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Exposure to airborne fungi, MVOC and mycotoxins in biowaste-handling facilities

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Abstract

Health impacts due to fungi in indoor air can only be estimated reliably, if both fungal propagules and fungal secondary metabolites are qualified and quantified. In the present study, the fungal species composition in a compost facility is compared to the spectrum of microbial metabolites in the air with regard to the physiological properties of different fungal species. A number of relevant fungi was tested for the production of both volatile and non-volatile metabolites on different substrata. The profiles of mycotoxins and microbial volatile organic compounds (MVOC) turned out to be specific for certain species in pure culture. Consequently, the fungi may have different toxicological health impacts, though information on the relevance of microbial volatiles is still limited.

Key words: bioaerosols – fungi – fungal spores – microbial VOC – mycotoxins

Introduction

Airborne fungal contaminants in compost facilities are widely discussed in regard to health hazards for workers. Apart from pathogenicity, the potential impact of bioaerosols on health is being widely discussed from both allergenic and toxicological points of view. Investigations in farm workers showed that an increased exposure to dust contaminated with mycotoxins can lead to hepatocellular carcinoma and mycotoxicoses of lung (Ghio and Roggli, 1995). The presence of mycotoxins in bioaerosols from compost facilities has not been investigated until now. Mycotoxins can be expected to be present in both living and dead spores of fungi or in organic dust. Potential impacts on health can not be estimated reliably, since neither qualities nor quantities of mycotoxins have been determined in airborne dusts and bioaerosols. MVOC are discussed to have effects on human health, such as lethargy, headache, and irritation of the eyes and mucous membranes of the nose and throat (Godish, 1995; Mølhave, 1990; Kjaergaard et al., 1991; Koren et al., 1992; Mølhave, 1993). The production of MVOC by fungi has been taken into account especially from the viewpoint of indoor pollution with microorganisms (Mølhave, 1991; Ström et al., 1990, 1994) but the relevance of fungal metabolites in working environments has not been sufficiently studied. Therefore, the capacities of relevant fungi to produce specific metabolites on different substrates and under varying environmental conditions must be investigated. The present investigations aimed at a differentiated exposure assessment of both airborne fungi and fungal secondary metabolites on working places in a compost facility.

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The different parts of a compost facility, where microbial and chemical exposure was estimated to be on a very high level, were regarded as model system to study relations between fungi and secondary metabolites.

Materials and methods

Sampling, quantification, and identification of airborne fungi

In January, April, July and October during a period of two years (eight sampling periods), sampling of airborne fungi was done in a compost facility using Sartorius MD 8 air sampler equipped with cellulose nitrate membrane filters (0.8 μm pore size, Sartorius). Sampling was done at three locations i. e. the loading area, the compost pile hall, and the storage area at approximately 1.50 m above ground. The sampling sites were located near a band conveyor, on which biowaste was transported to the sorting cabin (loading area), near screw conveyors in the compost pile hall and near a pile row in the storage area in downwind direction. Since the sampling locations were completely or at least partly housed, sampling was not heavily affected by the direction of wind. The air samples (six samples at each location) were plated by dilution in steps up to 10^{-4} on Dichloran Glycerol 18 % agar (DG 18) with 100 ppm chloramphenicol. The detection limits for numbers of cfu per m^3 varied for the different steps of dilution, being 2×10^2 cfu at 10^0; 2×10^3 cfu at 10^{-1}; 2×10^4 cfu at 10^{-2}; 2×10^5 cfu at 10^{-3}; and 2×10^6 cfu at 10^{-4}. Numbers of cfu presented here are mean values of six samples per location (sampling in succession) with 3 parallels each, derived from one step of dilution. Parallels were incubated for 6 days at 22 °C for the quantification and isolation of mesophilic fungi, and 3 days at 37 °C to quantify thermotolerant species. The outgrowing fungi were separately quantified (cfu) according to their colony morphology and exemplary transferred to current identification media. The media for identification were prepared according to Samson et al. (1995). As far as technically possible, an entire differentiation and quantification of each individual species was aimed at. Detailed descriptions of incubation procedures, distinction and scoring of species on the isolation medium (DG 18), and identification are given by Fischer (2000).

