MEDIATING EFFECTS OF HUMIC SUBSTANCES IN THE CONTAMINATED ENVIRONMENTS

Concepts, Results, and Prospects

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Abstract: A new concept for the mediating action of humic substances (HS) in the contaminated environment is developed. It defines three scenarios of mitigating activity of HS in the system "living cell-ecotoxicant". The first scenario refers to deactivation of ecotoxicants (ET) by HS due to formation of non-toxic and non-bioavailable complexes. It takes place outside of the cell and is defined as “exterior effects”. The second scenario refers to deactivation of ET due to HS adsorption onto the cell wall or membrane and is defined as "boundary effects": sorption takes place on the cell surface and implies changes in permeability and structure of the cell membrane. The third scenario refers to amelioration of contaminant toxicity due to activation of systemic resistance to chemical stress. This implies HS participation in immune response activation and is defined as "interior" effects. Viability of this concept was confirmed by the results of detoxification experiments. It was shown that chemical binding ("exterior effects") played a key role in ameliorating toxicity of ecotoxicants (Hg(II) and PAHs) strongly interacting with HS, whereas enhanced immune response ("boundary and interior" effects) was much more operative for a decrease in toxicity of atrazine weakly interacting with HS. The formulated concept provided satisfactory explanations for a vast pool of reported findings of mitigating activity of HS reviewed in the chapter. Few cases of amplified toxicity reported for weakly interacting contaminants in the presence of low molecular weight HS were related to facilitated penetration and follow up dissociation of humic-contaminant complexes in the cell interior. It is concluded that the developed concept can be used as a prospective tool for both predictive modelling of

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mediating effects of HS in the contaminated environments and designing new humic material suitable for a use as detoxicants and plant activators.

Key words: humic substances; mercury; polycyclic aromatic hydrocarbons; atrazine; detoxifying ability; mediating effects

1. INTRODUCTION

Humic substances (HS) compose from 60 to 80% of non-living organic matter in both soil and water ecosystems (Thurman and Malcolm, 1981). With the turnover of 2 Gt of carbon per year, humification is the second largest process after photosynthesis (20 Gt/a) contributing into the global carbon cycle (Hongve et al., 1981). HS are products of chemical-microbiological synthesis occurring during decomposition of mortal remains of living organisms. In contrast to the synthesis of biopolymers in living organisms, formation of humic molecules does not have a genetic code and proceeds stochastically: the only structures that survive are those which resist further microbial and chemical decomposition. As a result, intrinsic features of HS are non-stoichiometric elemental compositions, irregular structures, heterogeneous structural units, and polydisperse molecular weights (Kleinhempel, 1970). Evidently, there are no two identical molecules of HS. Despite that, HS of different origin have a very similar structural organization. A humic macromolecule consists of an aromatic core highly substituted with functional groups (among those dominant oxygen functionalities – carboxyls, hydroxyls, carbonyls) and of peripheral aliphatic units composed mostly of polysaccharidic and polypeptidic chains, terpenoids, etc. (Stevenson, 1994).

Complex structure provides for a very diverse reactivity of HS. They are able of ionic, donor-acceptor (including hydrogen bonding and charge-transfer complexes) and hydrophobic bonding. As a result, they can bind heavy metals as well as polar and highly hydrophobic organic compounds released into environment (e.g., pesticides, polycyclic aromatic hydrocarbons, polychlorinated compounds). The mitigating impact of HS prescribed to formation of non-toxic and non-bioavailable humic complexes was numerously reported in the literature (Giesy et al., 1983; Vymazal, 1984; Landrum et al., 1985; McCarthy et al., 1985; Morehead et al., 1986; Oris et al., 1990; Perminova et al., 1999; Misra et al., 2000). On the other side, hydrophobic aromatic core and hydrophilic peripheral moieties (e.g., polysaccharides) determine amphiphylic character and surface activity of

humics. This brings about biological activity of HS resulting from direct interactions with living organisms through adsorption on cell surface or by penetration into the cell (Müller–Wegener, 1988; Kulikova et al., 2005). These chemical-biological interactions provide for increasing interest to remedial uses of humic materials (Perminova and Hatfield, 2005).

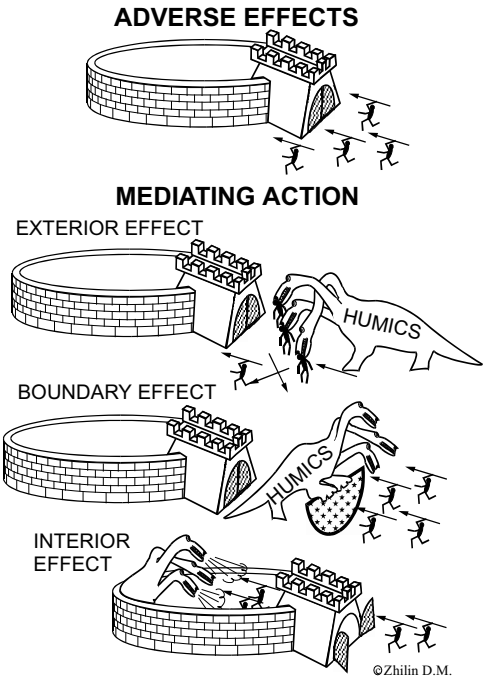

Figure 1. Conceptual model of mediating effects of HS in the system "living cell-ecotoxicant". Three proposed scenarios refer to "exterior", "boundary", and "interior" effects according to interactions involved. "Exterior effects" imply deactivation of ecotoxicant by HS due to formation of non-toxic complexes and take place outside of the cell. "Boundary effects" imply deactivation of ecotoxicant due to HS adsorption onto cell wall and take place on cell surface. "Interior effects" imply amelioration of contaminant toxicity due to activation of systemic resistance to chemical stress and take place inside of the cell due to HS participation in activating cell immune response.

