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Estimation of Uptake of Humic Substances from Different Sources by *Escherichia coli* Cells under Optimum and Salt Stress Conditions by Use of Tritium-Labeled Humic Materials[▽]

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The primary goal of this paper is to demonstrate potential strengths of the use of tritium-labeled humic substances (HS) to quantify their interaction with living cells under various conditions. A novel approach was taken to study the interaction between a model microorganism and the labeled humic material. The bacterium *Escherichia coli* was used as a model microorganism. Salt stress was used to study interactions of HS with living cells under nonoptimum conditions. Six tritium-labeled samples of HS originating from coal, peat, and soil were examined. To quantify their interaction with *E. coli* cells, bioconcentration factors (BCF) were calculated and the amount of HS that penetrated into the cell interior was determined, and the liquid scintillation counting technique was used as well. The BCF values under optimum conditions varied from 0.9 to 13.1 liters kg^{-1} of cell biomass, whereas under salt stress conditions the range of corresponding values increased substantially and accounted for 0.2 to 130 liters kg^{-1}. The measured amounts of HS that penetrated into the cells were 23 to 167 mg and 25 to 465 mg HS per kg of cell biomass under optimum and salt stress conditions, respectively. This finding indicated increased penetration of HS into *E. coli* cells under salt stress.

Humic substances (HS) are natural organic compounds comprising 50 to 90% of the organic matter of peat, coal, and sapropel (i.e., sludge that accumulates at the bottom of lakes), as well as of the nonliving organic matter of soil and water ecosystems (9, 34, 53). Being the products of stochastic synthesis, HS are characterized as polydispersed substances having elemental compositions that are nonstoichiometric and structures which are irregular and heterogeneous. Thus, it is not possible to assign an exact structure to HS. Instead, they are operationally defined using a model structure predicated on available compositional, structural, functional, and behavioral data: a model structure containing all the same basic structural units and types of reactive functional groups (46). HS have been demonstrated to contain a large amount of residues resembling the original building blocks (aromatic subunits, amino acids, carbohydrates, etc.) (51) as well as polyphenolic components with nonhydrolyzable C-C and ether bonds (51, 16). Since humic matter is a complex mixture of organic substances, HS yield extremely high polydispersity values (i.e., the ratio of weight-average molecular weight to the number-average molecular weight [M_w/M_n]), which vary within the range of 1.64 to 4.40 (38). These extremely high polydispersity values mean that even though they yield relatively high values of molecular weight, HS contain a low-molecular-weight fraction.

HS are known to play important roles in protecting microorganisms and higher plants from climatic and technogenic stresses, such as pollution, draught, UV irradiation, and pathogen and viral infections (2, 22, 31). However, mechanisms underlying protective functions of these natural systems are still poorly understood. The primary reason for that is a lack of experimental tools for tracking uptake and distribution of natural organic mixtures in living cells and tissues, which makes it extremely difficult to link structure and functions in systems of such high complexity. Besides, predicting HS behavior in biological systems is extremely arduous, as HS are complex mixtures with a number of concurrent properties, such as polyanionic and polyelectrolyte character, hydrophilic and hydrophobic moieties, different functional groups, etc.

The most straightforward hypothesis is that the biological activity of HS depends on both hydrophobic and hydrophilic characteristics of structural components (56). This hypothesis implies that the biological effects of HS are connected to membrane activity (12, 44, 56). Sorption of HS onto cells is the best documented phenomenon; numerous studies include phytoplankton (7, 15, 36, 57), isolated fish gill cells (7), bacteria (13, 14, 27, 57), fungi (60), and plants (12, 31, 44). This suggests that the sorption of HS onto biological membranes is a general process, but very few quantitative estimates are available (13, 36). Moreover, the penetration of HS into the living cells is still questionable, and to the best of our knowledge, only one study has reported a direct estimate of HS uptake by microorganisms (10).

The main complication that arises in the study of the interactions of HS with living cells is the lack of a reliable analytical technique for determination of HS in the presence of biomolecules (e.g., proteins, lipids, and saccharides). To overcome the problem, radioactive labeling of HS is being used widely for

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this purpose. However, the reported studies deal predominantly with synthetic rather than with native humic materials (49, 50, 58). This is because of the approaches used for the radioactive labeling of the humics used in those studies, which involve either composting of a labeled precursor (e.g., ^{14}C-glucose) with a soil sample (17) or the synthesis of model polymeric compounds. The polymeric compounds either are synthesized by enzyme-mediated oxidative polymerization of phenolic compounds, which is initiated by adding H_2O_2 in the presence of horseradish peroxidase (19), or proceed spontaneously in the presence of oxygen or other oxidants at an alkaline pH (48). When phenolics are polymerized with nonaromatic precursors (e.g., proteins, peptides, amino acids, carbohydrates, and amino sugars), the resulting humic-like materials are very similar to natural HS (19). These methods can be used for producing both ^{14}C- and ^{15}N-labeled humic-like substances. Their substantial disadvantage is that the resulting materials are similar but not identical to natural HS. Given high structural heterogeneity and irregularity inherent within HS, the availability of a broad set of labeled humic materials identical to their natural counterparts is a prerequisite for disclosing the mechanism of their interactions with living organisms on the cellular and organismal levels.

