STORAGE STABILITY OF VIABLE MOULD SPORES AND ENDOTOXIN IN HOUSE DUST SAMPLES

A Koch[1*], KJ Heilemann[2] and W Bischof[1]

[1] Friedrich-Schiller-University Jena, Institute of Occupational, Social and Environmental Medicine, Department of Indoor Climatology Erfurt, Germany
[2] Fachhochschule Erfurt - University of Applied Sciences, Department of Landscape Architecture

ABSTRACT
To assess indoor pollution exposure, due to mould spores and endotoxin, it is often necessary to analyse stored samples. But present knowledge about disintegration of biological agents during storage is insufficient. Dust sample aliquots were distributed randomly over a range of storage temperatures. Mould spore concentrations were analysed over a period of two years and endotoxin over one year. Mould spores: A disintegration of 10 % of the initial concentration of mould spores was found at a storage temperature of 4 °C and a storage time of two weeks. Cladosporium seems to be the storage time limiting component of the house dust samples. Lower storage temperatures have the advantage that the disintegration rates are lower than at 4 °C. Endotoxin: The concentration in house dust is constant over a storage period of one year at room temperature and at –20 °C.

INDEX TERMS
Mould spores, Endotoxin, House dust, Storage time, Temperature

INTRODUCTION
Moulds as well as mites, are the most important producers of indoor allergens. Based on the need for saving energy, increasing tightness of apartments has led to big changes in indoor climate during the last decades, including an increasing frequency of mould problems in apartments. Since more than 40 % of the population world-wide are sensitised to indoor allergens (Schober, 1999), studies on indoor mould exposure and related health effects are nowadays one of the main research goals. The role of endotoxins in house dust is also of interest and associations with respiratory symptoms and the protective effects of early exposure are being studied. In contrast to other indoor related agents, methodology of exposure measurements concerning mould spores and endotoxins are up to now not well defined although activities towards standardisation and/or harmonisation of such determinations have been reported (American conference of governmental industrial hygienists, 1989, Commission of the European Communities, 1993). At present WHO is launching activities within its programme “Health guidelines for biological agents in the indoor environment” (2000). One of these methodological aspects is the impact of storage conditions of house dust samples for further analysis on the exposure estimate. Storage – understood as keeping dust samples in a closed system for the time between sampling and analysis – is one of the main confounders of mould spore exposure assessment. This paper deals with storage aspects of mould spores and endotoxin at different temperatures and times.

* Contact author email: koch@ark.ef.uni-jena.de

METHODS

In each of six homes two dust samples were taken in a highly standardised way by vacuuming, using the same type of vacuum cleaner (Firm: Philips, Hamburg, Type: Flüsterjet Vitall 371, 1000 Watt) equipped with a special attachment (the ALK allergen mouthpiece, ALK, Hørsholm, Denmark) to collect dust on a paper filter. Dust was obtained from the carpet in the living room. For each floor, two areas of approximately 1m² were sampled for 2 minutes each. The dust samples were transported immediately to the laboratory. The 12 samples were mixed, sieved (500 μm), shaken horizontally on a lab shaker for an hour and by hand for 15 minutes. Afterwards, the dust was sieved again.

Dust sample aliquots of 0.03 g for mould spore analyses and 0.2 g for endotoxin analyses were placed in small glass tubes and sealed tightly. These glass tubes were distributed randomly into a series of four storage conditions: room temperature (approximately 20 °C = 68 °F), refrigerator temperature (4 °C = 39.2 °F), -20 °C (= -4 °F) and –80 °C (= -112 °F) for mould analyses and into two conditions (room temperature and –20 °C) for endotoxin analyses. The concentrations of mould spores were analysed 13 times over a period of two years and endotoxin 6 times over a period of one year. The analyses were carried out initially on 50 (mould) or 40 (endotoxin) parallel samples and later with 10 parallel samples of each series.

Mould spores: Dust aliquots were diluted in 3 ml 0.9 % NaCl and plated onto DG18 (dichloran-18 % glycerol agar) with added 0.100 g/l Chloramphenicol to prevent bacterial growth. The plates were incubated at 25 °C for 10 days. All analyses were done in triplicate. The numbers of colony-forming units (cfu) were counted by the naked eye and expressed as cfu/g dust. Colonies were identified to Genera Alternaria, Aspergillus, Cladosporium and Penicillium using high powered light microscopy (Carl Zeiss Jena, Ergaval).