The primary goal of the present paper is to develop a holistic concept of the mediating action of HS accommodating both chemical and biological interactions occurring in contaminated systems. It defines three major scenarios of mitigating activity of HS in the system "living cell-ecotoxicant", which are visualized in Figure 1 using the allegory of "dragon" (humics) taking three different tactics to protect its "fortress" (cell) from the attacking enemy (ecotoxicant). The first scenario refers to deactivation of ET due to

chemical binding to HS leading to formation of non-toxic and non-bioavailable complexes. It takes place outside of the cell and is defined as “exterior effects”. The second scenario refers to deactivation of ET due to HS adsorption onto cell wall or membrane and is defined as "boundary effects": sorption takes place on cell surface and implies changes in permeability and structure of cell membrane. The third scenario refers to amelioration of ET toxicity due to activation of systemic resistance to chemical stress. This implies HS participation in immune response activation and is defined as "interior" effects.

The mediating action of HS in reality might imply simultaneous involvement of all three mechanisms. However, their prevalence will depend on molecular properties of HS. So, chemical binding is the most probable way of mediating action of HS possessing high affinity for ET. For high molecular weight HS, which cannot penetrate cell walls because of steric hindrances, this can be the only mechanism of mediating action. The “boundary” mechanism is expected to be most operative for the hydrophobic HS. The third mechanism can be of particular importance for low molecular weight fractions of HS, which can penetrate the cells.

2. REVIEW OF MEDIATING EFFECTS OF HUMIC SUBSTANCES ON SELECTED CONTAMINANTS

2.1 Mercury

An overview of the effects exerted by HS upon mercury toxicity to and bioaccumulation by the aquatic test organisms demonstrates a purely mitigating action of HS regardless of its source and a type of the test-organisms. Decrease in mercury toxicity and bioaccumulation in the presence of natural and synthetic organic chelating agents is widely reported in literature (Oikari et al., 1992; Sjoblom et al., 2000; Hammock et al., 2003) and usually is related to a reduction in concentration of Hg(II).

2.2 Polycyclic Aromatic and Polychlorinated Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs), polychlorinated dioxins (PCDDs) and polychlorinated biphenyls (PCBs) belong to the large class of hydrophobic organic contaminants (HOC). Both uptake and bioaccumulation

of these compounds are strongly dependent on hydrophobicity of the chemical (as indicated by its octanol/water partition coefficient, K_{OW}) (Hawker and Cornell, 1985; Akkanen and Kukkonen, 2003) and environmental factors which can modify its physical-chemical state (Müller–Wegener, 1988). Among the factors determining speciation of HOC in aquatic systems, the most important is their partitioning to particulate and dissolved organic matter (Carlberg and Martinsen, 1982; Landrum et al., 1985; Chiou et al., 1987; Hur and Schlautman, 2004). The importance of this process for toxicity and bioavailability of PAHs, PCDDs and PCBs is demonstrated by numerous publications (Landrum et al., 1985; Carlberg et al., 1986; McCarthy and Bartell, 1988; Servos et al., 1989; Oikari and Kukkonen, 1990; Weber and Lanno, 2000; Reid et al., 2001). Hydrophobic binding is assumed to be a major mechanism of HOC association with DOM (Merkelbach et al., 1993; Perminova et al., 1999). Given that DOM in natural waters is predominantly composed of HS (about 50% of DOC (Thurman and Malcolm, 1981) many publications pay specific attention to chemical interaction between HS and HOC. It is found that humics – HOC association is the stronger, the higher K_{OW} both for humic and HOC compounds are (McCarthy et al., 1989; Merkelbach et al., 1993; Kopinke et al., 1995; Poerschmann et al., 2000). On the other hand, HOC sorption is greatly affected by chemical structure and composition of HS (Kang and Xing, 2005). Taken into consideration that HS hydrophobicity is determined by their structure (Kopinke et al., 1995), the binding constant for the same contaminant can greatly vary depending on the source of humics. For example, the most hydrophobic Aldrich HA displayed the highest affinity for HOC, whereas the most hydrophilic HS – surface water DOM were characterized with the lowest affinity for HOC (Landrum et al., 1985; Morehead et al., 1986).

Binding to HS controls accumulation of HOC by aquatic organisms. This is solidly confirmed by the results on HOC bioaccumulation from humics-containing solutions. All humic materials, regardless of their origin, caused a reduction in bioaccumulation of PAHs, PCBs and TCDD both by crustaceans and fish species. The reduction was proportional to hydrophobicity of HS present and generally consistent with the value of binding constants. Fractionation of DOM with a use of XAD-8 resin showed that it was hydrophobic anionic fraction of the DOM, which was responsible for the contaminant-DOM interaction (McCarthy et al., 1989; Kukkonen, 1991). Exceptions from the given observations were low hydrophobic PAHs (naphthalene, anthracene, phenanthrene) and extremely hydrophobic congeners of PCDD with more than six substituting chlorine atoms whose bioaccumulation was practically not affected by HS (Bruggeman et al.,

1984). The above results brought different researchers to a conclusion that a use of the commercially available Aldrich HA as a model humic material can greatly overestimate the potential of DOM to reduce HOC bioaccumulation by aquatic biota. It means that particular caution should be exercised while predicting mitigating impact of HS on bioaccumulation potential of PAHs and polychlorinated hydrocarbons in a real aquatic system. It is especially true given the fact, that influence of HS on toxicity of these chemicals is hardly studied at all.

