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Research Article

Spectral Characterization of Fungal Metabolites in Aqueous Medium with Humus Substances

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The work is targeted to confirm participation of microscopic fungi in transformation of humus substances in aquatic environments. The research is focused on the spectroscopic study of the collection of fungal strains with different pigmentation of mycelium. Spectral properties of fungal metabolites were measured and compared to that of natural aquatic nonliving organic matter and commercial humus substances in aqueous solutions. The experiments revealed that the effect of microscopic fungi growing in the culture medium with added humate appeared as changes in the humic-type fluorescence: its characteristics became more similar to that of nonliving organic matter in natural waters than to original humate preparation. The experiments demonstrated degradation of coal-originated humate due to microbial activity into compounds of smaller molecular size and increased heterogeneity. We resume that transformation of humus substances by fungal cultures can be monitored and characterized using spectral measurements.

1. Introduction

Aqueous nonliving organic matter (NOM) and soil humus substances (HSs) are natural organic compounds representing the largest pool of carbon on the Earth [1]. Being the products of stochastic synthesis, NOM and HS were characterized as polydispersed substances having nonstoichiometric elemental composition with irregular and heterogeneous structures [2–5]. These natural substances are polymeric aromatic oxyacids of irregular structure with chemical properties which allow neutralizing hazardous effects of various pollutants [6, 7]. Commercial HS products are widely used in industry, agriculture, and in different technologies of wastewater treatment. For instance, very promising types of water-dispersible sorbents for heavy metal removal include hybrids consisting of magnetite nanoparticles coated by HS products [8–10].

The problem of interaction of HS and microscopic fungi in aqueous and terrestrial biocenoses provoked wide scientific discussions. It is well known that fungi are among the most diverse organisms in the world [11] and represent very important functional and structural component of biological ecosystems [12]. Fungal communities play a significant role in human well-being and ecological processes [13, 14]. Fungal natural habitats include soil, water, and various organisms. They are present in almost all regions and climates, even under extreme conditions. However their role in geochemical cycling usually remains underestimated. Nowadays, these microorganisms are widely used in biomedical research and biotechnology. In addition to direct benefit (sources of antibiotics) or adverse effects (agents of disease), microscopic fungi can impact many environmental processes, particularly those associated with the turnover of organic matter. The processes of soil organic matter transformation and regularities of utilization of plant residues by fungi were described in [15–17]. Fungi affect the soil properties in different ways: via transformation of organic matter, via soil structure status, acidity, and temperature, as well as via the regulation of soil microbiota functioning. Fungi play an important role in plant litter decomposition through nutrient recycling because they

attack the lignocellulose matrix that other organisms are unable to assimilate. Biodiversity and potential functions of microscopic fungi in aquatic ecosystems were shown in [18, 19].

Although it is debated whether microscopic fungi are capable to produce humus substances directly [20, 21], undoubtedly they play essential role in lignin degradation and humus substances turnover [22, 23]. It was established that some fungi are capable to produce dark-brown polymers in the presence of dead plant biomass and thereby might contribute to the pool of humus or humic-like substances in salt marsh estuaries [24, 25]. The fungal polymers resemble humus substances obtained from dead plant biomass or from salt marsh sediments in their elemental composition and elemental ratios, as well as the UV, visible, and FTIR spectral characteristics [26]. Comparative studies of fungal polymers—melanin and humin-like substances produced by individual and mixed cultures of basidiomycetes—showed their similarity in elemental composition and physicochemical properties to natural HS [27, 28]. Melanins are polymers of phenolic and/or indolic nature. The position of humin-like substances among other natural polymers formed under similar conditions, such as melanins and HS, is still not determined [28]. Studies of the dynamics of laccase production suggested that the humin-like substances were produced via extracellular degradation of lignin macromolecules with involvement of laccase, an extracellular oxidase of basidiomycetes [29].

