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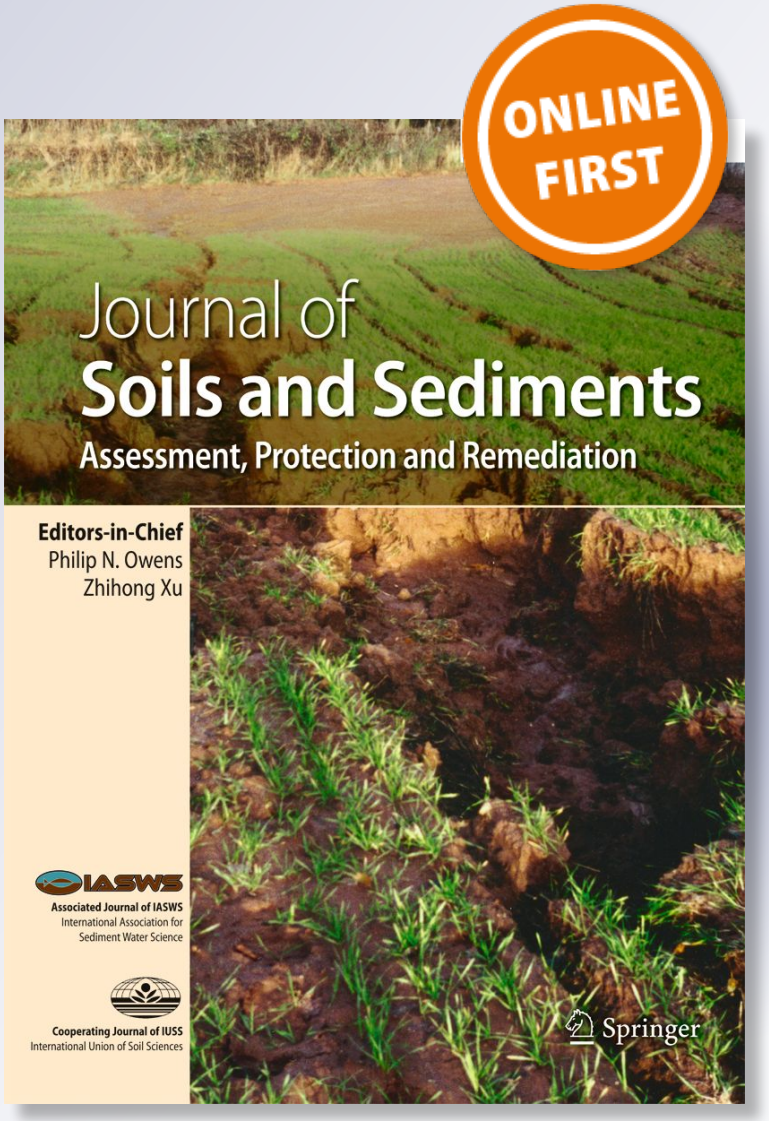

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HUMIC SUBSTANCES AND NATURE-LIKE TECHNOLOGIES

Biodegradation of humic substances by microscopic filamentous fungi: chromatographic and spectroscopic proxies

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Abstract

Purpose The study of interactions between humic substances (HSs) and soil filamentous fungi is the key to understanding the sustainable soil functioning. The present work aims to examine the decomposition of HSs by filamentous dark-pigmented fungus *Alternaria alternata* under the laboratory conditions and to determine the effect of easily assimilable organic carbon on this process. Analyzing such polydisperse substances like HSs by a complex integrated methodology makes it possible to explore the data on their decomposition by microorganisms.

Materials and methods To achieve the aforementioned goals, we used chromatographic and spectroscopic approaches: low-pressure size-exclusion and hydrophobic interaction chromatography accompanied by absorption and fluorescence spectroscopy. To determine the effect cometabolism conditions produced on HS decomposition, two types of carbon substrates were added to the nutrient media: easily assimilable organic carbon (standard 0.3% or reduced 0.03% sucrose content) and hardly assimilable organic carbon (HSs), as well as their combinations. Five HS samples of different organic matter origin have been inspected: potassium humates (HPs) and humic acids (HAs) from coal, peat, and lignosulfonate. Correlation matrix and principal component analysis (PCA) were calculated for comprehensive data analysis.

Results and discussion Transformations of the investigated HSs under fungal cultivation lead to the increase in the low molecular weight fraction, rise of hydrophilic fraction, enlargement of absorbance ratio A_{250}/A_{365}, shortening of the emission wavelength of the humic-type fluorescence, and growth in the fluorescence quantum yield measured with excitation at 355 nm. A positive correlation was observed between the accumulation of fungal biomass and the degree of HS decomposition. PCA analysis confirms that the difference in the results of HS decomposition largely depends on the sucrose content and the nature of HSs. We divided all the HS samples into four groups according to the degree of HS decomposition: original HS solutions, HPs altered using fungal cultivation at 0.03% sucrose, HAs after fungal cultivation at 0.03% sucrose, and finally, HSs (both HPs and HAs) after fungal cultivation at 0.3% sucrose.

Conclusions In the laboratory experiments, we showed that (1) the isolated HAs were more effectively degraded than the parent HPs, and this process was more pronounced at a reduced sucrose content, and (2) the decomposition of stable organic compounds (HSs) was activated by the easily assimilable carbon sources (especially 0.3% sucrose) being present. We assume that it is the easily assimilable organic carbon that most likely triggers the HS degradation working as the *priming effect* in natural environments.

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Abbreviations

A_{250}/A_{365}	Absorbance ratio at 250 nm and 365 nm
CDOM	Chromophoric dissolved organic matter
FAs	Fulvic acids
HA_{coal}	Humic acid isolated from HP_{coal}
HA_{peat}	Humic acid isolated from HP_{peat}
HAs	Humic acids
HIC	Hydrophobic interaction chromatography
HP_{coal}	Potassium humates produced from coal
HP_{lingo}	Potassium humates produced from lignosulfonate
HP_{peat}	Potassium humates produced from peat
HPs	Potassium humates
HSs	Humic substances
LPSEC	Low-pressure size-exclusion chromatography
PCA	Principal component analysis
QY_{355}	Fluorescence quantum yield at 355 nm
RP-HIC	Reversed-phase hydrophobic interaction chromatography

1 Introduction

Humic substances (HSs) play a crucial role in the biosphere, as they take part in various processes considered as prerequisites for the *well-being* of both terrestrial and aquatic ecosystems. Filamentous fungi are involved in HS turnover, including the processes of HS synthesis (humification), as well as their decomposition and mineralization (Gramss et al. 1999; Zavarzina et al. 2011). Various functional groups of filamentous fungi, especially the wood decomposers (soft-rot ascomycetes, brown-rot and white-rot basidiomycetes) or litter and soil-inhabiting fungi (soil filamentous fungi, saprotrophic basidiomycetes), perform a number of essential functions in HS turnover. However, it is still not clear which functions are performed by the particular groups of fungi.

