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SEWAGE SLUDGE PARTICLE SIZE FRACTIONS AFFECT KERATINOLYTIC AND KERATINOPHILIC FUNGI

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FRAKCJE MECHANICZNE OSADU ŚCIEKOWEGO WPŁYWAJĄ NA GRZYBY KERATYNOLITYCZNE I KERATYNOFILNE

Wcześniejsze badania wykazały, że skład ziarnowy wpływa na grzyby keratynolityczne i keratynofilne w osadach ściekowych. Niniejsza praca miała na celu określenie składu ilościowego i jakościowego powyższych grzybów we frakcjach osadu ściekowego (> 1, 1–0,5, 0,5–0,25, 0,25–0,125, 0,125–0,063, 0,063–0,032 i < 0,032 mm) w doświadczeniu modelowym. W oryginalnej próbce osadu ściekowego i w jej frakcjach skład grzybów keratynolitycznych i keratynofilnych oznaczano metodą przynęty włosowej. Z kolei skład grzybów aktidiono-opornych określono metodą rozcieńczeń, wykorzystując pożywkę Wieganda z dodatkiem chloramfenikolu (100 mg/dm^3) i aktidionu (500 mg/dm^3). Liczba grzybów keratynolitycznych i keratynolitycznych była niższa we frakcjach niż w próbce oryginalnej osadu ściekowego. Liczba grzybów aktidiono-opornych była natomiast wyższa we frakcjach niż w próbce oryginalnej. Obserwowano również różnice jakościowe. Wyciągnięto wniosek, że składniki odżywcze, głownie zawartość siarki i stosunek C:S miały większy wpływ na skład grzybów keratynolitycznych i keratynofilnych niż liczba propagul grzybowych (wielkość inokulum) w osadzie ściekowym.

Summary

Previous studies indicated that particle size distribution affects the composition of keratinolytic and keratinophilic fungi in sewage sludge. The present study was to determine the composition of these fungi in sludge particle size fractions (> 1, 1–0.5, 0.5–0.25, 0.25–0.125, 0.125–0.063, 0.063–0.032 and < 0.032 mm) in a model experiment. In the original sludge sample and its fractions, the composition of keratinolytic and keratinophilic fungi was determined by using the hair baiting method. The composition of actidione-resistant fungi was also determined by using the dilution method and the Wiegand medium supplemented with chloramphenicol (100 mg/dm^3) and actidione (500 mg/dm^3). The number of keratinolytic and keratinophilic fungi isolated by the hair baiting method was lower in fractions than in the original sludge sample. In contrast, fungal quantities obtained by the dilution method were higher in fractions than in the original sludge sample. Qualitative differences were also observed. The conclusion was that nutrient factors associated with sludge particle size fractions, chiefly total sulfur content and C:S ratio, affected the composition of keratinolytic and keratinophilic fungi in the sludge more than the fungal propagule quantities (inocula).

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INTRODUCTION

According to Majchrowicz and Dominik's definition [6], keratinolytic fungi are specialized in decomposition of keratin, being the main component of keratinous substrata, while keratinophilic fungi accompany keratinolytic fungi, utilizing non-protein components of these substrata or the products of keratin decomposition. Most of the above-mentioned fungi are resistant to actidione (cycloheximide). Since many keratinolytic and keratinophilic fungi have been found to be the agents responsible for human mycoses [4], and since these fungi occur in abundance in sewage sludge and sludge-amended soils, studies on fungal incidence in these environments are of hygienic, epidemiological and ecological significance. Recent reports [14, 15] have demonstrated that many environmental factors such as pH, temperature, ammonium nitrogen, proteolytic activity, organic carbon and total nitrogen, C:N ratio, total sulfur, C:S ratio and available phosphorus affected keratinolytic and keratinophilic fungi in sewage sludge. Additionally, the liming process considerably affected the composition of the fungi in the sludge environment [13]. It was suggested that particle size distribution also influenced fungal composition in sewage sludge. Therefore, the study aimed to determine the composition of keratinolytic and keratinophilic fungi in sludge particle size fractions in a model experiment.