The goal of this work was to study the behavior of humics in a simple microbial system using humic substances from various natural sources. For this purpose, six natural humic materials with different molecular features and properties were isolated from various natural sources and then labeled with tritium using a technique developed in previous studies (3, 4). A strain of the well-studied bacterium *Escherichia coli* was used as a model bacterial culture. The molecular weight of humics is quite high and is generally considered to be the leading factor restricting their uptake by living cells. In view of our study, prokaryotic bacteria seemed to be the most appropriate model, as recent evidence suggests that size differences among eukaryotic homologues of integral membrane proteins are consistently larger than their bacterial counterparts (8).

To demonstrate the potential strengths of using tritium-labeled HS for biological study, uptake of HS by bacterial cells under stress conditions was also studied. One way in which bacteria respond to environmental change is to regulate cell membrane permeability. Thus, another goal of this work was to monitor changes in HS-bacterium interactions under salt stress conditions. For quantitative indicators, bioconcentration factors, maximum adsorption, and the amount of HS that penetrated into the cell interior were used.

MATERIALS AND METHODS

Humic materials. Humic materials (six samples) used in this study were isolated from soil and peat and also included a commercial preparation of humic acids (HA) from brown coal. A sample of soil humic acids (SHA-CtL00) was isolated from meadow chernozem (related to the mollisols) sampled near Lipetsk (approximately 52°37′N, 39°35′E), Russia. The HA extraction was performed as described in Orlov and Grishina (35). It included preliminary treatment of soil with 0.1 M H_2SO_4 and alkaline extraction with 0.1 M NaOH followed by acidification of the extract to pH 1 or 2. The precipitated HA were desalted by dialysis. Soil fulvic acids (FA) were extracted from sod-podzolic soil (related to Spodosol) from a garden sampled near Moscow (approximately 55°45′N, 37°35′E), Russia (SFA-Pg96), and meadow chernozem (related to the mollisols) sampled near Lipetsk, Russia (SFA-CtL00). To isolate FA, supernatant from the precipitation of HA was passed through the Amberlite XAD-2 resin as described elsewhere for isolation of the aquatic HS (26). Peat humic materials were isolated from the lowland peat of Sakhtysh Lake deposit (Ivanovo region [approximately 52°13′N, 25°55′E], Russia). The isolation procedure of peat HA was as described elsewhere (24) and included preliminary treatment of a peat sample with ethanol-benzene mixture (1:1 [vol/vol]) to remove bitumen compounds followed by an alkaline extraction with 0.1 M NaOH and acidification of the extract to pH 1 or 2 with 0.1 M HCl. A sample of peat fulvic acids (PFA-Sk300) was extracted from the supernatant as described above for soil FA. The precipitated HA were then treated with ethanol to extract hymatomelanic acids. The residual precipitate was dialyzed and used for further experiments (PHA-Sk300). Coal humic acid (CHA-Pow) was isolated from potassium humate produced from leonardite (Powhumus) kindly provided by Humintech Ltd. (Germany). For this purpose, a weight of potassium humate was dissolved in distilled water and centrifuged to separate and discard any insoluble mineral components. The supernatant was then acidified to pH 2 with concentrated HCl and centrifuged. The precipitate of HA was collected, washed with distilled water, desalted using electrodialysis, evaporated at 60°C, and stored in a desiccator over P_2O_5.

Tritium-labeled humic materials. Tritium-labeled humic materials (^{3}H-HS) were prepared as described by Badun et al. (3, 4). In brief, HS solution in 0.005 M NaOH was uniformly distributed on the wall of the reaction vessel and frozen by liquid nitrogen and lyophilizing. The reaction vessel was vacuumed and filled with tritium gas (0.5 Pa), and the tungsten filament disposed in the central part of the reactor vessel was heated by electric current to 1,950 K. HS was treated by tritium atoms for 10 s. Residual gas was evacuated, and the new portion of tritium gas was used for labeling if it was necessary. The obtained ^{3}H-HS samples were dissolved in a phosphate buffer (0.028 M, pH 6.8) and purified by dialysis for a month at 4°C, allowing elimination of exchangeable tritium from the OH^-, $COOH^-$, and NH_n groups of HS. A dialysis membrane with a cutoff of 2 kDa (Merck, Germany) was used. The radioactivity of solutions of labeled substances was measured using a liquid scintillation spectrometer, RackBeta 1215 (Finland).

Elemental analysis. Elemental analyses (C, H, and N) of HS were conducted on a Carlo Erba Strumentazione analyzer (Carlo Erba, Italy). Ash content was determined manually. Oxygen contents were calculated as a difference. The contents of all the elements were calculated on an ash-free basis.