Soil filamentous fungi comprising ascomycetes, deuteromycetes, and zygomycetes constitute an important group of soil microbial community and predominate over the other fungal groups at the early stages of litter decomposition (Lindahl et al. 2007). In litter or mineral soil horizons, the soil filamentous fungi can synthetize humic acids (HAs) and modify lignin and HSs (Zavarzina et al. 2011). Noteworthy, white rot and litter-decomposing fungi are much more active in the processes of HS degradation and mineralization, whereas ascomycetes influence mainly the processes of modifying and polymerizing humic materials (reviewed by Grinhut et al. 2007). For a long time, the capability of soil filamentous fungi contributing to humification has been attributed mainly to the intracellular production of melanins, i.e., the high molecular weight polyphenol-like dark pigments (Valmaseda et al. 1989). Fungal melanins constitute one of the cornerstones for the hypothesis of soil humification processes, being considered as the presumable precursors of the soil HSs, since they both exhibit similar physical and chemical characteristics.

Soil filamentous fungi *Alternaria* are considered to be one of the most common saprophytic fungal genera on the planet (Dang et al. 2015). Hyphal cells are darkly pigmented with melanin, which protects hyphae and spores from the environmental stress (Carzaniga et al. 2002), and take part both soil humus formation and decomposition. Therefore, an experimental study of some aspects of interactions between HSs and *Alternaria* spp. is of a special environmental interest because it may elucidate the process and mechanisms of sustainable soil functioning.

Interactions between HSs and fungi are considered to be a cometabolic processes; i.e., HS decomposition is associated with metabolism of the easily assimilable organic carbon (Zavarzina et al. 2011). Moliszewska and Pisarek (1996) reported that in the case when HSs were the sole carbon source, *Alternaria alternata* could not consume it to build up the mycelium. Rezacova et al. (2006) showed that *A. alternata* is able to modify soil HSs and did not find any significant impact of glucose on utilization of either HAs or fulvic acids (FAs) by microfungi. At the same time, some other species of ascomycetes (for example, some representatives of the genus *Trichoderma*) can develop on media with HAs and coals, exploiting them as the sole source of carbon (Gramss et al. 1999; Silva-Stenico et al. 2007). These contradictory findings demonstrate that the role the assimilable organic carbon plays in the process of decomposing hard-to-reach C substrates, such as HSs, by *A. alternata* remains still unclear.

A set of laboratory experiments with pure cultures to study the ways and conditions under which these fungi can decompose HSs could shed light on this issue. To evaluate transformations of HS chemical structure, various methods have been developed and are being applied now. Since polydispersity of HSs significantly impedes the interpretation of the data on their composition and properties, it is fractionation techniques that are currently used as being comparatively more informative. Thus, conventional low-pressure size-exclusion chromatography (Perminova et al. 2003; Zavarzina et al. 2008) is often used to characterize HSs. Hydrophobic interaction chromatography (HIC) allows us to obtain the distribution patterns of hydrophobic and hydrophilic fractions at various degrees of hydrophobicity and to contribute to understanding the HS chemical structure (Stepanov 2005; Debska et al. 2007;

Stepanov 2008). Both of these methods can be highly valuable for analyzing HS in the fungal growth media, which can be considered as a complicated mixture of HS, melanin moieties, and fungi metabolites.

Among the basic tools used to study the natural organic matter, the absorbance and fluorescence spectroscopy are to be mentioned (Boyle et al. 2009; McKay et al. 2016; Yakimenko et al. 2018). In particular, they are often used to characterize HS and chromophoric dissolved organic matter (CDOM) which naturally occurs in waters (Patsayeva and Reuter 1995; Shubina et al. 2010; Patsaeva et al. 2018). Numerous studies in the field have shown direct correlations between the optical, physical, and chemical properties of CDOM, such as molecular size, lignin content, and aromaticity (Helms et al. 2008; Fichot and Benner 2012; Wünsch et al. 2018). Therefore, indexes such as absorbance ratios (A_{250}/A_{365}), fluorescence emission maximum at $\lambda_{Ex} = 355$ nm, and fluorescence quantum yield at 355 nm appeared to be very informative to characterize the molecular size, degree of aromaticity, type of fluorescence, transformation, and origins of CDOM (Coble 1996; Wünsch et al. 2015; Trubetskoj et al. 2018). This evidence allowed us to conclude that a set of spectroscopic analyses could be likewise applied to studying HS transformations in colored liquid growth media, since they have a lot of generic and chemical similarities with CDOM.

The major objective of this experimental work was to examine the decomposition of HSs by filamentous dark-pigmented fungus *A. alternata* under the laboratory conditions and to determine the effect produced by the easily assimilable organic carbon on this process. To evaluate the degree of HS decomposition, we used two groups of methodological proxies: chromatographic (low-pressure size-exclusion chromatography (LPSEC) and reversed-phase hydrophobic interaction chromatography (RP-HIC)) and spectroscopic (absorbance and fluorescence).

2 Materials and methods

2.1 Fungal culture

As a potential agent for HS decomposition, we used a strain of pure culture *Alternaria alternata* (Fr.) Keissl, i.e., the dark-colored species with a documented synthesis of melanin (Kirk et al. 2008). Aqueous suspension of the fungal spore was obtained by flushing off the surface of the 14-day colonies followed by filtration through a sterile fine steam sieve. Suspension of the fungal spores with the density of 1×10^6 (1.0 ml) was introduced into 100 ml of Czapek nutrient medium with the following mineral composition (g/l): 3.0 $NaNO_3$, 1.0 K_2HPO_4, 0.5 $MgSO_4$, 0.5 KCl, and 0.001 $FeSO_4$, pH 5.5–6.0. Stock pure culture *A. alternata* was cultivated on the Czapek agar medium with 15 g/l of agar content. All reagents had the highest grade available.

After 14 days of exposure, the cultural liquid was separated from the mycelium via filtration through a cellulose acetate membrane with 0.2 μm pore size (Chromafil, Macherey-Nagel, Germany). The cultural liquid was sampled and subjected to chromatographic and spectroscopic analyses.

Fungal mycelium dry mass was determined by weighting after the cultural liquid had been filtered through an ash-free paper filter, and the biomass had been dried at 120 °C to constant weight.