Endotoxin: Endotoxin was extracted under sterile and pyrogen-free conditions from dust samples in 5 ml aqua dist with 0.125 M NH_4HCO_3 + 0.05 % Tween (V/V) for 2 hours at room temperature by continuous shaking. Suspensions were centrifuged at 1,000 g, extracts were stored at –20 °C and analysed the following day. Endotoxin was assayed by the quantitative kinetic chromogenic Limulus Amebocyte Lysate (LAL) method with the KQCL of Bio Whittaker.

Mould spores: Assuming that decay of moulds is exponential, a non-linear model of the form

$$m_{it} = \alpha \cdot \exp(\beta_i \cdot t) + e_{it} \qquad (1)$$

was used to describe the relationship between the average number of mould spores and time, related to storage temperature, where t = storage time in weeks, m_{it} = average number of mould spores at time t for storage temperature i, $i \in \{-80, -20, 4, 20\}$, α = true spore number at the starting time (t = 0), β_i = disintegration rate for storage temperature i, e_{it} = random error at time t for storage temperature i.

Parameters α and β_i, $i \in \{-80\ °C, -20\ °C, 4\ °C, 20\ °C\}$, were estimated by means of weighted least squares using the number of parallel samples as weighting. All computations were performed using the statistical analysis package SAS for Windows version 6.12 (SAS-Institute Inc., 1988).

RESULTS

Mould spores

Composition of the house dust samples

The CFU-number in the samples at time t = 0, as well as the total spore number for each analysed genus, followed a POISSON distribution (χ^2-test of goodness of fit) with the following frequencies shown in Table 1. The composition of the dust sample at this time is shown in Figure 1.

Table 1. Frequencies of the POISSON distribution

	Total mould	*Alternaria*	*Aspergillus*	*Cladosporium*	*Penicillium*
λ	9.88	0.20	0.96	1.66	4.20

Analysis of disintegration

The disintegration rates, which were obtained by non-linear weighted regression analysis with respect to equation (1), are shown in Table 2; the common factor 10^{-4} in ß is factored out.

The probability that the real disintegration rate is outside the bounds of the intervals is 2.5 %.

Table 2. Disintegration rate b ($*10^{-4}$) of the fungus spores with respect to different storage temperatures and their 95 %-confidence intervals.

	-80 °C		***-20 °C***		***4 °C***		***20 °C***	
	β	*95 %-CI*	***B***	*95 %-CI*	***β***	*95 %-CI*	***β***	*95 %-CI*
Total mould	**4**	(2…7)	**4**	(1…6)	**7**	(6…8)	**25**	(18…31)
Alternaria	**2**	(-17…22)	**6**	(-13…24)	**9**	(-9…26)	**35**	(-12…80)
Aspergillus	**-3**	(-8…1)	**-6**	(-14…3)	**4**	(-6…13)	**10**	(-2…22)
Cladosporium	**7**	(-2…15)	**9**	(1…18)	**12**	(4…20)	**90**	(35…145)
Penicillium	**8**	(4…13)	**8**	(3…13)	**11**	(7…16)	**40**	(28…53)

The concentrations of the mould spores of genera Aspergillus and Alternaria were too low for the observed disintegration rate to be statistically significant. For the total spore number, you could determine the disintegration process statistically for storage at 20 °C for 2 months, at 4 °C for 6 months, at -20 °C for 18 months and at -80 °C for 24 months. Inserting into equation (1) the estimated value of the disintegration rate you obtain the following storage times (in weeks) for disintegration of the fungus spores to 90 % of the original spore number. The expectation values (E) and the lower limits of the 95%-confidence intervals (97.5 %) are shown (Table 3). Relating these values to the total spore count it should be noted that at time zero non-identified mould genera represent 28 % (Figure 1).