Chemical analysis and exposure assessment in situ

VOC and MVOC were sorbed on Tenax GR 60/80 (Supelco) by active sampling using suction pumps (SKC EEx ia IIC T4) and analysed by thermal desorption (Perkin Elmer ATD 400) in combination with GC/MS (Perkin Elmer 4000, Finnigan ITD 800) as described by Fischer et al. (1999a).

Airborne dusts and bioaerosols were sampled using a Gravikon VC 25 dust sampler equipped with a 3 μm cellulose nitrate membrane filter and analysed for secondary metabolites of the fungi using high pressure liquid chromatography with diode array detection (HPLC-DAD). Details on the analysis of samples from pure cultures and native bioaerosols were described by Fischer (1999b, 2000).

Analysis of volatiles and mycotoxins in laboratory experiments

The ability of different fungal species to produce MVOC and mycotoxins was tested in pure cultures. The production of MVOC was tested on yeast extract sucrose agar (YES), compost extract agar (CEA) with different supplements (i. e. yeast extract, sucrose and carboxy-methyl-cellulose in different combinations), and on compost as natural substratum according to Fischer (2000). The spore suspensions for inoculation of compost were supplemented with a solution containing yeast extract and sucrose to support fungal growth initially. To define species-specific profiles, the production of MVOC was tested for two or three strains of each species originating from different locations. Fungal strains, growth conditions, culture, analysis and details on the assay for sampling in pure culture on YES medium were described by Fischer (2000). The analysis of MVOC was done by thermal desorption and GC/MS.

The spectra of non-volatile metabolites in conidial extracts and culture extracts (containing also mycelium and medium) were compared for a limited number of relevant fungi. The production of mycotoxins was investigated on four synthetic media in combination (Czapek yeast autolysate agar (CYA), malt extract agar (MEA), oatmeal agar (OA), and YES) (Fischer et al., 1999b), and on compost extract agar (CEA) supplemented with sucrose, yeast extract, and carboxy-methyl cellulose in different combinations as described by Fischer (2000). The analysis of non-volatile metabolites and mycotoxins was done using HPLC-DAD according to Frisvad and Thrane (1987).

Results

Microbial exposure assessment

Highest numbers for cfu of mesophilic species were found in the loading area, where numbers of cfu/m^3 ranged between 10^4 and 10^7, followed by the compost pile hall (10^5 to 10^6 cfu/m^3) and the storage area (10^3 to 10^4 cfu/m^3). For the thermotolerant species a similar trend was observed during the two years period, but cfu values in the loading area were nearly as high as in the compost pile hall (10^5 to 10^6 cfu/m^3). In general, the seasonal variations found in 1997 were not observed in 1998 due to specific weather conditions.

A more differentiated picture resulted in regard to the species-specific spore counts. Among the mesophilic species a clear preference for the compost pile hall was observed only for *P. variabile* and *P. verruculosum*, whereas other penicillia such as *P. crustosum*, *P. cyclopium*, *P. glabrum* and *P. roqueforti* rather seemed to be characteristic for the loading area (Table 1). Other fungi did not show a clear preference for a certain location, but for some of them a seasonal variation was found. In the loading area, typically thermotolerant species such as *A. candidus*, *A. fumigatus*, *A. nidulans*, *A. niger*, *E. nidulans* and *Rhizopus oligosporus* were found with highest spore counts mainly in spring (Fig. 1A).

Table 1. Species with highest spore counts found either in the loading area or in the compost pile hall at different incubation temperatures.