MATERIALS AND METHODS

Sewage sludge from the Bytom-Miechowice wastewater treatment plant was used in the experiment. This plant treats part of the municipal wastewater from the town of Bytom. It was the excess sludge, after extended aeration (without primary settling tank) and the integrated biological C and N removal process, dewatered by centrifuging, and piled with plant residues for 1–2 years. Stabilized organic matter characterized the sludge. About 20 kg of dried and crumbled sludge was divided into seven particle size fractions: > 1, 1–0.5, 0.5–0.25, 0.25–0.125, 0.125–0.063, 0.063–0.032 and < 0.032 mm, using a high-power fractionation

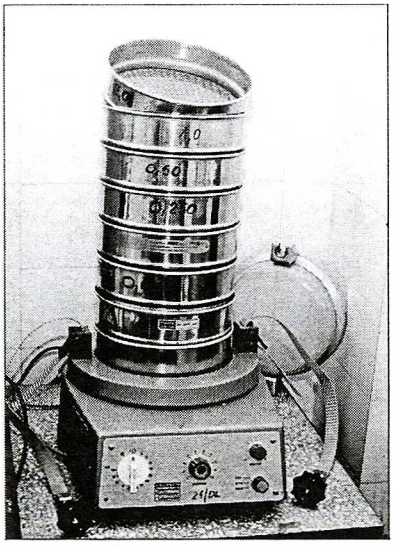

Fig. 1. A column shaker used for fractionation of open-air dried sewage sludge sample

column shaker (Fig. 1). The fractionation took about eight hours. The fractions were collected in sterile plastic containers, then thoroughly mixed, stored at 4°C for 12 hours and used for analyses and experiments.

In the original sludge sample and its fractions, the composition of keratinolytic and keratinophilic fungi was determined using the hair baiting method [17]. Small Petri dishes (5-cm diameter) were filled with 5 g of sludge or its fraction and covered with 0.15 g of detergent-defatted, fine cut, and autoclaved child hair each. The dishes were incubated in the dark at 23°C for four months. Ten dishes responded to the original sludge sample and each fraction. During incubation, stable moisture conditions (ca. 40%) by adding sterilized redistilled water were maintained in the samples. The fungal growth indices were as follows: number of isolates; isolation frequency (number of Petri dishes positive for fungal growth*100/total number of Petri dishes set up); and the number of species.

The qualitative and quantitative composition of actidione-resistant fungi was also determined in the original sludge sample and its fractions. Dilutions in autoclaced physiological saline and the Wiegand medium [8] supplemented with chloramphenicol (100 mg/dm^3) and actidione (500 mg/dm^3), with 10-day incubation in the dark at 23 and 37°C were used. Pure fungal isolates were identified to the species level. The geophilic dermatophytes were identified using the monograph by de Hoog *et al.* [4], while in identification of the fungi from the genera *Chrysosporium, Aphanoascus, Malbranchea* and allied genera the papers by van Oorschot [7], Cano and Guarro [1] and Sigler and Carmichael [11] were used. Subsequently, the monograph by Raper and Fennel [9] was used for identification of *Aspergillus* strains. Finally, the monograph on soil fungi by Domsch *et al.* [3] was used for identification of other fungi. The *in vitro* hair degradation test was that of Ulfig *et al.* [16]. The test relies on the incubation of fungal strains for one month on Sabouraud 1:10 + mineral salts [12] with sterilized child hair laid over its surface.

Particle size fraction distribution, pH in H_2O, pH in 1 M KCl, total nitrogen, organic carbon, C:N, total sulfur, C:S, heavy metals (Fe, Mn, Zn, Cd, Pb, Cu, Cr, Ni, and Hg) and total bacterial (on SMA) and fungal (on MEA supplemented with chloramphenicol in the quantity of 100 mg/dm^3) numbers also were determined in the original sludge sample and its fractions. The methods for determination of sludge physico-chemical parameters were described in a previous paper [14].

RESULTS

Trichophyton terrestre with its teleomorph *Arthroderma quadrifidum*, *Microsporum gypseum* and *Amauroascus mutatus* prevailed in the original sludge sample (Tab. 1). The first two fungi occurred in all fractions. In the fraction < 0.032 mm, *M. gypseum* grew well in ten dishes, whereas *T. terrestre* with its teleomorph *A. quadrifidum* was observed only in three dishes. The growth of the latter fungus was weak and the number of ascomata was low. Most isolates of *A. mutatus* were isolated from fractions >1 and 0.125–0.063 mm. The remaining keratinolytic fungi were sporadically isolated from the original sludge sample and its fractions. The number of keratinolytic fungi isolates was distinctly higher in the original sludge sample than in its fractions (Tab. 2). In the case of keratinophilic fungi, the number of isolates and the number of species were higher in the original sludge sample than in its fractions. No more significant differences between fungal compositions were observed.

