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ASSESSMENT OF MICROBIAL EXPOSURE RISKS FROM HANDLING OF BIOFUEL WOOD CHIPS AND STRAW - EFFECT OF OUTDOOR STORAGE

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Abstract: Handling of biofuels may release dust particles containing high concentrations of hazardous microorganisms, thus representing a potential occupational health problem. We analysed the microbial dustiness of baled straw (cultivated both conventionally and ecologically) and of wood chips from piles that had been stored outdoors for up to 11 months by using total spore counting, cultivation, and measuring of endotoxin and chemical markers of fungal biomass, lipopolysaccharide, and peptidoglycan. The bacterial dustiness of straw was much greater than of wood chips whereas the fungal dustiness did not differ much. In general, samples taken from the inner part of each biofuel material were dustier than samples taken from the surface, except for fungal and bacterial biomass in wood chips and total fungi and fungal biomass in ecological straw. A considerable increase of bacterial dustiness occurred during storage over summer. Dust from ecological straw contained considerably less of bacterial components than from conventional straw and, in addition, exhibited a less pronounced increase upon storage over summer. In summary, biofuels represent sustainable energy resources of growing economic importance but may at the same time pose significant health problems. We found that storage of biofuels outdoors over summer increased the microbiological dustiness and should therefore be avoided, and that ecological straw contained less of microbe-containing dust than conventional straw and should be preferred since it reduces the exposure to harmful microbiological agents.

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INTRODUCTION

Biofuels represent sustainable energy resources of growing economic importance. However, during handling of biofuels workers may be exposed to high levels of microorganisms. Such exposure is known to be associated with adverse health effects [1, 3, 10] including, e.g. respiratory symptoms and eye irritation [5, 6, 12, 13, 17, 18, 21], and should therefore be kept as low as possible. In a previous study we analysed the microbial dustiness of straw, wood chips, wood pellets and wood briquettes, all commonly used biofuels in Scandinavia. A rotating drum was used to generate the dust. We found that in particular straw released large amounts of dust containing high concentrations of bacteria and fungi. In comparison, wood pellets and briquettes showed much lower microbial dustiness [11].

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Table 1. Pearson correlations (r) between parameters of microbial dustiness of wood chip biofuel samples, p-values (*italic*), and numbers of observation. Significant correlation coefficients (after Bonferroni correction) in **bold**.

	LPS ng	Endotoxin EU	Cfu of bacteria	Total bacteria	Muramic acid	Cfu of thermophilic actinomycetes	Cfu of mesophilic actinomycetes	Ergosterol	Cfu of fungi	Total fungi	Dustiness
LPS ng	1	0.68908 *0.0032* 16	**0.87362** *<0.0001* 18	**0.69346** *0.0014* 18	**0.85461** *0.0002* 13	**0.84905** *0.0009* 11	0.62306 *0.0057* 18	0.02331 *0.9317* 16	**0.65864** *0.0030* 18	0.15515 *0.5387* 18	0.56262 *0.0151* 18
Endotoxin EU		1	0.56347 *0.0120* 19	**0.84761** *0.0001* 19	**0.72578** *0.0075* 12	0.54907 *0.0802* 11	0.34562 *0.1472* 19	0.15547 *0.5801* 15	0.64127 *0.0031* 19	0.54830 *0.0151* 19	**0.91967** *<0.0001* 19
Cfu of bacteria			1	0.46706 *0.0328* 21	**0.90398** *<0.0001* 14	**0.87708** *<0.0001* 13	**0.89517** *<0.0001* 21	0.04440 *0.8656* 17	**0.71169** *0.0003* 21	-0.02280 *0.9219* 21	0.38565 *0.0842* 21
Total bacteria				1	0.64679 *0.0124* 14	0.63170 *0.0206* 13	0.31148 *0.1693* 21	0.16984 *0.5146* 17	0.46688 *0.0329* 21	0.38781 *0.0824* 21	**0.65204** *0.0014* 21
Muramic acid					1	**0.89996** *0.0023* 8	0.70633 *0.0047* 14	-0.24589 *0.4411* 12	0.58751 *0.0272* 14	-0.11626 *0.6923* 14	0.44390 *0.1118* 14
Cfu of thermophilic actinomycetes						1	**0.83540** *0.0004* 13	0.68483 *0.0289* 10	0.73228 *0.0044* 13	0.11613 *0.7056* 13	0.17423 *0.5692* 13
Cfu of mesophilic actinomycetes							1	0.26585 *0.3024* 17	**0.69865** *0.0004* 21	-0.09692 *0.6760* 21	0.17605 *0.4453* 21
Ergosterol								1	0.60129 *0.0107* 17	**0.67954** *0.0027* 17	0.25863 *0.3162* 17
Cfu of fungi									1	0.61035 *0.0033* 21	0.59254 *0.0046* 21
Total fungi										1	**0.68758** *0.0006* 21
Dustiness											1