Loading area, incubation at 22 °C	Loading area, incubation at 37 °C	Compost pile hall, incubation at 22 °C	Compost pile hall, incubation at 37 °C
Absidia corymbifera,	*Aspergillus flavus*	*Aspergillus candidus*	*Aspergillus versicolor*
*Aspergillus fumigatus**	*A. fumigatus*	*A. eburneo cremeus*	*Paecilomyces variotii*
Cladosporium cladosporioides	*A. nidulans (sterile)*	*Paecilomyces variotii**	*P. islandicum*
Cladosporium herbarum	*Rhizopus oligosporus*	*Penicillium fellutanum*	
Doratomyces oligosporus		*P. variabile*	
Eurotium herbariorum		*P. verruculosum*	
Mycelia sterilia			
Penicillium brevicompactum			
P. clavigerum			
P. polonicum			
P. glabrum			
P. italicum			
P. janczewskii			
P. roqueforti			
P. spinulosum			
*Rhizopus oligosporus**			
Trichoderma citrinoviride			

Species listed here were significantly more frequent ($p \leq 0.01$) in the respective part of the facility compared to other locations when averaged over the whole period of the investigation.
* Species marked with an asterisk are themotolerant, but have also been scored at 22 °C.

The only thermotolerant species with a preference for summer was *Paecilomyces variotii* (Fig. 1B, pile hall).

In the compost pile hall species such as *P. brevicompactum*, *P. crustosum*, *P. cyclopium*, *P. fellutanum*, and *P. roqueforti* were most frequent in summer (Fig. 1B) and autumn, whereas highest counts of thermotolerant species were found in winter and autumn. The most striking effect was observed for *A. fumigatus*. *Aspergillus versicolor*, *Paecilomyces variotii*, and *Penicillium islandicum*, showed highest spore counts in Summer of the first year.

In the storage area spore counts were generally low compared to the other sampling sites. In October 1997, January and April 1998 cfu of *E. nidulans* were nearly as high or even higher than those of *A. fumigatus* due to widely deteriorated compost (high compost quality).

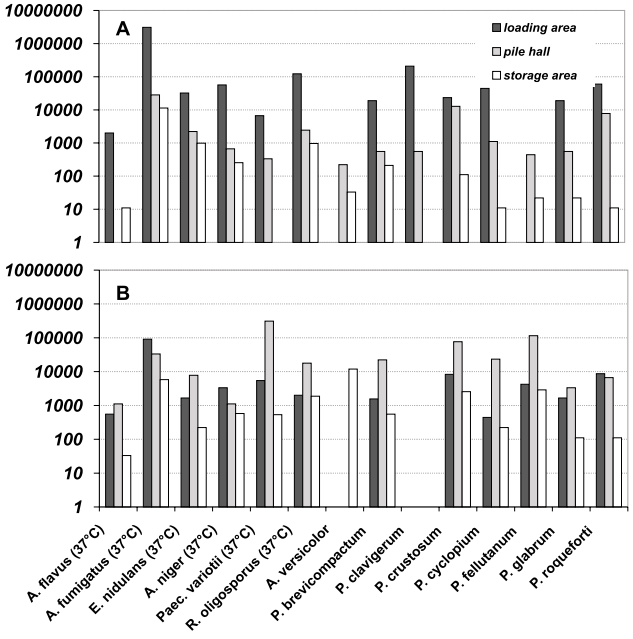

Fig. 1. Colony-forming units (cfu/m^3 air) of the commonest thermotolerant (37 °C) and mesophilic species in spring (A) and summer (B) 1997 in a composting facility with vessel composting technique. Legend: A. = *Aspergillus*, Paec. = *Paecilomyces*, D. = *Doratomyces*, P. = *Penicillium*.

Toxigenic potential of relevant fungi

The potential to produce mycotoxins and non-volatile secondary metabolites was investigated for approximately 250 freshly isolated strains. Among the eleven most relevant species, viz. *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. parasiticus*, *A. versicolor*, *Emericella nidulans*, *Paecilomyces variotii*, *Penicillium brevicompactum*, *P. clavigerum*, *P. crustosum*, and *P. cyclopium*, 37 metabolites partly of toxicological relevance were identified. Several unknown metabolites were found for the less frequent species, which were mainly investigated for chemotaxonomic delimitation from closely related species.

The number of metabolites for one species was somewhat reduced on semi-natural media (CEA) compared to the synthetic medium (YES) (Table 2). Some mycotoxins, such as sterigmatocystin in *Emericella nidulans*, were not present in the conidial extracts,

Table 2. Mycotoxin production of some relevant species investigated within the study.