2.3 Pesticides and Other Organic Xenobiotics

Another big group of hazardous organic chemicals is presented by pesticides, which vary greatly in their chemical structure. All pesticides are characterized by high toxicity to biota (Choudhry, 1983; Draber et al., 1991). A whole number of publications are devoted to examination of pesticide toxicity in the presence of HS (Stewart, 1984; Day, 1991; Oikari et al., 1992; Genevini et al., 1994; Steinberg et al., 1994; Freidig et al., 1997; Gensemer et al., 1998; Koukal et al., 2003; Mézin and Hale, 2003). However, there is a substantial disparity in the reported findings. This can be explained by diverse chemical and physiological mechanisms involved. Comparison of the HS effects exerted on toxicity of pesticides, substituted phenols and anilines shows that enhanced toxicity was mostly observed in the test solutions containing low molecular weight HS. For example, increase in acute toxicity of nine out of thirteen organic chemicals was found in the presence of DOM from different lakes (Oikari et al., 1992). Stewart (1984) reported on enhanced toxicity of eight methylated phenols and anilines to algae in the test solution containing commercially available soil fulvic acids with molecular weight ranging from 643 to 951. Comparison of HS effects of different origin – Aldrich HA (Na-form) and DOM – on toxicity of fenvalerate (Oikari et al., 1992) – shows that in the presence of high molecular weight hydrophobic Aldrich HA a reduction in toxicity takes place, whereas enhanced toxicity of fenvalerate is observed in the DOM-containing test solution. Very similar results were reported by Loffredo et al. (Loffredo et al., 1997). The authors observed a reduction in toxicity of three herbicides in the presence of soil HA, whereas sewage sludge HA (which are likely of lower molecular weight) resulted in an increased toxicity.

Summarizing the above data, it can be concluded that sensibilizing effect of low molecular weight fractions of HS on toxicity of some trace metals also takes place in case of pesticides and substituted phenols/anilines. These effects can be linked to long-term observations of soil scientists on the

specific physiological action of low molecular weight compartment of soil humics. According to numerous studies (Prát, 1963; Batalkin et al., 1982; Samson and Visser, 1989; Mazhul et al., 1993; Ermakov et al., 2000), HS can permeate or modify cell membranes. It is shown also, that FA are to be taken up to a larger degree than HA, and lower molecular weight HS (< 2500 D) to a greater extent than the higher molecular weight material (Prát, 1963; Führ, 1969; Führ and Sauerbeck, 1965). This allows a suggestion that membranotropic fraction of HS can facilitate penetration of bound to it low molecular weight compounds into the cell. These compounds can be heavy metals ions as well as molecules of pesticides or other toxic chemicals. It is the enhanced translocation of the toxic chemicals across biological membranes, which seems to be responsible for the discussed above increase in toxicity of some contaminants in the presence of HS with lower molecular weights.

The given contradictory findings show an importance of systematic studies on mediating effects of HS in the contaminated environments, which would couple chemical and toxicological interactions between ET and HS in the framework of a holistic conceptual model of the mediating action of HS.

3. CONCEPTUAL MODEL OF MEDIATING ACTION OF HS

To estimate contribution of chemical binding in mediating action of HS, an original approach has been undertaken described in detail in our previous publication (Perminova et al., 2001). According to this approach, interaction between HS and ET can be schematically described by the following equation:

$$\mathrm{HS} + \mathrm{ET} \leftrightarrow \mathrm{HS\text{-}ET} \tag{1}$$

To quantify this interaction, an equilibrium constant K is commonly used:

$$K = \frac{[\mathrm{HS\text{-}ET}]}{[\mathrm{ET}] \times [\mathrm{HS}]} \tag{2}$$

where [HS], [ET] and [HS-ET] are the equilibrium concentrations of the reagents and reaction product.

Given that the total HS concentration (C_{HS}) in natural system is much higher than that of ET (C_{ET}), [HS] in Eq. (1) can be substituted with C_{HS}. Because of the unknown stoichiometry of the reaction, C_{HS} is usually expressed on a mass rather than on a molar basis (Perminova , 1998; Swift, 1999). Due to that, the binding constant K can be rewritten in the form of sorption constant K_{OC} as follows:

$$K_{OC} = \frac{1-\alpha}{\alpha} \times \frac{1}{C_{HS}} \tag{3}$$

where α is the portion of freely dissolved ET in the presence of HS, $\alpha = [ET]/C_{ET}$, and C_{HS} is the total mass concentration of HS normalized to the content of organic carbon (OC), kgC/L.

The above expression allows us to estimate binding affinity of HS for ET by determining a portion of freely dissolved ET in the presence of HS. This can be done using common analytical techniques with or without preliminary separation of freely dissolved and HS-bound species of ET. On the other side, assuming that only freely dissolved ET is biologically active (toxic and bioavailable), the same binding constant can be determined from reduction in toxicity (T_{ET}) or bioaccumulation (BCF_{ET}) in the presence of HS. If the assumption were valid, binding constants determined from a reduction in concentration of freely dissolved ET using analytical techniques and from a reduction in toxicity and bioaccumulation using bioassay techniques would be equal. Thus, the similarity of the binding constants determined from chemical and toxicological experiments can be used for making a judgment on the contribution of chemical binding into mediating action of HS. Indeed, if only freely dissolved ET is toxic, then toxicity T in the presence of HS (T_{ET+HS}) can be expressed as follows:

$$T_{ET+HS} = \alpha \bullet T_{ET} \tag{4}$$

In this case, detoxification effect D exerted by HS can be defined as:

$$D = (T_{ET} - T_{ET+HS})/T_{ET} \tag{5}$$

If toxicity of ET is proportional to its concentration in the test system:

$$T_{ET} = k \bullet C_{ET} \text{ and } T_{HS+ET} = k \bullet [ET] \tag{6}$$

then D is numerically equal to a portion of ET bound to HS, ($1 - \alpha$). In this case, dependence of D on concentration of HS can be described by "detoxification" constant (K_{OC}^{D}) with a use of Eq. (3) as follows:

$$D = \frac{K_{oc}^{D} \times C_{HS}}{1 + K_{oc}^{D} \times C_{HS}} \tag{7}$$

The corresponding K_{oc}^{D} constant is an analogue of binding constant K_{oc} and can be calculated by fitting experimental relationships of D *versus* C_{HS} with a use of non-linear regression. To determine D from experimental data, ET toxicities in the absence and presence of HS were defined as follows:

$$T_{ET} = \frac{R_0 - R_{ET}}{R_0} \tag{8}$$

$$T_{ET+HS} = \frac{R_{HS} - R_{HS+ET}}{R_{HS}} \tag{9}$$

where R_0 is the response of a test organism in control solution, R_{ET} is the response of a test-organism in the ET solution, R_{HS} is the response of a test-organism in the HS solution; R_{HS+ET} is the response of a test-organism in the solution of ET with HS present.