The present study acknowledges the fact that both straw and wood chips are usually stored outdoors for longer periods of time (months) before used as biofuels. The aim was to evaluate how such storage might affect the tendency of these materials to release dust containing potentially hazardous microorganisms and microbial components. Dust from wood chips and straw that had been stored outdoors for up to 11 months was generated by the same rotating drum as used previously in order to mimic actual handling of the biofuels [11]. The microbiological analyses of the dusts included total spore counting, cultivation, determination of endotoxin by the *Limulus* method, and quantification of ergosterol (marker of fungal biomass), 3-hydroxy acids (3-OH FAs, markers of lipopolysaccharide (LPS)), and muramic acid (MuAc, marker of peptidoglycan) by using gas chromatography-mass spectrometry (GC-MS). The correlations between the different microbiological parameters were studied.

MATERIALS AND METHODS

Biofuels. The biofuels studied included wood chips and straw from both conventional ("conventional straw") and organic ("ecological straw") farming. The straw was collected from the field within 1 week after harvest. Ecological straw was defined as straw that had been cultivated using natural fertilizers and without any use of chemicals. The chips came from pine (40%) and fir (60%). The straw was baled in 250 kg round bales, and the chips were generated and piled, in October year 1, and thereafter the materials were stored, uncovered, outdoors. Samples of the biofuels were taken for analysis immediately before storage started (October year 1), as well as after different time periods of storage (February, April, and September year 2). In April and September, biofuel samples were taken both from the surface and from the middle of each straw bale and wood chip pile.

Generation and collection of dust aerosols. A rotating drum was used to generate dust from the biofuel samples. In brief, samples (3–6 kg) were loaded onto the bottom of a rotating (7 rpm, 5 min) drum [11]. Dust used to evaluate the total dustiness was sampled on a 140 mm diameter 8 µm pore size cellulose nitrate membrane filter (Sartorius, Göttingen, Germany) whereas dust for microbiological analysis was sampled on 6 filter cassettes. Four

Table 2. Pearson correlations (r) between parameters of microbial dustiness of straw biofuel samples, p-values (*italic*), and numbers of observation. Significant correlation coefficients (after Bonferroni correction) in bold.