2.2 Humic substances

Five samples of HSs with different organic matter origins were used (Table 1):

- Three commercially available water-soluble potassium humates produced from coal (HP_{coal}), peat (HP_{peat}), and lignosulfonate (HP_{ligno})
- Two HAs isolated from the abovementioned HP_{coal} and HP_{peat} (HA_{coal} and HA_{peat})

HA_{coal} and HA_{peat} were isolated from the corresponding HSs after precipitation of HA-like fraction by acidification of the filtered alkaline solution of initial humates. Precipitate was centrifuged, dialyzed, and dried. In all the samples, the elemental content was determined using a CHN analyzer (Carlo Erba) and ash content was determined after ignition at 900 °C in a muffle oven.

To be introduced into a culture medium, the HS samples were sterilized before fungal inoculation. Sterilization was carried out in two ways: by a photochemical sterilization using UV treatment for 25 min and by mechanical sterilization, i.e., the filtration using membrane filters (Millipore) with the pore diameter of 0.22 μm.

Table 1 Some characteristics of humic substances (HSs), % of dry matter

Humic substance	HS source	Ash	C	N	H	S
Potassium humates						
HP_{ligno}	Lignosulfonate	40.0	34.96	0.37	3.65	3.97
HP_{peat}	Peat	25.8	42.27*	1.06	4.34	0.74
HP_{coal}	Coal	32.1	26.20	1.32	3.58	0.55
Humic acids						
HA_{peat}	HP_{peat}	2.4	50.22	2.41	4.29	0.31
HA_{coal}	HP_{coal}	5.1	55.64	1.21	6.44	0.47

*C content in the original liquid product is 3.26%

2.3 Experimental design

To determine the effect of cometabolism conditions on HS decomposition during *A. alternata* cultivation, two types of carbon substrates were introduced into a nutrient medium: easily assimilable organic carbon (as sucrose), hardly assimilable organic carbon (as HSs), and their combinations. In the course of several sets of experiments, HSs were added into 100-ml flasks with liquid Czapek medium at varying concentrations (as described below) into a standard (0.3%) or reduced (0.03%) sucrose content. The sample was used as a pure control, and the fungal culture was cultivated on a nutrient medium containing 0.3% sucrose without HSs (pure control). As analytical controls, the HS solutions were used at the same concentrations as those introduced into the nutrient medium before the spore inoculation for *A. alternata* cultivation (control no *A. alternata*).

In two sets of experiments, HSs were added to the growth medium in the following way:

1. For the experiments with LPSEC and spectroscopy, the amount of HSs to be added was normalized by C content or matter content. Potassium humates (HPs) (HP_{coal}, HP_{peat}, and HP_{lingo}) were added to the growth medium at the concentrations capable of providing the content of carbon equal to the one in 0.03% sucrose (0.126 g C/l) (0.48 g/l HP_{coal}, 3.87 g/l HP_{peat}, and 0.32 g/l HP_{ligno}). HAs (HA_{peat} and HA_{coal}) were applied at 100 mg/l, since this concentration is considered to be of high biological activity (Tikhonov et al. 2010).
2. To analyze the cultural liquid using RP-HIC, HSs were added to the nutrient medium at their biologically active concentration (200 mg/l for HP_{coal} and HP_{peat} and 100 mg/l for HA_{peat} and HA_{coal}).

Before the stage of spectral and chromatographic measurements, the cultural liquids of all samples were filtered through a cellulose acetate membrane with 0.2 μm pore size (Chromafil, Macherey-Nagel, Germany).

2.4 Measurements

2.4.1 Low-pressure size-exclusion chromatography

Before and after incubation with a fungal culture, the molecular weight distributions of initial HSs and their decomposition products were studied using LPSEC (chromatograph BioLogic LP, Bio-Rad).

After cultivating *A. alternata* at various concentrations of HSs and sucrose, as described in Section 2.2, the samples of filtered cultural liquids were applied to a 10 × 560 mm chromatography column filled with Sephadex G-50 gel. Tris-HCl buffer (pH 8.2) was used as an eluent; the flow rate was 0.50 ml/min, and the sensitivity was 0.05, 0.1, or 0.2, depending on the concentration of the sample solution. To obtain elution profiles, the absorption of the solution was measured using UV detection at a wavelength of 208 nm in a flow quartz cuvette. Apparent molecular weights were determined by Determan's formulae for globular proteins (Osterman 1985). Percentage of each fraction in HAs was evaluated by calculating chromatographic peak areas as described in Zavarzina et al. (2002). The weight average molecular masses were calculated as described in Kudryavtsev et al. (2000).

2.4.2 Reversed-phase hydrophobic interaction chromatography

Distributions of hydrophobic and hydrophilic fragments were determined in cultural liquids using RF-HIC operated on Octyl Sepharose CL-4B (Pharmacia, Sweden) with 0.05 M Tris-HCl buffer (pH 8.2) as an eluent. After cultivating *A. alternata* at different ratios of sucrose and HSs as described in Section 2.2, filtered cultural liquids were fractionated. The sample volume was 0.5 ml; the rate of filtration was 1 ml/min; the eluate absorbance was monitored at 280 nm; a 1 × 10 cm column (Amicon) was used. Adding salt (20% ammonium sulfate) in the initial buffer increased the polarity of the eluent and hindered the hydrophobic fragments of separated molecules to interact with hydrophobic gel matrix (Osterman 1985; Milanovskii 2006). The first two fractions (A and B) eluted from the column in the presence of ammonium sulfate had predominantly hydrophilic properties.

To increase the step gradient of 0–0.2%, sodium dodecyl sulfate (SDS) was used as a detergent. The gradient profile was formed by an Ultrograd 11300 device (LKB). While the detergent gradient was increasing, the elution of hydrophobic fractions occurred: hydrophobic fraction C was eluted weakly at 0.1% SDS and hydrophobic fraction D at 0.2% SDS (Stepanov 2005, 2008). To evaluate the percentage of each hydrophobic and hydrophilic fraction in HSs, chromatographic peak areas were calculated on similarity as described in Zavarzina et al. (2002).