SEC analysis. Size exclusion chromatography (SEC) analyses of HS were performed according to Perminova et al. (37). The SEC system Abimed (Gilson, France) included a high-pressure liquid chromatography (HPLC) pump, autosampler, and glass column and was equipped with a UV detector. The column, 25 mm by 20 cm and packed with Toyopearl TSK HW-55S gel (Toso Haas, Japan), was used for separation. The 0.028 M phosphate buffer (pH 6.8) was used as a mobile phase. The absorbance of eluate was detected at 254 nm. Sodium salts of polystyrenesulfonic acids with molecular weights (in thousands) of 2.29, 4.48, 14, 20.7, 45.1, and 80.84 (Polymer Standard Service, Germany) were used as markers for molecular weight calculations. HS solutions were set at a concentration of 40 mg liter^{-1} by equilibrating with the mobile phase prior to the analysis. Based on the data obtained, weight-average molecular weight, M_w, was calculated using GelTreat software (21).

^{13}C solution-state NMR analysis. Quantitative ^{13}C solution-state nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avalance 400 spectrometer operating at 100 MHz. An HS sample with a weight of 70 mg was dissolved in 0.7 ml of 0.1 M or 0.3 M NaOD and transferred into a 5-mm NMR tube. ^{13}C NMR spectra were acquired with a 5-mm broadband probe, using the CPMG pulse program with an 8-s relaxation delay and acquisition time of about 0.2 s. These conditions were shown to provide quantitative determination of carbon distribution among the main structural fragments of HS (20). The assignments were as follows (in ppm): 5 to 108, aliphatic nonsubstituted and O-substituted C atoms (ΣC_{Alk}); 108 to 165, aromatic nonsubstituted and O-substituted C atoms (ΣC_{Ar}); 165 to 187, C atoms of carboxylic and esteric groups (ΣC_{COO}); and 187 to 220, C atoms of quinonic and ketonic groups ($\Sigma C_{C=O}$).

Surface activity characterization. Surface activity is among the most important properties determining largely the biological activity of organic compounds, since this parameter influences the ability of substances to interact with biological membranes. The above property of HS was determined following adsorption isotherms of HS at the toluene-water interface using the scintillating phase procedure (5). In brief, 3 ml of toluene containing 2,5-diphenyloxazole (0.045% [wt]) was carefully placed on the surface of 0.8 ml aqueous solution of labeled HS. The radioactivity of the resultant mixture was then measured using a RackBeta 1215 (Finland) liquid scintillation spectrometer. After 5 or 6 days, the radioactivity approached a steady value. Next, 2 ml of the organic phase was transferred into another vial, and the counting rate estimated for both the combined organic phase and mixture residues. Counting rate I is a sum of two constituents: $I = I_S + I_V$, where I_S is the counting rate due to the radioactivity at the toluene-water interface and I_V is the counting rate due to labeled HS in toluene alone, assuming that $I_V = \varepsilon_V \cdot a_V \cdot V$, where ε_V is the counting

TABLE 1. Elemental composition, M_w, and content of carbon in the structural fragments of the humic materials used in this study

HS index	Elemental composition[a]			% Ash	M_w[b]	Content of carbon in the structural fragments (%)[c]:			Γ_{max} (mg m^{-2})[d]
	H/C	O/C	C/N			$\Sigma C_{C=O}$	ΣC_{COO}	$\Sigma C_{Ar}/\Sigma C_{Alk}$	
CHA-Pow	0.87	0.50	53	7.8	9.4	5.7	19.0	5.0	0.71
PFA-Sk300	1.18	0.89	27	11.2	9.2	3.2	16.9	0.6	1.50
PHA-Sk300	1.15	0.66	17	3.3	18.6	1.9	16.5	0.7	2.80
SFA-CtL00	0.81	0.52	16	8.9	11.8	2.6	21.4	0.4	0.15
SFA-Pg96	0.88	0.61	19	5.7	11.3	3.1	18.0	1.1	0.08
SHA-CtL00	0.79	0.35	14	6.0	15.9	3.2	15.6	1.9	0.30

[a] H/C, O/C, and C/N are atomic ratios.
[b] M_w is expressed in thousands.
[c] $\Sigma C_{C=O}$, quinonic and ketonic groups; ΣC_{COO}, carboxylic and esteric groups; ΣC_{Ar}, aromatic nonsubstituted and O-substituted moieties; ΣC_{Alk}, aliphatic nonsubstituted and O-substituted moieties.
[d] Γ_{max}, the maximal adsorption value on toluene-water interface.

efficiency in toluene, a_V is the volume-specific radioactivity of HS in toluene, and V is the volume of toluene. By combining the above two equations, the following relationship is derived: $I_S/\varepsilon_V = I/\varepsilon_V - a_V \cdot V$. The term I_S/ε_V can be expressed as $I_S/\varepsilon_V = 0.5 \cdot S \cdot \Gamma \cdot a_M$ (5), where S is the area of the toluene-water interface, Γ is the water/toluene adsorption coefficient for HS on the toluene-water interface, and a_M is the mass-specific radioactivity of HS.

Experiments examining HS adsorption on the toluene-water interface were conducted over an HS concentration range of 0.1 to 100 mg liter^{-1}. The maximal adsorption value Γ_{max} for the toluene-water interface, derived from assuming a Langmuir isotherm, was used as a quantitative measure of HS surface activity.