	LPS ng	Endotoxin EU	Cfu of bacteria	Total bacteria	Muramic acid	Cfu of thermophilic actinomycetes	Cfu of mesophilic actinomycetes	Ergosterol	Cfu of fungi	Total fungi	Dustiness
LPS ng	1	**0.71777** *<0.0001* 35	**0.52548** *0.0012* 35	**0.66996** *<0.0001* 35	**0.75034** *<0.0001* 33	0.74698 *0.0130* 10	-0.27040 *0.1911* 25	0.18473 *0.2881* 35	0.37096 *0.0282* 35	0.44239 *0.0078* 35	**0.80363** *<0.0001* 35
Endotoxin EU		1	**0.69785** *<0.0001* 36	**0.86457** *<0.0001* 36	**0.55768** *0.0006* 34	0.59125 *0.0718* 10	0.20591 *0.3234* 25	0.09541 *0.5799* 36	0.41978 *0.0108* 36	**0.74518** *<0.0001* 36	**0.96890** *<0.0001* 36
Cfu of bacteria			1	**0.82509** *<0.0001* 36	**0.72652** *<0.0001* 34	**0.82266** *0.0035* 10	**0.66009** *0.0003* 25	0.07443 *0.6662* 36	**0.89289** *<0.0001* 36	**0.68529** *<0.0001* 36	**0.70283** *<0.0001* 36
Total bacteria				1	**0.56293** *0.0005* 34	0.65536 *0.0397* 10	0.25518 *0.2183* 25	0.07271 *0.6735* 36	**0.53206** *0.0008* 36	**0.74333** *<0.0001* 36	**0.83889** *<0.0001* 36
Muramic acid					1	**0.78057** *0.0077* 10	0.37234 *0.0802* 23	0.29998 *0.0848* 34	**0.73643** *<0.0001* 34	0.44998 *0.0076* 34	**0.64556** *<0.0001* 34
Cfu of thermophilic actinomycetes						1	0.69539 *0.1250* 6	-0.24318 *0.4984* 10	**0.95076** *<0.0001* 10	0.46772 *0.1728* 10	0.58790 *0.0739* 10
Cfu of mesophilic actinomycetes							1	-0.02145 *0.9189* 25	**0.74175** *<0.0001* 25	0.46799 *0.0183* 25	0.12525 *0.5508* 25
Ergosterol								1	0.05083 *0.7684* 36	0.35763 *0.0322* 36	0.15227 *0.3753* 36
Cfu of fungi									1	0.49455 *0.0022* 36	0.44386 *0.0067* 36
Total fungi										1	**0.69998** *<0.0001* 36
Dustiness											1

of the cassettes contained Teflon filters (25 mm diameter, 3 µm pore size; Millipore, Bedford, USA) and 2 contained polycarbonate filters (25 mm diameter, 0.4 µm pore size, Nucleopore, Cambridge, MA, USA). The mass of the collected dust was determined by weighing the filters both before and after sampling. Before weighing, the filters were equilibrated at constant air temperature and humidity for 24 h. A particle counter (Grimm model 1200) was used for collection of data of dust particles >0.75 µm [11].

Determination of microbial composition. A modified CAMNEA method [15] was used for identifying and quantifying the cultivable and non-cultivable microorganisms in the dust on the polycarbonate filters [11]. The following microorganisms were measured (in cfu, after cultivation for 7 days): fungi (Dichloran Glycerol agar, Oxoid, Basingstoke, England) incubated at 25ºC and at 45ºC, bacteria (Nutrient agar, Oxoid, Basingstoke, England) with actidione (cycloheximide; 50 mg l^{-1}) incubated at 25ºC, mesophilic actinomycetes (10% Nutrient agar) with actidione (50 mg l^{-1}) incubated at 25ºC, and thermophilic actinomyctes (10% Nutrient agar) with actidione (50 mg l^{-1}) incubated at 55ºC.

Total numbers of bacteria and fungal spores were determined after staining in 20 ppm acridine orange (Merck) in acetate buffer. Fungi and bacteria were counted at a 1,250 × magnification by epifluorescence microscopy (Orthoplan; Leitz Wetzlar). The numbers of microorganisms were determined in 40 randomly chosen fields or until a number of at least 400 spores were counted.

Endotoxin was determined from extracts of Teflon filters. 5.0 ml sterile 0.05% Tween 20 aqueous solution was added to each filter, which was then shaken (300 rpm) at room temperature for 60 min and centrifuged (1,000 × g) for 15 min. The supernatant was then analysed (in duplicate) by a kinetic *Limulus* Amebocyte Lysate test (Kinetic-QCL endotoxin kit, BioWhittaker, Walkersville, Maryland, USA) as described earlier [11].

