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Mycotoxins and other fungal metabolites in grain dust from Norwegian grain elevators and compound feed mills

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RESEARCH ARTICLE

Abstract

Employees at grain elevators and compound feed mills are exposed to large amounts of grain dust during work, frequently leading to airway symptoms and asthma. Although the exposure to grain dust, microorganisms, β -1 \rightarrow 3-glucans and endotoxins has been extensively studied, the focus on the mycotoxin content of grain dust has previously been limited to one or few mycotoxins. Our objective was therefore to screen settled grain dust from grain elevators and compound feed mills for fungal metabolites by LC/MS-MS and explore differences between work places, seasons and climatic zones. Seventy fungal metabolites and two bacterial metabolites were detected. Trichothecenes, depsipeptides, ergot alkaloids, and other metabolites from *Fusarium*, *Claviceps*, *Alternaria*, *Penicillium*, *Aspergillus*, and other fungi were represented. The prevalence of individual metabolites was highly variable, and the concentration of each metabolite varied considerably between samples. The prevalence and concentration of most metabolites were higher in grain elevators compared to compound feed mills. Differences between seasons and climatic zones were inconclusive. All samples contained multiple mycotoxins, indicating a highly complex pattern of possible inhalational exposure. A mean exposure of 20 ng/m³ of fungal metabolites was estimated, whereas a worst case scenario estimated as much as 10 μ g/m³. Although many of these compounds may be linked to toxicological and immunological effects through experimental or epidemiological studies, it still remains to be determined whether the detected concentrations implicate adverse health outcomes when inhaled.

Keywords: inhalation, occupational health, occupational exposure, mycotoxin mixture, settled dust, co-occurrence

1. Introduction

Employees at grain elevators and compound feed mills are exposed to large amounts of grain dust during work, frequently leading to airway symptoms and asthma (Broder *et al.*, 1984). Grain dust is a heterogeneous mixture of inorganic soil particles, plant fragments, insects and mite body parts, viable and nonviable microorganisms, and their bioactive components such as endotoxins, β -1 \rightarrow 3-glucans and mycotoxins (Halstensen *et al.*, 2007, 2013) that all may exert health effects (Smith, 1989). The exposure to viable and non-viable microorganisms and endotoxin has been extensively studied, and both experimental and epidemiological evidence of health effects have been

reported (Health Council of the Netherlands, 2011). However, the significance of fungal metabolites in dust particles on human health is currently unclear.

Mycotoxins are fungal metabolites that may exert immunosuppressive, endocrine, carcinogenic and toxic effects on humans and animals (CAST, 2003). The health risks from ingesting mycotoxin-contaminated agricultural products are widely acknowledged and to a certain extent controlled, but it is unclear whether the inhalation of mycotoxin containing dust during crop handling represents an occupational health risk. Trichothecenes are a diverse group of sesquiterpene mycotoxins that are highly toxic (Creasia *et al.*, 1990; Pang *et al.*, 1988;

Ren *et al.*, 2007; Schiefer and Hancock, 1984), and their inhalation may result in higher toxicity than after dermal or oral exposure (Amuzie *et al.*, 2008; Creasia *et al.*, 1990; Schiefer and Hancock, 1984), presumably because of higher bioavailability (Amuzie *et al.*, 2008; Petzinger and Ziegler, 2000). Epidemiological studies have, furthermore, implicated that adverse health effects including cancer and reproductive outcomes are caused by inhalation of mycotoxins (Autrup *et al.*, 1991; Kristensen *et al.*, 2000; Mclaughlin *et al.*, 1987; Nordby *et al.*, 2006).

The major mycotoxin classes of concern are trichothecenes, aflatoxins, fumonisins, zearalenone (ZEA) and ochratoxin A (OTA), which are produced by three genera of fungi, namely *Fusarium*, *Aspergillus* and *Penicillium* (CAST, 2003). *Fusarium*-related trichothecenes have drawn a lot of attention due to their toxicity and to the fact that their producers are plant pathogens, causing plant diseases reducing grain quality and leading to crop losses in grain cultivation. The most commonly occurring trichothecenes in grain worldwide are deoxynivalenol (DON) and its acetylated derivatives, nivalenol (NIV), T-2 toxin (T-2) and HT-2 toxin (HT-2). The mycotoxins that may occur in grain dust are identical to those that are commonly found in grain, although in higher concentrations (Halstensen *et al.*, 2006a; Kryszynska-Traczyk *et al.*, 2007; Sanders *et al.*, 2013, 2014).

The mycotoxin content of the grain dust will indirectly reflect which fungi that have been active at one or several stages in the grain production chain. Fungal growth and production of mycotoxins are firstly depending on climatic conditions, secondly on crop type and its fungal resistance, and thirdly on the handling and storage conditions of harvested crops. Handling of grain from different sources, grain elevator and compound feed mills technology and operator routines will additionally influence the generation of dust and subsequently the exposure risk of the workers. At least twenty different mycotoxins have been detected in settled and airborne grain dust (Halstensen *et al.*, 2008). Previous studies are mostly limited to one or few mycotoxins, whereas crops and dust often are contaminated with many mycotoxins and other fungal metabolites produced either from the same or from different fungal species. As the combined exposure to multiple mycotoxins may have additive, interaction or synergistic effects (Grenier and Oswald, 2011), the co-occurrence of mycotoxins is of great interest in the assessment of health risks from exposure to grain dust. The exposure to combined mycotoxins has in some cases been shown to exert greater toxicity and carcinogenicity than exposure to single mycotoxins (Bouaziz *et al.*, 2013; Kouadio *et al.*, 2007).

State-of-the-art LC-MS instrumentation allows simultaneous analysis of a large number of different compounds, e.g.

fungal metabolites (Abia *et al.*, 2013; Sulyok *et al.*, 2006, 2010; Vishwanath *et al.*, 2009). In order to obtain an overview over the real contamination of a variety of samples, a targeted multiplex LC-MS assay has been developed over the last decade that allows for simultaneous quantification or semi-quantification of more than 300 different metabolites primarily of fungal origin (Shephard *et al.*, 2013; Uhlig *et al.*, 2013; Vishwanath *et al.*, 2009).