Table 1. The number of keratinophilic and keratinophilic fungi isolates in the original sludge sample and its fractions

Fungal species	Number of fungal isolates							
	Original sludge sample	Fractions						
		> 1 mm	1–0.5 mm	0.5–0.25 mm	0.25–0.125 mm	0.125–0.063 mm	0.063–0.032 mm	< 0.032 mm
Keratinolytic fungi								
Trichophyton terrestre Durie & Frey	10	10	5	8	9	10	9	3
Teleomorph *Arthroderma quadrifidum* Dawson & Gentles	10	10	6	8	9	10	9	3
Microsporum gypseum (Bodin) Guiart & Grigorakis	9	4	8	8	7	7	6	10
Amauroascus mutatus (Quelet) Rammeloo	8	3	0	0	0	1	0	0
Trichophyton ajelloi (Vanbreuseghem) Ajello	1	1	0	1	0	0	0	0
Chrysosporium indicum (Randhawa & Sandhu) Garg	0	0	0	0	2	0	0	0
Tel. *Aphanoascus terreus* (Randhawa & Sandhu) Apinis	0	0	0	0	2	0	0	0
Chrysosporium keratinophilum D.Frey ex Carmichael	0	0	0	0	1	0	0	0
Keratinophilic fungi								
Verticillium lecani (Zimm.) Viegas	10	9	9	8	10	7	9	10
Paecilomyces lilacinus (Thom) Samson	6	3	2	4	1	3	5	4
Pseudallescheria boydii (Shear) McGinnis et al.	2	1	0	1	0	0	1	0
Fusarium oxysporum Schlecht.	2	1	0	0	1	0	0	0
Aspergillus fumigatus Fresenius	1	0	0	0	0	0	1	0
Aspergillus alutaceus Berk. & Curt.	1	0	0	0	0	1	0	0
Mycelia sterilia (white)	1	0	0	0	1	0	0	0
Penicillium janthinellum Biourge	0	0	0	0	1	1	0	0
Trichoderma koningii Oudem.	1	0	0	0	0	0	0	0

The highest bacterial numbers were observed in fractions 0.5–0.25 and 0.063–0.032 mm, whereas the highest fungal numbers were found in fractions 0.063–0.032 and <0.032 mm (Tab. 3). The number of actidione-resistant fungi at both 23 and 37°C was higher in fractions 0.125–0.063, 0.063–0.032 and <0.032 mm than in larger fractions.

Table 2. The keratinophilic and keratinophilic fungi growth indices in the original sludge sample and its fractions

Fungal growth indices	Original sludge sample	Fractions						
		> 1 mm	1–0.5 mm	0.5–0.25 mm	0.25–0.125 mm	0.125–0.063 mm	0.063–0.032 mm	< 0.032 mm
Keratinolytic fungi								
Number of isolates	38	28	19	25	30	28	24	16
Frequency of isolation (%)	100	100	90	90	90	100	100	100
Number of species	4	4	2	3	4	3	2	2
Keratinophilic fungi								
Number of isolates	24	14	11	13	14	12	16	14
Frequency of isolation (%)	100	100	100	100	100	90	100	100
Number of species	8	4	2	3	5	4	3	2

Table 3. Microbiological characteristics of the original sludge sample and its fractions

Microbiological parameters	Original sludge sample	Fractions						
		> 1 mm	1–0.5 mm	0.5–0.25 mm	0.25–0.125 mm	0.125–0.063 mm	0.063–0.032 mm	< 0.032 mm
Total bacterial number*	5.7×10^6	8.9×10^6	1×10^7	2.7×10^7	2.9×10^6	6.2×10^6	2.5×10^7	2.8×10^6
Total fungal number	3×10^5	4.1×10^5	2.5×10^5	2.6×10^5	4×10^5	7.5×10^5	2.7×10^6	1.9×10^6
Number of actidione - resistant fungi at 23°C	3.1×10^4	5×10^4	5.4×10^4	5.9×10^4	9.6×10^4	9.4×10^4	1.2×10^5	1.9×10^5
Number of actidione-resistant fungi at 37°C	2.3×10^3	1.5×10^3	3.5×10^3	1.8×10^3	3.3×10^3	1.1×10^4	1.8×10^4	1.4×10^4