Species	Some important toxins found in pure cultures	Toxicological relevance	Numbers of cfum^3 in situ	Toxins present in the spores	Toxin produced on CEA
A. flavus	Aflatoxin	carcinogenic †	10^3	–	n. t.
A. fumigatus	Fumitremorgens	tremorgenic †	10^6	+	–
	Gliotoxin	immunotoxic, cytotoxic †		–	–
	Trypacidin**	unknown		+	+
	Tryptoquivaline	tremorgenic †		+	+
	Verruculogen	tremorgenic ‡		+	–
A. giganteus	Patulin	nephro-, hepatotoxic ‡	up to 10^3	+	n. t.
A. niger	Tetracyclines	antibiotic †	up to 10^4	+	+
A. versicolor	Sterigmatocystin	carcinogenic †	up to 10^4	n. t.	n. t.
	Versicolorin	unknown			
E. nidulans	Sterigmatocystin	carcinogenic †	up to 10^4	–	n. t.
Paecilomyces variotii	Viriditoxin	–	up to 10^6	n. t.	n. t.
P. clavigerum	Patulin	nephro-, hepatotoxic ‡	up to 10^7	+	n. t.
	Penitrem A	tremorgenic †		+	
P. crustosum	Penitrem A	tremorgenic†	10^5	+	+
P. polonicum	Verrucofortine**	unknown	10^4	+	+
	Verrucosidine**			+	+
P. islandicum	Islanditoxin*	–	up to 10^4	n. t.	n. t.
P. roqueforti	RoquefortinC	neurotoxic †	10^5	n. t.	n. t.

Only a limited number of species was tested on semi-natural medium (CEA) to reduce the number of samples within this screening. CEA = compost extract medium, n. t. = not tested, * = described in References (Reiß, 1981), not found in the present investigation, ** = toxicological relevance unknown, † = cited in Falbe and Regitz (1997), ‡ = cited in Reiß (1981).

Table 3. Volatiles exclusively produced by two test strains of each species tested (after Fischer et al., 1999a).

Species	Specific volatile compounds
Aspergillus candidus	Hexanoic acid ethyl ester, methoxybenzene, 3-cycloheptene-1-one, 1,3,6-octatriene
A. fumigatus	*p*-Mentha-6,8-diene-2-ol acetate, camphene, *trans*-β-farnesene, α-pinene, three unknown terpenes
A. versicolor	1-(3-Methylphenyl)-ethanone, 6-methyl-2-heptanone
Emericella nidulans	2,3-Dimethyl-butanoic acid methyl ester, 4,4-dimethyl-pentenoic acid methyl ester, 2-methyl-butanoic acid methylester, α-humulene like, α-terpinolene, β-fenchylalcohol, three unknown terpenes
Paecilomyces variotii	δ-4-Carene, megastigma-4,6(E),8(Z)-triene, neo-allo-ocimene, β-phellandrene
Penicillium crustosum	2-Ethylfuran, 2-ethyl-5-methylfuran, isopropylfuran
P. clavigerum	β-Caryophyllene
P. polonicum	2-Methyl-2-bornene, germacrene A
P. expansum	1-Methoxy-3-methylbenzene (3-methyl-anisol), aromadendrene, bicycloelemene

The species mentioned here were among the most frequent fungi in the air of composting plants. Isolates of *Penicillium brevicompactum* and *Penicillium glabrum* are not included since the production of metabolites was inconsistent within the two different strains of the same species.

though produced by most strains. Fumigaclavine C, tryptoquivaline, and trypacidin, characteristic for *A. fumigatus*, were found in conidial extracts, but highly toxic compounds such as gliotoxin and fumitremorgen were not present. Finally, compounds such as cyclopenol, cyclopenin, and penitrem A as characteristic for some penicillia, were found in conidial extracts on semi-natural media and are assumed to occur in native bioaerosols.