The wood- and leaf-litter-decaying activity of basidiomycetes has been well known for a long time. Recently a great interest in studying the processes of organic matter turnover in soil and aqueous ecosystems is focused on nonbasidiomycetous microfungi, other important groups of soil and amphibious microscopic fungi like Deuteromycetes or ascomycetes. This interest is caused not only due to their ability to affect basidiomycetes activity using a set of exoenzymes [15], but as well as due to the direct participation of nonbasidiomycetous microscopic fungi in destruction of biopolymers and organic matter turnover [30]. Being destroyers of litter, dead branches, and tree trunks, including their constituent cellulose inaccessible to most organisms, microscopic fungi contribute significantly to the biological cycles of carbon. Interaction of microorganisms with NOM in aqueous and terrestrial biocenoses is an intriguing problem for our environment [13, 31]. However the interaction of HS and microscopic fungi has not been studied much. The influence of commercial HS potassium humate on some physiological characteristics of microscopic fungi for the first time has been described in [32] for the cultures with differently pigmented mycelium. It was shown that addition of potassium humate in concentration of 0.1% or 0.02% by weight to the medium has accelerated the growth rate for the light-colored colonies and decelerated for the colonies with black and deep-brown pigmentation.

It is well established that fluorescence spectroscopy is an effective method for environmental monitoring and hydrographic measurements [33, 34], characterization of benthic organisms [35], algal cells [36, 37], corals [38] and higher plants [39–46], specification of photosynthetic pigments [47–49], and other biological molecules [50, 51]. Spectral methods were successfully employed for studying NOM in natural water [52–59], as well as commercial HS preparations [60–63].

The results on fluorometric investigation of HS transformations caused by some microscopic fungi were reported in [64]. It was determined the ability of eight soil microfungal species, *Alternaria alternata, Clonostachys rosea* f. rosea, *Exophiala* cf. *salmonis, Fusarium* cf. *coeruleum, Fusarium redolens, Paecilomyces lilacinus, Penicillium canescens,* and *Phoma* sp., as well as two basidiomycetes *Trametes versicolor* and *Phanerochaete chrysosporium* to modify fluorescence properties of fulvic acids and humic acids.

Our work is aimed to confirm participation of microscopic fungi in transformation of humus substances. It is focused on fluorescence spectroscopic study of the collection of microfungal strains with different pigmentation of mycelium (from uncolored to dark colored), for which we previously revealed the dependence of growth rate on presence of humus substances in the medium [31]. The objective of the work is a detailed study of fluorescence properties (change of emission maximum, fluorescence quantum yield) of fungal metabolites in the medium without and in presence of commercial HS.

2. Experimental

We have analyzed six soil microfungal strains with different pigmentation of mycelium: *Fusarium moniliforme* (noncolored), *Alternaria alternata* (dark pigmented), *Phoma glomerata* (dark-brown pigmented), *Cladosporium cladosporioides* (dark pigmented), *Geomyces pannorum* (brown pigmented), and Mycelia sterilia (orange-brown pigmented). The fungi species were cultivated in the liquid Czapek medium with addition or without addition of potassium humate Powhumus, produced from brown coal leonardite, in concentration of 0.2 g/L. All cultures grew up in 200 mL glass flasks in darkness at temperature of 25 degrees. Several strains of micromycetes in parallel were studied in each experiment using at least three replicates for each strain. Time of cultivation varied from 2 to 6 weeks. Filtered culture fluid was used for further spectral investigations. Significant changes in fluorescence spectra due to the growth of microorganisms have been observed two-three weeks after beginning of fungi cultivation. Experiments with different cultures were repeated several times within two years period. Experiments with *C. cladosporioides, Ph. glomerata,* and M. sterilia were carried out 3 times; other cultures were investigated twice. Each repetition of the experiment with the same strain showed similar basic trends (the tendency to change the maximum of emission spectrum to shorter wavelengths, changes in the fluorescence quantum yield), although those quantitative values varied in up to 10%.

Absorption spectra were measured using UV-Vis spectrophotometer Unico-2804 for filtered samples without dilution in relation to the Czapek medium without HS. For fluorescence measurements fungal metabolites were diluted in 10 times. Fluorescence emission spectra were registered with excitation at 270, 310, or 355 nm using luminescence

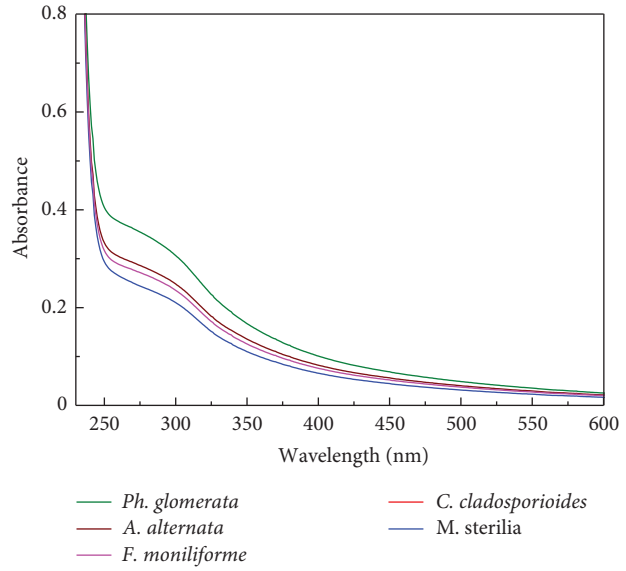

Figure 1: Absorption spectra of fungal metabolites for different cultures grown without humate in the medium.