When studying the exposure and health risks of grain handlers it is important to consider the composition of the mycotoxin mixture in the dust and any possible differences between work places. Knowledge of commonly co-occurring metabolites in grain dust can further provide the basis for future toxicological and epidemiological studies on combined effects. The objective of this study was therefore to determine the co-occurrence of more than 300 fungal metabolites in grain dust from Norwegian grain elevators and compound feed mills and explore differences between work places, seasons and climatic zones.

2. Materials and methods

Sampling and sampling sites

Thirty-three samples of settled grain dust (1.5-15 g) were collected from 20 Norwegian grain elevators and compound feed mills in 19 municipalities in 9 counties during winter 2008 (n=9), autumn 2008 (n=15) and winter 2009 (n=9). In the grain elevators, various grain was loaded into the elevator, sorted, winnowed, dried, rotated, moved, stored and unloaded on a continuously basis. Grain moving was either air-driven (suck-and-blow), elevator-driven, or done by passive emptying by gravity. In the compound feed mills, the grain was milled and mixed with other nutrients such as maize, calcium carbonate, fat, vitamins, and amino acids, pressed into feed pellets and filled into sacks or tanks. In autumn a large part of the grain loaded into the grain elevator came from local producers and could include humid batches that needed drying. In winter season the delivered grain had been dried at the farms before delivery, and the amount of imported grain was higher than in autumn. The activity was highest in autumn. Samples of grain dust that had newly settled on the surroundings were gently collected with a spoon and/or by brushing the dust into petri dishes and transferred to tubes after arrival at the lab. Twenty-two of the samples were from grain elevator departments whereas 8 of the samples were from compound feed mill departments. Two of the samples were not possible to categorise in one of the two departments, and one sample was taken nearby the feeding system of a wood chip heating plant of the company. These samples were excluded from comparisons between departments, but were included in all other data analyses. The sampling sites span over three geographically and climatically different districts; central coastal Norway (Nord-Trøndelag, Sør-Trøndelag), south-

eastern Norway (Oslo, Østfold, Vestfold, Telemark) and eastern inland of Norway (Buskerud, Hedmark, Oppland). All districts are located north of 59 degrees northern latitude. The samples were stored at -20 °C until analysis.

Semi-quantitative multi-mycotoxin analysis using LC-MS/MS

Dust samples were weighed and transferred to 50-ml centrifuge tubes (Greiner Bio-One GmbH, Frickenhausen, Germany). The analytical method consisted of three steps: extraction using a mixture of acetonitrile:water:acetic acid (79:20:1, v/v/v), dilution using a mixture of acetonitrile:water:acetic acid (20:79:1, v/v/v) and direct analysis using a QTRAP 5500 LC-MS/MS System (AB SCIEX, Foster City, CA, USA) equipped with a Turbo Ion Spray electrospray ionisation (ESI) source and a 1290 Series HPLC System (Agilent Technologies, Inc., Santa Clara, CA, USA). The weight of individual dust samples varied between 0.5 and 5.0 g. The matrix-to-solvent ratio was 1:4, i.e. a 0.5 g dust sample was extracted with 2 ml and a 5 g dust sample was extracted with 20 ml of extraction solvent. Details on the analysis method can be inferred from the literature (Sulyok *et al.*, 2010; Taubel *et al.*, 2011; Vishwanath *et al.*, 2009). The validation and thus the determination of the limits of detection/quantification (LOD/LOQ) for grain dust samples were far beyond the scope of our study. However, the method's LOD for mycotoxins in maize has been determined to be in the lower µg/kg or ng/kg-range (Malachova *et al.*, 2014). For example, the LOD for aflatoxin B₁ in maize has been determined to 0.6 µg/kg, that of ochratoxin A to 0.7 µg/kg and that of DON to 5.4 µg/kg. It is reasonable to anticipate that the LOD's for individual fungal metabolites in grain dust are comparable.

Data analysis

Non-parametric statistics were applied to the data set due to the skewed distribution of metabolite concentrations. Percentages of positive samples (=prevalence), median of the positive samples and maximum values were calculated for each metabolite. Prevalence differences between groups were tested with Pearson's Chi-square test. Subsequently, the positive samples were selected and differences in the metabolite concentration between groups were tested by the Mann-Whitney U test (two groups) or Kruskal Wallis t-test (more than two groups). Differences with *P*-level ≤0.05 were regarded significant. Exposure estimates were computed for metabolites with prevalence >80%. The concentrations in samples that did not contain quantifiable concentrations of these metabolites were estimated as the lowest observed value divided by the square root of 2 in order to include all samples in the data analyses. The grain workers' mean and worst case inhalational exposure was estimated by multiplying the arithmetic mean and maximum concentration of individual metabolites in the settled dust with the arithmetic mean and maximum

concentration of airborne grain dust. The workers exposure to airborne grain dust was measured at the same time point as the collection of settled grain dust, and is reported elsewhere (Halstensen *et al.*, 2013).

3. Results

Occurrence of fungal metabolites in settled grain dust

Seventy fungal metabolites and two bacterial metabolites were detected in the samples (Table 1). Trichothecenes, depsipeptides, ergot alkaloids, and other metabolites from *Fusarium*, *Claviceps*, *Alternaria*, *Penicillium*, *Aspergillus*, and other fungi were represented. The prevalence of individual metabolites was highly variable, and the concentration of each metabolite varied considerably between samples. Most samples contained both type A and B-trichothecenes, and the concentrations of the type-B trichothecene DON was in the mg/kg range. Fungal depsipeptides were detected in all samples, with particularly high concentrations of Enniatin A₁ (ENN A₁) and B₁ (ENN B₁). The *Fusarium* metabolites ZEA, aurofusarin, avenacein Y, moniliformin (MON), culmorin and equisetin, the *Alternaria* metabolite alternariol methyl ether and the *Penicillium* metabolite mycophenolic acid were also present in all samples. Furthermore, a high proportion of the samples contained fumonisins, which is especially important to note as these fungal polyketides are usually absent in Norwegian grain due to the lack of the producing *Fusarium* species. Also other fungal metabolites, such as apicidin, emodin, monocerin, tryptophol and skyrin were present in nearly all or all samples. The largest quantities of detected metabolites in the grain dust were related to the genus *Fusarium*.