* – all numbers in CFU/g d.w.; CFU – colony forming units

From the actidione-resistant medium by the dilution method, ten fungal species were identified in the original sludge sample (Tab. 4). The number of species in the fractions ranged between 13–17. The highest number of species (17) was identified in the fraction 1–0.5 mm. *Arthrographis alba*, *T. terrestre*, *Phialemonium dimorphosporum*, *Paecilomyces lilacinus*, *Verticillium lecani* and *Penicillium nigricans* prevailed in the original sludge sample. These species also occurred abundantly in all fractions. However, the more diverse composition of actidione-resistant fungi characterized the fractions. The keratinolytic species identified were as follows: *T. terrestre*, *Aphanoascus reticulisporus*, *Sporothrix schenckii*, *Trichophyton ajelloi* and *Chrysosporium* anamorph of *Aphanoascus clathratus*. The highest number of *T. terrestre* propagules was observed in fractions 0.125–0.063, 0.063–0.032 and < 0.032 mm (range 2000–2360 CFU/g d.w.). The remaining species occurred infrequently in the samples examined.

Table 4. The number of actidione-resistant fungi in the original sludge sample and its fraction

Fungal species	Number of fungal propagules (CFU/g d.w.)							
	Original sludge sample	Fractions						
		> 1 mm	1–0.5 mm	0.5–0.25 mm	0.25–0.125mm	0.125–0.063mm	0.063–0.032mm	< 0.032 mm
Arthrographis alba Gené. Ulfig & Guarro	$2.2x10^3$	$2.6 x 10^3$	$3.5x10^3$	$2.7x10^3$	$3.1x10^3$	$2.7x10^3$	$4.5x10^3$	$6.4x10^3$
Trichophyton terrestre Durie & Frey*	$8.5x10^2$	$3x10^2$	$5.5x10^2$	$3.8x10^2$	$4.1x10^2$	$2.3x10^3$	$2.0x10^3$	$2.4x10^3$
Phialemonium dimorphosporum W.Gams & W.B.Cooke	$1x10^2$	$1.2 x 10^2$	$2.1x10^2$	$1.1x10^3$	$1.7x10^3$	$1.3x10^3$	$4.2x10^2$	$8.8x10^2$
Paecilomyces lilacinus (Thom) Samson	$1.7x10^2$	$5.3x 10^1$	$1.7x10^2$	$2x10^2$	$1.6x10^2$	$8.0x10^2$	$2.5x10^3$	$1.2x10^3$
Verticillium lecani (Zimm.) Viegas	$2.8x10^2$	$5.3 x 10^1$	$1.1x10^2$	$8.4x10^2$	$4.2x10^2$	$2.2x10^2$	$1.2x10^3$	$1.2x10^3$
Penicillium nigricans Bain. ex Thom	$1.2x10^2$	$2.5 x 10^2$	$2x10^2$	$2.2x10^2$	$1.4x10^2$	$5.5x10^2$	$1.7x10^3$	$6.5x10^2$
Candida spp.	0	0	0	0	$1.3x10^3$	$7.5x10^2$	0	0
Penicillium verrucosum v. *cyclopium* (West.) Samson. Stolk & Hadlok	0	0	0	$1.3x10^2$	$1.3x10^2$	$7.5x10^2$	0	$2.5x10^2$
Sporothrix sp.	0	$4.6 x 10^2$	$5x10^1$	$3.8x10^2$	0	0	0	$2.5x10^2$
Scopulariopsis candida (Guéguen) Vuill.	$5.8x10^1$	$8x10^0$	$5x10^0$	$1.3x10^1$	$5.7x10^1$	0	$5.5x10^2$	$9.0x10^1$
Geomyces v. *pannorum* (Link) Sigler & Carmichael	0	0	$1x10^2$	0	0	$3.8x10^2$	0	0
Mycelia sterilia (white)	$6x10^0$	$2x10^0$	$1.1x10^2$	$1.1x10^2$	$2.6x10^1$	$4.3x10^1$	$1.5x10^1$	$6.7x10^1$
Aspergillus versicolor (Vuill.) Tiraboschi	0	0	$1.0x10^2$	0	0	$2.5x10^2$	0	0
Nectria inventa Pethybr.	0	0	0	0	0	0	0	$2.5x10^2$
Aspergillus fumigatus Fresenius	$2x10^0$	$3x10^0$	$3x10^0$	$4.3x10^1$	$3x10^0$	0	$3.3x10^1$	$1.4x10^2$
Aphanoascus reticulisporus (Routien) Hubálek*	0	$2x10^1$	$3.4x10^1$	$2.3x10^1$	$2x10^1$	$5x10^1$	$2.5x10^1$	$3.3x10^1$
Arthrographis kalrai (Tewari & Macpher.) Sigler & Carmichael*	0	0	0	0	0	$5x10^1$	$1.3x10^2$	0
Sporothrix schenckii Hektoen & Perkins*	0	$1.3x 10^2$	0	0	0	0	0	0
Trichophyton ajelloi (Vanbreuseghem) Ajello*	0	0	$1x10^2$	0	0	0	0	0
Pseudallescheria boydii (Shear) McGinnis et al.	$1x10^0$	0	$1.7x10^1$	$5x10^0$	0	0	0	$4.0x10^1$
Chrysosporium anamorph *Aphanoascus clathratus* Cano & Guarro*	0	$3x10^0$	0	0	0	0	$3.3x10^1$	0
Chrysosporium keratinophilum D.Frey ex Carmichael*	0	$1x10^0$	$1x10^0$	0	0	0	$3.3x10^1$	0
Acremonium sp.	0	0	$5x10^0$	0	0	0	0	0
Fusarium oxysporum Schlecht.	0	0	0	0	$5x10^0$	0	0	0