Production of volatiles by fungi

A wide range of MVOCs (alkanes, alcohols, ketones, aldehydes, esters, ethers, terpenes and terpene derivatives) was found to be synthesized by the fungi in pure culture. The MVOC profiles resulted in characteristic fingerprints for most species tested. Data on the abundant species were already published by Fischer et al. (1999a). 2-Methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and limonene were found for nearly all species under test. 1-Octen-3-ol was found in four species, i. e. *A. candidus*, *A. versicolor*, *E. nidulans* and *Penicillium brevicompactum*. In addition to these, many volatiles turned out to be species-specific when the fungi were grown on YES agar in pure cultures (Table 3, Table 4). Likewise, spectra of volatiles on CEA and native compost were to some extent species-specific. The spectra of volatiles changed with differing

nutritional conditions, but some compounds were produced on all substrata (Fischer, 2000).

Mycotoxins in native dusts and bioaerosols

In the extract of total dust and bioaerosols from the compost pile hall tryptoquivaline and trypacidin were found (Fig. 2). The two compounds were produced by all strains of *A. fumigatus* investigated in pure culture. Fumitremorgin A and gliotoxin were not detected in the respective sample, though they were found in extracts from pure cultures. The corresponding viable spore counts for *A. fumigatus* in the compost pile hall were approximately 3.2×10^7 per m^3 air resulting in a total number of 3.6×10^9 viable spores collected with the high volume air sampler (Fischer et al., 1999b).

Spectrum of microbial VOC in situ

Among the volatiles occurring at each season and on every sampling site we could detect 2-methylfurane, α-pinene, camphene, and limonene. Especially α-pinene and limonene are likely to derive from plant material in higher amounts, although these compounds were regularly found to be produced by a number of differ-

Table 4. Volatiles characteristic for a small number of species (two or three taxa) (after Fischer et al., 1999a).

Compound	Spesies
Ether:	
Anisol (methoxybenzene)	*Aspergillus candidus, Penicillium olsonii*
1,2-Dimethoxybenzene	*A. versicolor, P. fellutanum*
Hydrocarbons:	
3-Methyl-1-heptene	*A. candidus, P. roqueforti*
Terpenes and derivatives:	
2-Methyl-bornane	*A. niger, P. crustosum, P. polonicum*
2-Methyl-2-bornene	*A. niger, P. polonicum*
γ-Cadinene	*Paecilomyces variotii, P. polonicum, P. fellutanum*
Camphene	*A. fumigatus, P. brevicompactum*
β-Caryophyllene	*P. clavigerum, P. roqueforti*
Elemol	*P. expansum, P. glabrum*
β-Elemol	*P. roqueforti, P. clavigerum*
β-Fenchylalcohol	*Emericella nidulans, P. roqueforti*
α-Phellandrene	*Paec. variotii, P. roqueforti*
α-Pinene	*A. fumigatus, A. niger, P. brevicompactum*
α-Terpinene	*Paec. variotii, P. clavigerum, P. roqueforti*
α-Terpinolene	*E. nidulans, P. brevicompactum, P. roqueforti*
a-Thujone-like	*P. brevicompactum, Trichoderma citrinoviride*