Grain elevator versus compound feed mills

The type A-trichothecene neosolaniol (NEO), several ergot alkaloids, OTA, viomellin and melegarin were detected in samples from grain elevators only (Table 1). The prevalence of the ergot alkaloid chanoclavine, the *Fusarium* metabolite FUM B3, the *Penicillium* and *Aspergillus* metabolites asteric acid, cyclophenol, cyclophenine, 3-methoxy-viricadin, the fungal metabolites calphostin C and skyrin, as well as the bacterial metabolites nonactin and monactin was higher in grain elevators than in compound feed mills. All samples in both grain elevators and compound feed mills contained *Fusarium*-related ENNs and beauvericin (Table 1), but the concentrations of ENNs were significantly higher in the grain elevators compared to the compound feed mills (*P*<0.001 to *P*=0.006; table 1). Similarly, the concentration of avenacein Y, apicidin, curvularin and emodin was highest in grain elevators (*P*=0.003 to 0.02), whereas the concentration of dechlorogriseofulvin and brevianamid F were higher in compound feed mills (*P*=0.005 and *P*<0.001, respectively).

Table 1. Fungal and bacterial metabolites in dust from grain elevators and compound feed mills.

Metabolite	All samples (n=33)			Grain elevators (n=22)			Compound feed mills (n=8)			Significance ²	
	Positive (%)	Median ¹ (µg/kg)	Max (µg/kg)	Positive (%)	Median ¹ (µg/kg)	Max (µg/kg)	Positive (%)	Median ¹ (µg/kg)	Max (µg/kg)	MW	χ ²
Type-A trichothecenes											
T-2 tetraol	82	5.4	30.8	86	5.4	31	63	3.5	6		
T-2 toxin	91	29	187	91	24	186	88	28	96		
HT-2 toxin	97	147	1,283	68	112	1,283	50	148	231		
NEO	3	29	29.0	4	29	29	0	0	0		
Type-B trichothecenes											
Nivalenol	97	32	160.3	94	35	160	100	31	137		
Deoxynivalenol	100	1,057	10,279	100	1,133	10,279	100	413	2,167		
Deoxynivalenol-3-glucoside	100	39	263	100	39	263	100	53	176		
3-acetyl-deoxynivalenol	61	61	317	100	55	317	38	109	121		
Depsideptides											
Enniatin A	100	61	1,227	100	78	418	100	29	59	***	
Enniatin A ₁	100	416	7,279	100	485	3,010	100	205	439	**	
Enniatin B	100	783	2,189	100	919	2,055	100	406	783	***	
Enniatin B ₁	100	934	9,098	100	1,371	4,906	100	496	773	***	
Enniatin B ₂	100	43	419	100	59	238	100	21	43	***	
Enniatin B ₃	100	0.3	0.8	100	0.3	0.8	100	0.2	0.3	**	
Beauvericin	100	32.4	255	100	32	96	100	51	255		
Zearalenone and related compounds											
Zearalenone	100	65	339	100	69	339	100	49	218		
β-zearalenol	39	11	54	27	12	16.5	50	10	54		
Zearalenone-4-sulphate	88	7.4	38.5	91	8	38.5	75	6	11		
Ergot alkaloids											
Chanoclavine	85	0.7	15	94	0.7	15	50	0.6	0.8		**
Agroclavine	6	3.4	5.5	9	3.4	5.5	0	0	0		
Fumigaclavine	9	1.3	2.3	14	1.3	2.3	0	0	0		
Ergometrine	82	2.6	54.6	77	3	55	100	1.8	6		
Ergometrinine	30	9.4	27.1	46	9.4	27	0	0	0		*
Ergocristine	15	9.2	22.5	23	9.2	23	0	0	0		
Ergocristinine	33	3.2	10.5	46	2.6	11	0	0	0		*
Ergosine	42	3.5	10.7	46	4.1	11	25	1.6	2.5		
Various <i>Fusarium</i> metabolites											
Chlamydosporols	45	69	406	50	108	406	50	62	148		
Aurofusarin	100	5,515	44,760	100	7,597	44,760	100	3,311	9,454		
Avenacein Y	100	3,149	10,596	100	4,826	10,596	100	854	6,619	**	
Monoliformin	100	151	2,700	100	181	2,700	100	161	779		
Butenolide	91	68	3,276	91	87	3,276	88	65	550		
Culmorin	100	1,702	10,450	100	1,760	10,450	100	826	3,072		
15-OH-culmorin	82	337	4,606	77	492	4,606	88	268	862		
Equisetin	100	169	954	100	250	954	100	144	547		
Fumonisin B ₁	58	225	2,513	88	209	2,513	88	361	938		
Fumonisin B ₂	61	81	910	50	72	910	75	198	732		
Fumonisin B ₃	24	74	195	14	82	195	50	64	129		*
Various <i>Alternaria</i> metabolites											
Alternariol	91	55	434	91	121	434	88	9.5	229		
Alternariol-OMe	100	6.3	31.4	100	7.6	29	100	6.4	31		
Altertoxin-I	82	14	67.2	91	22	67	63	9.6	32		
Tentoxin	76	3.6	16.2	73	5	16	88	3.4	12		

Table 1. Continued.

