiment (within 4 days). However, experiments conducted on deionized water allowed us to detect the difference in the influence of concentrations of HA on the survival of daphnia (see Table 1). The nature of the influence of concentration of HA on the survival of daphnia is shown in Fig. 2. As can be seen, additives of HA in the investigated concentration range (0-15 mg•L⁻¹) exert a nonlinear extreme effect on the survival of these objects. Thus, in a sample containing 1 mg•L⁻¹ of HA, after 24 hours all hydrobionts died, in contrast to a sample containing 15 mg•L⁻¹, in which survival was almost unchanged throughout the experiment. Changes at 5 mg•L⁻¹ are close to deionized water.

When investigating the influence of HA on *A. cepa L.* all model solutions with different contents of HA did not show toxic effects. In all cases, the phytotoxic effect was less than 50%. However, these bio objects react to the concentration of additives of the HA, like daphnia (Fig. 3), namely, they have extreme values at a concentration of HA 1 mg•L⁻¹. It should be noted that for the investigated solutions prepared on deionized water, the extreme point on concentration dependencies corresponds to min, and for saline solutions to max. In the first case, at concentrations of HA up to ~5-10 mg•L⁻¹ there is a suppression of growth of *A. cepa L.* observed, and in the other – its

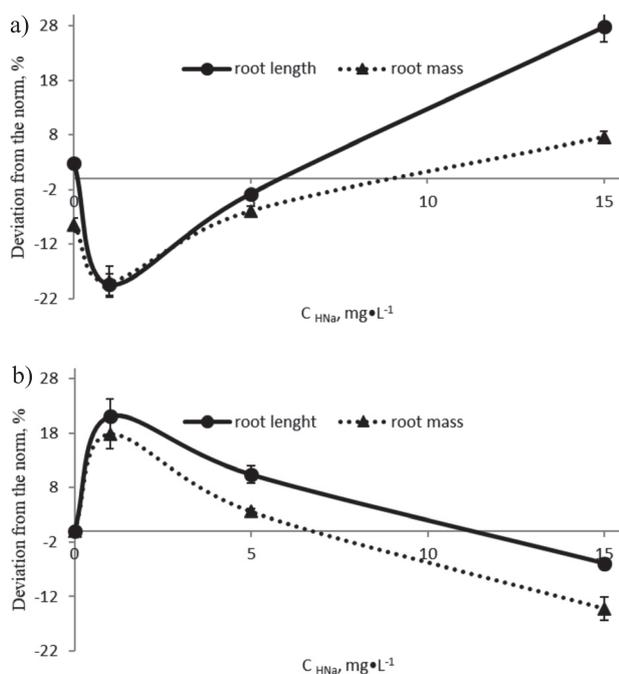


Fig. 3. Influence of HA concentration c) on changes in length and mass of roots of *A. cepa L.* in deionized water a) and in model solution b).

stimulation. The revealed multi-directional effect of the HA correlates well with known literary data on the influence of low concentrations of HA on living organisms [65].

In explaining of the multi-directional action of the HA, depending on their concentration in the solution from the point of view of open non-equilibrium systems, it can be assumed that before and after 5-10 mg•L⁻¹ HA are in different states characterized by sometimes opposite properties. Concentration range of 5-10 mg•L⁻¹ is critical, which reflects the transition of HA from one state to another. If this assumption is correct, then this concentration range should be critical for the concentration changes of any other system properties.

Fig. 4 shows changes in the optical and sorption (4a) properties, ζ-potential and electrical conductivity (4b), as well as the average size of optical in homogeneities (4c) recorded in aqueous solutions of HA by the method of dynamic light scattering, and the number of available acidic groups in a molecule of HA, determined by the

