Detection of an aflatoxin-like substance in an office building

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ABSTRACT
Some months after a water damage incident in an office building a few employees began suffering from different symptoms. The complaints increased in the following two years. We investigated the office using various methods. Analyses of fungi and bacteria were made with material samples and air measurements. Some mycotoxins were analysed with the ELISA test and endotoxins were analysed with the LAL test in dust. Fungi were detected in different building materials, e.g. *Chaetomium* sp., *Aspergillus* spp. and *Penicillium* spp. Dust samples were investigated with a cytotoxicity test. High cytotoxicity was found. In the sediment dust high concentrations of an aflatoxin-like substance were detected, described as ELISA-aflatoxin. ELISA-aflatoxin could be found in one isolated *Aspergillus* strain. This strain probably belongs to a new species that is similar to *Aspergillus ustus*. It is called *Aspergillus pseudoustus* sp. nov.

INDEX TERMS
Aflatoxin; Cytotoxicity; Mycotoxins; *Aspergillus ustus*; *Chaetomium* sp.

INTRODUCTION
At our indoor investigations in more than a 1000 buildings every year, we experienced that very often there is no correlation between the amount of CFU in the air or dust and the strength of the symptoms. Also, the extent of the microbial damages in floors, walls or ceilings and the spectrum of species detected in the mouldy material do not always correlate with the symptoms. But we observed repeatedly that the health complaints occur or increase continually after water damages if the damages dried slowly without removing the mouldy materials.

Because of this observation we suspected that during slow drying either the microorganisms produce increasing amounts of toxins or that the emission of toxins increases.

One typical case was investigated with different methods: water damage arose in the upper floor of an office building, caused by a hole in the roof. Water entered the building and stood some centimetres high on the floor in all rooms. The hole in the roof was repaired and the water was pumped out. But the floor materials were not dried using technical drying equipment.

As time went by the employees suffered increasingly from different complaints such as respiratory problems, headaches, skin problems and sore throats. Three years after the water damage we were charged with the task of investigating the office rooms to find the reason for the symptoms. Because of the long period of time that had passed between the damage and the investigations we suspected pollution with toxins. Beside the standard methods like cultivating microorganisms in air samples, dust samples and material samples, we analysed endotoxins and some mycotoxins in dust and materials.

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METHODS

First, the office was inspected and humidity was measured in walls, floors and ceilings using the Protimeter Surveymaster SM. Airborne cultivable microbial particles were sampled with the LKS 30 impactor (100 l, 30 l/min) using three different agar media: DG 18 with chloramphenicol, 3% malt extract agar (MEA) with chloramphenicol and casein soy peptone agar (CASO) with cycloheximide. Two different methods were used to take dust samples. Some dust samples were collected with a fibre glass filter, mounted at the tube of a vacuum cleaner, and the other samples were collected in the bag of the vacuum cleaner. Dust was collected from an area of 2 m^2.

To search for hidden microbial damage we used a mould dog (Lorenz and Diederich, 2001).

Cultivable microorganisms in paper were analysed by the direct contact method using DG18, MEA and CASO agar. The mineral wool and dust samples were analysed by the dilution plate method. Parts of the sample were suspended in a buffer solution (peptone with 0.05% Tween 80), then shaken and diluted. From the original suspension up to the fourth dilution step 0.1 ml was plated in duplicate on the three agar media. The plates were incubated at 24 ± 0.5°C and analysed after 3, 6 and 8, respectively, 12 (dust samples) days. Additionally, MEA plates were incubated at 37°C and analysed after 2 and 4 days. Pure cultures of *Aspergillus pseudoustus* sp. nov. were obtained by isolation on malt agar at 24 ± 0.5°C.

Endotoxins were analysed with the LAL test (Limulus-Amoebozyten-Lysat test). Mycotoxins were analysed with the ELISA test (Enzyme-linked Immunosorbent Assay).

For the evaluation of the cytotoxic properties the MTT-cell culture bioassay was used, as described previously (Gareis, 1995). The MTT assay was carried out with swine kidney cells as target cells, which are known to react sensitively towards a wide range of mycotoxins.