Metabolite	All samples (n=33)			Grain elevators (n=22)			Compound feed mills (n=8)			Significance ²	
	Positive (%)	Median ¹ (µg/kg)	Max (µg/kg)	Positive (%)	Median ¹ (µg/kg)	Max (µg/kg)	Positive (%)	Median ¹ (µg/kg)	Max (µg/kg)	MW	χ ²
<i>Penicillium</i> and <i>Aspergillus</i> metabolites											
Sterigmatocystin	56	4	11.4	64	3.9	11.4	38	4.3	4.6		
Mycophenolic acid	100	45	165	100	63	165	100	29	125		
Ochratoxin A	24	9	18.6	37	9	19	0	0	0		*
Dechlorogriseofulvin	64	42	483	100	35	83	75	108	483	**	
Averufin	0	0	0	0	0	0	0	0	0		
Asterric acid	45	19	186	64	20	37	13	19	19		**
Cyclophenol	88	141	3,732	95	182	3,732	63	139	362		*
Cyclophenine	70	6.4	143	82	6.3	143	38	7.6	9.6		*
Viridicatin	45	8	35.5	50	7.6	36	13	7.7	7.7		*
3-OMe-viridicatin	88	4	24.2	100	3.2	24.2	50	4.1	6.3		***
Viomellin	39	167	1,726	45	164	1,726	0	0	0		*
Terphenyllin	42	38	232	50	38	232	25	41	66		
Cyclopeptine	39	3.5	15.9	45	3.8	16	25	2.6	2.9		
Brevianamid F	97	11	149	95	6.8	50	88	65	149	***	
Meleagrins	21	31	56.7	32	31	57	0	0	0		
Other fungal metabolites											
3-nitropropionic acid	79	16	93.6	73	12	77	88	22	94		
Monocerin	97	5	24.6	100	4.8	8.6	88	5.5	25		
Tryptophol	91	45	281	86	34	281	100	79	187		
Rubellin D	55	23	226	54	27	226	50	19	88		
Apicidin	100	48	766	100	61	182	100	39	109		*
Secalonic acid D	42	92	342	50	120	342	38	42	99		
Curvularin A	55	33	186	59	44	186	50	7	25	**	
Cyclosporin C	36	76	1,030	45	76	1,030	12	48	48		
Calphostin C	55	79	452	68	61	452	25	79	85		*
Methylsulochrin	61	1.5	10.3	59	1.4	10.3	75	1.6	3.7		
Emodin	100	84	298	100	94	298	100	73	105	**	
Chrysophanol	67	29	88.6	73	39	89	38	29	45		
Skyrin	94	13	51.4	100	16	51	75	6.7	28		*
Physcion A	91	239	4,531	95	289	4,531	75	193	428		
Bacterial metabolites											
Nonactin	88	1.4	61.7	95	1.5	14.6	63	1.3	2.3		*
Monactin	76	5	56.7	85	5.2	57	50	4.8	10.2		*

¹ Median of positive samples.

² Level of significance of Mann-Whitney U test (MW) or Person Chi square (χ²) between grain elevators and compound feed mills; * P≤0.05; ** P≤0.01; *** P≤0.001.

Season and climatic zones

The prevalence of ergometrin and asterric acid was significantly higher in dust collected in the winter season compared with the autumn season (χ²; P=0.04 and P=0.05, respectively), whereas the prevalence of cyclopeptine was higher in autumn season (P=0.04). NEO was not detected in autumn samples. Significant seasonal differences in fungal metabolite concentrations could not be observed among

the positive samples. However, the concentration of the bacterial metabolite nonactin was significantly higher in autumn (median 1.8 µg/kg, max. 61.7 µg/kg) compared with winter season (median 0.8 µg/kg, max. 3.5 µg/kg) (P=0.02). The distribution across the three different climatic zones was significantly different for twenty of the metabolites (result not shown). However, neither the distribution of depsipeptides nor *Aspergillus* and *Penicillium* metabolites differed between climatic zones.

Co-occurrence of fungal metabolites

There was high degree of co-occurrence of different metabolites in all samples (Figure 1).

Some of the metabolites were strongly correlated within their group. Strong correlations were observed between the detected type A trichothecenes ($r=0.62-0.88$, $P=0.001$ to $P>0.001$), except NEO, which was present in only 3% of the samples and did not correlate with any of the other type A trichothecenes. NEO was not present in compound feed mills. All type B trichothecenes were strongly correlated ($r=0.48-0.78$; $P=0.006$ to $P<0.001$).

The spearman correlation between DON and DON-3-glucoside was 0.78, $P<0.001$ (Figure 2A). Strong correlations were observed between all depsipeptides ($r=0.64-0.98$, $P<0.001$), except beauvericin, which did not correlate with any of the ENNs, although all depsipeptides were present in all samples. The correlation between ZEA and ZEA-4-sulphate was 0.94, $P<0.001$ (Figure 2B). Culmorin correlated strongly with its 15-OH-derivative ($r_s=0.68$, as well as the unrelated aurofusarin ($r_s=0.82$), (Figure 2C). All *Alternaria* metabolites were strongly mutually correlated ($r=0.51-0.86$, $P=0.01$ to $P<0.001$). Particularly strong was the correlation between alternariol-methylether and alternariol, as well as altertoxin-1 and alternariol (Figure 2D).