Bacterial culture. The bacterial culture used was a strain of *Escherichia coli* XL1 with tetracycline resistance. Bacteria were first incubated in 2YT medium and then grown in M9 medium for experiments. The 2YT medium was composed of yeast extract (10 g liter^{-1}), tryptone (16 g liter^{-1}), and NaCl (5 g liter^{-1}). The M9 medium was composed of $Na_2HPO_4 \cdot 12H_2O$ (15.1 g liter^{-1}), KH_2PO_4 (3.0 g liter^{-1}), NaCl (0.5 g liter^{-1}), NH_4Cl (1.0 g liter^{-1}), $MgSO_4 \cdot 12H_2O$ (0.5 g liter^{-1}), $CaCl_2$ (5.6 mg liter^{-1}), and glucose (0.25 g liter^{-1}). Cultural media were autoclaved at 120°C for 30 min. The solutions of glucose, salts of calcium and magnesium, tetracycline, and HS were sterilized by filtration through a 20-μm filter and added to the broth separately. Tetracycline was added to 2YT or M9 media to a final concentration of 30 mg liter^{-1} immediately before inoculation. The medium pH was adjusted to 7.5 using NaOH.

For cultivation, 1,000-ml Erlenmeyer flasks containing 200 ml of 2YT medium were inoculated with a chip from the frozen 2YT culture (kept at −70°C, glycerol was added as a cryoprotector) and cultivated on an orbital shaker at 37°C for 10 h. Sorption studies were performed in Erlenmeyer flasks with 50 ml M9 medium supplied with nonlabeled HS and tritium-labeled HS. The M9 medium was inoculated with 0.5 ml of 2YT culture and cultivated on an orbital shaker at 37°C overnight. To subject bacteria to salt stress, NaCl was added to the M9 media before sterilization to a final concentration of 18 g liter^{-1}, with the osmotic potential equaling −1.2 MPa (47).

Bacterial growth was monitored by measuring the optical density of the culture at 600 nm (OD_{600}). To determine a wet weight of cells, 10 ml of broth was centrifuged at 5,000 rpm for 30 min, and then the pellet was washed twice with distilled water. The obtained OD_{600} values of the cells were correlated with the cell wet weight concentration via the calibration. Detection and discrimination of live and disintegrated cells were performed using propidium iodide-based tests (43).

Uptake experiments were conducted by growing *E. coli* cells overnight at 37°C in M9 medium supplied with nonlabeled HS at concentrations of 5, 10, 15, 25, and 50 mg liter^{-1} and 18 g liter^{-1} NaCl for salt stress, if required. Tritium-labeled HS (100 μl) was added to the cultivation flasks to make the final radioactivity of the solution about 45 nCi ml^{-1}. After overnight cultivation, the cells were centrifuged (5,000 rpm, 30 min), and the radioactivity of supernatant was measured. The value obtained was assigned to the equilibrium concentration of HS. Then the supernatant was replaced with an equal amount of M9 media, the cells were resuspended, and radioactivity of the suspension was measured. The radioactivity value corresponded to the amount of HS taken up by the cells, i.e., the sum of HS adsorbed on the surface of the cells and HS that penetrated into the cells. To determine the amount of HS that penetrated into the cells, cell lysis was performed using chloroform (2 or 3 drops per 10 ml of suspension) and centrifugation (5,000 rpm, 30 min) was performed to separate debris. The radioactivity of the supernatant was assigned to the amount of HS that penetrated the cells. The experiments were performed in triplicates.

Bioconcentration factors (BCF) were calculated as a slope of the relationship of an amount of HS taken up by *E. coli* cells versus HS equilibrium concentration in accordance with the guidelines of the U.S. Environmental Protection Agency (11). The agency defines BCF "as the ratio of chemical concentration in the organism to that in surrounding water." Hence, the slopes of the obtained relationships yield the values of BCF. This follows from the following equation: $HS_{uptake} = BCF \cdot [HS]$, where HS_{uptake} is the amount of HS taken up by bacteria and [HS] is the equilibrium concentration. The BCF index reflects the sum of HS both sorbed onto cell membranes and penetrated into the cells. The amount of HS penetrated into the cells was calculated at 50 mg liter^{-1}, which was the highest concentration used. The amount of adsorbed HS per squared meter of bacterial surface was calculated assuming a radius of a cell equal to 0.6 μm (28). Means separation was conducted using the least significant difference (LDS).

RESULTS

Properties of humic substances used. The obtained structural-molecular characteristics of the HS samples used are presented in Table 1. As shown in Table 1, the studied HS differed significantly in their structural-molecular characteristics. The atomic ratio H/C varied from 0.79 (soil HA SHA-CtL00) to 1.18 (peat FA PFA-Sk300), whereas O/C values ranged from 0.35 (soil HA SHA-CtL00) to 0.89 (peat FA PFA-Sk300). In general, soil HS used in our study were characterized with lower H/C and O/C ratios than those of peat HS. This was evident for the lower content of saturated structures and for the higher content of oxygen-containing functional groups in peat compared to soil HS. The latter was confirmed also by ^{13}C NMR data. In particular, the sum of C atoms of carboxylic, esteric, quinonic, and ketonic groups (i.e., sum of ΣC_{COO} and $\Sigma C_{C=O}$) was higher in the case of soil humics than for peat preparations. The minimum aromaticity degree, calculated as a ratio of carbon content in aromatic nonsubstituted ΣC_{Ar} structures to that of nonsubstituted ΣC_{Alk} structures, was observed for soil FA SFA-CtL00, and the maximum degree was observed for coal HA CHA-Pow.