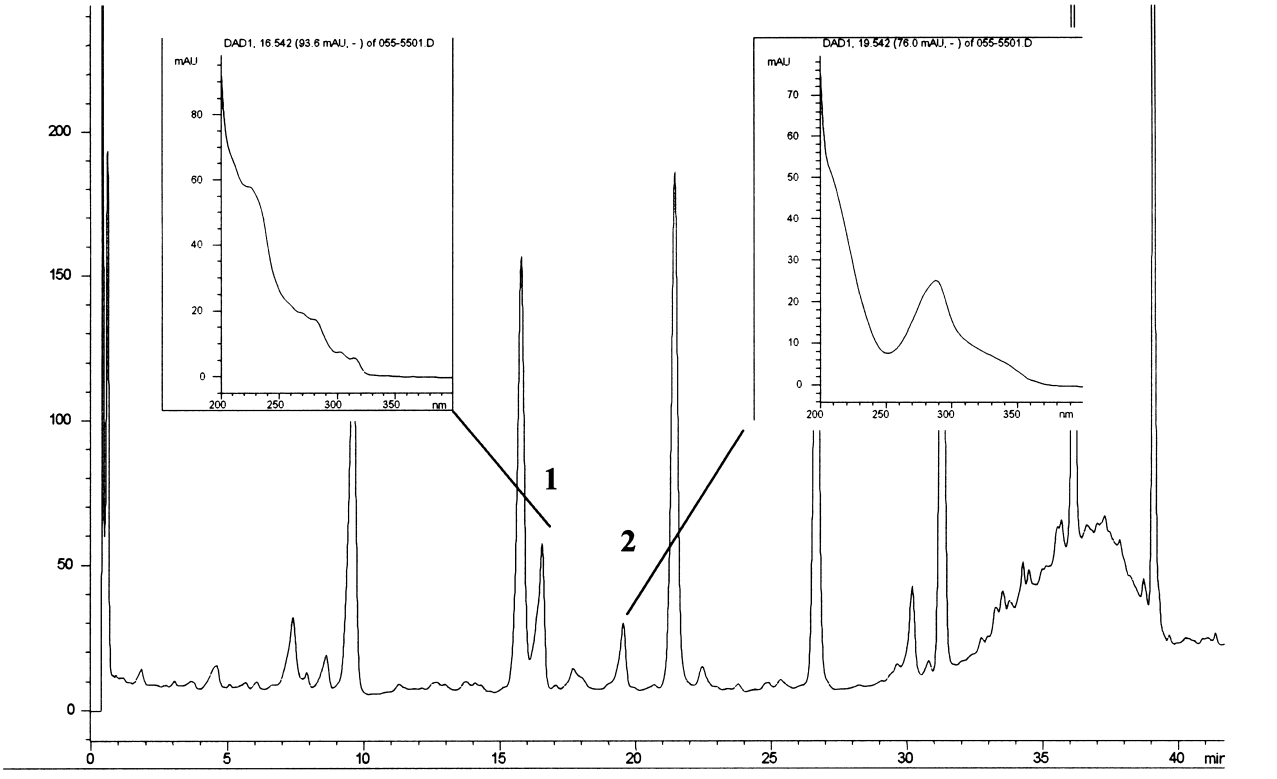

Fig. 2. HPLC-traces of an extract of total dust and bioaerosols sampled in the compost pile hall. Tryptoquivaline [1] and trypacidin [2], for which the UV-vis spectra are given, were characteristic for *A. fumigatus* among a number of relevant species regularly found in compost facilities.

Table 5. Volatiles occurring in the compost plant and possibly deriving from fungi.

Sampling site	Loading area:				Compost pile hall:				Storage area:			
Season Compound	Apr 97	Jul 97	Oct 97	Jan 98	Apr 97	Jul 97	Oct 97	Jan 98	Apr 97	Jul 97	Oct 97	Jan 98
2-Methylfurane	+	+	+	+	+	+	+	+	+	+	+	+
2-Pentanol	+		+	+	+	+		+				+
3-Octanol [1]	+				+	+	+	+	+		+	
2,5-Dimethylfurane					+	+	+	+				
3-Methyl-1-butanol [2]	+	+	+	+	+	+	+	+				
2-Methyl-1-butanol [3]	+	+	+	+	+	+	+	+		+*		
Dimethyldisulfide [4]	+	+	+	+	+	+	+	+	+	+	+	
2-Heptanone												+
α-Pinene	+	+	+	+	+	+	+	+	+	+	+	+
Camphene [5]	+	+	+	+	+	+	+	+	+	+	+	+
3-Octanone[6]	+	+	+		+	+	+	+				+
2-Octanone												+
2-Pentylfurane									+	+	+	+
Limonene [7]	+	+	+	+	+	+	+	+	+	+	+	+
Camphor	+	+	+		+	+	+	+	+	+		+

Some of the terpenes probably derive from plant material. Most compounds were also found in pure cultures.
* = coincides with highest spore counts in the storage area compared to other seasons, [ ] see Fig. 3.