RESULTS

At the inspection in one of the office rooms (room no. A), we found visible microbial growth on the wall paper behind the base board. Except for this no visible mould was found. In all rooms no enhanced moisture was measured in walls, ceilings and floors (see Table 1).

Table 1 Wall paper with visible mould, room no. A

Conditions	Species	CFU/20 cm^2
DG 18, 24°C	*Penicillium* spp.	>300[a]
	Cladosporium spp.	>30[a]
	Alternaria sp.	4
MEA, 24°C	*Chaetomium* sp.	>300[a]
	Alternaria sp.	>10[a]
	Cladosporium spp.	>10[a]
MEA, 37°C	*Aspergillus pseudoustus* sp. nov.	100

[a]Not completely countable because of overgrowth.

The airborne cultivable microorganisms were measured in two rooms (nos. A and B) and in the corridor. An outdoor measurement was made as a reference. High quantities of *Penicillium* spp. were found in room no. A. Different indicator organisms for indoor sources (Anonymus, 2001) were found with low concentrations in room nos. A and B and in the corridor, these were *Aspergillus fumigatus*, *Aspergillus restrictus* group, *Chaetomium* sp., *Trichoderma* sp. and *Wallemia sebi*.

Noticeable concentrations of *Chaetomium* sp. were found during dust analyses. The quality and quantity of the other microorganisms found are typical for house dust. The quantity of *Aspergillus versicolor* and *Eurotium* sp. obviously was increased (see Tables 2 and 3).

Table 2 Airborne cultivable microorganisms (CFU/m^3)

Conditions	Microorganism	Corridor	Room no. A	Room no. B	Outdoor air
MEA, 24°C	*Chaetomium* sp.	–	70	10	–
	Cladosporium spp.	–	–	20	40
	Penicillium spp.	50	370	40	–
	Phoma sp.	10	–	10	–
	Trichoderma sp.	10	–	–	–
DG18, 24°C	*Asp. fumigatus*	–	–	10	–
	Aspergillus restrictus group	20	–	–	–
	Botrytis sp.	10	–	–	–
	Cladosporium spp.	10	–	10	60
	Eurotium sp.	–	–	–	10
	Penicillium spp.	20	880	10	10
	Wallemia sebi	10	–	–	–
CASO, 24°C	Unidentified bacteria	250	430	80	380
	Bacillus sp.	–	10	–	–

Table 3 Cultivable microorganisms in dust samples (CFU/g)

Conditions	Species	Room no. C	Room no. D
DG 18, 24°C	*Cladosporium* spp.	13 0000	250 000
	Aspergillus versicolor	25 000	15 000
	Penicillium spp.	10 000	13 000
	Eurotium sp.	15 000	12 000
	Aspergillus ochraceus	–	<1000
	Yeasts	10 000	–
MEA, 24°C[a]	*Chaetomium* sp.	200 000	400 000
	Aspergillus niger	<1000	–
MEA, 37°C	Aspergillus fumigatus	2500	4000
	Aspergillus niger	<1000	1000
	Trichoderma sp.	<1000	<1000

[a]List of fungi without those obtained on DG18.

Dust was collected in four rooms and analysed for endotoxins and three different mycotoxins. Endotoxins were not detected. The concentrations of ochratoxin A and citrinin were less than the reference values of 20 µg/kg for ochratoxin A and 100 µg/kg for citrinin. Using the ELISA test in three dust samples we found high concentrations of aflatoxin. The reference measurements normally contained less than 5 µg/kg aflatoxin, mostly less than 2 µg/kg were detected in house dust. A distinction between the different kinds of aflatoxin cannot be made with the ELISA test and since we are not sure that this test is really specific for aflatoxins, we called the detected substance ELISA-Aflatoxin (see Table 4).

Table 4 Analysis of sediment dust taken with fiber glass filter adapted at a vacuum cleaner

	Endotoxin, EU/g	ELISA-Aflatoxin, µg/kg	Ochratoxin A, µg/kg	Citrinin, µg/kg
Corridor	<1.25	35.0	1.4	43.8
Room no. A	<1.25	n.d.	0.7	16.1
Room no. B	<1.25	43.8	2.6	34.0
Room no. D	<1.25	53.1	3.9	43.4

n.d. = not detected.