Values of determined weight-average molecular weight ranged from 9.2 (peat FA PFA-Sk300) to 18.6 (peat HA PHA-Sk300) and matched well data published for similar humic preparations (37).

The maximum adsorption value Γ_{max} ranged between 0.08 mg m^{-2} (soil FA SFA-Pg96) and 2.8 mg m^{-2} (peat HA PHA-Sk300). The experiments performed showed that the

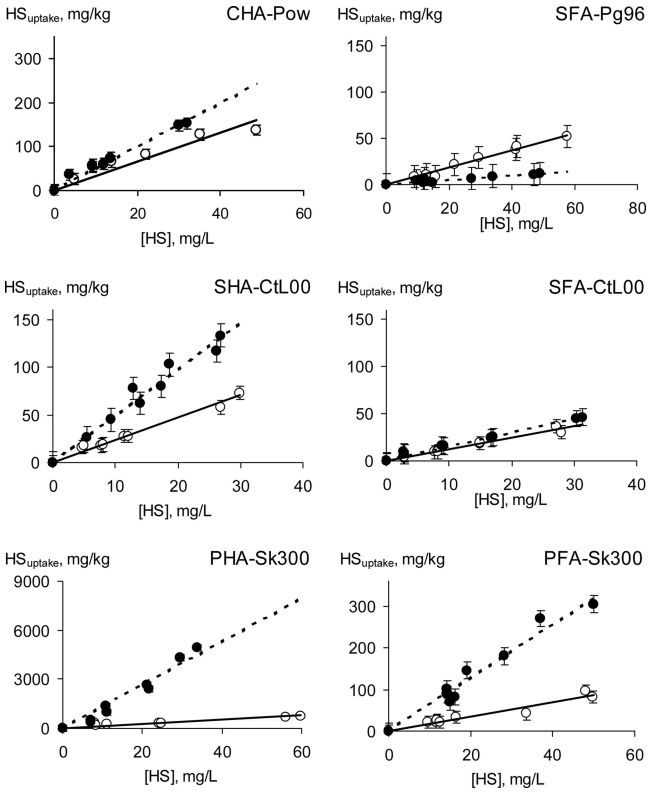

FIG. 1. HS uptake by *E. coli* cells at different HS concentrations under optimum (open dots) and salt stress (black dots) conditions. Bars represent standard deviations. Error bars do not extend outside some symbols.

surface activity of studied HS preparations increased in the following order: soil FA < soil HA < coal HA < peat FA < peat HA.

Bioconcentration of humic materials by bacterial cells under normal and stress conditions. Uptake of humic materials by bacterial cells was studied under normal and stress conditions. For this purpose, bacterial cells were cultivated on ^{3}H-HS amended media. From the resultant distribution of radioactivity between cell biomass and aqueous phase, the amount of uptaken HS and the equilibrium concentration of HS were measured, respectively. These parameters were determined for six humic materials used in this study, nominally, leonardite HA, low moor peat HA and FA, mollisol HA and FA, and sod-podzolic soil FA. To simulate environmentally relevant conditions, all experiments were conducted with HS concentrations ranging from 5 to 50 mg liter^{-1} (53). To simulate stress conditions, salt stress that was expected to alter membrane permeability was used.

Results are shown in Fig. 1 as plots of the amount of HS taken up by bacterial cells versus the HS concentration in aqueous phase. Results demonstrate that substantial uptake was observed for the humic acids tested. The highest uptake was observed for samples derived from peat (PHA-Sk300), and the lowest for soil fulvic acids (SFA-CtL00 and SFA-Pg96). A second distinct feature was the much higher uptake under salt stress conditions as opposed to optimum conditions of incubation.

The bioconcentration factors (BCF) and maximum sorption values of HS calculated from the above relationships are summarized in Table 2.

Penetration of humic materials into the cell interior. There are two different processes which might contribute to the bioconcentration of HS: the first is adsorption of humic materials onto the cell surface, while the second is their accumulation in the cell interior. We expected that HS might cross the cell membrane, and a particular set of the experiments was devoted to estimation of the amount of HS that penetrated into the cells. For this purpose, the cells were first cultivated with ^{3}H-labeled humic materials and then destroyed using chloroform. By measuring the radioactivity of supernatant in the cell suspension, the amount of HS that penetrated the cell wall was determined. The obtained results are shown in Table 3.

TABLE 3. Estimates of amount of HS that penetrated the cell wall under optimum and salt stress conditions at HS concentration of 50 mg liter^{-1}

HS	HS penetrated at 50 mg liter^{-1} (mg [kg cells]$^{-1}$)[a]	
	Optimum	Stress
CHA-Pow	63 ± 12a	102 ± 11c
PFA-Sk300	93 ± 12a	256 ± 18b
PHA-Sk300	167 ± 21a	465 ± 25a
SFA-CtL00	27 ± 15a	34 ± 9c
SFA-Pg96	23 ± 8a	25 ± 9c
SHA-CtL00	65 ± 14a	82 ± 16c

[a] Values represent means ± SD (n = 3). Values in the same column followed by different letters are significantly different ($P \leq 0.05$).