Chemical analysis was used to quantify MuAc, 3-OH FAs of 10–18 carbon chain lengths, and ergosterol. In brief, dust-containing Teflon filters were subjected to alkaline hydrolysis to liberate ergosterol, and to acid methanolysis to liberate MuAc and 3-OH FAs. The marker analytes were then purified, derivatised, and analysed by using gas chromatography-tandem mass spectrometry (GC-MSMS) employing an ion-trap type of GC-MS

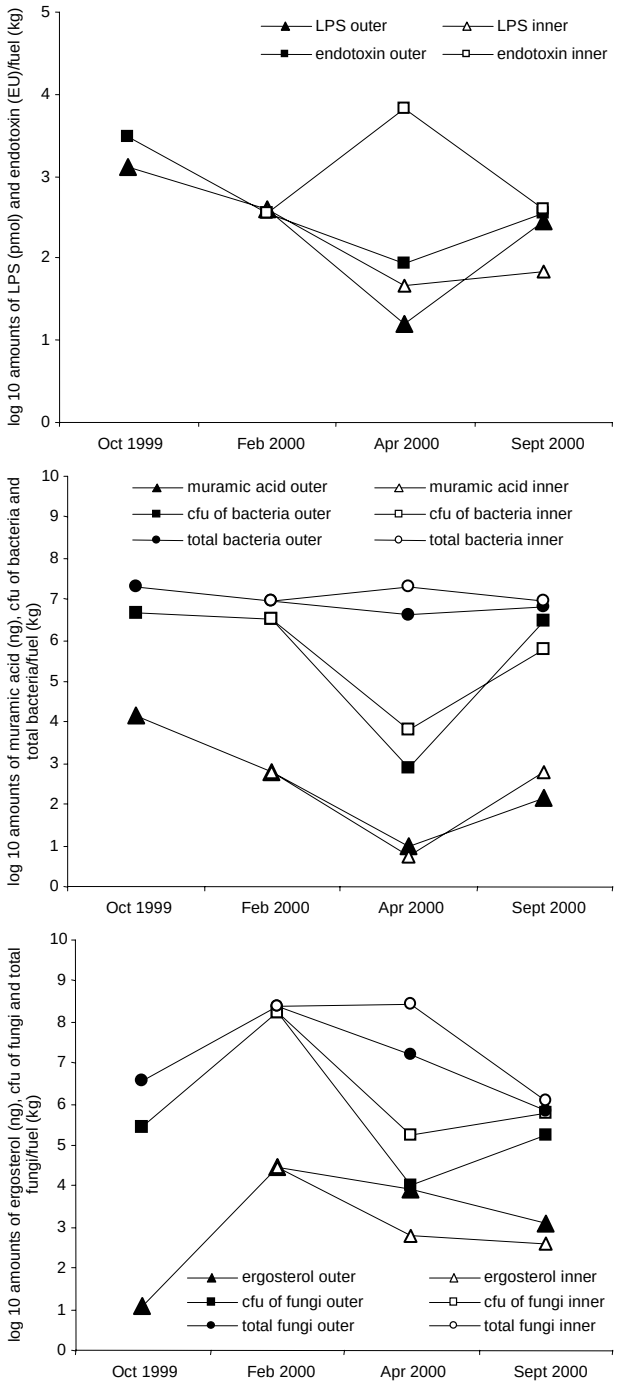

Figure 1. Microbiological characteristics of dust from wood chips taken from the surface and middle of a pile immediately after pile erection and after 4, 6, and 11 months of storage outdoors.

instrument. LPS was determined by summarizing the number of moles of the 3-OH FAs and dividing by 4 [11].

Statistics. Test of correlations between variables was based on Pearson correlation coefficients for data following a log normal distribution. Acceptances of 0 hypotheses after Bonferroni correction of variables, expressed as $< \alpha$(number of variables), were respectively 0.00833 (6), 0.00625 (8), 0.00500 (10), 0.00455 (11), 0.00417 (12), 0.00385 (13), 0.00357 (14), 0.00313 (16), 0.00294 (17), 0.00278 (18), 0.00263 (19), 0.00238 (21), 0.00200 (25), 0.00152 (33), 0.00147 (34), 0.00143 (35), and 0.00139 (36).