To determine a reduction in ET toxicity provided exclusively by chemical binding to HS, its toxicity in the presence of HS (T_{ET+HS}) was normalized not to the control, but to the response of a test-organism in the presence of HS. The purpose was to take into account a possible stimulation effect of HS, while a decrease in ET toxicity in the presence of HS reflects a combined action of two effects: first, *toxicity sequestration* caused by a reduction in the concentration of freely dissolved ET due to binding to HS; and second, *stimulating effect* of HS on the test organism.

The corresponding detoxification effect D can be defined as follows:

$$D = \left(1 - \frac{R_{HS} - R_{HS+ET}}{R_{HS}} \Big/ \frac{R_0 - R_{ET}}{R_0}\right) \times 100\% \tag{10}$$

It means that each experimental point on detoxification curve results from four measurements of different responses of the target organism. To

ensure validity of the given model for toxicity data, it should be additionally assumed that sensitivity of test-organisms both to toxic action of freely dissolved ET and direct effect of HS does not change under impact of HS and ET present in the test system, respectively.

Bioconcentration factor (*BCF*) of ET can be defined with a use of the following expression:

$$BCF_{ET} = \frac{\text{concentration of ET in test - organism } (\mu\text{g/g w/w})}{\text{concentration of ET in the test solution } (\mu\text{g/ml})} \qquad (11)$$

In contrast to toxicity, BCF is not strongly affected by direct effect of HS, and depends mostly on the equilibrium concentration of ET in the presence of HS. It can be calculated with a use of the following expression:

$$BCF_{ET+HS} = \frac{\text{concentration of ET in test - organism } (\mu\text{g/g w/w})}{\text{equilibrium concentration of ET in the presence of HS } (\mu\text{g/ml})} \qquad (12)$$

In this case, the portion of freely dissolved ET can be written as follows:

$$\alpha = \frac{BCF_{ET+HS}}{BCF_{ET}^{0}} \qquad (13)$$

The corresponding "bioaccumulation" constant (K^B) can be determined by fitting an experimental dependence of a reduction in *BCF* equal to the portion of ET bound to HS ($1\text{-}\alpha$) versus concentration of HS by the model yielding from Eq. (3):

$$B = \frac{K_{oc}^{B} \times C_{HS}}{1 + K_{oc}^{B} \times C_{HS}} \qquad (14)$$

To test validity of the proposed approach, a corresponding experimental set up was developed. It implied determination of ET binding constants using both analytical and biotesting techniques. To encompass as broad range of mediating effects of HS as possible, three classes of ET greatly differing in chemical properties and biological activity were used for our studies, nominally: Hg(II) (the most toxic heavy metal), PAH – pyrene, fluoranthene, anthracene (highly hydrophobic organic contaminants) and atrazine (sim-triazine herbicide, specific inhibitor of photosynthesis). Of

particular importance is, that all studies were conducted using representative sets of structurally diverse HS samples from main natural sources (coal, peat, soil, fresh water) and of different fractional composition (humic *versus* fulvic acids). To assure statistical significance of the obtained results, each experimental set accounted for 20-25 HS samples.

4. EXPERIMENTAL PART

4.1 Ecotoxicants

For all experiments, *mercury(II)* salts of pure grade were used. The concentrations of mercury were $0.8 \cdot 10^{-6}$ M (as $HgCl_2$) and $(0.1–0.8) \cdot 10^{-6}$M (as $Hg(NO_3)_2$) for toxicity tests and analytical techniques, respectively.

PAHs used were *anthracene* (Aldrich, 98% pure), *fluoranthene* (Aldrich, 97% pure), *and pyrene* (Aldrich, 97% pure). The batch technique described elsewhere (Perminova et al., 1999) was used for preparation of aqueous solutions of the selected PAHs. For toxicity tests, the concentrations were $1.7 \cdot 10^{-7}$, $7 \cdot 10^{-7}$ and $5 \cdot 10^{-7}$M for anthracene, fluoranthene and pyrene, respectively.

Atrazine (99.97%) was purchased from Dr. Ehrensdorf Ltd. A stock solution of atrazine ($4.6 \cdot 10^{-5}$M) was prepared in distilled water and stored in the dark at 4 °C.

4.2 Humic Materials

Humic materials used were isolated from different natural sources (fresh water, soil, peat) using techniques described below.