Table 3. Possible storage times (up to the 10 % disintegration) in weeks; Expectation value (E) and low 95 % -CI-bound (97.5 %)

	-80 °C		***-20 °C***		***4 °C***		***20 °C***	
	E	*97.5 %-CI*	***E***	*97.5 %-CI*	***E***	*97.5 %-CI*	***E***	*97.5 %-CI*
Total mould	**26**	15	**26**	18	**15**	13	**4**	3
Alternaria	**53**	5	**18**	4	**12**	4	**3**	1
Aspergillus	**-**	105	**-**	35	**26**	8	**11**	5
Cladosporium	**15**	7	**12**	6	**9**	5	**1**	1
Penicillium	**13**	8	**13**	8	**10**	7	**3**	2

Analyses of the maximum storage time at room temperature for at most 10 % decays yielded the following expected values (as well as the lower limits of the 95 % -CI): Alternaria 20 (9) days, Aspergillus 75 (34) days, Cladosporium 8 (5) days, Penicillium 18 (14) days, total moulds 30 (22) days. Tolerating a disintegration up to 10 % of the initial number of spores,

storage at 4 °C is possible for two weeks. The analyses pointed out that especially Cladosporium and Penicillium are insufficiently predictable at room temperature over a longer time. Cladosporium seems to be the storage time limited component of the house dust sample. Figure 1 shows the composition of the dust sample after storage for 2 weeks at 4 °C. No statistically significant difference could be proven for the composition compared to time t = 0 (exact Fisher-test.)

If the dust sample has to be stored for a longer time, a disintegration process will become verifiable at each of the examined storage temperatures. Since the disintegration rates of the particular types differ, the composition of the dust sample changes with increasing storage time. For example not only the CFU count of total moulds has declined to 71 % after two years of storage at -20 °C, but also the composition of the fungus differs significantly compared to the initial sample (Figure 1, exact Fisher-Test).

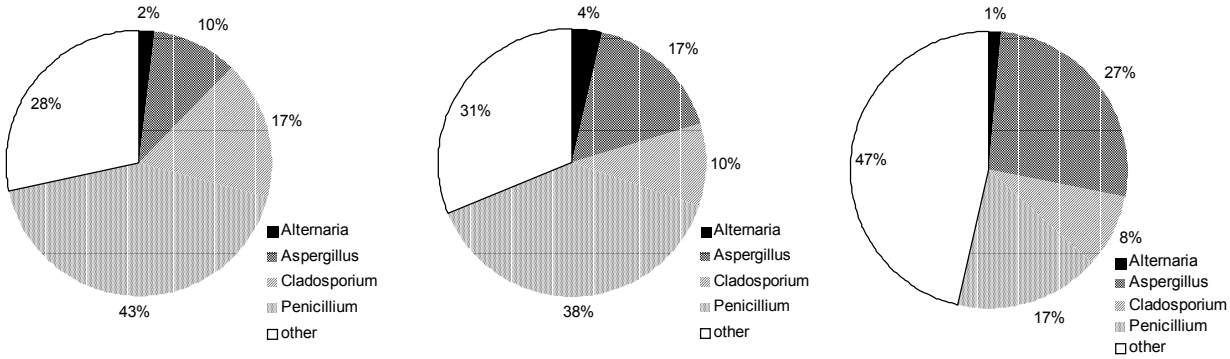

Figure 1. Composition of the dust-sample at t = 0, t = 2 weeks at storage temperature 4°C and t = 2 years at storage temperature –20 °C

Endotoxin:

The concentration of Endotoxin in house dust is constant over a storage time of one year at room temperature and at –20 °C (Figure 2).