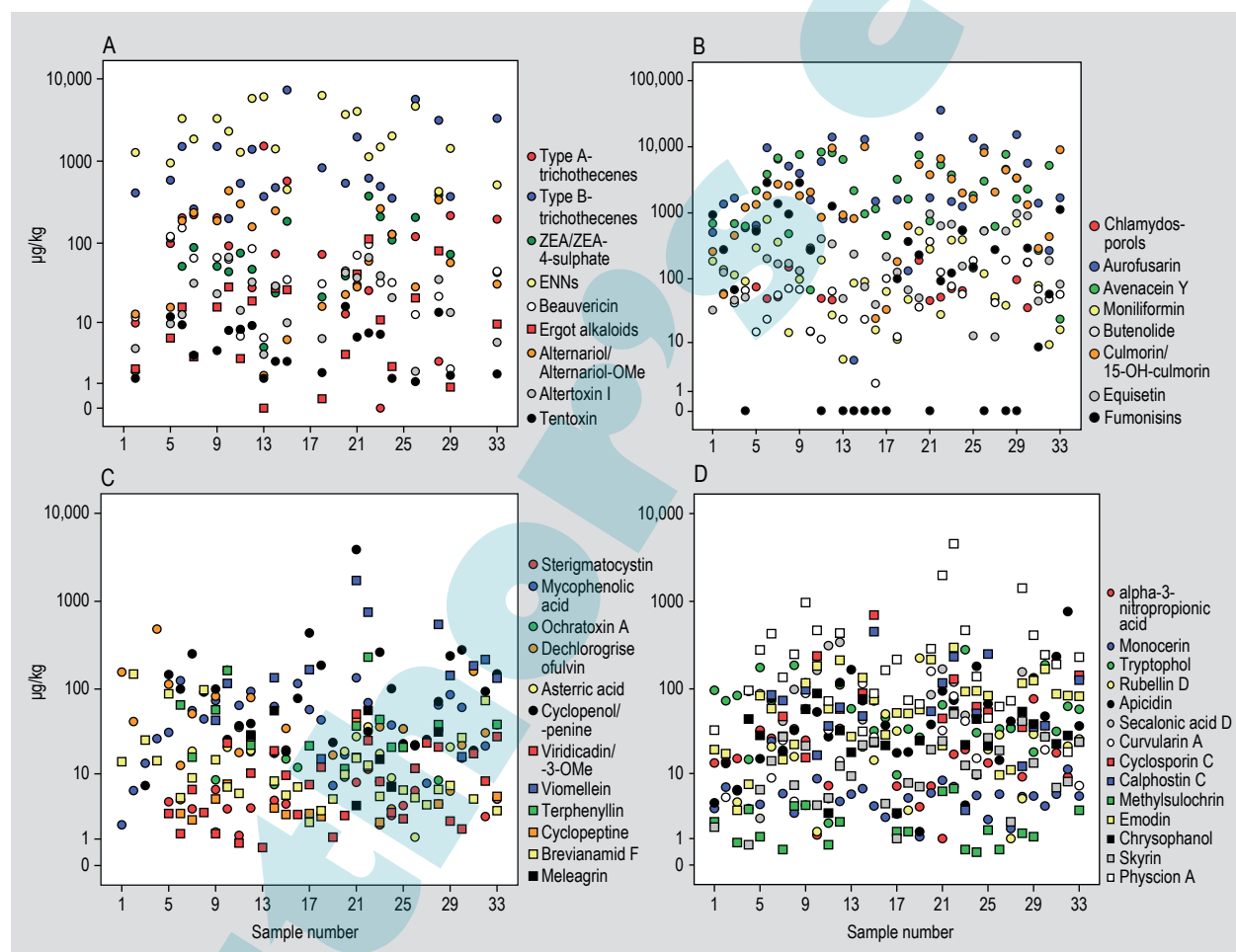


Figure 1. Co-occurrence plots of mycotoxins and fungal metabolites in dust from grain elevators and compound feed mills. Samples 1-8 were collected in compound feed mills, samples 9-30 were collected in grain elevators. Samples 31-32 were not categorised, and sample 33 was collected nearby the feeding system of the wood chip heating plant of one of the companies. Co-occurrence of mycotoxins and fungal metabolites in each sample was plotted according to importance and toxicity; major *Fusarium* mycotoxins, ergot alkaloids, depsipeptides and *Alternaria* metabolites (A), various other *Fusarium* metabolites (B), various *Penicillium* and *Aspergillus* metabolites (C) and various other fungal metabolites (D). Several closely related metabolites were combined to improve readability: In plot A, all type A trichothecenes, all type B trichothecenes, zearalenone (ZEA) and ZEA-4-sulphate, enniatins, ergot alkaloids, alternariol and alternariol-methyl-ether, respectively, were grouped together to make the plots clearer. In plot B, culmorin and 15-hydroxy-culmorin, and all fumonisins, respectively, were grouped together. In plot C, cyclophenol and cyclophenine, viridicadin and viridicadin-3-methyl-ether, respectively, were grouped together.

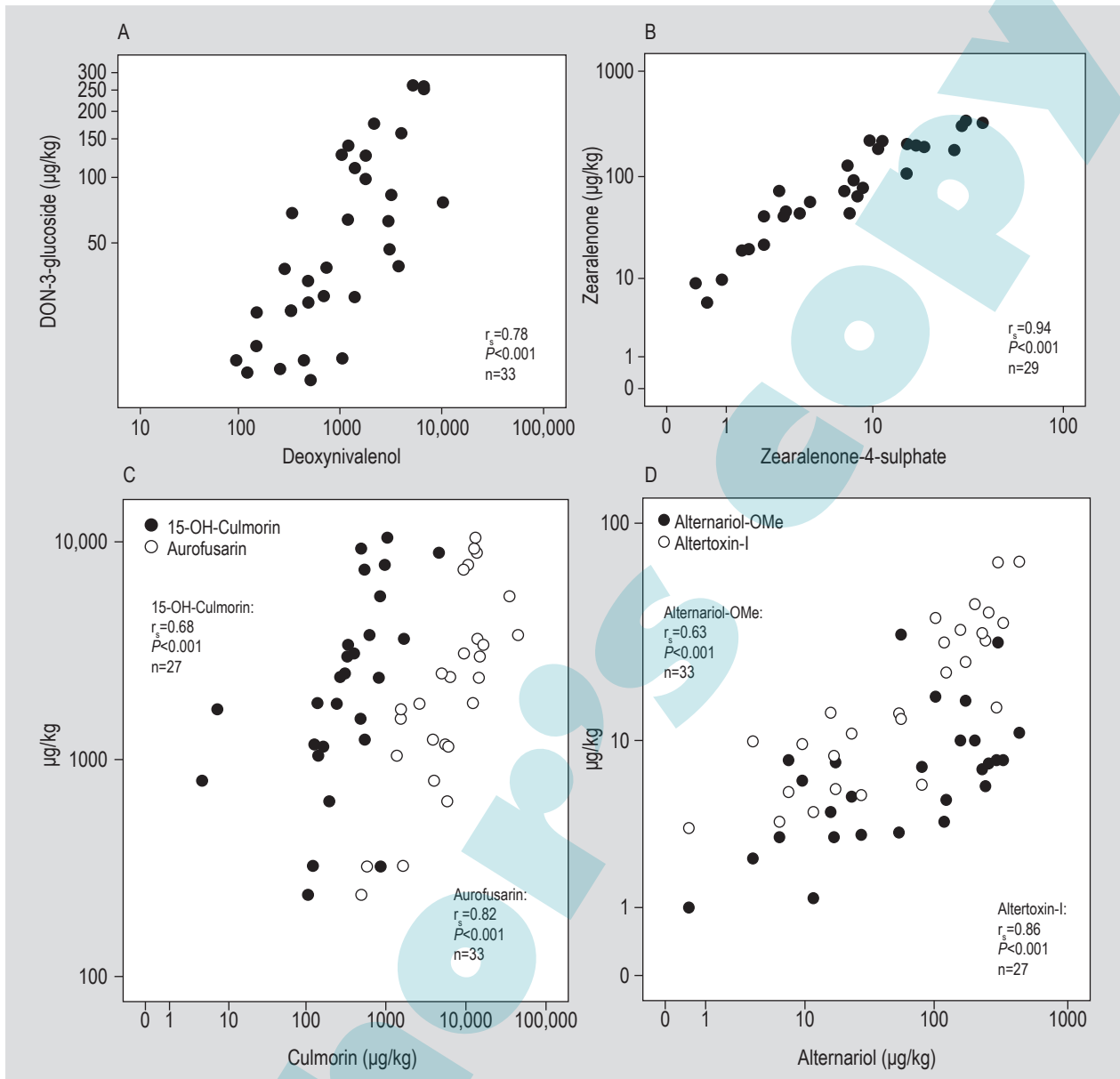


Figure 2. Scatter plots of selected metabolite correlations. (A) Deoxynivalenol (DON) versus DON-3-glycoside; (B) Zearalenone (ZEA) versus ZEA-4-sulphate; (C) culmorin versus 15-hydroxy-culmorin and aurofusarin, respectively; (D) alternariol versus alternariol-methyl ether and altertoxin 1, respectively. The spearman correlation coefficient r_s , P -value and number of samples is given for each correlation plot.