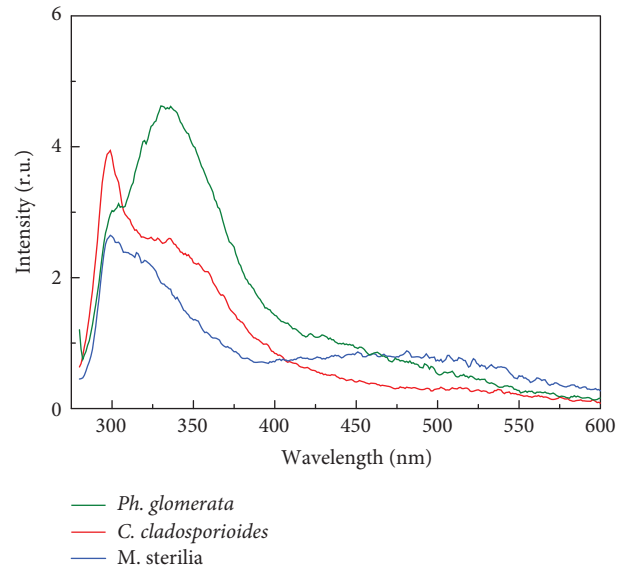

Figure 2: Fluorescence spectra of fungal metabolites for cultures grown without humate in the medium (excitation at 270 nm).

spectrometer Solar CM2203. The choice of excitation wavelength was based on previous reports on studying spectral components of chromophoric NOM [56–58]. All spectra were registered in standard quartz cells with 1 cm optical path. The fluorescence quantum yield (FQY) for each excitation wavelength was calculated using a reference sample with known quantum yield. As a reference sample the solution of quinine sulphate was used, because of similarity of its fluorescence band to NOM or HS spectral band, both in shape and maximum position. The FQY of quinine sulphate dissolved in aqueous solution of sulphuric acid in water with concentration 0.05 mole/L is equal to 0.546 [65]. Instrumental error of the absorbance values was 0,005. In fluorescence measurement, the instrumental error was less than 5% for fluorescence intensity at a specific wavelength and less that 0,04% for its integral value. The error in finding fluorescence peak position was about 5 nm. The relative error in calculation of FQY did not exceed 10% of its value.

3. Results and Discussion

3.1. Absorption Spectra. Figure 1 shows absorption spectra of filtered fungal cultural liquid measured in relation to the Czapek medium. Each spectral curve is an average of three culture replicates grown in the liquid medium without HS during three weeks.

Absorption spectra of fungal metabolites typically are featureless, with a monotonic decline with wavelength increasing from 200 to 600 nm. In certain wavelength ranges, there is appearance of natural chromophoric groups. We attribute them as absorption band of phenolics and quinones (a shoulder located around 280–290 nm).

3.2. Fluorescence Spectra. Fluorescence of fungal metabolites was observed in the spectral range of 280–600 nm (Figures 2 and 3). Typical fluorescence spectrum of fungal metabolites excited at 270 nm consists of two overlapping bands: (i) the UV fluorescence band within 300–350 nm, attributed to phenolics and proteins and (ii) the blue fluorescence of fungal biopolymers within 400–600 nm.

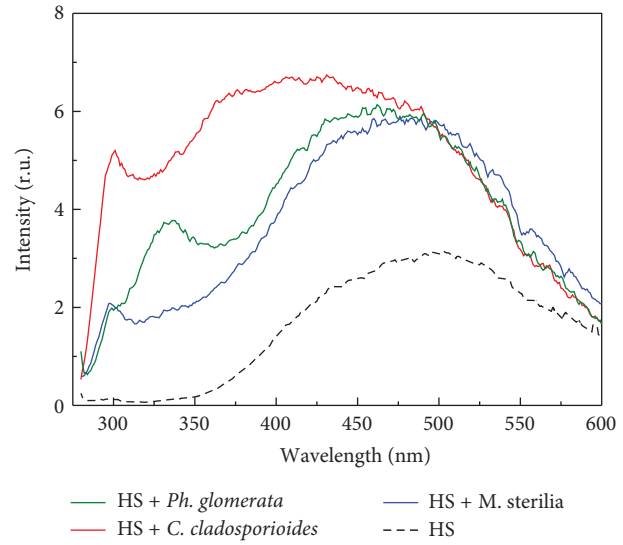

Figure 3: Fluorescence spectra of fungal metabolites for cultures grown in presence of humate in the medium (excitation at 270 nm).