Aquatic humic materials were isolated with a use of sorption on XAD-2 resin and follow-up elution with 0.1 M NaOH (Mantoura and Riley, 1975). The alkali extracts were desalted and used without further fractionation as a mixture of FA and HA. They were designated as *AHF*. Samples of native swamp water served as preparations of the aquatic dissolved organic matter (*ADOM*). *Peat humic materials* were isolated from 5 highland and 4 lowland peats of different geobotanical composition. The isolation procedure was as described elsewhere (Lowe, 1992) and included a preliminary treatment of a peat sample with an ethanol-benzene (1:1) mixture followed up by an alkaline (0.1 M NaOH) extraction. The alkali extracts were desalted and used without further fractionation as a mixture of FA and HA. They were designated as *PHF*. One sample was a concentrated water extract of woody-

herbaceous peat (*PDOM*). *Soil humic acids (SHA)* were extracted from soils of different climatic zones: Sod-podsolic soils (Moscow and Novgorod regions), gray wooded soils (Tula region), chernozems (Voronezh region). Sod-podzolic soils were also of various agricultural uses: virgin, plough, garden. The HS extraction was carried out according to Orlov and Grishina (Orlov and Grishina, 1981). This included pre-treatment of a soil sample with 0.1 M H_2SO_4, follow up alkaline extraction (0.1 M NaOH), and acidification of the extract to pH 1-2. The precipitated HA were desalted by dialysis. *Soil fulvic acids (SFA)* were extracted from 3 sod-podzolic soils of different agricultural use, virgin grey wooded soil and typical chernozem. To isolate FA, the supernatant after precipitation of HA was passed through Amberlite XAD-2 resin. Further treatment was as described for aquatic HS. *Non-fractionated mixtures of HA and FA* of soil (*SHF*) and fresh water bottom sediments (*BHF*) were isolated by alkaline extraction (0.1 M NaOH) of soil or bottom sediment sample without further fractionation of the extract. To isolate *non-fractionated mixtures of water-soluble soil HA and FA* (*SDHF*), the acidified (pH 1–2) water extract (1:2) of three sod-podzolic soils (virgin, plough, and garden) was passed through Amberlite XAD-2 resin and follow up elution with 0.1 M NaOH. Then the alkali extracts were desalted. Commercial samples of *coal HA* (*CHA*) – *Aldrich Humic Acid* (*CHA-AHA*) and *Activated Coal Humic Acid* (*CHA-AGK*) (Biotechnology Ltd., Moscow, Russia) were used as purchased from the suppliers. Concentrated stock solutions of HS (100–500 mgOC/L) were prepared by evaporation of the corresponding desalinated isolates or by dissolution of a weight of a dried material.

Structural characterization of HS. The target humic materials were characterised with the data of elemental analysis, size-exclusion chromatography (SEC) and ^{13}C NMR spectroscopy. The corresponding characteristics are given in our previous publications (Perminova et al., 1999, 2001). In brief, the humic materials used were characterized with the following parameters: contents of elements and atomic ratios (C, H, N, O, H/C, O/C), molecular weight, molar absorptivity at 280 nm (ABS_{280}) and contents of carbon in the main structural groups as measured using ^{13}C NMR under quantitative conditions (Kovalevskii et al., 2000). The assignments in the ^{13}C NMR spectra were made after (Kovalevskii et al., 2000) and were as follows (in ppm): 5-50 – aliphatic H and C-substituted C atoms (C_{Alk}), 50-108 – aliphatic O-substituted C atoms (C_{Alk-O}), 108-145 – aromatic H and C-substituted atoms ($C_{Ar-H,C}$), 145-165 – aromatic O-substituted C-atoms (C_{Ar-O}), 165-187 – C atoms of carboxylic and esteric groups (C_{COO}), 187-220 – C atoms of quinonic and ketonic groups ($C_{C=O}$).

For the experiments with *Hg(II)*, a set of 24 humic materials was used including 2 ADOM, 5 AHF, 3 BHF, 6 PHF, 3 SHA, 3 SHF and 2 CHA. For the experiments with *PAH* a set of 26 humic materials was used including 4 AHF, 7 PHF, 1 PDOM, 8 SHA and 5 SFA. For the experiments with *atrazine*, a set of 25 humic materials was used including 1 AHF, 3 PHF, 2 PDOM, 9 SHA, 5 SFA, 1 SHF and 3 SDHF.

4.3 Determination of the Hinding Constants Using Analytical Techniques

Mercury. Chemical binding of Hg(II) to humic materials was quantitatively characterized with an amount of the mercury-binding sites (BS) in the humic material and with the values of the stability constants of the Hg-HS complexes. To determine an amount of the BS in humic materials, the saturated Hg-HS precipitates were obtained at pH 2 under conditions described in (Zhilin et al., 1996). The content of Hg in the saturated humates (meq/g) was treated as equal to the BS content in the humic material. Basing on this parameter, Hg-equivalent concentration of humic preparation was determined and used for calculation of the stability constant of Hg-HS complexes instead of the molar concentration of HS. To determine the corresponding stability constant K_{BS}, ligand exchange technique with a use of adsorption was applied (Yudov et al., 2005). For this purpose, Hg(II) adsorption on polyethylene surface from 0.0025 M hydrocarbonate buffer was studied in the presence of HS. Total Hg(II) concentration accounted for 50–250 nM, concentration of HS 2-40 mg/L, pH 6.9–7.2. Hg(II) concentration in the presence of HS was determined using cold vapor AAS technique as described in (Zhilin et al., 2000).

PAH. Binding of PAH to dissolved humic materials was characterized by partition coefficients determined with a use of fluorescence quenching technique (K_{OC}^{fq}) as described in (Perminova et al., 1999). The PAH solutions below the water solubility limit (0.6×10^{-7}, 1×10^{-7} and 5×10^{-7} M for pyrene, fluoranthene and anthracene, respectively) were prepared by the solubilisation technique. The concentration of HS was in the range of (0.2-6) $\times10^{-6}$ kgC/L. The slopes of the obtained Stern-Volmer plots yielded the K_{OC}^{fq} values.

Atrazine. Binding of atrazine to humic materials was characterized with the binding constant K_{OC}. Binding experiments were conducted at pH 5.5 as described in (Kulikova and Perminova, 2002). The concentration of HS was in the range of $(0.2–0.8)\times10^{-3}$ kgC/L, initial concentration of atrazine was $9.3\cdot10^{-6}$ M. For the separation of the freely dissolved and the bound to HS fractions of herbicide, the batch ultrafiltration technique was applied using a

membrane filter with a molecular weight cut-off of 1000 D. Atrazine was determined in the ultrafiltrate with a use of a HPLC technique.

