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MULTILEVEL ANALYSIS OF THE IMPACT OF ENVIRONMENTAL FACTORS AND AGRICULTURAL PRACTICES ON THE CONCENTRATION IN HAY OF MICROORGANISMS RESPONSIBLE FOR FARMER'S LUNG DISEASE

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Abstract: Farmer's lung disease (FLD) is common in eastern France. It is the main form of occupational hypersensitivity pneumonitis, caused by chronic inhalation of microorganisms (antigens) from mouldy hay, straw, or grain. The purpose of this study was to assess, with a panel of data collected between 1997–2003, environmental factors and agricultural practices that independently modify concentrations in hay of microorganisms potentially responsible for FLD. A total of 629 hay samples from 86 farms were included in statistical analyses using linear multilevel regression models allowing to consider the nested structure of the data: individual-level (batch of hay) and group-level (farm). The outcome variable of these models was the concentration in hay (logarithmic value of concentration+1) of microorganisms incriminated in FLD (*Absidia corymbifera*, *Eurotium* spp., thermophilic actinomycetes). The simultaneous analysis of batch of hay- and farm-level factors showed that bad climatic conditions of harvest, high-density hay-packing modes, (especially round bales) and altitude (2nd plateau,]700–900] m) were the main factors associated with high concentrations of these microorganisms in hay. This study allowed clarification of the factors that influence the microbial concentration of hay with etiological agents of FLD.

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Key words: farmer's lung disease, moulds, actinomycetes, hay, multilevel models.

INTRODUCTION

Farmer's lung disease (FLD) is the most common form of occupational hypersensitivity pneumonitis (extrinsic allergic alveolitis) caused by chronic inhalation of microorganisms (antigens) from mouldy hay, straw, or grain [1]. Its clinical expression is characterized by symptoms of dyspnea, cough, tiredness, headaches, occasional fever/night sweats and general feeling of sickness. Any one or all of the symptoms may be apparent depending on the severity of FLD: acute, sub-acute or chronic. Diagnostic criteria generally include: 1) respiratory symptoms suggestive of the diagnosis; 2) evidence of exposure to antigens (by history or detection of precipitating antibodies); 3) bronchoalveolar lavage lymphocytic alveolitis; 4) low carbon monoxide-diffusion capacity and 5) consistent chest radiograph or high-resolution computed tomography [3, 9, 18]. Prevalence and distribution of FLD vary widely according to countries and geographical locations due to agricultural practices and climatic conditions (0.2%–1.5% of the farming population).

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Thus, occurrences of FLD are most important in cattle-rearing areas, cold and rainy during the indoor feeding season (winter) [1, 19]. Thermophilic actinomycetes such as *Saccharopolyspora rectivirgula* were the first microorganisms identified as etiological agents of FLD [10]. The involvement of other bacterial and fungal species such as *Aspergillus* spp. have since been demonstrated [5, 8]. In Franche-Comté, a region in eastern France, a prospective case-control study showed that *Absidia corymbifera* and *Eurotium amstelodami* play a role in FLD [13]. In contrast, *S. rectivirgula* was very rarely isolated in hay samples. Other studies carried out in the same region partially dealt with the question of factors influencing the concentration in the hay of microorganisms incriminated in FLD [14, 16]. All these studies (Tab. 1) yielded a large number of hay samples from many farms. Collected data allowed us to perform a more powerful statistical analysis, by a global approach which took into account the nested hierarchical structure of the data (batches of hay within farms). The aim of our study was to assess environmental factors (including altitude, temperature and rainfall) and agricultural practices that independently modify concentrations in hay of microorganisms potentially responsible for FLD.

MATERIALS AND METHODS

Location of farms, study period and hay sampling. The farms were located in Franche-Comté at altitudes between 200–1,450 m. Hay sampling was carried out between 1997–2003 according to a standardized procedure. A batch of hay was defined as a set of bales or bulk harvested from the same meadow, the same day, and stored using the same procedure.