RESULTS

Effect of storage on dust particles and microbiological parameters. Levels of LPS and MuAc in wood chips dust decreased considerably during the winter storage (October–April) and increased again over summer regardless whether the chips samples had been collected from the inner part or from the surface of the pile (Fig. 1). The same trend was found for endotoxin (except for samples collected from the inner part of the pile), colony forming units (cfu) of bacteria and cfu of fungi. The total bacterial concentration did not change markedly over the storage period whereas levels of both total fungi and of ergosterol showed an increase between October and February and a decrease from February to September.

Aspergillus fumigatus was not found in the dust released in October. In February, 3.04×10^7 cfu of *A. fumigatus*/kg wood chips were released on average. In April, the levels were 1.16×10^3 and 2.71×10^4 cfu/kg, and in August they increased to 2.34×10^5 and 5.07×10^5 cfu/kg, for respectively "outer" and "inner" chips.

Levels of all bacterial parameters (LPS, endotoxin, MuAc, cfu of bacteria and total bacteria) and of cfu of fungi in dust from samples of conventional straw collected from the surface of the bale all decreased over the winter (October–April) and increased again over the summer (Fig. 2). However, just as for wood chips, levels of ergosterol and total fungi increased from October until February, decreased during spring, and then increased again over summer. Samples from the inner part of each stored material were not taken for analysis in September; however, the over-all trend was similar to the surface samples, except that the endotoxin levels of dust from inner samples increased slightly between February and April. The bacterial and fungal markers for ecological straw in general showed a similar trend as for conventional straw except that the levels of LPS (outer) continued to decrease over summer and the levels of ergosterol and LPS (inner) increased over the spring (Fig. 3).

A. fumigatus was found only in dust released from 3 of the 30 studied straw samples, and only in ecological straw.

Release of particles (>0.75 μm/m^3) from straw decreased after the winter storage and increased again over the summer except for samples taken from the inner part of each stored material where the dustiness increased between February and April. Wood chips taken from the outer part of each pile were less dusty than any of the straw samples except samples taken from the inner part of the wood chips pile which released just as many particles as ecological straw (Fig. 4).

Correlations between microbial dustiness parameters. Total wood chip dustiness correlated with endotoxin and total bacteria and fungi; cfu of bacteria correlated with LPS, MuAc, cfu of thermophilic and mesophilic