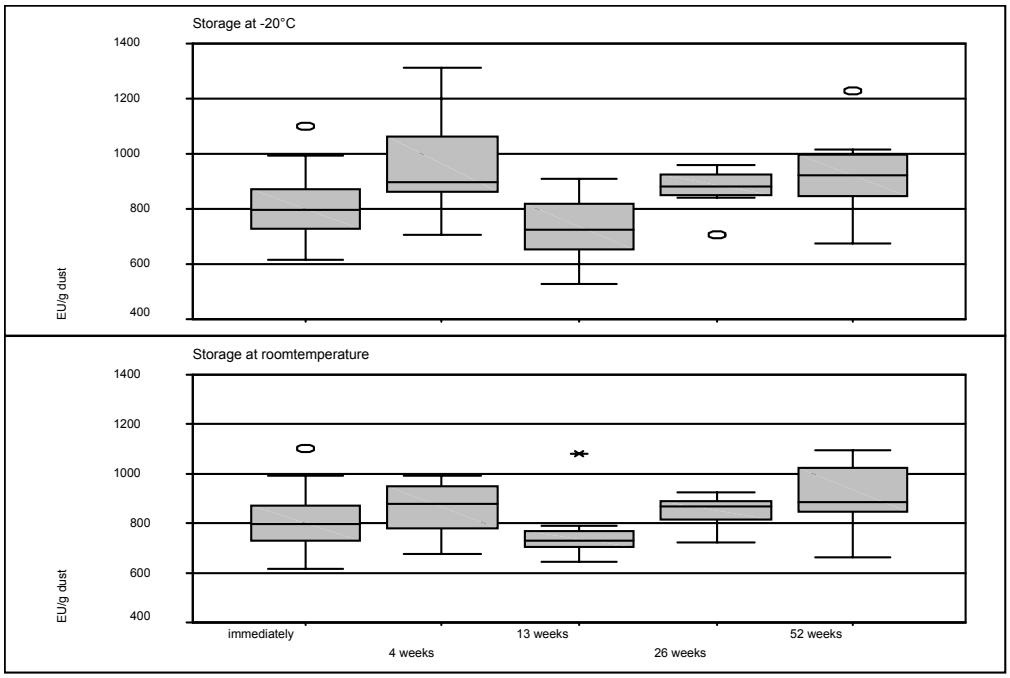

Figure 2. Endotoxin concentration in house dust samples stored at –20 °C and at room temperature respectively over a period of one year.

The concentrations of Endotoxin of the chilled samples and the non-chilled samples do not differ significantly at each time of measurement. There is no advantage of lower storage temperatures over room temperature. Significant differences between the concentrations of Endotoxin at each time of measurement, both at –20°C and at room temperature (homogeneity test of Kruskal-Wallis) are mainly due to high test-variability. In addition even slight changes of ambient temperature during sampling-preparation and analyses seems to influence endotoxin concentrations. Investigations to check this impact are in progress.

DISCUSSION

In the current literature, different preservation technologies for moulds have been described and compared. The aim of these preservation procedures is the conservation of the viability of a genus, generally i.e. new growth should be possible after certain periods. Absolute conservation of all viable parts of the sample for the time of preservation did not stand up to discussion (Fennell, 1960, Onions et al., 1983). Described technologies are periodic transfer, oil-, silicagel-, soil- and sand-cultures, freezing up at different temperatures and with lyophilisation. Fennell (1960) reported that dried cultures survive in the refrigerator longer than at room temperature. If a long-time-preservation is required, lyophilisation will be the method of choice today (Bosmans, 1974, Rybnikár, 1989, 1995). With lyophilisation a genera-specific reduction of detectable viable spores was noticed. In recent reports concerning exposure to indoor mould spores, little information is given about storage conditions of the house dust samples up to the time of analysis. Wickman et al. (1992) as well as Verhoeff et al. (1994) stored house dust samples, before analysis, for 2 weeks in Petri-dishes with Silicagel, in darkness and at room temperature. Koch et al. (2000) also stored the samples at room temperature, and the analyses were carried out within 10 days. Ostrowski et al. (1997) emphasised that dust samples were analysed as soon as possible. Buttner and Stetzenbach 1993 stored the spores in dryness at 4 °C up to their application. These meagre facts on the storage of house dust samples show that the impact of storage was either ignored or has been dealt with empirically and that long storage times were avoided far as possible to prevent a source of error.

Likewise, little information is given about storage temperatures and procedures for endotoxin. Most authors reported storage at -20°C (Douwes, 1995, Richter, 1999, Bischof, 2002) or refrigerated (Milton, 1996) until extraction or they did not make any statement. Unlike epidemiological investigations, chilled transport and – if possible – immediate preparation without storage is stipulated for determination of the concentration of Endotoxin in the context of occupational medicine and occupational safety and health. In exceptional cases transport and storage are allowed for up to one week at 2 - 6 °C and above this period at -20 °C (BIA-Arbeitsmappe, 1997).

CONCLUSION AND IMPLICATIONS

Mould spores: Each storage process is associated with disintegration processes. Storage at normal refrigerator temperatures is sufficient for a storage time up to 14 days. Lower storage temperatures have the advantage that the disintegration rates are lower than at 4 °C. Furthermore it is possible to detect single genera for a longer time and at lower concentrations.