Microbiological analyses. Microbiological analyses were performed according to methods previously described [13] and remained unchanged during the study period. Briefly, after freezing at -18°C overnight to kill mites, samples were cultured on 5 culture media in adequate temperatures as follows: Dichloran-Glycerol (Oxoid, Unipath, Basingstoke, UK) with 0.5% chloramphenicol (Merck, Darmstadt, Germany) at 30ºC for mesophilic mould isolation, 3% malt-agar (Oxoid, Unipath, Basingstoke, UK) with 10% salt and 0.5% chloramphenicol, at 20ºC for osmophilic fungal species, actinomycete isolation agar Bacto medium (Difco, Detroit, MI, USA) at 30ºC for mesophilic actinomycetes and at 52ºC for thermophilic actinomycetes,

Table 1. Previous studies on farmer lung's disease (FLD) in Franche-Comté (1997–2003).

Study	Objective	Design	Study period	No. of subjects and/or hay samples	Conclusion
Reboux *et al.* [13]	To determine etiological agents of FLD	Prospective paired case-control	1998–2000	11 FLD cases/11 control farmers and 22 urban subjects (non-exposed control group)	*Absidia corymbifera*, and maybe *Eurotium amstelodami* and *Wallemia sebi* can be considered as causative agents of FLD.
Roussel *et al.* [15].	To assess microbiological evolution of hay and its relation with the risk of FLD cases or relapses	Sequential microbiological analyses of hay and clinical/serological surveillance of farmers	2000–2001	10 farmers (5 with a past history of FLD) and 76 batches of hay (only 48 sampled 3 times)	Proliferation of causative agent of FLD in the hay: in case of bad harvesting conditions (humidity), and with peak concentration in January. Strong relation between concentration of *A. corymbifera* in the hay and FLD occurrences.
Roussel *et al.* [17]	To compare FLD cases and controls in terms of agricultural practices and microbiological composition of hay	Unpaired case-control	2000–2003	10 FLD cases/42 controls and 178 batches of hay	Higher microbiological concentration of hay (including *A. corymbifera*) from FLD farms. No difference in agricultural practices.
Roussel *et al.* [16]	To evaluate salting as a hay preservative against FLD agents	Paired case-control (salted/unsalted hay)	2003	2 × 51 batches of hay	Ineffectiveness of salting.
Reboux *et al.* [14]	To determine impact of climatic factors and agricultural practices on microbiology of hay	Comparison between hay from Finnish (Kuopio) and French (Doubs) farms	2002	92 batches of hay: 29 (Kuopio) and 63 (Doubs)	Haymaking method appeared to be the main factor influencing microbiology of hay: lower concentration of *A. corymbifera* and *Eurotium* spp. in low density square bales.

R8 medium at 52ºC [2] and Muller-Hinton (Becton Dickinson®, Cockeysville, MD, USA) at 37ºC for thermotolerant aerobic bacteria. Identification (based upon macroscopic and microscopic morphological criteria) and counting of microbial colonies were carried out after 3 and 7 days of incubation. Concentrations of fungi and actinomycetes were expressed in colony forming units per gram (cfu/g) of hay.

Data structure

Batch of hay (level 1). The batch of hay corresponded to the lowest level of data nested within a farm. For each batch of hay, individual-data were collected: hay type (hay from the 1st crop was harvested in May or June; hay from the 2nd and 3rd crops, later), moisture of field (0="no"; 1="yes" [flood ground, heavy, on a river]), presence of voles or moles (0="no"; 1="yes" [soil in the hay due to tumuli]), meadow type (natural; artificial; mixed), year of harvest (1997–1999, 2001 and 2002), climatic conditions of harvest (0="good"; 1="bad" [wet weather]), salting (0="no"; 1="yes"), drying time (in days) and relative humidity of the batch of hay (in %) converted into binary data using the median cut-point (0="less or equal than median"; 1="higher than median"), use of a hay-packing machine (0="no"; 1="yes"), hay-packing mode (high density round bales [HDRB]; high density cubic bales [HDCB]; medium density cubic bales [MDCB], low density cubic bales [LDCB] and in bulk), storage location (0="open barn"; 1="closed barn") and sampling periods (P1="December–January"; P2="February–March"; P3="April").