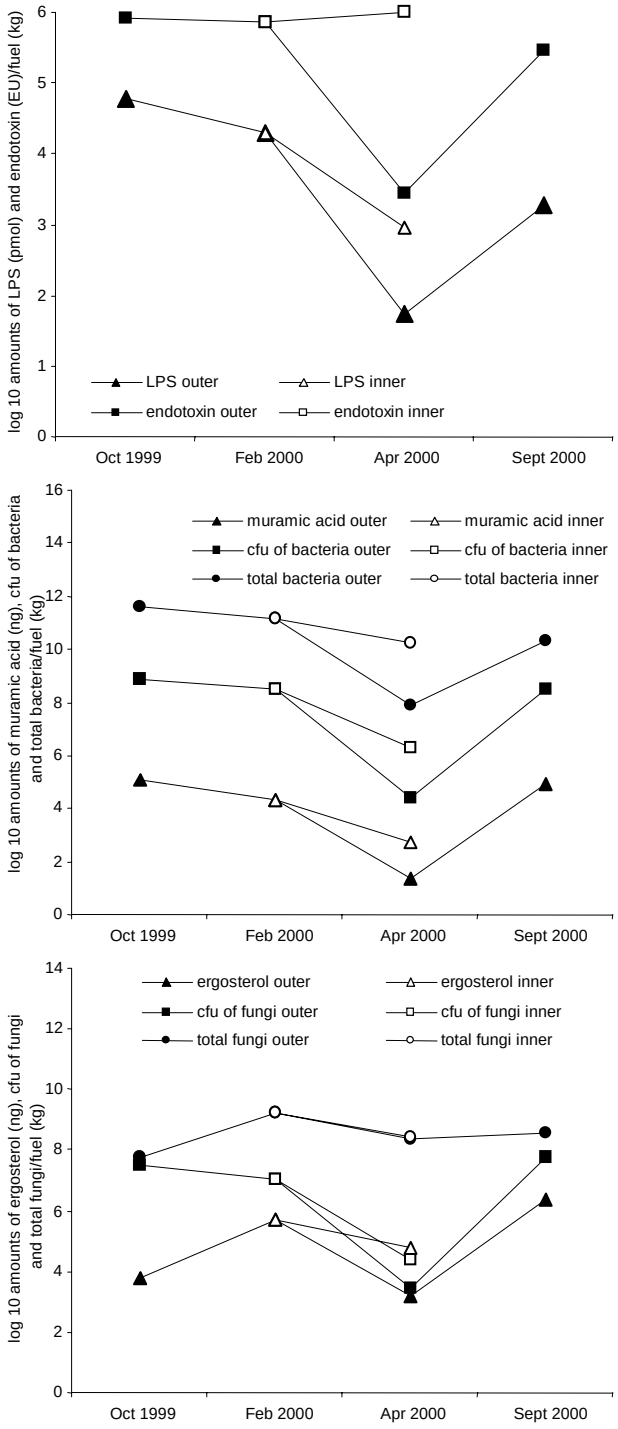

Figure 2. Microbiological characteristics of dust from conventional straw taken from the surface and middle of a bale immediately after baling and after 4, 6, and 11 months of storage outdoors.