As it was preciously shown in experiments with natural water samples and aqueous soil extractions, typical fluorescence of natural HS (humic-type fluorescence) has emission maximum in the range of 420–460 nm [56–58], while fluorescence emission band of commercial HS products is shifted to the region of 500–520 nm [58, 63], longer wavelengths compared to natural aquatic HS.

Table 1: FQY and λ_{em} for commercial HS solution and fungal metabolites of cultures grown in presence and absence of HS.

FQY and λ_{em} (for given λ_{ex})	HS solution	*C. cladosporioides*		*Ph. glomerata*		M. sterilia	
		Without HS	With HS	Without HS	With HS	Without HS	With HS
FQY (λ_{ex} = 270 nm)	0.6%	2.4%	3.3%	1.3%	1.9%	1.5%	1.4%
FQY (λ_{ex} = 310 nm)	0.5%	1.7%	5.2%	0.5%	1.9%	0.9%	2.1%
FQY (λ_{ex} = 355 nm)	0.4%	5.0%	7.2%	1.0%	2.1%	2.3%	3.4%
λ_{em}, nm (λ_{ex} = 270 nm)	498	UV	414	UV	423	UV	459
λ_{em}, nm (λ_{ex} = 310 nm)	501		423		435		446
λ_{em}, nm (λ_{ex} = 355 nm)	500		451		465		473

Emission maximum position of natural aquatic HS depends on excitation wavelength λ_{ex}. The wavelength of maximum emission excited at λ_{ex} = 310 nm shifts towards shorter wavelength compared to that excited at 270 nm or 355 nm. This phenomenon was observed for all types of natural HS [56–58, 66, 67] and is known as "blue shift" of DOM fluorescence [66, 67]. In contrast, the position of emission maximum for commercial HS does not depend on excitation wavelength [58, 63].

After fungi growing in the humate-containing medium, their fluorescence spectra consist of two broad overlapping bands: (i) the UV fluorescence of phenolics or proteins and (ii) the wide band around 425–470 nm conditional on fungal metabolite products (Figure 3). With λ_{ex} = 310 nm the position of the second band is shifted towards shorter wavelengths compared to fluorescence of the same sample excited at 270 nm or 355 nm. This resembles the spectral features of dissolved organic matter occurring in natural water [56–58]. This spectral behavior cannot be explained by simple addition of fluorescence bands for humate and fungal metabolites, since the blue fluorescence of fungal biopolymers is not strong enough to give such an effect.

3.3. Fluorescence Quantum Yield (FQY). The values of FQY for the humic-type fluorescence reside between 2–4% for NOM in natural water, 0.1–0.3% for aqueous soil extractions, and 0.4–1.2 for commercial HS preparations in water [26, 27]. The value of FQY of most of natural HS increases along with the excitation wavelength rising from 270 to 355 nm [27]. Our experiments revealed that the effect of fungi growing with HS in the culture medium appeared as significant changes in the humic-type fluorescence (see Table 1 and Figure 3). The value of quantum yield of humic-type fluorescence for HS modified by fungi increased and its emission maximum were essentially shifted towards shorter wavelengths compared to that for original humate solution.

Moreover, the spectral behavior of humic-type fluorescence along with excitation wavelength has been changed after growing of microfungal cultures. For original humate solution FQY was slightly decreasing with λ_{ex} (Table 1). But due to fungi growing, the HS-containing culture medium demonstrated increase of FQY with λ_{ex}. The peak wavelengths for the initial HS solution were about 500 nm and practically identical under different excitation wavelengths (λ_{ex} = 270, 310, or 355 nm), while after culture growing its emission maximum became essentially dependent on excitation wavelength.

Significant transformation of humus substances due to the growth of microorganisms has been observed not earlier than two-three weeks after the start of cultivation. Table 1 summarizes the results for three fungal cultures which demonstrated the best reproducibility in our experiments after 3 weeks of cultivation. Other fungal cultures showed similar basic tendencies in spectral behavior after the same period of cultivation. Longer growing of fungal cultures caused the effects of mycelium autolysis.