2.4.3 Spectral measurements

Absorption was measured for the samples of filtered cultural liquids after cultivating *A. alternata* in various concentrations of HS and sucrose. These samples had been placed in a quartz cuvette with a 1-cm optical path with the spectrophotometer Solar PV 2201 (Belarus) within the spectral range 200–1000 nm using distilled water as blank. Fluorescence spectra were measured by the luminescence spectrometer Solar CM2203 (Belarus) at several

wavelengths of the exciting radiation (270 nm, 310 nm, and 355 nm) for aqueous solutions and cultural fluids in quartz cuvettes at a 90° geometry. To reduce the inner filter effect on fluorescence measurements, the samples were diluted by distilled water to get absorbances < 0.1. The absorbance ratio at 250 nm and 365 nm (A_{250}/A_{365}) was calculated as described in Trubetskoj et al. (2018). Based on both the wavelength-integrated fluorescence intensities and absorbance values at an excitation wavelength (measured for diluted samples), the fluorescence quantum yield (QY) values were calculated using quinine sulfate solution in 0.1 N H_2SO_4 as a reference with a fluorescence QY value of 0.55 as described in Milyukov et al. (2007).

2.5 Statistics

Correlation matrix and the Pearson correlation coefficient were calculated, and principal component analysis (PCA) was carried our using XLSTAT software, 2014. An aggregate of 16 samples characterized by seven parameters each was incorporated into PCA. These parameters included pH; increment of biomass % to pure control; proportion of low MWF, %; absorbance ratios (A_{250}/A_{365}); fluorescence emission maximum at $\lambda_{Ex} = 355$ nm; fluorescence quantum yield at 355 nm (QY_{355}), %; and proportion of hydrophilic to hydrophobic fractions as shown in Tables 2, 3, and 4.

3 Results

3.1 Effect of *A. alternata* on molecular weight distribution and spectral characteristics of HSs in growth medium at different contents of sucrose as easily assimilable organic carbon

Relying on both the literature and our results, the effectiveness of HS decomposition during the fungal growth was evaluated by means of an integrated approach including the following analyses: biological (biomass increment in relation to pure control samples), spectral (increase of A_{250}/A_{365} ratio, decrease of the humic-like emission maxima, and increase in QY_{355} values relative to analytical control samples), and chromatographic (increase in the proportion of the low molecular weight fractions and change in the molecular weight of the low molecular weight fractions in relation to analytical control samples). For a full list of indicators, see Tables 2 and 3 for HPs and HAs, respectively.

Molecular mass fractionation of the cultural liquid with different sources of carbon in treatments before and after fungal growth revealed that all the investigated samples contained the compounds of different molecular weight, which can be roughly divided into two fractions: that of high and that of low molecular weight. The sum of low molecular weight fractions and high molecular weight fractions is 1 (or 100%). The presence of these fractions is clearly seen on chromatograms as two peaks (Table 2). Thus, high molecular weight fractions were represented by the compounds of molecular weight

Table 2 Effect of different rates of sucrose and humates on *A. alternata* biomass increment, molecular weight distribution, absorbance ratios (A_{250}/A_{365}), fluorescence emission maximum at λ_{Em} 355 nm (Em_{max}), and fluorescence quantum yield at 355 nm (QY_{355}) in cultural liquids

Sample	pH	Increment of biomass % to pure control	Low molecular weight fraction		A_{250}/A_{365}	λ_{Em}, nm (λ_{Ex} = 355 nm)	QY_{355}, %
			MW range, Da	Mass %			
Aa_0.3% sucrose	8.3	–	3600 ÷ 6700	65	47.1	438	5.9
HP_{ligno}							
Control: no Aa	9.0	–	4800 ÷ 6200	20	5.1	432	0.42
Aa_0.03% sucrose	6.3	− 46	4600 ÷ 6100	20	2.3	439	0.12
Aa_0.3% sucrose	7.8	20	3400 ÷ 5900	67	5.8	425	1.58
HP_{peat}							
Control: no Aa	8.6	–	2100 ÷ 3600	30	2.9	460	0.31
Aa_0.03% sucrose	6.8	nd	nd	nd	2.7	450–447	0.17
Aa_0.3% sucrose	8.6	− 18	2100 ÷ 3600	30	3.2	437	0.61
HP_{coal}							
Control: no Aa	8.8	–	4800 ÷ 6200	8	2.3	480	0.27
Aa_0.03% sucrose	7.0	− 11	4800 ÷ 7500	28	2.1	448–460	0.40
Aa_0.3% sucrose	8.7	16	4800 ÷ 6600	39	9.2	438	5.46

Aa Alternaria alternata, *nd* no data

Table 3 Effect of different rates of sucrose and humic acids on *A. alternata* biomass increment, molecular weight distribution, absorbance ratios (A_{250}/A_{365}), fluorescence emission maximum at λ_{Em} 355 nm (Em_{max}), and fluorescence quantum yield at 355 nm (QY_{355}) in cultural liquids

Sample	pH	Increment of biomass % to pure control	Low molecular weight fraction		A_{250}/A_{365}	λ_{Em}, nm (λ_{Ex} = 355 nm)	QY_{355}, %
			MW range, Da	Mass %			
HA_{peat}							
Control: no Aa	8.6	–	1640 ÷ 3400	34	2.6	454	0.67
Aa_0.03% sucrose	6.9	14	2300 ÷ 3400	73	9.2	455–465	1.06
Aa_0.3% sucrose	8.3	22	3100 ÷ 6600	77	17.0	430	6.8
HA_{coal}							
Control: no Aa	8.5	–	2900 ÷ 5300	33	4.2	489	2.13
Aa_0.03% sucrose	6.1	− 19	3600 ÷ 5300	57	11.1	456–465	0.69
Aa_0.3% sucrose	8.3	19	3400 ÷ 6700	73	11.9	430	4.25

Aa Alternaria alternata

ranging from 19,900 to 23,400 Da for all the studied samples. It may be represented by both high molecular metabolites of *A. alternata* and compounds of humic or melanin nature. The proportion of high molecular weight fractions decreased in the process of fungal growth in the samples with HS introduction (such as HPs and those isolated from HAs) in relation to the analytical control samples. At the same time, the proportion of low molecular weight fractions increased. Therefore, the molecular weight of the compounds representing low molecular weight fractions varies (Tables 2 and 3). Thus, the molecular weight of *A. alternata* cultural liquid in the Czapek medium is 3600 ÷ 6700 Da, HP_{coal} and HP_{ligno} are characterized by peaks corresponding to 4800 ÷ 6200 Da, while HP_{peat} has molecular weight fluctuating in the range of 2100 Da and 3600 Da. This is evidence for the set of low molecular weight fractions in the composition of the studied HSs being significantly diverse.