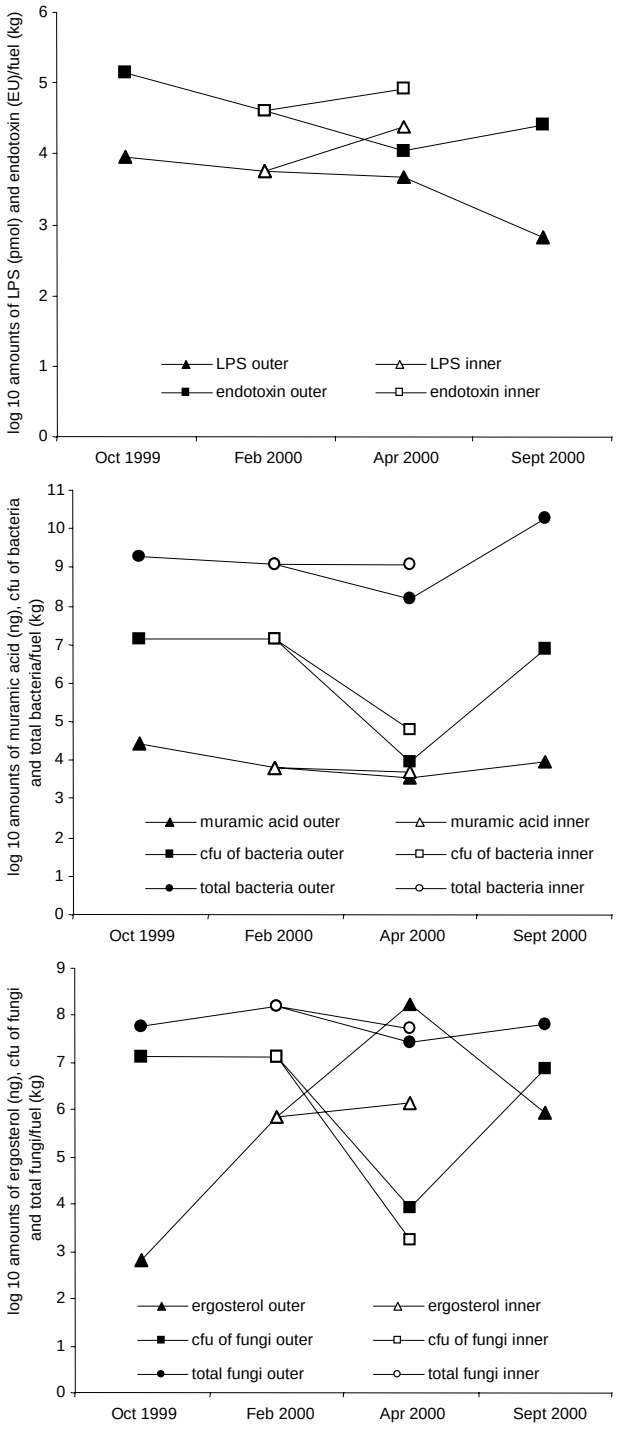

Figure 3. Microbiological characteristics of dust from ecological straw taken from the surface and middle of a bale immediately after baling and after 4, 6, and 11 months of storage outdoors.

actinomycetes, and cfu of fungi; ergosterol correlated only with total fungi; MuAc correlated with LPS, cfu of bacteria, endotoxin, and cfu of thermophilic actinomycetes; LPS correlated with cfu of bacteria and total bacteria, MuAc, cfu of thermophilic actinomycetes, and cfu of fungi (the correlation with endotoxin was nearly significant); endotoxin correlated with total bacteria, MuAc, and total dustiness; total fungi correlated with ergosterol and total dustiness; cfu of fungi correlated with cfu of mesophilic actinomycetes, cfu of bacteria, and LPS; total bacteria correlated with endotoxin, LPS, and total dustiness; cfu of thermophilic actinomycetes correlated with cfu of mesophilic actinomycetes, cfu of bacteria, MuAc, and LPS; cfu of mesophilic actinomycetes correlated with cfu of thermophilic actinomycetes and cfu of bacteria and fungi (Tab. 1).

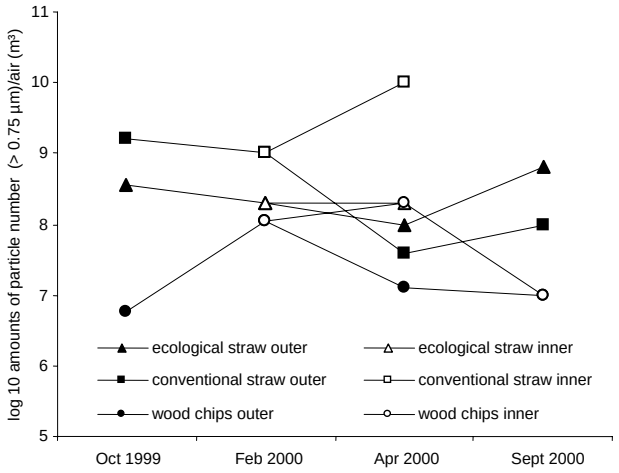

Figure 4. Release of particles (>0.75 µm/m^3) from samples of studied biofuels taken from the surface and middle of each stored material at the beginning of storage period, and after 4, 6, and 11 months of storage outdoors.

Total straw dustiness correlated with endotoxin, total bacteria and fungi, LPS, cfu of bacteria, and MuAc; cfu of bacteria correlated with all parameters except ergosterol (which did not correlate with any studied parameter); MuAc correlated with LPS, endotoxin, cfu of fungi, cfu of thermophilic actinomycetes, dustiness, cfu of bacteria, and total bacteria; cfu of thermophilic actinomycetes correlated with cfu of fungi, MuAc, and cfu of bacteria; LPS correlated with MuAc, endotoxin, dustiness, cfu of bacteria, and total bacteria; endotoxin correlated with LPS, MuAc, cfu of bacteria, total bacteria, total fungi, and dustiness; total fungi correlated with endotoxin, dustiness, cfu of bacteria, and total bacteria; cfu of fungi correlated with cfu of thermophilic and mesophilic actinomycetes, cfu of bacteria, total bacteria, and MuAc; total bacteria correlated with endotoxin, LPS, MuAc, cfu of bacteria, dustiness, cfu of fungi, and total fungi; cfu of mesophilic actinomycetes correlated with cfu of fungi and bacteria (Tab. 2).