Farm (level 2). The farms were the highest level of data and were classified into 3 categories according to grassland surface (in hectares, ha): 0="<50 ha"; 1="[50–100] ha"; 2=">100 ha". The geographical and climatic data of the location of each farm were taken into account as independent variables: annual average of temperatures (°C) and rainfall (mm) enabled us to define 2 temperature areas (0="≤+8°C"; 1=">+8°C") and 2 rainfall areas (0="≤800 mm"; 1=">800 mm"), and the farms were grouped into 4 altitude (m) categories (0="plain, <500 m"; 1="first plateau, [500–700] m"; 2= "second plateau, [700–900] m"; and 3="mountain, >900 m").

Statistical analysis. Data were analyzed using multilevel linear regression models with the concentration (logarithmic value of concentration+1) of a given microorganism in hay as the outcome variable: one model for each microorganism or group of microorganisms (*Absidia corymbifera*, *Erotium* spp., *Wallemia sebi*, mesophilic *Streptomyces* and thermophilic actinomycetes). This approach allowed us to take the correlated structure of the data into account. Indeed, conventional linear regression models make the untenable assumption that batches of hay from the same farm are unrelated. Moreover, these single-level regression models tend to underestimate standard errors of coefficients of explanatory variables in the case of clustered data and hence, may lead to biased statistical interpretations [7]. Initially, each independent variable was tested in a univariate model, and secondly, a reference model was built by introducing variables with $p<0.20$ in the univariate analysis. The reference model was built in 3 steps. First, a null model that included only the constant (i.e. no explanatory variables) was assessed to determine the initial distribution of the variance of the dependant variable (total variance) between the 2 levels: the intra-farm correlation (correlation among observations within a farm) was calculated by dividing the variance at the farm-level by the total variance. Secondly, a model simultaneously assessed individual- and group-level variables in order to estimate their joint effect on

Table 2. Characteristics of batches of hay (n = 629).

Variables	No. (%)
Hay type	
• 1st crop	437 (69.5)
• 2nd or 3rd crop	192 (30.5)
Meadow type	
• natural	475 (75.5)
• artificial or mixed	154 (24.5)
Moist field	62 (9.9)
Presence of voles or moles	34 (5.4)
Year of harvest	
• 1997–1999	65 (10.3)
• 2001	437 (69.5)
• 2002	127 (20.2)
Good climatic conditions of harvest	604 (96)
Drying time in days, mean (SD)/median (range)	2.9 (0.7)/ 3 (1–9)
Salting	106 (16.9)
Use of a hay-packing machine	241 (38.3)
Hay-packing mode	
• High density round bales	428 (68)
• High density cubic bales	20 (3.2)
• Medium density cubic bales	38 (6)
• Low density cubic bales	15 (2.4)
• Bulk	128 (20.4)
Relative humidity in %, mean (SD)/median (range)	19.2 (0.5)/ 19.1 (18.0–23.3)
Storage location	
• Open barn	170 (27)
• Closed barn	459 (73)
Period of hay sampling	
• P1=December–January	484 (76.9)
• P2=February–March	117 (18.6)
• P3=April	28 (4.5)

SD – standard deviation.

Table 3. Characteristics of farms (n = 86).

Variables	No. (%)
Grassland surface	
• < 50 hectares	20 (23.3)
• [50–100] hectares	53 (61.6)
• > 100 hectares	13 (15.1)
Temperature area[a]	
• low (≤ +8° C)	31 (36)
• high (> +8° C)	55 (64)
Rainfall area[b]	
• low (≤ 800 mm)	49 (57)
• high (> 800 mm)	37 (43)
Altitude	
• Plain (<500 m)	28 (32.6)
• 1st plateau ([500–700] m)	17 (19.8)
• 2nd plateau (]700–900] m)	26 (30.2)
• Mountain (> 900 m)	15 (17.4)

[a]Average annual temperature; [b]Average annual rainfall.

the concentration of microorganisms in hay. Finally, a full model estimated all main effects and interactions between individual-level variables and each group-level variable. Modelling employed iterative generalized least-squares (IGLS) estimation using MlwiN v. 2.02 software [12]. Likelihood ratio tests were performed to choose a parsimonious model between 2 alternative models: the significance of cross-level interactions and random coefficients for variables (*vs.* fixed coefficients) with selection of the best model between a model that contains these parameters and one that does not. The final model was analyzed thoroughly with respect to satisfying statistical assumptions, including normal distribution of residuals that was graphically checked. A p-value less than 0.05 was considered significant.