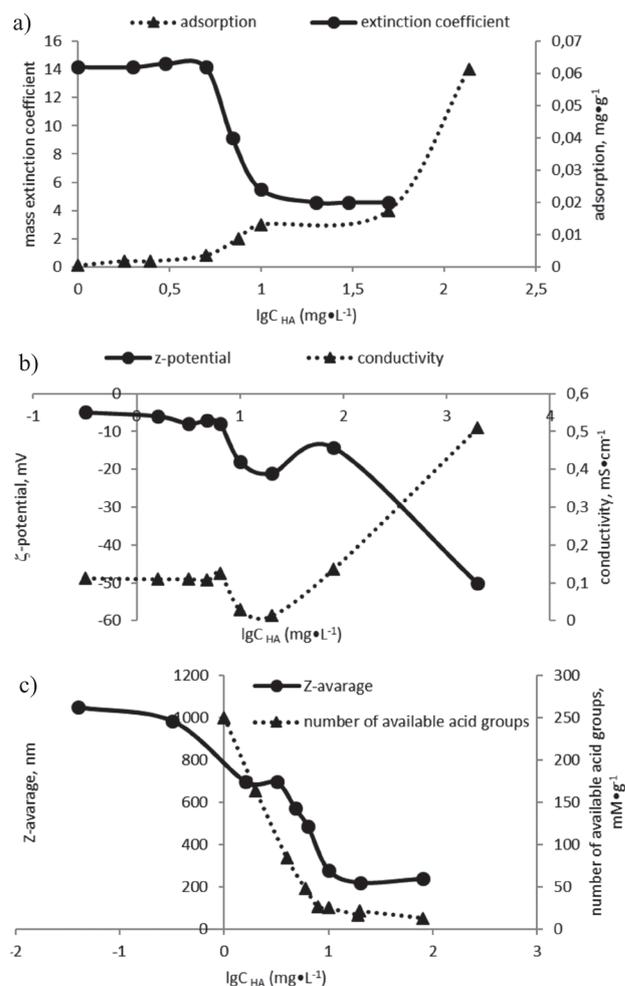


Fig. 4. Dependence of physicochemical properties of HA on their concentrations. Optical and sorption properties a), ζ-potential and electrical conductivity b), average size of optical homogeneities c).

method of potentiometric titration. As can be seen, all the presented dependences in the concentration range of 5-10 mg•L⁻¹ are characterized by sharp deviations from linearity, which is characteristic of phase transitions. As we previously suggested in [63], this concentration range should be considered as a critical concentration of the formation of supramolecular associates.

Our results confirm the structural changes in aqueous solutions of HA with an increase in their concentrations. Due to the association (self-organization) of HA molecules in an aqueous solution, the spatial arrangement of atoms and groups, the conformation of molecules and their enlargement, structure deformation change. As a result, a part of the active groups of molecules becomes enclosed inside the associate, as well as shielded fragments of other molecules that are practically inaccessible for chemical interaction. In addition, increasing the concentration of HA reduces the distance between molecules. Functional groups can enter into intermolecular interactions, form intramolecular and intermolecular hydrogen bonds, which can lead to shielding not only with acidic groups, but also with phenolic ones, which are responsible for radical processes.

Thus, the concentration dependences of the physicochemical parameters of aqueous solutions of HAs confirm the possibility of the existence in the system at least two states of HA characterized by different properties. The concentration range of 5-10 mg•L⁻¹ can be considered a transition between these states. At the same time, their complex-forming ability with metal cations, radical-oxidative activity [60, 63], and biological activity significantly change. It should be noted that the biological activity of HA is more pronounced for dilute solutions.

Conclusions

This paper proposes a new approach to assessing the biological activity of HA, namely considering them as open non-equilibrium systems in which properties are determined by the state or level of organization of the system. It is shown that changes in various properties of aqueous solutions of HA, including biological activity, are characterized by non-linear concentration dependencies with the presence of intermittent changes, indicating qualitative rearrangements in the system. This fact determines the possibility of using the HA concentration to establish the level of organization of the system. Determining critical concentrations is especially important, since it will allow for managing the structural organization of these objects and predicting their properties. Such an approach, in contrast to the generally accepted assessment of biological activity by identifying relationships with the source of origin of HA, explains the inconsistency of literary data, as well as the ability of all HAs to perform the same biosphere functions.

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Conflict of Interest

The authors declare no conflict of interest.

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