DISCUSSION

Health hazards due to inhalation of dust from materials used as biofuels are widely acknowledged. However, it is still largely unknown which components of the dust are responsible for the adverse health effects. Of the different microbial components that may be present, endotoxin has been the most thoroughly investigated. Thus, elevated levels of endotoxin at worksites have been associated with the development of several symptoms including chest tightness, fever, fatigue, and diarrhoea [2, 4, 16, 21]. In the present study, we found that straw dust endotoxin correlated significantly with LPS, cfu of bacteria, and total fungi; in addition, endotoxin both in wood chip and straw dust correlated significantly with total dustiness, MuAc, and total bacteria. Since total dustiness correlated positively with all of the microbiological parameters, reducing the general dustiness may be efficient in reducing adverse health effects. At the same time, however, since the correlations between total dustiness and cfu of fungi, fungal biomass (ergosterol), cfu of actinomycetes, and (in case of wood chips) MuAc, cfu of bacteria and LPS were not significant, reducing the overall dust concentrations may only have a limited effect on these microbiological parameters.

Straw dust exhibited more close correlations between the different microbiological parameters than wood chips dust. This may reflect the different microbial communities in straw and wood chips, and may also be partly explained by the fact that the overall concentrations of microorganisms were much higher in straw than in wood chips [11]. Certain significant correlations were found both for straw and wood chip dusts; between LPS, MuAc, and cfu of bacteria and total bacteria, between endotoxin, MuAc, and total bacteria and dustiness, between cfu of bacteria, MuAc, and cfu of fungi, and between total fungi and dustiness. Interestingly, endotoxin correlated significantly with LPS only in the case of straw dust, possibly because there was much more fungal material in dust from wood chips than from straw, and that the *Limulus* method used to measure endotoxin (unlike the GC-MSMS method used to measure LPS) may cross-react with glucans which are major fungal cell wall components. The cross reaction with glucans, however, can be easily overcome by using β-glucan blocker [20] which, however, was not used in the present study.

The bacterial dustiness of straw was much greater than of wood chips, whereas the fungal dustiness did not differ much between the different biofuels. In general, samples taken from the inner part of each biofuel pile were dustier than samples taken from the surface, except for ergosterol and MuAc in wood chips and ergosterol and total fungi in ecological straw. Thus, it may be advisable to store biofuels as flat rather than high piles. A considerable increase of bacterial dustiness occurred between April and September. Dust from ecological straw contained considerably less bacterial components than dust from conventional straw. In addition, ecological straw exhibited a less pronounced increase upon storage over summer.

Storage of the wood chips resulted in a considerable growth of *A. fumigatus*, an allergenic and infective mould [9, 22], especially in the inner part of the pile. This may have been due to a spontaneous heat production, as has previously been found in self-heating wood chips piles [14].

Several studies have addressed the potential hazardness of occupational exposure to bioaerosols by studying the released microorganisms. For example, hay was found to release more fungi and bacteria if stored baled than if stored loose [8]. Hospitalization of farmers due to farmer's lung was most prevalent during the period (springtime) when the release of microorganisms was highest [7, 19]. Mould exposure from chips was also studied [6]. However, to the best of our knowledge this is the first systematic study on the microbial dustiness of biofuels during storage. Our data indicate that workers

handling biofuels may be exposed to high levels of dust rich in microorganisms. Since the correlations between endotoxin, total bacteria and fungi, and total dustiness were significant both for straw and wood chips, dustiness may be a good indicator of endotoxin and total bacterial and fungal levels in these biofuels. Therefore, reducing the dust levels will in general reduce the microbial exposure. Storage of biofuels outdoors over summer results in increased dustiness and should therefore be avoided if possible. We also found, in accordance with our previous study [11], that ecological straw contained less microorganisms than conventional straw and should be preferred since it reduces the exposure to harmful microbiological agents.

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