Potential inhalational exposure

The highest potential inhalational exposure came from various *Fusarium* metabolites, which were present in highest concentrations in the dust (Figure 3). The estimated mean exposure to *Fusarium* metabolites, including trichothecenes and aurofusarin (not included in Figure 3), was below 20 ng/m^3 , whereas the worst case exposure was nearly $10 \text{ }\mu\text{g/m}^3$.

4. Discussion

The present study is to our knowledge the broadest characterisation of mycotoxin occurrence in settled grain dust from grain elevators and compound feed mills. Several fungal metabolites that had previously never been detected in grain or grain dust were demonstrated, in addition to mycotoxins commonly occurring in grain, such as DON, T-2 and HT-2. Trichothecenes, fumonisins, ZEA and OTA, mycotoxin groups of major concern, were represented. While aflatoxins, representing highly carcinogenic compounds, were not detected in the samples, the related

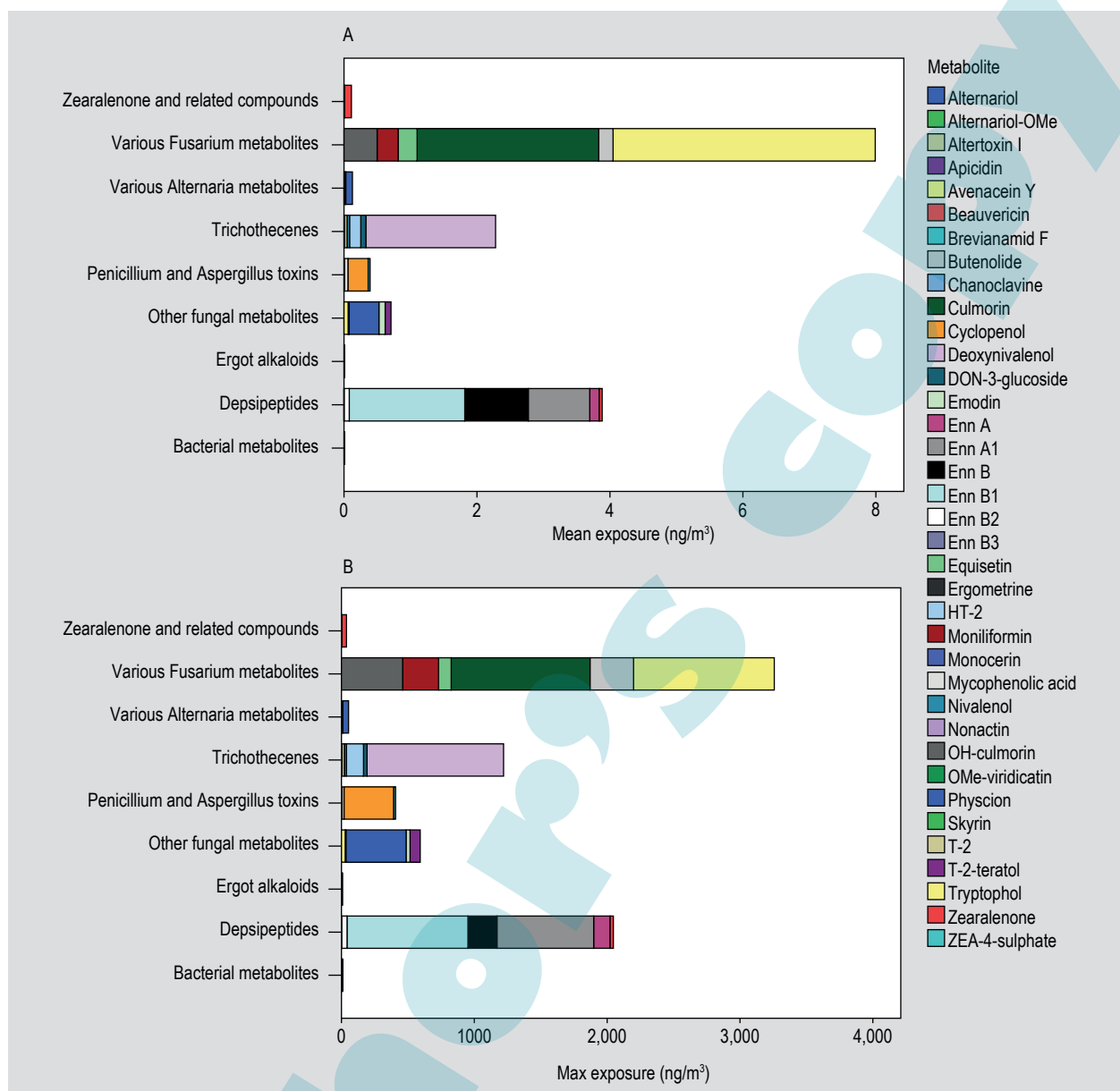


Figure 3. Estimated inhalable exposure to mycotoxins and fungal metabolites during work in grain elevators and compound feed mills. Mycotoxins and metabolites with prevalence above 80% were included in the estimates and stacked in group-based bars. Aurofusarin was excluded from the figure due to a very high mean and maximum concentration that precluded the contribution from all other mycotoxins in the stacked bar chart.

compound sterigmatocystin was detected in several of the samples. Sterigmatocystin is an aflatoxin precursor in some *Aspergillus* spp, while the compound is apparently not converted to aflatoxins in *Aspergillus versicolor* (Alkhayyat and Yu, 2014).