4.4 Toxicity Tests

Depending on ET type and media (aquatic or soil), different test organisms were chosen to carry out toxicity tests. Toxicity tests in aquatic media were conducted using as target organisms green algae *Chlorella* (Hg and atrazine) and Crustacea *Daphnia* (PAH). For soil bioassays with atrazine, wheat plants were used.

According to the proposed approach, each experimental series included the following treatments: control (water or nutrient solution), ET solution at the working concentration, HS solutions at five different concentrations, solutions of ET+HS at the working concentration of ET and above concentrations of HS. The obtained data were used for calculation of *D* using Eq. (10) at each concentration of HS.

Toxicity tests with algae. For the toxicity tests with Hg and atrazine, green algae *Chlorella pyrenoidosa* was used as described in (Polynov, 1992). The relative yield of variable fluorescence F_v/F_m was used as a target response. It is indicative of the quantum yield of chlorophyll fluorescence and can be derived from the induction fluorescence curve (Kautski effect). The algae were cultured in 20% nutrient solution (Tamiya et al., 1961) in temperature-controlled vessels at 35±2 °C under continuous irradiance of 60 μmol photons/m^2s provided by fluorescent lamps and bubbling with moisturized air. Prior to toxicity tests, algal cells were concentrated by centrifugation and resuspended in 10% Tamiya nutrient solution without phosphate or EDTA to yield an initial concentration of about 10^6 cells/ml. Then ET or HS or their mixture was added to algae cells. F_v/F_m ratio was determined after 4 (in case of Hg) or 3 hours (in case of atrazine) exposure under the same conditions as for algae growing. Fluorescence induction curve parameters (F_v/F_m and F_i/F_m) of algae chlorophyll were calculated after subtraction of the self-fluorescence of humic materials.

$HgCl_2$ was used as a toxic Hg(II)-species for conducting toxicity tests. The working concentration of $HgCl_2$ was $0.8 \cdot 10^{-6}$ M; concentration of HS varied in the range of 2.5–12.5 mg C/L; pH 7.2. Toxicity tests with atrazine were conducted at its working concentration of $6.7 \cdot 10^{-7}$ M. The concentration of HS varied in the range of 0.6-5 mg C/L; pH 6.8.

Toxicity tests with Daphnia. Acute toxicity tests with PAHs were performed according to Perminova et al. (2001). The Crustacea *Daphnia magna* was used as a test organism, its grazing activity (averaged filtration

rate per *Daphnia*, ml/hr) – as a target response. Daphnids were cultured in tap water at 20 °C with a light: dark rhythm of 16 h:8 h. A suspension of the green algae *Chlorella vulgaris* was used to feed *D. magna* daily. For the toxicity tests, 5–6 day old animals were used. The working solutions of PAH were prepared at the maximum achievable concentrations of, nominally, $5 \cdot 10^{-7}$, $7 \cdot 10^{-7}$, and $1.7 \cdot 10^{-7}$ M for pyrene, fluoranthene and anthracene, respectively. The concentration of HS varied in the range from 1.5 to 25 mg C/L. Three replicates were made for each assay.

Toxicity tests with plants. Three sod-podzolic soils of different agricultural use (garden, plough, and virgin) were used for testing. The commercial preparation of brown coal HA (AGK) was used as a detoxifying agent for soil tests. Wheat plants *Triticum aestivum* served as a test-object and wet biomass of plants as a target response. The application rate of atrazine was 1 mg/kg, the rate of CHA-AGK ranged from 35 to 105 mg/kg. The plants were harvested after 30 days of growth. Calculation of the detoxification coefficient D and binding constant K_{OC}^{D} was performed as described above.

5. RESULTS AND DISCUSSION

5.1 Aquatic Media

Typical dependencies of Hg, PAHs, and atrazine toxiticies upon HS concentration in the test system are given in Figure 2. Toxicity of all model ET decreased with an increase in HS concentration. Detailed explanations of the results obtained for each ET are presented below.

Mercury. The bioassays with $HgCl_2$ implied both bioaccumulation and toxicity measurements in the presence of HS. Both toxicity and bioaccumulation of $HgCl_2$ decreased along with an increase in HS concentration (Figure 2). This is in agreement with the findings of other investigators (Oh et al., 1986).

From the data on toxicity and *BCF* changes, the detoxification (K_{BS}^{D}) and bioaccumulation (K_{BS}^{B}) constants of Hg-HS complexes were calculated as described above. For this purpose, the Hg-equivalent concentration of HS was estimated (Zhilin, 1998). To calculate the K_{BS}^{D}, it was assumed that Hg(II) was distributed between $HgCl_2$ and Hg-BS complexes (BS is a binding site of a humic molecule). This allowed us to express K_{BS}^{D} as follows:

$$K_{BS}^{D} = K_{BS/Cl} \bullet K_{HgCl_2} \qquad (15)$$

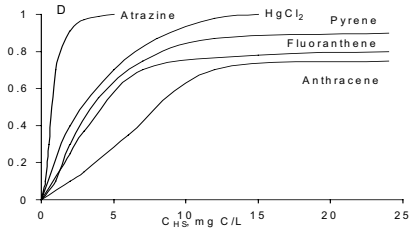

Figure 2. Typical detoxification curves - relationships of detoxification coefficient D versus HS concentration for different model ecotoxicants: PAHs, Hg(II), and atrazine.

where $K_{BS/Cl}$ is the constant of ligand exchange, K_{HgCl2} is stability constant of $HgCl_2$.