RESULTS

Batches of hay and farms. A total of 629 batches of hay from 86 farms were included in the study with the number of hay samples per farm ranging from 2–24. Most batches were harvested in 2001 and 2002 (90%) from the 1st crop (69.5%), from natural meadows (75.5%), and packed in HDRB (68%) (Tab. 2). Characteristics of farms in terms of grassland surface, climatic area and altitude are presented in Table 3.

Multilevel analyses

Results from the multilevel analyses are reported in Table 4 (only coefficients of variables entered in the full model are shown).

Absidia corymbifera. The full model for *A. corymbifera* indicated that concentrations of this fungal species were statistically higher in hay samples from HDRB than in those from lower density packing: MDCB ($p<0.05$), LDCB ($p<0.05$) and bulk ($p<0.01$). Hay harvested on the 2nd plateau (]700–900] m) was significantly more loaded ($p<0.01$) with *A. corymbifera* than hay from the plain (<500 m). A significant cross-level interaction was detected between climatic conditions of harvest and altitude: the effect of bad climatic conditions of harvest on concentrations of *A. corymbifera* in hay (positive association) was stronger for the 2nd plateau ($p<0.01$) and mountain ($p<0.05$) than for the plain (not significant).

Eurotium spp. Hay from the 2nd or 3rd crop was significantly less loaded with *Eurotium* spp. ($p<0.01$) than hay from the 1st crop. Bad climatic conditions of harvest and a relative humidity of batches of hay greater than 19.1% were significantly and positively associated with a high concentration of *Eurotium* spp. ($p<0.05$) in hay. As for altitude, hay from the 2nd plateau had higher concentrations of *Eurotium* spp. than hay from the plain ($p<0.01$).

Wallemia sebi. The model developed for *W. sebi* showed that bad climatic conditions of harvest were positively associated with high concentrations of this mould in hay ($p<0.01$). In addition, hay samples from P2 (February–March) contained lower concentrations of *W. sebi* ($p<0.001$) than those from P1 (December–January). Moreover, unlike previous microorganisms, the concentration of *W. sebi* in hay was significantly and negatively associated with the altitude of farms, that is, lower in batches of hay from the 2nd plateau ($p<0.05$) and the mountain ($p<0.01$) than in those from the plain.

Mesophilic *Streptomyces*. For mesophilic *Streptomyces*, only bad climatic conditions of harvest were significantly and positively associated with higher microbial concentration in hay ($p<0.01$).

Thermophilic actinomycetes. The thermophilic actinomycetes model showed that batches of hay from the 2nd or 3rd crop, artificial or mixed meadows, and harvested in bad weather were significantly more loaded with thermophilic actinomycetes than those respectively from the 1st crop ($p<0.05$), natural meadows ($p<0.01$), and harvested in good weather ($p<0.05$). In addition, concentrations of thermophilic actinomycetes in hay were higher during P2 than during P1 ($p<0.05$). Moreover, hay from the 2nd plateau had higher concentrations of thermophilic actinomycetes than hay from the plain ($p<0.01$). Concentrations of thermophilic actinomycetes were statistically higher in samples from HDRB than in those from hay in bulk ($p<0.01$).

Three of the 5 models showed a significant random effect of one variable: *A. corymbifera* (relative humidity), *Eurotium* spp. (use of a machine for hay-packing) and *W. sebi*

Table 4. Multilevel regression analyses[a] for concentration (logarithmic value of concentration+1) of *Absidia corymbifera*, *Erotium* spp., *Wallemia sebi*, mesophilic *Streptomyces* and thermophilic actinomycetes in hay (n = 629). Significance: * p<0.05, ** p<0.01, *** p<0.001.