TABLE 2. Bioconcentration of HS by *E. coli* cells under optimum and stress conditions[a]

HS	BCF (liters kg^{-1})		HS sorbed at 50 mg liter^{-1} (mg m^{-2})		HS sorbed at 50 mg liter^{-1} (mg [kg cells]$^{-1}$)	
	Optimum	Stress	Optimum	Stress	Optimum	Stress
CHA-Pow	3.2 ± 0.9b	5.0 ± 0.6b	14 ± 3b	17 ± 4b	132 ± 7b	150 ± 3c
PFA-Sk300	1.8 ± 0.7b	6.9 ± 0.8b	8 ± 1b	32 ± 5b	89 ± 11b	288 ± 24b
PHA-Sk300	13.1 ± 0.5a	130 ± 15a	62 ± 12a	546 ± 40a	720 ± 47a	4631 ± 426a
SFA-CtL00	0.9 ± 0.5b	1.5 ± 0.5c	1 ± 1b	3 ± 1c	33 ± 4b	45 ± 1d
SFA-Pg96	0.9 ± 0.5b	0.2 ± 0.5c	4 ± 1b	1 ± 1c	47 ± 8b	47 ± 10d
SHA-CtL00	2.3 ± 0.6b	5.3 ± 0.3b	6 ± 1b	11 ± 2b	66 ± 11b	125 ± 11c

[a] Values represent means ± SD (n = 3). Values in the same column followed by different letters are significantly different ($P \leq 0.05$).

DISCUSSION

Bioconcentration of humic materials by bacterial cells under normal and stress conditions. The observed BCF values of HS under optimum conditions varied in the range from 0.9 to 13.1 liters kg^{-1} of cell biomass. The lowest BCF values were obtained for soil FA SFA-CtL00 and SFA-Pg96, whereas the highest one was for peat HA PHA-Sk300. For the pairs of HA and FA extracted from the same source (PHA-Sk300/PFA-Sk300 and SHA-CtL00/SFA-CtL00), the tendency of an HA bioconcentration higher than that of FA was registered. Similar results were obtained for all pairs of HA and FA extracted from the same source (peat PHA-Sk300/PFA-Sk300 and soil SHA-CtL00/SFA-CtL00), with BCF values of HA substantially exceeding those of FA. The given trends are consistent with differences in molecular features of HA and FA. So, HA possess much higher hydrophobicity and molecular weight values compared to FA. These differences are demonstrated in Table 1, which shows values of aromaticity and weight-average molecular weight (M_w) for HA samples much higher than those for FA. Hence, it might be concluded that bioconcentration of HS increased along with an increase in their aromaticity and a decrease in the content of aliphatic moieties and enrichment in oxygen (O/C values). This finding is consistent with the results reported by Vigneault et al. (56), who studied adsorption of Suwannee River FA and HA onto green alga *Selenastrum capricornutum* and revealed the larger adsorption of HA than of FA. Similar results were also reported by Moura et al. (30), who demonstrated the adsorption of humic materials onto *Bacillus subtilis* followed the trend of HA > FA, which is also reasonable from the mechanistic point of view. Considering the hydrophobic character of lipid biomembranes, it could be expected that more hydrophobic humic materials may display higher affinity for biomembranes as opposed to less hydrophobic, more oxidized structures. The same is true for humic materials of higher molecular weights (HA) as opposed to smaller molecules (FA).

The calculated values of HS sorbed under optimum conditions at the concentration of 50 mg $liter^{-1}$ varied from 1 to 62 mg m^{-2}, and for the FA studied, it ranged from 1 to 8 mg m^{-2}, which was in accordance with Parent et al. (36), who reported FA sorbed to microalga *Chlorella* cell surfaces at the amount of 17 mg m^{-2} at pH 5. The observed slight difference in the amount of FA sorbed was probably because of differences in biochemical characteristics and surface areas of *E. coli* and *Chlorella* as well as the higher pH used in our study (pH 7.5). *E. coli* is a prokaryote, while *Chlorella* is a eukaryotic organism. The size of the *Eucarya* membrane channels (including both integral membrane proteins from over 20 ubiquitous families of channels, secondary carriers, and primary active transporters) varies from 111 to 280% of the size of those of the *Bacteria* (8). Perhaps the more important point is that the *Chlorella* membrane is surrounded by a cell wall composed of cellulose, whereas the outer membrane of *E. coli* is a lipid bilayer containing lipids, proteins, and lipopolysaccharides (LPS). Hence, the levels of affinity for humics will differ between organisms.