K_{BS}^{D} values determined at I = 0.019M (ionic strength of the cultivation medium) were then adjusted to I = 0.0 M using the Davies equation. The log K_{BS}^{D} and log K_{BS}^{B} values laid in the range of 13.0–14.4 and 13.0–15.3, respectively. These were on the same order of magnitude as K_{BS} values determined using analytical technique. Of particular interest was the statistically significant correlation (P>95%) found between K_{BS}^{D}, K_{BS}^{B} and log K_{BS}. The r values were 0.64 and 0.69 for the pairs of variables "K_{BS}^{D}-log K_{BS} " and "K_{BS}^{B}-log K_{BS}", respectively. According to the criteria set for the proposed approach, a conclusion was made that the main mechanism of $HgCl_2$ toxicity and bioconcentration sequestration by HS is formation of non-toxic and non-bioavailable Hg-HS complexes.

PAH. Typical relationships of PAH toxicities versus HS concentration are given in Figure 2. The observed toxicities decreased along with an increase in HS concentration. These effects were observed for all humic materials except for soil FA that did not influence PAH toxicities. The calculated K_{OC}^{D} values for pyrene, fluoranthene and anthracene have been reported (Perminova et al., 2001). Among PAHs studied, comparable K_{OC}^{D} values were obtained for pyrene ($1.1 \cdot 10^5$–$6.0 \cdot 10^5$ L/kg C) and fluoranthene ($0.4 \cdot 10^5$–$8.0 \cdot 10^5$ L/kg C), and the lower ones were observed for anthracene ($0.3 \cdot 10^5$–$6.0 \cdot 10^5$ L/kg C). This can be related to lower hydrophobicity of anthracene (log K_{ow} = 4.45) compared to pyrene and fluoranthene (log K_{ow} = 4.88 and 5.16, respectively) (Hansch et al., 1995). Similar trends were previously reported for PAH bioconcentration in the presence of HS (Steinberg et al., 2000).

Based on the measured K_{OC}^{D} values, the target humic materials can be arranged in the following descending order: Aldrich HA ≅ chernozemic HA>> sod-podzolic and grey wooded soil HA ≅ peat HF >> aquatic HF ≅ chernozemic FA >> sod-podzolic and grey wooded soil FA. These trends

with the source of HS are in agreement with those observed in our previous studies on partition coefficients determined with a use of the fluorescent quenching technique (K_{OC}^{fq}) (Perminova et al., 1999). They are also consistent with the findings of other investigators. For example, very high binding affinity of Aldrich HA was reported in (McCarthy et al., 1989; Chin et al., 1997). Higher K_{OC} values for soil HA than for soil FA were reported in (Gauthier et al., 1987; De Paolis and Kukkonen, 1997).

Statistical data treatment showed rather strong positive correlation between K_{OC}^{D} and K_{OC}^{fq} (r = 0.86, 0.85, and 0.76 for pyrene, fluoranthene and anthracene, respectively). Intercepts of regression lines were equal to zero and slopes – significantly different from one (mean±CI, P = 95%):

$$\text{pyrene: } K_{OC}^{D} = (2.6 \pm 0.3) \bullet K_{OC}^{fq} \quad (16)$$

$$\text{fluoranthene: } K_{OC}^{D} = (4.6 \pm 0.6) \bullet K_{OC}^{fq} \quad (17)$$

$$\text{anthracene: } K_{OC}^{D} = (6.0 \pm 1.4) \bullet K_{OC}^{fq} \quad (18)$$

Thus, the $K_{OC}^{fq}s$ account for about 74, 72 and 58% of variability in the $K_{OC}^{D}s$ for pyrene, fluoranthene and anthracene, respectively. However, slopes of the regression lines indicated a constant bias between the partition coefficients estimated by two techniques used. As was shown in (Perminova et al., 2001), the observed bias between the K_{OC}^{fq}s and K_{OC}^{D}s, both of those are *conditional constants*, resulted from differences in conditions of their determination. The fluorescence quenching determinations were conducted in distilled water, whereas the toxicity tests – were in filtered tap water. The presence of Ca^{2+} and Mg^{2+} in tap water at the concentration of 4 mM seems to be the most probable reason of substantially higher K_{OC}^{fq} values in comparison with those determined in distilled water. An increase in K_{OC}^{fq} value in the presence of Ca^{2+} was reported by Schlautman and Morgan (1993) for pyrene and anthracene. Hence, the detoxification effect of HS could be mostly attributed to chemical binding, nominally, to formation of non-toxic PAH-HS complexes.

Atrazine. The typical relationship of atrazine toxicity versus HS concentration is shown in Figure 2. The toxicity decreased along with an increase in HS concentration. Among humic materials studied, HS of soil solution displayed the highest detoxifying ability; coal and chernozemic HA had comparably high detoxifying ability, whereas soil FA did not affect significantly herbicide toxicity. The calculated K_{OC}^{D} values laid in the range from $5.0 \cdot 10^4$ to $2.1 \cdot 10^6$ L/kg C being about three orders of magnitude higher than those obtained using ultrafiltration technique (87–580 L/kg C)

(Kulikova and Perminova, 2002). This means that the observed detoxification effects cannot be explained only by the chemical binding of atrazine. Of particular importance is, that there was no correlation ($r = 0.12$) found between the values of K_{OC} and K_{OC}^{D}.

This made us to hypothesise another mechanism for the mediating effect of HS in case of atrazine, nominally, direct stimulating impact of HS displayed under stress conditions. A similar phenomenon was numerously reported in the literature for the higher plant experiencing draught conditions, temperature stress, unfavourable pH, and others (Kulikova et al., 2005 and citations in it). To test this hypothesis, additional experiments were conducted under temperature stress conditions. For this purpose, the thermophilic stain of algae *Chlorella pyrenoidosa* was cultivated at 25 °C instead of the optimum 35 °C during three hours in the presence of HS. After three hours of incubation, photosynthetic activity of algae in the absence of HS (water soluble HS extracted from the forest SDHF1 and garden SDHF3 sod-podzolic soils were used) almost equalled zero. Increase in HS concentration was accompanied with enhanced photosynthetic activity of algae. When HS concentration reached 2 mg C/L, F_v/F_m values equalled to those recorded under optimum temperature conditions (Figure 3). The obtained data show that HS greatly stimulated photosynthetic activity of algae cells under conditions of the temperature stress. Of particular interest is that there was no stimulating activity of HS in relation to algae tested under optimum temperature conditions. Hence, it seemed to be feasible that the detoxification effect of HS is rather a consequence of the beneficial effect of HS than that of the chemical binding of atrazine to HS.