* – keratinolytic species; CFU – colony forming units

In the original sludge sample and in the first four fractions (< 1–0.125 mm), the C:N ratio ranged between 7.4–8.5, while in the last two fractions (0.125–0.032 mm) the C:N range was 9.1–10.7 (characteristics in Table 5). In the original sludge sample and first four fractions, the total sulfur content ranged between 0.52–0.57% d.w. The total sulfur contents for the last two fractions were 1.09 and 1.45% d.w. In the original sludge sample, the C:S ratio was 34.9. In fractions, the C:S ratio ranged between 12 and 42.6. The C:S ratios were distinctly lower in fractions 0.125–0.063, 0.063–0.032 and < 0.032 mm (12–21.2) than in other fractions. Higher concentrations of iron, manganium, zinc and cadmium were detected in the last three fractions. Lead was „accumulated" in the fraction < 0.032-mm. The highest nickel concentration was observed in the fraction >1 mm. The contents of these heavy metals in the original sludge sample and in its fractions were determined using ICP spectrometry (with mineralization in nitrohydrochloric acid in the microwave MDS 2000 system). No significant relationships were found for other microbiological and physico-chemical parameters.

Table 5. Physico-chemical characteristics of the original sludge sample and its fractions

Physico-chemical parameters	Units	Original sludge	Fractions						
			> 1 mm	1–0.5 mm	0.5–0.25 mm	0.25–0.125 mm	0.125–0.063 mm	0.063–0.032 mm	<0.032 mm
Fraction distribution	%	–	40.8	29.3	16.4	8.6	2.8	1.3	0.8
pH in H_2O	–	5.8	6.0	5.8	5.8	5.8	5.7	5.8	6.0
pH in 1 M KCl	–	5.7	5.9	5.7	5.7	5.7	5.6	5.6	5.8
Total nitrogen	%	2.5	2.9	2.7	1.9	2.0	2.3	2.2	1.7
Organic carbon	%	19.9	23.8	22.6	16.3	14.9	20.8	23.8	17.4
C:N	–	7.8	8.2	8.5	8.5	7.5	9.1	10.7	10.0
Total sulfur	%	0.57	0.56	0.53	0.52	0.55	0.98	1.09	1.45
C:S	–	34.9	42.4	42.6	31.3	27.0	21.2	21.8	12.0
Iron	mg/kg d.w.	11093	12800	10393	8485	10018	12967	14177	15130
Manganium	mg/kg d.w.	1013	913	818	761	1053	1698	2123	2545
Zinc	mg/kg d.w.	2247	2109	1843	1478	1726	2178	2094	2432
Cadmium	mg/kg d.w.	8.9	8.9	8.1	6.7	7.7	9.5	9.4	10.4
Lead	mg/kg d.w.	161.6	178.4	162.6	133.2	143.6	174.1	166.3	195.5
Copper	mg/kg d.w.	145.8	143.0	124.7	95.7	107.5	128.6	125.2	136.6
Chromium	mg/kg d.w.	47.8	86.4	45.8	33.1	36.8	46.8	46.3	51.7
Nickel	mg/kg d.w.	28.1	191.6	27.9	25.2	21.7	27.9	29.5	29.9
Mercury	mg/kg d.w.	1.6	1.5	1.4	1.0	1.1	1.1	1.2	1.3