The work [64] describes that some microfungal species shifted the emission maximum of fulvic and humic acids to longer wavelengths. The opposite effect (shift of the humic acids emission maximum to shorter wavelengths) caused by microscopic fungi was observed in [64] for humic acids complemented by glucose. In our research we did include sugar in the medium; however, we cannot directly compare our results with those of [64] because of the following two reasons. First, for fluorescence excitation we used the UV wavelengths ranging from 270 to 355 nm, while in the authors of [64] excited fluorescence at 470 and 468 nm. Second, in our study we have used commercial HS preparation from leonardite, while the paper [64] deals with fulvic and humic acids purified by column chromatography.

Our findings were explained by transformation of refractory HS from bigger to smaller macromolecules by fungi cultures during their growing. Microscopic fungi utilize big macromolecular compounds with longwave fluorescence emission and produce smaller ones with emission shifted towards shorter wavelengths. Dependence of the values of FQY and λ_{em} on λ_{ex} reflects heterogeneity of composition of substances in the culture medium. Thus, spectral characteristics of humate solution became more similar to that of NOM of natural water or soil HS.

For confirmation of the hypothesis we have made experiments to compare fluorescence of natural NOM of different molecular size and commercial humic acids. Test samples were taken from two rivers in Moscow region. These NOM-containing samples were filtered through cellulose acetate filter with 5 nm pore size to get low-molecular weight fraction and analyzed with a fluorescence spectrometer. As a result of investigations we submit the list of spectral characteristics (see Table 2) of NOM in initial water sample and its low molecular weight fraction. Humic acid Aldrich was dissolved in water to receive the samples of commercial HS.

Table 2: FQY and λ_{em} for natural NOM and its low molecular weight fraction in comparison to solutions of commercial humic acids in water.

FQY and λ_{em} (for given λ_{ex})	NOM of natural water			Low molecular weight fraction of NOM			Commercial humic acids	
FQY (λ_{ex} = 270 nm)	1.8%	2.7%	2.6%	2.2%	4.0%	3.9%	1.3%	1.3%
FQY (λ_{ex} = 310 nm)	2.5%	3.2%	3.4%	3.0%	4.8%	3.9%	1.2%	1.2%
FQY (λ_{ex} = 355 nm)	3.6%	3.9%	5.0%	5.0%	7.2%	5.7%	1.0%	1.1%
λ_{em}, nm (λ_{ex} = 270 nm)	446	446	438	450	436	439	478	477
λ_{em}, nm (λ_{ex} = 310 nm)	428	421	420	424	420	423	475	475
λ_{em}, nm (λ_{ex} = 355 nm)	448	445	445	451	447	445	476	477

Once again we have observed that emission maximum of commercial HS solutions is shifted to longer wavelength region compared to that of natural HS in water. The shift of emission to shorter wavelengths, or so-called "blue shift" was observed for natural water with change in excitation wavelength from 270 to 310 nm, and it did not occur for humic acid solutions. We can resume that for low-molecular weight fraction of NOM the value of FQY is higher than the same value for the initial NOM, which proves the hypothesis that microscopic fungi break macromolecular HS compounds producing smaller ones with higher values of FQY.

4. Conclusions

Chromophoric organic matter released by fungi strains into aqueous medium without added humate showed similar spectral features for various fungi strains: absorbance values decreasing towards longer wavelengths (with a shoulder at 280–290 nm) and fluorescence emission spectra consisting of two overlapping bands (the UV peak of phenolics and proteins and the blue-green fluorescence of fungal polymers).

The experiments revealed that the effect of microscopic fungi growing in the culture medium with added humate (concentration 0.2 g/L) appeared as changes in the humic-type fluorescence: its characteristics became more similar to that of nonliving organic matter in natural waters. Fluorescence quantum yield of humus substances modified by fungi increased, and emission maximum was essentially shifted towards shorter wavelengths compared to that for original solution of commercial humate. Significant transformations of humus substances due to the growth of micro-organisms have been observed typically three weeks after the start of the experiment. Wavelength of emission maximum and quantum yield of humic-type fluorescence became excitation wavelength dependent. The experiments revealed degradation of coal-originated commercial humate due to microbial activity into compounds of smaller molecular size and increased heterogeneity. We resume that transformation of humus substances by fungal cultures can be monitored and characterized using spectral measurements.

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