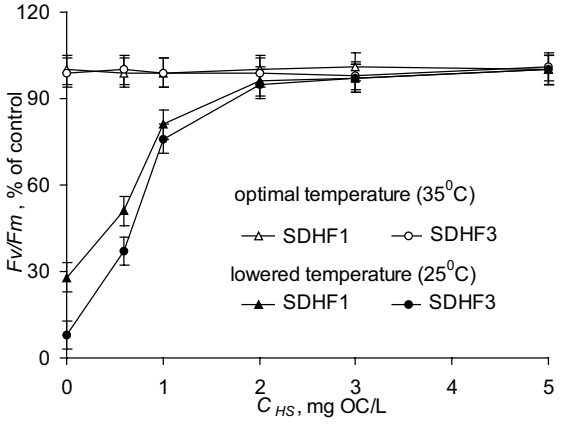

Figure 3. Dose-dependent stimulating effects of HS exerted on algae cultivated under stress conditions (lowered temperature).

As a possible mechanism of the stimulating impact of HS on living organisms under non-optimum conditions, an increase in permeability of the cell membrane could be proposed. In terms of Figure 1, we are facing here with the "boundary", rather than with the "exterior" effects of HS. It can be surmised that HS can be used as "building material" for repairing the cell membrane under stress conditions. This can improve physiological state of the stressed algae and, as a consequence, its photosynthetic activity. To gain deeper understanding of the mechanism of detoxifying action of HS in relation to atrazine, additional studies are needed. From our point of view, the first priority should be given to studies on penetration of HS into the plant cells.

5.2 Mediating Effects of HS in the Soil System

To study mediating effects in soil, laboratory experiments with atrazine were carried out. For this purpose, soil samples were contaminated with atrazine (1 mg/kg) and mixed with different amounts of HS preparation. Three sod-podzolic soils of the different agricultural use (virgin, plough and garden) were used in these studies. Coal HA was used as a humic material. The prepared soils were further used for wheat growth experiments.

Addition of HA to all three soils resulted in a decrease in atrazine toxicity. The calculated D values varied depending on the soil type. Maximum and minimum D values of 0.52 and 0.14 were observed in virgin and plough soils, respectively. D values correlated well with actual atrazine toxicity in the soils under study. So, wet biomass of the test plants treated with atrazine was 75, 10 and 20% of the control values in the virgin, plough and garden soils, respectively. For all soils studied, an application of HA at the highest rate of 105 mg/kg led to a (50±6)% increase in the plant biomass compared to the variant with atrazine alone.

The K_{OC}^{D} values calculated from a reduction in atrazine toxicity induced by HA additions accounted for $2.1 \cdot 10^4$, $0.3 \cdot 10^4$ and $0.7 \cdot 10^4$ L/kg C for virgin, plough and garden soils, respectively. These values are considerably smaller that those obtained for dissolved HS. However, similar to dissolved HS, the obtained K_{OC}^{D} values were about two orders of magnitude higher than those obtained for atrazine binding to HS immobilized onto kaolin clay (Celis et al., 1998). The obtained results allowed us to conclude that amelioration of atrazine toxicity in soil is determined by both direct stimulating impact of HS on plants and chemical binding of atrazine to HS.

6. CONCLUSIONS

A new concept of mediating action of HS in the system living cell – ecotoxicant is developed. It defines mechanisms underlying mediating effects of HS in the context of intra- and extra-cellular processes. The first category is related to chemical binding and defined as “exterior effects”: the corresponding processes take place outside of the cell and imply formation of non-bioavailable and non-toxic HS-ET complexes. The second category is related to HS adsorption on cell walls or membranes and defined as "boundary effects": sorption takes place on cell surface and implies changes in permeability and structure of the cell membrane. The third category is related to penetration of HS alone or HS-ET complexes into the cell and defined as “interior effects”. The corresponding processes mostly take place in stressed organisms and imply participation of penetrated HS in the cell metabolism (e.g., induction of systemic acquired resistance and enhanced system immunity) or break down of the penetrated HS-ET complexes inside of the cell and increase in toxicity.

Viability of this concept was demonstrated both by reviewing literature data and by results of our own detoxification experiments. The reported findings revealed uniformly mitigating effects of humic substances on toxicity of the contaminants having high affinity for binding to HS (e.g., Hg, PAHs). For contaminants of weak or intermediate affinity for HS (e.g., chloranilines, terbutylazine), both ameliorated and amplified toxicity was observed in the presence of HS. This allowed us to suggest that chemical binding played a key role in detoxification of contaminants capable of forming stable complexes with HS, whereas direct interaction of HS with living cells including changes in membrane surface and permeability contribute the most in mediating effects exerted upon contaminants weakly bound to HS.

This suggestion was confirmed by toxicity assays conducted in the presence of HS with contaminants of different binding affinity. Strong correlation between binding affinity and detoxifying ability of HS was observed for PAHs and Hg(II), whereas no correlation with binding affinity, but highest detoxifying effects were observed for weakly interacting atrazine. Stimulating impact of HS on living cells under stress conditions was proposed as leading detoxifying mechanism in case of atrazine. This was confirmed by the results of same bioassays with alternative stress factor – temperature. As in case of atrazine presence, dose-dependent stimulating effects were observed in HS-containing test-systems cultivated under lowered temperature. The conducted studies allow a conclusion on promising applications of HS as detoxicants and plant activators in the fields of bioremediation and plant protection.

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