Mycotoxins have been found in settled grain dust from corn processing plants in Georgia, USA (aflatoxins) (Silas *et al.*, 1987), in grain elevators in New Orleans (ZEA, but no aflatoxin or OTA) (Palmgren *et al.*, 1983), from threshing on Polish farms (MON, NIV, DON and OTA) (Krysinska-Traczyk *et al.*, 2001, 2007), in German grain elevators (OTA,

DON and ZEA) (Mayer *et al.*, 2007), and from threshing and storage work on Norwegian farms (HT-2, T-2, DON, NIV, diacetoxyscirpenol and OTA) (Halstensen *et al.*, 2004; Nordby *et al.*, 2004). The median concentrations of the majority of these mycotoxins were 2-70 times higher in the present study.

The detection of a number of *Penicillium*, *Aspergillus* and *Fusarium* toxins in farms and storage facilities in Belgium was one of the first studies that utilised a multi-mycotoxin LC/MS-MS approach (Tangni and Pussemier, 2007) in an occupational setting. The development of the LC/MS-MS

method for the detection of microbial metabolites used in the present study started in 2006 (Sulyok *et al.*, 2006) and has previously been used to screen the metabolic profile in the waste management sector (Mayer *et al.*, 2011) and indoor air (Peitzsch *et al.*, 2012; Taubel *et al.*, 2011). Higher numbers of microbial secondary metabolites and at higher levels were found in settled dust derived from moisture damaged damp schools compared to schools not affected by moisture damage and dampness (Peitzsch *et al.*, 2012). It was suggested that the normal fungal metabolite spectrum shifted due to increased water activity following building dampness.

The metabolites detected in the grain dust reflect the commonly observed infection of Norwegian grain with certain fungal species. As expected, a majority of the detected metabolites were from *Fusarium* typically found in grain in Norway. *Fusarium langsethiae* is the main producer of type A trichothecenes in Norway, whereas type B trichothecenes and ZEA are produced by *Fusarium graminearum* and *Fusarium culmorum*. *Fusarium avenaceum* is likely responsible for the observed contamination with MON, ENNs and avenacein Y (Uhlig *et al.*, 2007). The detection of ENNs in all samples demonstrate the widespread contamination of grain with the latter species, and the concentration of ENN A₁ and B₁ was considerably higher than previously found in samples from waste recycling plants (Mayer *et al.*, 2011). The contamination with DON, DON-3-glucoside and 3-acetyl-DON is most likely related to *F. graminearum* and *F. culmorum* (Langseth *et al.*, 2001). Culmorin is also produced by these species, and the co-occurrence with DON in all samples was therefore expected and in accordance with previous reports showing that culmorin compounds often occur together with type-B-trichothecenes (Ghebremeskel and Langseth, 2001). More exotic for Scandinavian samples was the detection of fumonisins, as the producers, *Fusarium verticillioides* and *Fusarium proliferatum*, are rarely observed in Norway. Fumonisins are commonly contaminating maize crops, and were in this connection most likely derived from imported maize used in the compound feed production. The higher fumonisin concentration in compound feed mills most likely reflects the use of the contaminated imported maize in the compound feed production. Periodical storage of maize in the grain elevator departments could be a reason for the presence of fumonisins also in grain elevators, and not only the compound feed departments. The occurrence of several *Alternaria* metabolites was high in the analysed samples (76%-100%). Although the observed occurrence in our study was significantly higher than the 6% prevalence of alternariol-methylether reported in a recent EFSA opinion on *Alternaria* toxins in food and feed (EFSA, 2011), it is in accordance with the prevalence previously reported from Norwegian grain (Uhlig *et al.*, 2013). The concentrations were, however, higher in grain dust than in grain. This was also observed for several other mycotoxins. Higher

concentrations of mycotoxins in grain dust compared to the grain itself have been reported previously (Halstensen *et al.*, 2006b; Krysinska-Traczyk *et al.*, 2007; Sanders *et al.*, 2013, 2014), and is probably because the grain dust is enriched with particles from the outer shell layer of the grain where the mycotoxin concentrations are higher than the whole grain. A relatively high number of samples (56%) contained sterigmatocystin, a metabolite that is structurally closely related to the aflatoxins and has similar toxic effects as aflatoxin B₁, but is considered to be less potent (EFSA, 2013). A similar high prevalence of sterigmatocystin, but with a lower mean concentration, has been found in Norwegian oats, whereas the content in barley and wheat was significantly lower (Uhlig *et al.*, 2013). The concentration of nonactin and monactin in the present study was in the same range as previously found in settled dust from paper waste, but lower than found in municipal waste samples (Mayer *et al.*, 2011).

It is difficult to explain all the observed differences in prevalence and concentration of metabolites seen between grain elevators and compound feed mills. However, the main contributors are likely to be the different raw materials being processed in the two departments. The higher concentration levels of many grain-related mycotoxins in grain elevators compared with compound feed mills was expected since the dust in compound feed mills are not only generated from grain, but also from other raw materials. On the other hand, the other raw materials may contain other metabolites not present in the grains. The ergot alkaloids, produced by *Claviceps purpurea* that parasitises the ears of grain, especially rye, the type A trichothecene NEO, produced by *F. langsethiae* and *Fusarium sporotrichioides*, and OTA, produced by the so-called storage fungi *Aspergillus ochraceus* and *Penicillium verrucosum*, was found in grain elevators only. Dechlorogriseofulvin was found in significantly higher concentration in the compound feed mills compared to the grain elevators. This metabolite is produced by *Penicillium griseofulvum* and other *Penicillium* species and have mycostatic activity against a variety of fungi, but is also commercially produced. Dechlorogriseofulvin has previously been found in one settled dust sample of municipal waste (Mayer *et al.*, 2011).

The observed differences between seasons and climatically different zones for certain metabolites were difficult to explain, and could be due to coincidence. The growth of fungi and their production of mycotoxins are generally dependent on weather, humidity, temperature, climate or geographic locations, as well as agricultural factors such as fungicide usage, ploughing routines and plant resistance. The mycotoxin prevalence may vary from year to year according to these determinants. The samples in the present study are collected in three seasons and three climatic zones and should thus include a fairly representative variation in this regard. The dominant fungal species and

the mycotoxins they produce may vary from one part of the world to another, depending on differences in topography and climate, but also on the local level (Amend *et al.*, 2010; Kryszynska-Traczyk *et al.*, 2001, 2007; Mayer *et al.*, 2007; Nordby *et al.*, 2004; Peitzsch *et al.*, 2012). As Norwegian grain elevators and compound feed mills commonly also use imported grain, this adds to the complexity of seasonal and climatic variability that determines mycotoxin presence.