An increase in pH, in turn, was demonstrated to result in a decrease in HS adsorption on the cell surface (56). According to a conclusion made by Fein et al. (13), who characterized the adsorption of HA onto the bacterial surface of Gram-positive *B. subtilis*, the interaction between HA and bacteria was strongest under low pH conditions, wherein both the bacterial surface and the HA molecule were less negatively charged. It is well known that the outer membrane of a Gram-negative bacterium is composed mainly of LPS, phospholipids, and lipoproteins, whereas the distinguishing characteristic of Gram-positive bacterium is the presence of several peptidoglycan layers and teichoic acids in the cell wall. These differences appear not to be important, given that reported values for HA sorption were very similar (30). The amount of HA adsorbed onto the *B. subtilis* biomass at pH 7.0 was about 0.8 mg kg^{-1}, which coincides well with the values obtained in the current study, 0.066 to 0.720 mg kg^{-1}.

Penetration of humic materials into the cell interior. Our experiments demonstrated clearly that a significant amount of HS could penetrate the cell wall. The measured amounts of HS found in the cell interior ranged from 23 to 167 mg kg^{-1}, accounting for about 20% of total HS uptake by the cells in the case of HA and reaching 100% in the case of FA. This finding is consistent with the data reported by Doblin et al. (10) on accumulation of tritium-labeled seawater HS by dinoflagellate *Alexandrium catenella.* The microautoradiography studies showed that most of the radioactivity was located inside the cell, with the remainder being associated with the cell surface.

The obtained results are also in accordance with the data reported by Wang et al. (58), who showed that ^{14}C-labeled HS were found inside the cells and even in DNA. Nardi et al. (31) reported that HS with molecular masses less than 3.5 kDa could easily penetrate the cell membrane of higher plants. Bioconcentration of HS in freshwater organisms, e.g., macrophyte *Ceratophyllum demersum* L. and invertebrate *Gammarus pulex* L., was demonstrated by Steinberg et al. (50). The variety of organisms which were demonstrated to be able to uptake HS shows the widespread occurrence of this process.

Of particular interest is that under salt stress conditions both total uptake and the amount of HS penetrating the cell wall increased considerably in most cases except for soil FA SFA-Pg96 and SFA-CtL00, for which no significant difference was observed. For those samples, the lowest uptake was observed, which could complicate acquisition of statistically relevant data from the bioassay.

A feasible explanation for increased uptake of humic materials under salt stress conditions might be alterations in HS behavior caused by an increase in ionic strength. Nominally, increased ionic strength might enhance the HS sorption affinity for a negatively charged cell surface due to partial compensation of the negative charge of humic polyanion, which lowers electrostatic repulsion. The above-stated hypothesis might explain the anomalous behavior of soil FA SFA-Pg96 and SFA-CtL00, for which no increase in sorption was detected under salt stress conditions. Among humics studied, those samples were characterized as having the highest concentration of O-containing functional groups (Table 1), which resulted in less pronounced compensation of the negative charge in the presence of NaCl.

Still, there might be other reasons connected to an increase in permeability of the cell wall under stress conditions: short-term formation of additional membrane pores (23). Pore formation in an artificial membrane induced by osmotic stress was demonstrated by Taupin et al. (52). Similar results were re-

ported by Hallett et al. (18), who observed the leakage of dyes from artificial membrane vesicles under osmotic stress. Data on increased membrane permeability for water molecules under hyperosmotic stress conditions were also reported for eukaryotic cells by Maheswari et al. (25) and van der Weerd et al. (55). Consistent data resulted from the NMR study of shoot apical regions of pearl millet *Pennisetum americanum* L. under hyperosmotic stress conditions, which showed a substantial increase in membrane permeability for water compared to a nonstress situation (55). In prokaryotic organisms such as *E. coli*, nonspecific diffusion transmembrane pores in the outer membrane are formed mainly by matrix porin OmpF and osmoporin OmpC being differentially expressed, depending on environmental conditions. Porins possess sizes similar to those of HS, and the general diffusion porins allow for the uptake of molecules with an M_w of up to 600 (6, 32). The pore sizes of OmpF and OmpC are 1.2 nm and 1.1 nm, respectively, and other porins can be 1.2 to 2.5 nm in diameter (29, 41). Reported values for HS, in turn, vary from 1.5 to 5.8 nm (40). The latter value is evidence of the opportunity of HS (or at least their low-molecular-weight fractions) to penetrate the *E. coli* outer membrane. The production of the porins is strictly controlled by environmental conditions. Under environmental conditions of low osmolarity, the low level of OmpR-P leads to increased transcription from the *ompF* promoter and a level of the OmpF porin higher than that of the OmpC porin. In a medium of high osmolarity, however, the high level of OmpR-P leads to greater production of OmpC than OmpF, as a result of the repression of transcription of the *ompF* gene and activation of the *ompC* gene (54). Changes in the physicochemical properties in response to variations in environmental conditions have also been demonstrated for another prokaryotic porin, OprB of *Pseudomonas aeruginosa*. OprB expression increased with increasing levels of NaCl, suggesting that the *oprB* promoter was positively responsive to changes in the medium ionic strength. In this situation, glucose diffusion into the periplasm through the OprB porin was probably facilitated (1). More important was the fact that both *E. coli* porins OmpF and OmpC prefer cations over anions., i.e., solutes carrying negative charges diffuse through these channels more slowly than their uncharged counterparts and solutes with double-negative charges diffuse even more slowly (33). As addressed above, partial compensation of the negative charge of humic polyanion occurred under increased ionic strength, resulting in enhanced HS sorption affinity. Stress conditions could also result in the production of outer membrane vesicles, which are often associated with porin proteins. Given that these vesicles present additional feasible surface area for interaction with humic materials, they could lead to an overestimation of the amount of HS inside the bacterial cell.