Table 4 Proportions of hydrophilic and hydrophobic fractions in *A. alternata* cultural liquids

Sample	HPI (A + B)	HPO (C + D)	HPI/HPO (A + B)/(C + D)
Aa_0.3% sucrose	90	10	9.0
HA_{coal}			
Control: no Aa	59	41	1.4
Aa_0.3% sucrose	72	28	2.6
HA_{peat}			
Control: no Aa	34	66	0.5
Aa_0.3% sucrose	70	30	2.3
HP_{coal}			
Control: no Aa	33	67	0.5
Aa_0.3% sucrose	67	33	2.0
HP_{peat}			
Control: no Aa	37	63	0.6
Aa_0.3% sucrose	79	21	3.8

HPI is the hydrophilic fraction, and HPO is the hydrophobic fraction

Aa Alternaria alternata

Tables 2 and 3 show that high molecular weight fractions predominate in analytical control samples achieving 66–92%. In all the cases when the fungal biomass growth was observed in the medium with HSs, a relative decrease in the proportion of high molecular weight fractions and accumulation of low molecular weight fractions in 2.2–4.8 times simultaneously occurred, which can be interpreted as HS decomposition performed by the fungi.

This tendency appeared to be especially pronounced at higher concentrations of easily assimilable organic carbon in the nutrient medium (0.3% sucrose) (Fig. 1a). The biomass increment also appeared to be the highest in the samples cultivated on 0.3% sucrose. HS decomposition is also carried out with a decrease in sucrose concentration in the medium to 0.03%, but less intensively.

Apart from the sucrose concentration in medium, the chemical nature of HSs affects the patterns of HS transformation. Thus, the decomposition patterns of water-soluble potassium humates and those of protonated HAs are different. In the experiments with HAs, sharper regularities were observed (Table 3). The compounds of molecular weight 3300 ÷ 6600 Da, typical of fungi metabolites, appeared in the medium with 0.3% sucrose after HA_{peat} was transformed. Yet, in a nutrient medium with 0.03% sucrose, the molecular weight of low molecular weight fraction compound is lower (2300 ÷ 3400 Da), which indicates a slower decomposition of HA_{peat}.

In experiments with HPs, the data vary for different samples (Table 2), potentially accounting for the differences in their origin and chemical composition (Table 1). The most

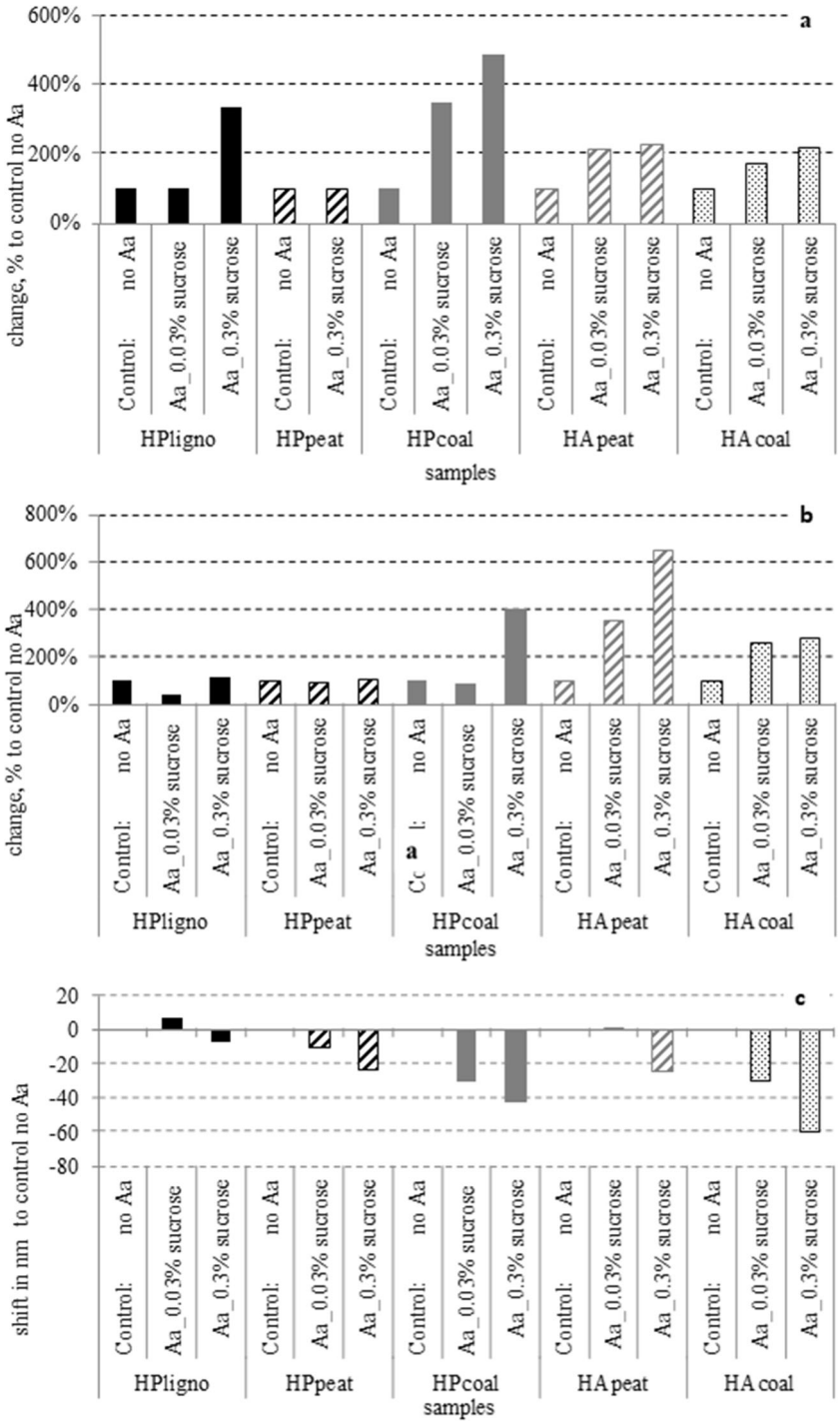

Fig. 1 Changes in share of low molecular weight fractions (**a**), absorbance ratios (A_{250}/A_{365}) (**b**), and fluorescence emission maximum at λ_{Em} 355 nm (Em_{max}) (**c**) as affected by *A. alternata* growth (normalized by corresponding control value for each HS—control no *A. alternata*)

expressed accumulation of low molecular weight fractions was observed for samples with HP_{coal}; namely, its level was increasing as the content of easily assimilable organic carbon in the medium increased. In the HP_{ligno} sample after the growth of *A. alternata*, the compounds with a molecular weight of less than 4000 Da (which are characteristic of micromycete metabolite and are not characteristic of HP_{ligno}_no Aa) appeared in low molecular weight fractions. This effect was revealed only when the sucrose content was standard; with the decrease in the content, the accumulation of low molecular weight fractions was not observed.