Correlations within groups of metabolites are likely due to more or less simultaneous production of these metabolites by the same producer (e.g. DON and acetylated derivatives). DON is partly converted to the less toxic DON-3-glucoside both by the producer and *in planta*, but the possibility of this 'masked' mycotoxin DON-3-glucoside to be 'unmasked' *in vivo* makes it important to include in the mycotoxin analyses and risk assessments (Nagl *et al.*, 2014). Likewise, ZEA-4-sulphate may be converted back to the oestrogen-binding ZEA *in vivo*, and exert hormonal effects (Plasencia and Mirocha, 1991). Our observation of a strong correlation between DON and DON-3-glucoside and between ZEA and ZEA-4-sulphate may in this regard be useful. As many *Fusarium* species produce aurofusarin, the very high concentration of this pigment in all samples could be expected, and this was probably the reason for the strong correlation with many of the other metabolites (data not shown). The correlation was particularly strong with other metabolites with high concentrations, such as culmorin.

Although the relationship between ingestion of mycotoxin and human health effects has been clearly established, health effects from inhalation are still under debate. However, toxicological studies indicate strong toxic effects of trichothecenes after inhalation (Creasia *et al.*, 1990; Pang *et al.*, 1988; Schiefer and Hancock, 1984). Furthermore, the basic mode of action of many of the detected metabolites is known, and may therefore indicate potential effects after inhalation. Mycophenolic acid is an immunosuppressant that was present in all samples, but in low levels. Sterigmatocystin is genotoxic *in vitro* similar to aflatoxin, although less hepatogenic (Jaksic *et al.*, 2012). Sterigmatocystin has been reported only once in Norway previously (Uhligh *et al.*, 2013). Two of the detected *Alternaria* metabolites, altertoxin-1 and alternariol OMe are known to have genotoxic potential. The possible inhalational effect of the high concentration of ENNs observed in all grain dust samples is uncertain since their significance *in vivo* is unknown. However, the compounds have recently been demonstrated to interfere with lysosomes (Ivanova *et al.*, 2012) and immunological responses (Gammelsrud *et al.*, 2012) *in vitro*. Thus, a potential effect of ENNs alone or in combination with other mycotoxins affecting the same parameters cannot be excluded.

Exposure to multiple mycotoxins can lead to extremely complicated biological responses within a relatively simple

system like a single cell. Understanding the mode of action of individual mycotoxins in simple *in vitro* systems can provide a rational basis for studying or predicting effects of mycotoxin mixtures (Speijers and Speijers, 2004; Wan *et al.*, 2013). Different toxicological parameters and experimental procedures have been used in animal studies (Huff *et al.*, 1986; Smith *et al.*, 1997; Theumer *et al.*, 2003; Wangikar *et al.*, 2004) and *in vitro* tests (Bensassi *et al.*, 2014; Bernhoft *et al.*, 2004; Braunberg *et al.*, 1994; Ndossi *et al.*, 2012; Solhaug *et al.*, 2013; Tammer *et al.*, 2007; Wan *et al.*, 2013) of combined effects of multiple mycotoxin exposure. Several prediction tools have been investigated (Heussner *et al.*, 2006; Li *et al.*, 2014; Tajima *et al.*, 2002). In general, most of the mycotoxin mixture studies have observed additive and/or synergistic interactions, depending on the mixture and chosen endpoint. The effect of a mycotoxin mixture cannot therefore be predicted from the effect of the individual mycotoxins (Tajima *et al.*, 2002). Mycotoxins can also act synergistically with other bioactive microbial components such as allergens, endotoxins and microbial volatile organic components, to amplify cellular responses *in vitro* and *in vivo* (Islam and Pestka, 2006; Kankkunen *et al.*, 2009; Zhou *et al.*, 2000). As grain dust also consists of several bioactive components, grain handlers may be affected by such interactions. Settled dust from grain elevators has been shown to exert moderate to high cytotoxicity in the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mayer *et al.*, 2007). However, the cytotoxicity was not correlated with OTA or DON concentrations, indicating the presence of other cytotoxic agents in the grain dust.

The estimated personal mean exposure of all detected fungal metabolites in the present study was below 20 ng/m³. This may seem low, but the effect level of each metabolite is not known and neither is the combined effect of all metabolites. In a worst case scenario, the workers may inhale up to 10 µg/m³ of fungal metabolites during a work shift. The actual inhaled dose will depend on the work intensity. To exemplify, an adult will breathe around 30 l/min during moderate physical activity. During an 8 h shift with moderate activity, a worker will thus inhale 14 m³ of air, and the worst case daily inhaled metabolite dose will be 144 µg. Presently, we cannot decide whether adverse levels can be reached under different scenarios of inhalational exposure, as no studies of human effects of airborne mycotoxins are known. Furthermore, the exposure data in the present study should be used with caution since they are estimations, and not direct exposure measurements. However, as no studies of airborne multiple mycotoxin exposure so far exists, this may be a useful proxy. The grain dust samples in the present study also contained a range of fungal metabolites for which very little toxicological information is available. The potential effects on human health both from oral and inhalational

exposure to these potentially bioactive compounds should therefore be subject for further study.

At present it is not feasible to assess the precise effects of the multiple exposures that grain dust represents. However, an important first step is to characterise the contents of the dust to be able to evaluate the existence of potential combination effects. Characterisation of the exposure patterns of fungal metabolites in different occupations will not only create knowledge of indicative concentrations of prevalent compounds, but also give a basis for evaluating the exposure differences qualitatively. This may further be used in a more focused risk assessment in the various occupational surroundings.

5. Conclusions

Over 70 microbial metabolites were detected in settled dust from grain elevators and compound feed mills. The main mycotoxins found were from the genus *Fusarium*. Particularly large quantities of DON, depsipeptides, aurofusarin, avenacein Y and culmorin were found. Most of the metabolites have previously not been detected in grain dust, and for some very little toxicological information is available. The prevalence and concentration of most metabolites were higher in grain elevators compared to compound feed mills. All samples contained multiple mycotoxins, indicating a highly complex pattern of possible inhalational exposure. Although many of these compounds may be linked to toxicological and immunological effects through experimental and epidemiological studies, it remains to be determined whether the detected concentrations of the microbial metabolites are of toxicological relevance and may implicate adverse health outcomes when inhaled.

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