	Absidia corymbifera	*Erotium* spp.	*Wallemia sebi*	Mesophilic *Streptomyces*	Thermophilic actinomycetes
Parameter			Estimates (SE[b])		
Fixed part					
Intercept	1.687 (0.246)***	2.626 (0.252)***	3.294 (0.330)***	2.524 (0.201)***	1.774 (0.190)***
Batch of hay factors					
Hay type (reference: 1st crop)					
2nd or 3rd crop	/	**-0.309 (0.114)****	/	/	**0.234 (0.111)***
Meadow type (reference: natural)					
Artificial or mixed	/	0.272 (1.162)	/	/	**0.439 (0.166)****
Moist field	/	0.186 (0.188)	/	/	/
Presence of voles or moles	/	/	/	/	0.434 (0.259)
Climatic conditions of harvest (reference: good)					
Bad	-0.243 (0.472)	**0.593 (0.275)***	**1.065 (0.400)****	**0.826 (0.300)****	**0.579 (0.265)***
Drying time (reference: ≤ 3 days)					
> 3 days	/	-0.135 (0.144)	-0.445 (0.262)	-0.225 (0.168)	/
Salting	/	/	0.424 (0.222)	/	/
Use of a machine for hay packing	/	0.061 (0.160)	/	0.267 (0.179)	/
Hay-packing mode (reference: high density round bales)					
High density cubic bales	0.002 (0.417)			-0.593 (0.445)	-0.505 (0.399)
Medium density cubic bales	**-0.633 (0.311)***	/	/	-0.247 (0.320)	-0.204 (0.287)
Low density cubic bales	**-1.079 (0.441)***	/	/	-0.860 (0.456)	-0.525 (0.404)
Bulk	**-0.600 (0.199)****			-0.005 (0.190)	**-0.539 (0.167)****
Relative humidity (reference: ≤ 19.1%)					
>19.1%	-0.306 (0.178)	**0.325 (0.135)***	/	/	/
Period of hay sampling (reference: P1 = Dec–Jan)					
P2 = Feb–Mar	-0.409 (0.212)	/	**-0.895 (0.271)*****	-0.392 (0.220)	**0.509 (0.200)***
P3 = April	0.458 (0.434)		-0.172 (0.521)	-0.211 (0.473)	0.716 (0.431)
Farm factor					
Altitude (reference: plain = < 500 m)					
1st plateau = [500–700] m	0.338 (0.287)	0.489 (0.266)	-0.596 (0.333)		0.525 (0.280)
2nd plateau =]700–900] m	**0.821 (0.264)****	**0.699 (0.248)****	**-0.607 (0.305)***	/	**0.776 (0.254)****
Mountain = > 900 m	0.349 (0.306)	0.506 (0.286)	**-0.928 (0.367)****		0.506 (0.300)
Cross-level interactions					
Bad climatic conditions of harvest × 1st plateau	1.393 (0.758)				
Bad climatic conditions of harvest × 2nd plateau	**2.322 (0.714)****	/	/	/	/
Bad climatic conditions of harvest × Mountain	**2.043 (0.968)***				
Random part					
Level 2: farm					
σ^2_{uo}: variance of intercept	0.525 (0.204)**	0.731 (0.182)***	1.164 (0.487)*	0.678 (0.148)***	0.570 (0.121)***
σ^2_{u1}: variance of random coefficient for a [variable]	0.329 (0.285) [hygrometry]	0.125 (0.240) [machine for hay packing]	0.851 (0.641) [drying time]		
σ_{uo1}: covariance	-0.110 (0.196)	-0.356 (0.184)	-0.697 (0.480)		
Level 1: batch of hay					
σ^2_e: variance between batches of hay	1.585 (0.099)***	1.429 (0.087)***	3.220 (0.199)***	1.749 (0.106)***	1.356 (0.082)***
Intra-farm correlation[c]	0.29	0.09	0.16	0.28	0.30

[a] Only coefficients of variables from the full model are shown; [b] SE – standard error; [c] Intra-farm correlation = $(\sigma^2_{uo} + \sigma^2_{u1} + 2\sigma_{uo1})/(\sigma^2_e + \sigma^2_{uo} + \sigma^2_{u1} + 2\sigma_{uo1})$

(drying time). This means that respective coefficients for "relative humidity", "use of a machine for hay-packing", and "drying time" varied significantly across farms. For these models, the model with a random coefficient fitted better data than the model with a fixed coefficient. No significant effect of "moist field", "presence of voles or moles", "drying time", "salting", "use of a machine for hay-packing", "year of harvest", "storage location", "grassland surface", "temperature area" and "rainfall area" was identified on the concentration of the 5 microorganims in hay (the 5 latter variables are not mentioned in Table 4).