Spectral characteristics also indicate HS decomposition by filamentous fungi. Absorbance ratios A_{250}/A_{365}, which appeared to correlate negatively with molecular size (Trubetskoj et al. 2018), in most cases, increased in a raw "analytical control (no *A. alternata*)–*A. alternata*_0.03% sucrose–*A. alternata*_0.3%

sucrose" (Tables 2 and 3). This fact confirms the decrease in the molecular weight of CDOM; at that, it is more expressed for HAs rather than for HPs. Changes in the position of the fluorescence maximum correspond to HS decomposition triggered by fungal activity; namely, in the analytical control of HPs or HAs, the emission of maximum wavelength is the highest (460–500 nm), and it is decreased in the process of fungal growth. Moreover, the shift of HS fluorescence band to the short-wave side is stronger with a higher content of sucrose. That is, under the fungal action, HS decomposition affects mainly the compounds with a lower molecular weight (and appears to be more hydrophilic, according to the chromatographic data).

In addition, QY_{355} value increases (Tables 2 and 3) in a raw "analytical control (no *A. alternata*)–*A. alternata*_0.03% sucrose–*A. alternata*_0.3% sucrose." Taking into account the previously obtained results, according to which low molecular weight CDOM fractions were observed to have higher QY values (Milyukov et al. 2007), the increase in QY_{355} value may implicitly indicate the decrease in both the molecular weight and the size of fractions during the fungal growth.

A partial deviation from the trends described above to the ones of spectral indices was observed in the experiments with HP_{ligno}. It should be noted that the analytical control HP_{ligno} differs from the other HS analytical controls with higher values of A_{250}/A_{365} ratio (5.1) and QY_{355} value (0.42) and with a shift of the fluorescence maximum upon excitation at 355 nm to shorter wavelengths (432 nm). It may be caused by low molecular weight phenolic compounds releasing from the source of organic material (lignosulfonate) during the manufacturing process (Yakimenko and Terekhova 2011; Fedoseeva et al. 2018).

The above-described patterns are presented in Fig. 1. The biomass of the fungal culture positively correlated with other indices indicating HS transformation under fungal metabolites. The proportion of low molecular weight fractions, A_{250}/A_{365} ratio, and QY_{355} values were the highest for the samples cultivated with a higher sucrose concentration. It should be mentioned also that of all HS samples, it is the isolated HAs rather than the initial HPs that are most effectively degraded. Thus, the sample Aa_HA_{peat}_0.3% sucrose is characterized by the following indicators: the proportion of LMFW is 77%; the A_{250}/A_{365} ratio is 17; the QY_{355} value is 6.8. To compare, here come the indicators of analytical control of HA_{peat}: the proportion of LMFW is 34%; the A_{250}/A_{365} ratio is 2.6; the QY_{355} value is 0.67. A sample of HA_{peat} can be considered as the most susceptible to microbial transformation under the proposed experimental conditions.

Thus, the following regularities can indicate the HS transformation under fungal metabolites: the decrease in the proportion of high molecular weight fractions and the change in the molecular weight of low molecular weight fractions in relation to analytical control, the increase in A_{250}/A_{365} ratio and the decrease in the humic-like emission maxima, and finally, the increase in QY_{355} values.

3.2 The effect of *A. alternata* on distribution of hydrophobic and hydrophilic fragments of HSs in growth medium

Experiments on hydrophilic-hydrophobic distribution of HS components may prompt a better understanding of the probable ways and common patterns typical of HS transformation induced by *A. alternata*. The entire spectrum of the fractions distributed according to the degree of hydrophilicity/hydrophobicity shown in the increasing SDS gradient is represented by the following components: the most hydrophilic A and B (elution time 0–60 min), relatively weak hydrophobic C (60–100 min), and relatively hydrophobic D (100–150 min). The selected HIC profiles are presented in the Electronic Supplementary Material.

Distribution of amphiphilic components in *A. alternata* cultural liquids (Electronic Supplementary Material) indicates the dominance of hydrophilic components (fraction A: labile, easily soluble organic compounds). It can be assumed that this fraction is represented by residual nutrients (carbohydrates) of nutrient medium and metabolites of fungal culture (water-soluble proteins, fungal carbohydrates, hydrophilic components of melanins) which accounts for 90% of the total sum of the substances (Table 4).

By contrast, in HS solutions, hydrophobic fractions predominate and achieve 41–67% of the total sum of fractions (Table 4). At the same time, these samples exhibit a more complex composition of hydrophilic fractions; namely, several fractions eluting up to 60 min of analysis (i.e., not interacting with the hydrophobic matrix) are observed on HIC profiles (Electronic Supplementary Material). Probably, a wide spectrum of hydrophilic fractions may occur because of a complex composition of HS polar organic moieties (amino acids, carbohydrates, fulvates, and other organic compounds with a high content of functional groups on the surface of the molecules).

HSs decomposed by *A. alternata* are characterized by a decrease in hydrophobic fractions as compared to the corresponding HSs without fungal growth (Table 4). During cultivation, the easily assimilable moieties of HS molecules seem to be degraded, then associate, and a relative accumulation of soluble (hydrophilic) organic compounds accumulate relatively. Apparently, hydrophilic fractions of initial HP_{peat} and HA_{peat} appear to be decomposed almost completely. This assumption is supported by a drastic change in the way the hydrophilic components distribute among HIC profiles in the samples before and after the process of fungal culture incubation. Thus, three powerful hydrophilic maxima (which were missing on HIC profiles of cultural liquids and the initial HP_{peat}) appear at the elution time of 10 min, 13 min, and 14.3 min for the sample Aa_HP_{peat} (Electronic Supplementary Material).

Table 4 presents the proportion of respective fractions and the ratio of hydrophilic to hydrophobic fractions.

The proportion of hydrophilic fractions in the initial samples of HSs varied from 33 to 59% of the total composition of the fractions, and the degree of hydrophilicity (based on the ratio of hydrophilic to hydrophobic fractions) for most samples is 0.4–0.6. The exception is HA_{coal}, in which the proportion of hydrophobic fractions was markedly reduced.

During the growth of *A. alternata* in a medium with HS addition, hydrophilic, more polar low molecular compounds accumulate in all samples. At the same time, during the growth of *A. alternata*, a decrease in the proportion of hydrophobic fractions (C + D) is observed. This trend can be traced to the increase in the proportion of hydrophilic to hydrophobic fractions in the samples from 0.5–1.4 to 2.0–3.7 after cultivation of *A. alternata*. This can be regarded as an indicator of HS decomposition, since the sample of fungal cultural liquid without HSs is characterized by the absence of hydrophobic fractions (C + D).