Endotoxin: Valid analyses of endotoxin concentration in house dust samples stored at room temperature for up to one year are not distorted by disintegration.

ACKNOLEDGEMENTS
Supported by a grant from the Bundesministeriums für Bildung, Wissenschaft, Forschung und Technologie (BMBF / FKZ 01 EE 9301/6).

REFERENCES
American conference of governmental industrial hygienists. 1989. *Guidelines for the assessment of bioaerosols in the indoor environment.* Cincinnati, Ohio.
BIA-Arbeitsmappe. 1997. Verfahren zur Bestimmung der Endotoxinkonzentration in der Luft am Arbeitsplatz. 19. LFG. XI/97 *(9450)*.
Bischof W, Koch A, Gehring U, et al. 2002. Predictors of high endotoxin concentrations in the settled dust of German homes. *Indoor Air*, in press.
Bosmans J. 1974. Ten years lyophilisation of pathogenic fungi. *Mycopath Mycol Appl*, Vol 3, pp 13-23.
Buttner MP, and Stetzenbach LD. 1993. Monitoring airborne fungal spores in an experimental indoor environment to evaluate sampling methods and the effects of human activity on air sampling. *Appl Environ Microbiol*, Vol 59, pp 219-226.
Commission of the European Communities. 1993. *Indoor air Quality & its impact on man (COST 613),* Report No.12: Biological particles in indoor environments. EUR 14988 EN, 32 pp.
Douwes J, Versloot P, Hollander A, *et al.* 1995. Influence of various dust sampling and extraction methods on the measurement of airborne endotoxin. *Appl Environ Microbiol* , Vol 5, pp 1763-1769.
Fennell DI. 1997. Conservation of fungous cultures. In *Botanical Review* Vol 26, pp 79-141.
Koch A, Heilemann, KJ, Bischof W, *et al.* 2000. Indoor viable mould spores - a comparison between two cities, Erfurt (estern Germany) and Hamburg (western Germany). *Allergy*, Vol 55, pp 176-180.
Milton DK, Walters MD, Hammond K, *et al* 1996. Worker exposure to endotoxin, phenolic compounds, and Formaldehyde in a fiberglass insulation manufacturing plant. *AIHA Journal*, Vol 57, pp 889-896.
Onions DS, and Onions AH. 1983. A comparison of some preservation techniques for fungi. *Trans Br Mycol Soc*, Vol 81, pp 535-540.
Ostrowski R, Meyer B, Bey M, *et al.* 1997. Occurrence of air-borne and dust-bound moulds in indoor environment. *Poster, Tagung d Gesellsch f Hyg u Umweltmed*, Aachen.
Richter K, Heinrich J, Bischof W, *et al.* 1999. Innenraumfaktoren und Asthma bronchiale in Hamburg und Erfurt als Teil der INGA-Studie. *Allergologie,* Vol 22, pp 14-26.
Rybnikár A. 1989. Ten years storage of the vaccination strain of Trichophyton verrucosum in the lyophilized state. *Acta Vet Brno*, Vol 58, pp 363-367.
Rybnikár A. 1995. Long-term maintenance of lyophilized fungal cultures of the genera Epidermophyton, Microsporum, Paecilomyces and Trichophyton. *Mycoses*, Vol 39, pp 145-147.
SAS-Institute Inc. 1988. *SAS/STAT™ User's Guide,* Release 6.03 Edition, Cry NC SAS Institute, Inc.
Schober G. 1999. Schimmelpilze in Innenräumen. Allergologie, Vol 22, pp 339-345.
Verhoeff AP, van Reenen-Hoekstra ES, Samson RA, *et al.* 1994. Fungal propagules in house dust. I. Comparison of analytic methods and their value as estimators of potential exposure. *Allergy*, Vol 49, pp 533-539.
WHO-Report. 2000. *meeting of the WHO Steering Committee on the health guidelines for biological agents in the indoor environment,* WHO, Espoo, Finland, unpublished.
Wickman MMD, Gravesen S, Nordvall SL, et al. 1992. Indoor viable dust-bound microfungi in relation to residential characteristics, living habits, and symptoms in atopic and control children. *J Aller*gy Clin Immunol, Vol 89, pp 752-759.