Because of the high concentrations of ELISA-Alfatoxin additional dust samples were collected in three rooms using another sampling method. Following this, not endotoxins but

the mycotoxins zearalenon and DON were analysed. The ELISA-Aflatoxin concentrations were now lower than in the samples collected with the fibre glass filters, but they were also high. The same dust samples were also analysed with the Cytotoxicity test. Very high cytoxicity was found in all three samples (see Tables 5 and 6).

Table 5 Sediment dust collected in the bag of a vacuum cleaner

	ELISA-Aflatoxin	Ochratoxin A	Zearalenon	DON	Citrinin
Room no. B	10.2	n.d.	1.3	1.0	32.8
Room no. D	10.5	n.d.	2.5	6.5	11.6
Corridor	7.3	n.d.	5.3	10.5	42.9

Table 6 Cytotoxicity analyses

	Dilution factor	IC_{50} mg/ml[a]	Valuation of toxicity[b]
Room no. B	11	0.98	+++
Room no. D	11	0.98	+++
Corridor	12	0.49	+++

[a]Inhibitory concentration IC_{50} (minimum concentration which reduces the MTT-cleavage activity by 50%).
[b]–, no toxicity; +, low toxicity; ++, medium toxicity; +++, high toxicity.

Based on these results the following question arose: Which species produces the ELISA-Aflatoxin? Because the concentration of ELISA-Aflatoxin in the wall paper with visible mould growth was not high (see Table 7), a search for hidden microbial damage was performed using a mould dog. The dog marked the floor in all office rooms. Different materials taken from the floor were analysed with the Aflatoxin ELISA test. In waxy paper (the layer between upper concrete layer and insulation layer, see Figure 1) the highest concentrations of ELISA-Aflatoxin were found. Therefore, this waxy paper was analysed for cultivable fungi.

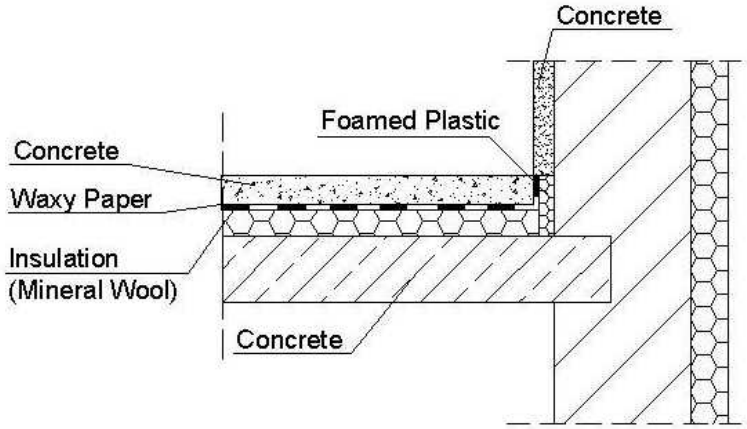

Figure 1 Construction of the floor.

Within the cultivated fungi only an *Aspergillus* strain could be the producer of the ELISA-Aflatoxin. The morphology of this strain was very similar to *Aspergillus ustus*. But further investigations showed that this strain is not *Aspergillus ustus* but a new species. We called it *Aspergillus pseudoustus* sp. nov. (Samson *et al.*, 2003) (see Table 8).

Table 7 Aflatoxin ELISA test of different floor materials and of wall paper

	Styropor®	Mineral insulation	Waxy floor paper	Carpeting	Concrete	PVC	Wall paper
ELISA-Aflatoxin	3.0	3.0	44.7	2.7	2.3	n.d.	4.4

Table 8 Cultivable fungi in the waxy floor paper

Conditions	Species	CFU/20 cm^2
DG 18, 24°C	*Penicillium* spp.	55
	Cladosporium spp.	6
MEA, 24°C	*Penicillium* spp.	23
	Aspergillus pseudoustus sp. nov.	12
	Sterile colonies	3
MEA, 37°C	*Aspergillus pseudoustus* sp. nov.	1

The *Aspergillus pseudoustus* nov. strain was isolated and cultivated on MEA under different conditions. One culture was incubated at 37°C in order to simulate optimum conditions. Another culture was incubated at 10°C for simulation of temperature stress conditions. In one culture salt was added to the MEA for simulating reduced humidity. In another case paraffin was added in order to simulate the conditions on the waxy floor paper. It is known that fungi normally cannot crack paraffin; therefore, it could be a stress factor. To control the influence of pure MEA, three pure plates were analysed as reference samples (see Table 9).