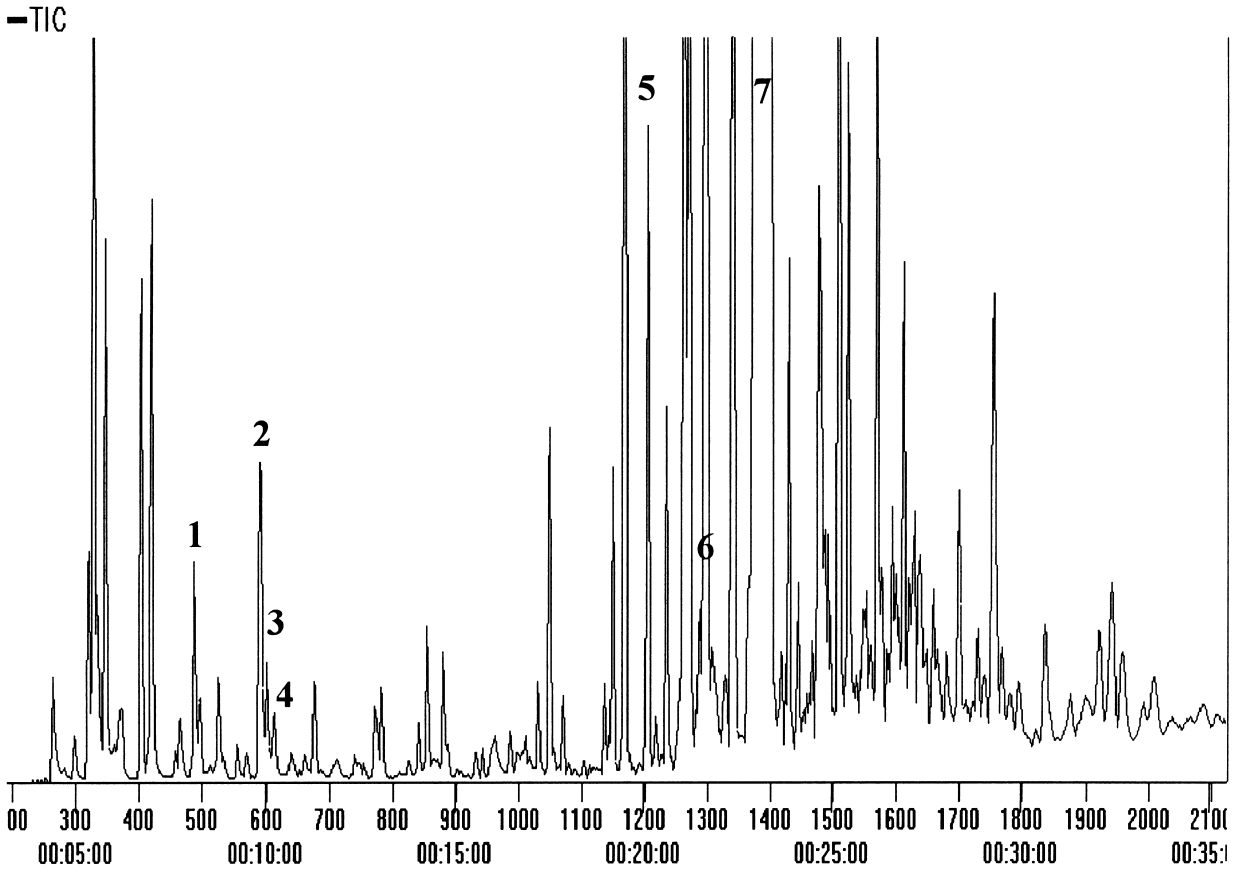

Fig. 3. GC-chromatogram from a sample of the compost pile hall. 3-Octanol [1] was specific for *P. crustosum* among the species tested in the laboratory experiment. 3-Methyl-1-butanol [2] and 2-methyl-1-butanol [3] were regularly found in situ and occurred in several species, whereas 3-octanone [6] could only be found in a limited number of species. Although camphene [5] and limonene [7] were produced by a wide range of species, these compounds are likely to originate to a large extent from plant material. Numbers refer to those given in square brackets in Table 5.

ent fungal species (Fischer et al., 1999a). In the storage area 2-methyl-1-butanol was only once found in July 97 (Table 5), whereas it was found consistently in the loading area and the compost pile hall (Table 5, Fig. 3). The occurrence in the storage area coincides with very high spore counts for a series of fungi during that season. It may therefore be an indication for increased emission of fungal propagules from the storage area.