d.w. – dry weight

DISCUSSION

In a screening study [14], *T. terrestre* with its teleomorph *A. quadrifidum* favored sludges with colloidal loam content <15%. No relationships between particle size distribution and *M. gypseum* were found. However, the number of *Pseudallescheria boydii* isolates positively correlated with colloidal loam content. In addition, positive relationships were found between the number of *M. gypseum* and *P. boydii* isolates with total sulfur and

sulfate contents. The lowest C:S ratio (20.5:1) characterized the sludges, in which both the species prevailed. In the present study, the fraction < 0.032 mm had the highest sulfur content and the lowest C:S ratio (12:1). On Wiegand medium, the number of *T. terrestre* propagules was high in this fraction. In spite of such a high „inoculum", the numbers of the fungus isolates and ascomata were low and the growth was weak. Subsequently, *M. gypseum* was not isolated on Wiegand medium. This testified to the low inoculum of the fungus in the original sludge sample and in its fractions (below the detection limit for the dilution method). However, the number of *M. gypseum* isolates was high and the growth on hair was abundant. Thus, it was evidenced that the fraction < 0.032 mm did not favor the growth of *T. terrestre* but provided favorable conditions for growth of *M. gypseum*. The number of *P. boydii* isolates was low. It can be explained with that the experiment was conducted at 23°C and this temperature did not favor the growth of the fungus.

Organic sulfur constitutes most of the sludge total sulfur. Sewage sludge contains high amounts of keratinous substrata, which are carbon, nitrogen and sulfur sources for keratinolytic fungi [5]. Moreover, sulfur is a component of sludge fulvic acids [10]. It can be assumed, therefore, that apart from high organic carbon content [2] both high organic sulfur content and low C:S ratio favored the growth of *Microsporum gypseum* in the fraction < 0.032 mm and in sewage sludge generally.

No influence of heavy metals on fungal populations in sewage sludge was observed in a screening study [14]. In the present study, higher concentrations of some metals were detected in the last three (small) fractions. There is, however, no evidence that these contaminants affected the fungi examined.

Fungal propagule quantities determined by the dilution method were higher in fractions than in the original sludge sample. Also, the number of actidione-resistant fungi at both 23 and 37°C was higher in fractions 0.125–0.063, 0.063–0.032 and < 0.032 mm than in large fractions. The small fractions also showed higher fungal diversity. The highest number of the total fungal population (on MEA) was also observed in small fractions. The phenomena may be explained by the fact that the contributions of the fractions to the original sludge sample were different. The small fractions had much smaller distributions than the large fractions did. The smaller contribution implied higher sludge mass analyzed and higher fungal quantities. In addition, the „cumulation" of the propagules in small fractions due to the sludge fractionation in a column shaker was also possible.

In contrast to fungal quantities determined by the dilution method, the numbers of keratinolytic and keratinophilic fungi isolated by the hair baiting method were lower in fractions than in the original sludge sample. Some qualitative differences between fractions were also found. The qualitative and quantitative composition of keratinolytic and keratinophilic fungi in the fraction > 1 mm mostly resembled the fungal composition in the original sludge sample. It is understandable, since this fraction represented as much as 40.8% of the total sludge mass. The differences in fungal compositions certainly resulted from different selectness of the methods applied. The hair baiting method is more selective in isolation of keratinolytic fungi than the dilution method but also more „sensitive" to environmental factors of the sample under examination. The data suggest that, generally, individual fractions contributed to the total population of keratinolytic and keratinophilic fungi in the sludge. It appears, however, that more nutrient factors associated with the fractions than the propagule quantities (inocula) affected the composition of these fungi in the sludge.

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