Finally, salt stress might be disrupting the cellular integrity of *E. coli* such that humic materials are actually interacting with debris rather than living cells. However, this study did not demonstrate significant differences in the amounts of disturbed cells determined using propidium iodide-based tests. For example, the total amount of cells exposed to 0.3 M NaCl was about three times less than that of the blank; yet, 2.8% and 3.3% of the cells were disintegrated cells under optimum and salt stress conditions, respectively.

The data obtained in this study (Table 3) show that an increase in BCF under salt stress conditions (a factor of 3.4) was almost doubly high compared to the increase in the amount of HS that penetrated the cell wall (a factor of 1.8). This can be indicative of a more pronounced contribution of increased HS sorption on the cell surface to bioconcentration of HS by bacterial cells than that of an osmotic pressure-induced increase in membrane permeability or another bacterial response under salt stress conditions.

Bioconcentration of humic materials by bacterial cells related to the structure. To compile a set of the structural data to be used for the correlation analysis, the approach described in detail in our previous publication (39) was undertaken. The approach involves a numerical description of the structure of HS in terms of composition, using a combination of the molecular descriptors of elemental, fragmental, and molecular weight composition. The atomic ratios (H/C, O/C, and C/N) were used as the descriptors of elemental composition, and percentages of carbon in the main structural groups ($\Sigma C_{C=O}$, ΣC_{COO}, ΣC_{Ar}, and ΣC_{Alk}) were used as the descriptors of fragmental composition. Hydrophobicity-hydrophilicity of HS under study was roughly estimated using a value of the ΣC_{Ar}-to-ΣC_{Alk} ratio as described in our previous publication (39). The weight-average molecular weight, M_w, was used as a descriptor of the molecular weight composition; as a parameter, it was shown to determine considerably interaction of HS with biota (45). The maximal adsorption (Γ_{max}) value on the toluene-water interface derived from the Langmuir equation was used as a quantitative characteristic of the surface activity of HS (5). The corresponding data are summarized in Table 1.

Among the examined properties of HS, the only statistically significant values of the correlation coefficient ($r > 0.82$, $P = 0.05$) were observed for the pairs of Γ_{max} values for all the parameters of HS interaction with bacteria, namely, BCF, HS sorbed, and HS penetrated under both optimum and salt stress conditions. This indicates that surface activity was a leading property of HS influencing their interaction with *E. coli*. This seems to be an easily explicable fact, as the phenomenon of surface activity is closely related to the process of sorption onto phase interfaces.

The relatively high values of the correlation coefficient ($r = 0.75$) were observed for the pair "BCF and M_w" that was statistically significant for the sample size studied at a P value of 0.10 and confirmed our finding that humic materials of a higher molecular weight may display higher affinity for biomembranes than those of HS of a lower molecular weight. That finding coincided with the observations of Rosenstock et al. (42). They studied decomposition by heterotrophic bacteria of different fractions of humic dissolved organic matter (DOM) in pelagic ecosystems and showed preferential bacterial growth on a 3-kDa-size fraction of humic DOM compared to one of >3 kDa.

Our study was devoted to quantifying the interaction of HS with living cells under optimum and salt stress conditions. A novel approach based on the use of tritium-labeled HS was developed and tested. The bacterium *E. coli* was used as a model microorganism. The lowest BCF values were obtained for FA (SFA-Pg96, SFA-CtL00, and PFA-Sk300), whereas the highest one was for peat HA (PHA-Sk300). For the pairs of HA and FA extracted from the same source (PHA-Sk300 and PFA-Sk300, and SHA-CtL00 and SFA-CtL00), the tendency of

an HA bioconcentration higher than that of FA was registered, which was probably connected with higher aromaticity, lower contents of aliphatic fragments, and oxygen in HA. For the whole data set, however, statistically significant values of the correlation coefficient between characteristics of HS sorption and their structural-molecular properties were observed only for Γ_{max}. This indicates that surface activity was a leading characteristic of HS influencing their interaction with *E. coli*. The established relationship between BCF and M_w demonstrated also that humic materials of higher molecular weight may display higher affinity for biomembranes than HS of lower molecular weight.

The conducted study showed that BCF values of HS under optimum conditions varied in the range from 0.9 to 13.1 liters kg^{-1} and increased drastically under salt stress conditions up to 0.2 to 130 liter kg^{-1}. The same was true for the amounts of HS that penetrated into the cells, which were determined as 23 to 167 mg and 25 to 465 mg HS per kg of cell biomass under optimum and salt stress conditions, respectively. Increased uptake of humic materials under salt stress conditions should be attributed to both alteration of HS behavior and an osmotic pressure-induced increase of membrane permeability.

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