Table 9 ELISA—analyses with the Aflatoxin test under different incubation conditions

Parameter	ELISA-Aflatoxin
Aspergillus pseudoustus nov., MEA, 37°C	3.8
Aspergillus pseudoustus sp. nov., MEA 10°C	5.7
Aspergillus pseudoustus sp. nov., MEA 24°C, + 3 % paraffin	7.5
Aspergillus pseudoustus sp. nov., MEA 24°C + 10 % NaCl - solution	2.1
Pure MEA without microorganisms	0.4–0.6

The highest concentration of ELISA-Aflatoxin was found in the MEA culture with paraffin and the second highest concentration after incubation at 10°C. Low temperature and suboptimum food are probably stress factors and can trigger the production of mycotoxins.

Reference analyses showed that basic concentrations in MEA are less than 1 µg/kg. Therefore, higher amounts denote a clear detection. These analyses were repeated. In not one of the samples more than 1 µg/kg ELISA-Aflatoxin was found. The *Aspergillus pseudoustus* nov. strain had stopped producing ELISA-Aflatoxin completely.

DISCUSSION

There was no chronological correlation between health complaints of the employees in the office and the growing of the microorganisms in the investigated case, but while the material gradually dry. The symptoms may not depend on pollution with cultivable airborne spores, since the quantities are not high enough. Possibly the high concentrations of *Chaetomium* sp. in the sediment dust play an important role.

The complaints were not typically allergic and could be better described as toxic irritation effects. Therefore, the toxins are probably the causative agents and not the spores. We assume that during gradual drying increasing amounts of toxins emit. It could be shown that the house dust is very cytotoxic. In the dust samples high amounts of *Chaetomium* were found, therefore the cytotoxicity can be partly caused by *Chaetomium* toxins, which could not be analysed in this work. But the detected ELISA-Alfatoxin probably will have an important influence on the cytotoxicity of the dust.

The producer of ELISA-Aflatoxin must be *Aspergillus pseudoustus* (sp. nov.). This species produced ELISA-Alfatoxin first in the laboratory, especially under stressing conditions, but lost this ability with prolonged cultivation.

Now there is a new question: What substance is the ELISA-Aflatoxin exactly? For the analysis larger amounts of these substances are needed. Therefore, we now are trying to trigger *Aspergillus pseudoustus* (sp.nov.) to produce this toxin again.

CONCLUSION AND IMPLICATIONS

It was shown that in the indoors we can expect microorganisms which produce very toxic metabolites under stressing conditions. These toxins can significantly contaminate the house dust, even if the source is not located at an open place but hidden, in this case in floor material under the concrete.

The question how the transportation of the toxins from the hidden place into the house dust can take place is very interesting. Surely there will be a chance of air between the insulation layer under the concrete and the room, but probably also animals can transport the toxins. Gareis and Göbel (1998) showed that mites, which use microorganisms as food, can excrete ochratoxin A with their excrements and will not be injured by the mycotoxin. The tests in the laboratory showed that under stressing conditions it is possible to trigger microorganisms to produce toxins. But it was also shown that they may stop the toxin production under laboratory conditions. We must expect higher concentrations and/or other kind of toxins in the normal microbial wildlife than in the laboratory. Investigations on actinomycetes have shown that only fine differences at culturing conditions will lead to a completely different kind of secondary metabolites (Puder, 1998). Why should it not be the same for fungi?

Therefore, quantity and quality of mycotoxins produced by microorganisms in laboratories cannot be the basis for calculating exposition scenarios in mouldy buildings.

For practical application these results show that water damages must be dried completely and quickly before microorganisms can grow up to relevant amounts. But if microorganisms had been grown in materials and had reached high amounts these materials must be removed, even if it is located in hidden places.

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