DISCUSSION

This statistical approach took into account the clustering of data and thus allowed us to evaluate factors modifying the microbiology of hay with a simultaneous analysis of batch of hay- and farm-level factors. In this presentation, the pertinence of using a multilevel modelling was justified *a posteriori* by the fact that in the 5 models, the residual variance at the farm-level remained significantly different from zero in the full model. Moreover, intra-farm correlations were not negligible (between 0.09–0.30). This means that variance at the farm-level accounted for between 9%–30% of the total variance of the concentration of microorganism in hay. Thus, the implementation of multilevel models for clustered data has the following advantages: the correction of underestimation of standard errors, the examination of cross-level interactions and the estimation of the variability of coefficients at the cluster level. In this study, we had a large number of hay samples, which made it possible to confirm or reverse some findings from previous studies and identify new facts. Indeed, this work confirmed the deleterious effect of bad climatic conditions of harvest on the microbiological quality of hay [15] with a proliferation of harmful microorganisms (*A. corymbifera*, *Eurotium* spp., *W. sebi*, mesophilic *Streptomyces*, thermophilic actinomycetes). This positive association was found for thermophilic actinomycetes with hay from 2nd or 3rd crops (*vs.* 1st crop) and from artificial or mixed meadows (*vs.* natural meadows). Moreover, the negative association between concentrations of *Eurotium* spp. and hay from 2nd or 3rd crops (*vs.* 1st crop) was confirmed. The findings of previous studies [11, 14, 15] concerning the ineffectiveness of salting as a hay preservative and the influence of high-density hay-packing modes were reinforced. This was especially true for HDRB which presented higher concentrations of *A. corymbifera* and thermophilic actinomycetes because this hay-packing mode retains moisture more easily and longer. As for the microbiological evolution of hay, only *W. sebi* and thermophilic actinomycetes showed a significant peak of concentration, respectively during P1 (Dec–Jan) and P2 (Feb–Mar) periods. Our study highlighted the influence of altitude on the microbial concentration of hay with etiological agents of FLD (*A. corymbifera*, *Eurotium* spp., and thermophilic actinomycetes): a gradient of microbial concentration from the plain to the mountain was identified. This gradient is a reflection of the humidity (higher in mountain than in plain) and is consistent with the results of Dalphin *et al.* [4], who demonstrated a positive correlation between altitude and the prevalence of FLD in Franche-Comté. Regarding *W. sebi*, an osmophilic fungal species, this gradient of microbial concentration was reversed, from the mountain to the plain. The role of humidity may again be involved (drier climate in the plain), given the ability of this mould to grow under relatively dry conditions (i.e. low water activity, a_w=0.65). This result is not in contradiction with high concentrations of *W. sebi* in hay harvested in bad climatic conditions, given the wide range of water activity in which it can grow and of the temperature, higher in plain than in mountain [6, 20]. This singular pattern of *W. sebi* compared to others microorganisms, notably *A. corymbifera* and *Eurotium* spp. is in line with recent studies [15, 17] that argue for excluding this mould from etiological agents of FLD in the region. A previous study [15] evoked the role of voles on the proliferation of harmful microorganisms in hay; this finding was not confirmed in our work. However, the number of batches of hay harvested in a meadow with voles was relatively low (n=34). Moreover, no effect of the year of harvest was detected. This should, however, be taken with caution given that most of the batches (90%) were harvested in 2001 and 2002.

Our study has several limitations. Firstly, the sampling of hay was not performed at equal duration of the harvest. Indeed, batches of hay harvested between May–September were sampled between November–April. However, this potential bias was partly controlled by 2 variables: "hay type" and "period of hay sampling". Secondly, the composition of plant species constituting hay varies depending on the altitude; this was not taken into account. This variability of plant species can influence the composition of the soil microbial species [21], and therefore hay. Thirdly, to date, many applications of multilevel analysis in the health field have used data collected for other purposes, usually during traditional single-level studies; that is a limitation *de facto*.

In conclusion, this study, by examining simultaneously the effect of batch of hay-level and farm-level factors, enabled us to complete, consolidate and clarify previous findings about the factors that influence the microbial concentration of hay with etiological agents of FLD. Finally, our study performs a synthesis which we hope will contribute to the implementation of appropriate strategies to prevent FLD, such as the promotion of low risk hay-packing modes (low density cubic bales), artificial drying systems, and use of respiratory protection in the case of handling mouldy materials.

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