Accumulation of hydrophilic compounds during *A. alternata* growth takes place due to hydrophilic metabolites of fungal increases, and the fungal culture consumes carbohydrates in nutrient medium. An inverse correlation is observed between the data sets for biomass increment and proportion of hydrophilic to hydrophobic fractions (the Pearson correlation coefficient is − 0.92). The reverse correlation is explained by the fact that the least increase in biomass is observed when the consumption of carbohydrates in nutrient medium and the accumulation of fungal metabolites are as the least active as possible; hence, the efficiency of HS decomposition is reduced. For instance, the sample $Aa\_HP_{peat}\_0.3\%$ sucrose is characterized by a high proportion of hydrophilic to hydrophobic fractions (3.8) with negative biomass growth (− 18%) and the lowest values of spectral HS decomposition parameters (A_{250}/A_{365} and QY_{355}, %) in relation to the samples after growth of *A. alternata* at 0.3% sucrose with other HSs.

Thus, the efficiency of HS decomposition by fungal metabolites increased drastically in the proportion of hydrophilic fractions, while the most difficultly degraded hydrophobic components of HSs decreased.

4 Discussion

A set of spectral and chromatographic indicators allowed to elucidate various decomposition patterns of the HSs during the fungal growth. They revealed that the changes in the initial solutions of HSs resulted in the molecular weight transformation accumulating the low molecular weight fractions and the increased hydrophilic fractions. Since all these transformations triggered the modifications of humic-like fluorescence as changing the spectral properties, these chromatographic and spectral indicators correlate differently with each other and are characterized by different information contents (Fig. 2). It seems that the most informative indicator for assessing the HS decomposition is the one exhibiting A_{250}/A_{365} ratio and QY_{355} values, since these parameters are positively correlated with the majority of other indicators.

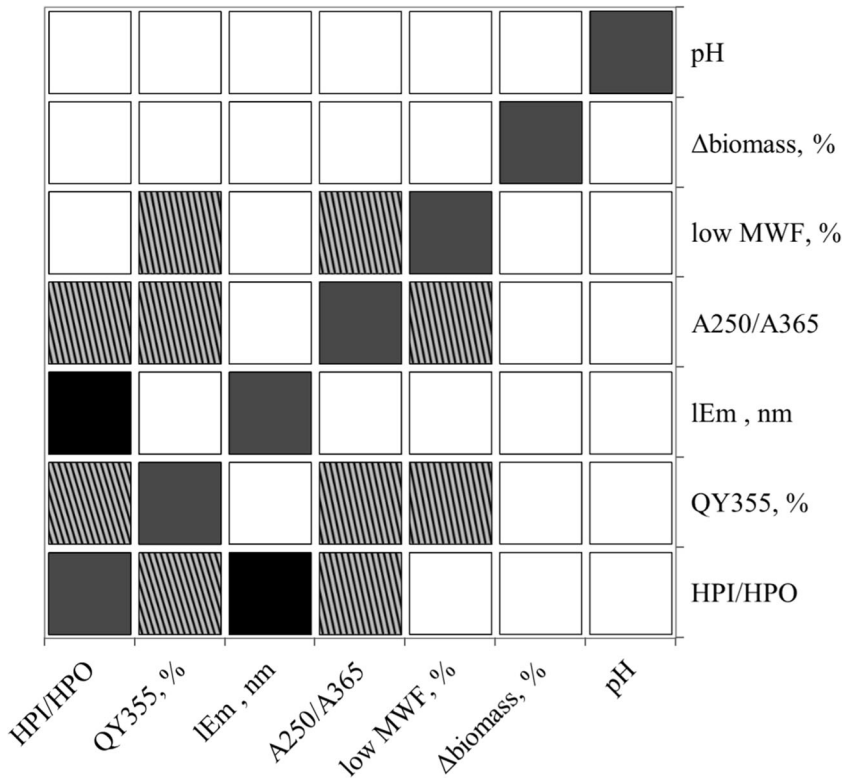

Fig. 2 Correlation map (p = 0.05): absence of correlation (white boxes), negative correlation (dark boxes), and positive correlation (striped boxes)

Table 5 Squared cosines of the variables and variance percentages corresponding to the principal components

Variables	PC1	PC2	PC3	PC4
pH	0.011	*0.752*	0.225	0.002
Δbiomass, %	*0.633*	0.136	0.009	0.109
Low molecular weight fraction, mass %	*0.741*	0.151	0.011	0.052
A_{250}/A_{365}	*0.805*	0.042	0.005	0.001
λ_{Em}, nm (λ_{Ex} = 355 nm)	0.318	0.077	0.250	*0.348*
QY_{355}, %	*0.821*	0.050	0.001	0.002
HPI/HPO	0.258	0.200	*0.371*	0.135

Values in italics correspond for each variable to the factor for which the squared cosine is the largest

In the course of our research, different patterns were found in the transformation of various HS samples (HPs and HAs from coal, peat, and lignosulfonate) performed by the melanin-containing fungus *A. alternata*. The type of transformation largely depended on the presence of sucrose as an easily assimilated carbon source. This was discovered while studying the results of PCA that yielded four principal components (PCs) with the highest values of squared cosines (Table 5). PC1 explains 51% of total variance and shows strong loadings for Δbiomass, low molecular weight fractions, A_{250}/A_{365}, and QY_{355}. PC2 explains 20% of the total variances and shows strong loading for pH values.

Figure 3 illustrates the distribution of the HS samples before (initial HS samples) and after (cultural liquids) *A. alternata* cultivation. All the samples are divided into four groups, depending on sucrose concentration and the nature of HSs:

- Original HS solutions (both HPs and HAs) with negative loadings on PC1 and PC2 with the exception of HP_{ligno} with slightly positive loadings on PC1. The exception is HP_{ligno}, which differs from other HS samples with following parameters: higher values of A_{250}/A_{365} ratio, QY_{355} value, and the shift of the fluorescence maximum upon excitation at 355 nm to the shorter-wavelength region
- HSs (both HPs and HAs) after fungal cultivation at 0.3% sucrose with positive loadings on PC1. The only exception is $HP_{peat\_Aa\_0.3\%}$ (the fourth group), characterized by a high proportion of hydrophilic to hydrophobic fractions