Among the frequently occurring volatiles, only dimethyldisulfide was found to coincide perfectly with the occurrence of *A. niger*, for which the production was also described in pure culture. However, a number of compounds that was not found in laboratory experiments coincided perfectly with some fungi. Some of these volatiles still have to be identified, others belonged to the group of terpenes and sesquiterpenes (Fischer, 2000). Further research on the production of MVOC on semi-natural and natural substrata will have to elucidate if these derive from plant material or from the fungal metabolism.

Discussion

The results indicated that besides *A. fumigatus* other pathogenic species, i. e. *Paecilomyces variotii* or toxinogenic moulds such as *Penicillium clavigerum*, can become prevalent species depending on the season. Furthermore, the species composition depends on factors such as composition of biowaste, weather conditions and type of process engineering (Fischer et al., 1998; Fischer, 2000). Consequently, a quantitative differentiation of species is needed to estimate health hazards reliably. If species-specific volatiles are to be found on natural substrata, these may serve as marker compounds for the selective detection of fungal species in indoor domestic and working environments. Some rather non-specific MVOC, which have already been described in References, were found at all sampling sites and in each season. The composition of the fungal spectrum and chemical exposure changed throughout the seasons, but relations between individual volatiles and fungi have to be studied further.

Until now, health effects as described for farmers exposed to mycotoxins in corn dust have not been observed in workers of biowaste handling facilities. But effects of long term exposure to mycotoxins have to be studied, since metabolites of *A. fumigatus* were found in bioaerosols when the number of cfu ranged above 10^7 cfu per m^3 air. Mycotoxins of other species can be expected to occur in trace amounts in bioaerosols and dusts in cases of lower spore numbers and, thus, more sensitive techniques for detection of mycotoxins e. g. HPLC-FLD or immunoassays must be used to detect relevant mycotoxins. Moreover, the analysis of non-volatile secondary metabolites can lead to a reliable identification of closely related species, especially in the *Penicillium aurantiogriseum* complex or in synnematious terverticillate penicillia.

Some of the findings are of practical relevance for occupational hygiene or process engineering in composting facilities. The fungal species composition depended on factors such as biowaste composition, weather conditions, intervals of biowaste collection, and type of process engineering. The multiplication of thermotolerant species, mainly *A. fumigatus*, is accelerated by high ambient temperatures, so that high spore counts already occurred before the intensive phase of biodegradation. High temperatures in summer in combination with a bi-weekly collection of biowaste favoured the development of *Paecilomyces variotii* during the main phase of decomposition. Thus, apart from *A. fumigatus* the pathogenic *Paecilomyces variotii* must also be regarded as an indicator of composting processes. A quantitative differentiation of individual species is considered crucial to estimate health hazards reliably, since the species composition may change throughout the year. Changed species spectra were also observed during the different phases of composting. Even in plants using different composting techniques the species spectra varied, since the degree of decomposition depends on the type of process engineering. Consequently, the presence of spores of certain species can be indicative of the degree of decomposition. Along with this observation, the hygienic quality of the compost may be determined.

An inventory of microbial metabolites in addition to fungal propagules has led to a more detailed identification of potential health hazards at the working place. Mycotoxins were found to occur in native bioaerosols. Thus, in addition to the pathogenic and allergological relevance, airborne fungi are of toxicological concern. However, for the mycotoxins found in bioaerosols an estimation of the toxicity is difficult because no toxicological data on the compounds are available.

Correlations between single volatiles and certain fungi were found, but these findings did not match with the species-specific volatiles obtained from pure cultures on YES (Fischer, 2000). A number of non-specific MVOC was found at all sampling sites or in each season, some of which showed preferences for certain sampling sites. They seemed to be correlated with a certain species composition or with microbial activity, and may thus be used to describe a certain state of decomposition. The apparently species-specific volatiles produced on compost as natural substrate can only be regarded as specific marker compounds for the detection of these species, when more species are screened for the production of MVOC. Further re-

search should concentrate on quantitative analyses of (M)VOC and more sensitive detection techniques for mycotoxins.

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