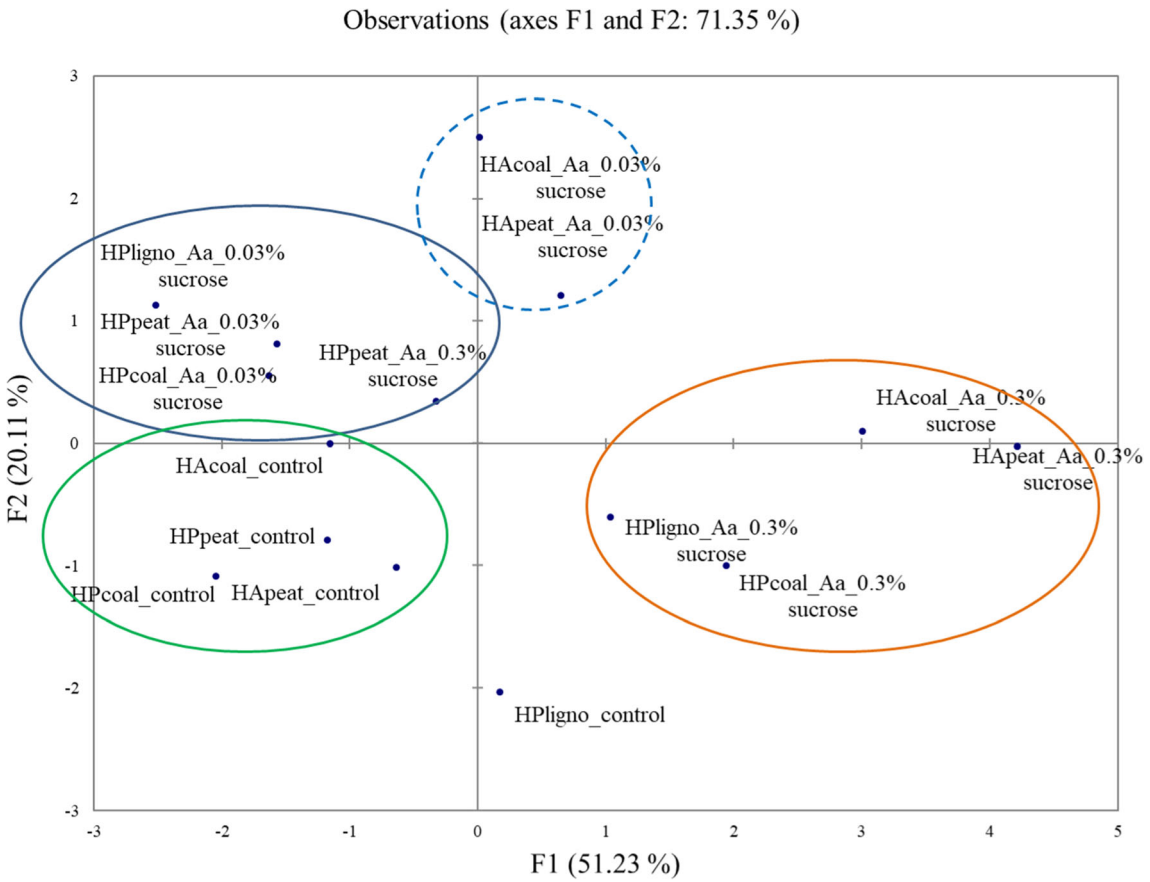

Fig. 3 Factor scores plot for the initial samples of humic substances and for the cultural liquids after growth of filamentous fungal culture in the principal components 1 and 2

with negative biomass increment and the lowest values of spectral parameters of HS decomposition (A_{250}/A_{365} и QY_{355}, %)
- HAs after fungal cultivation at 0.03% sucrose with positive loadings on PC2
- HPs after fungal cultivation at 0.03% sucrose with positive loadings on PC2 and negative loadings on PC1

The obtained results confirmed a positive role of assimilable organic carbon in the process of decomposition of hard-to-reach C substrates as HSs by *A. alternata*. The most effective degradation occurs under the conditions of saturation of the medium with easily assimilable organic carbon (in our experiments with 0.3% sucrose). At the same time, the degree of HS decomposition positively correlated with the accumulation of fungal biomass. Thus, easily assimilable organic carbon plays a triggering role in HS degradation by the fungi and enhances the accumulation of fungal biomass. This is in a clear accord with the recent views on the degradation of soil stable organic compounds. It was hypothesized first and experimentally confirmed later that the easily assimilable organic carbon is required to trigger the mechanism of *priming effects* in order to degrade them (Bingeman et al. 1953; Hardie et al. 2009; Kuzyakov 2010).

Among the HS samples under scrutiny, the isolated HAs were degraded more effectively than the original parent HPs. The same effect was observed for both sucrose concentrations, although under lower content (0.03%) conditions, it was much more pronounced. The main difference between HPs and corresponding HAs lies in their composition: the former are HP salts with a certain share of acid-soluble compounds (fulvic-like fraction and non-humic organic substances), whereas the latter are pure HAs (Table 1). It should be noted that HAs are more readily degraded by *A. alternata* and other microfungi than the smaller FA molecules, as reported earlier (Gramss et al. 1999; Rezacova et al. 2006). The present work shows for the first time the differences in degradation patterns for acid and salt forms of the same HSs.

5 Conclusions

Detailed multifaceted laboratory experiments on the transformation of various HS samples (HPs and HAs of different genesis of coal, peat, and lignosulfonate) by the melanin-containing fungus *A. alternata* were carried out using a set of contemporary techniques (namely, chromatographic and spectral analyses). The obtained results enable us to identify the contribution of ascomycetes to HS decomposition with greater precision and gain a complex picture. The following characteristics can indicate that HS transformation takes place: a decrease in the proportion of high molecular weight fractions and a change in the molecular weight of low molecular weight fractions relative to analytical controls of HS samples, an increase in the proportion of hydrophilic fractions to hydrophobic fractions, the increasing of A_{250}/A_{365} ratio and the decreasing of the humic-like emission maxima, and finally, an increase in QY_{355} values.

The samples of both groups (HAs in protonated form and HPs) were transformed under metabolites of pure culture of microscopic filamentous fungi *A. alternata*. The isolated HAs were degraded more effectively than the original parent HPs that were much more pronounced at a lower sucrose content (0.03%). It should be brought out specially that the most noticeable changes in HS composition in the Czapek nutrient medium with the growth of *A. alternata* occurred only in the presence of an easily assimilated carbon source (especially at 0.3% sucrose). Thus, it can be concluded that the easily assimilable organic carbon can stimulate the processes of fungal decomposition of stable organic compounds like HSs. Probably, in natural media (soil, water), the content of assimilable organic carbon may trigger the mechanism of priming effects (Hardie et al. 2009; Kuzyakov 2010), yet this is to be investigated in further studies.

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