

The Effect of Ionization on Air Quality in a Horse Stable

Utrecht University – Faculty of Veterinary Medicine

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Drs. Milou Anthonisse

Dr. C.M. Westermann
Dr. F.J.C.M. van Eerdenburg
Dr. Ir. I.M. Wouters

Index

Summary	Fout! Bladwijzer niet gedefinieerd.
Introduction.....	4
Methods	6
Study design.....	6
Exposure measurements.....	8
Computational and statistical analysis.....	9
Results	11
Dust filters and endotoxins.....	11
Airborne cultivatable fungal colonies	16
Discussion.....	19
Literature.....	22
Appendix 1.....	24
Appendix 2.....	26
Appendix 3.....	27

Summary

Horses kept in stables are likely to be exposed to high levels of organic dust. Organic dust plays a role in increased risk of inflammatory reactions and is associated with respiratory diseases. There are multiple ways to decrease the amount of organic dust in the horse stable, of which the most common are a change in bedding and feeding regime and an increase in natural ventilation. The aim of this study was to investigate the effect of the ionization of air on dust, endotoxin and fungus in horse stables, and the differences between those levels in stables with horses kept on shavings eating haylage and stables with horses kept on straw eating dry hay.

Four units (96m² each) of six boxes were used. Each unit was equipped with an ionization installation, composed of a ionization box connected to electricity network, 3 corona wires and 4 earthed wires (all wires had a length of 12m). In 2 units, horses were kept on shavings and were fed haylage and in the other 2 units horses were kept on straw and were fed dry hay. Ambient inhalable dust samples were collected on a fixed, similar position within each unit. Samples were taken in each unit with and without activated ionization and during daytime and during nighttime, repeatedly over the course of a week. In addition, sampling was performed on 5 different positions in each stable unit to verify if there are locations within stable units that have consistent higher dust levels than other locations. The dust samples were analyzed for endotoxin by the Limulus Amebocyte Lysate assay. Culturable fungi were collected with an Anderson impactor on DG-18 Agar plates, with a sampling time of 30 seconds per sample.

156 samples were examined for dust levels and 154 samples were examined for endotoxin levels. Highest dust and endotoxins levels were found in the units where the horses were kept on straw, being fed dry hay. Comparing day and night, higher dust and endotoxins levels were found during daytime. 86 samples were taken for the examination of growth of fungal colonies. Samples taken in straw units showed higher growth of fungal colonies than samples taken in units where horses were kept on shavings. Nighttime sampling showed less fungal growth than daytime sampling.

No difference in dust, endotoxins or fungal growth were found when ionization was activated. Dust, endotoxins and fungal spores do tend to be significantly lower in stables where horses are being kept on shavings, being fed with haylage, compared to stables where they are being kept on straw and being fed with hay.

Introduction

Most sport horses are kept in stables and spend only a part of the day in the pasture or paddock. Keeping horses stabled decreases the risks of all kinds of injuries, but increases the risks of respiratory diseases due to inhalation of high levels of organic dust (Fleming, Hessel, & Van den Weghe, 2008, Vol 28, No 7). Organic dust is composed of materials from microbial, plant and animal sources and may contain pathogenic or non-pathogenic living or dead bacteria and fungi, high molecular weight allergens, bacterial endotoxins, $\beta(1\rightarrow3)$ glucans, pollen, and plant fibers (Douwes, 2005). In humans the part of dust that reaches the airways is divided in three fractions, 'inhalable', 'thoracic' and 'respirable' dust fractions. The inhalable fraction is the part of the dust cloud that can be inhaled into the nose, and can cause nasal infections. The thoracic fraction is the part that can penetrate the head airways and enter the airways of the lung causing upper airway inflammation. The respirable fraction can penetrate beyond the terminal bronchioles into the gas-exchange region of the lungs and can cause lower airway infection and irritation (Hazard Prevention and Control in the Work Environment: Airborne Dust, 1999). The size of dust particles that are considered to be respirable, differs between 0,5 and 5 μm (Clemens & Pirie, 2007) Therefore, respirable dust is considered as a good index to evaluate the health hazard of airborne dust inhalation (Vandenput, Istasse, Nicks, & Lekeux, 1997). Endotoxin is a component of the cell wall of Gram negative bacteria and a ubiquitous component of organic dusts (Douwes, 2003). Endotoxin has proven to be a powerful inflammatory agent and much of its toxicity is associated with the lipid A component, a part of the cell wall of Gram negative bacteria. Several occupational studies in humans have demonstrated that workers who come into contact with high levels of endotoxin have an increased risk of inflammatory reactions that are associated with respiratory diseases such as asthma, chronic bronchitis, and organic dust toxic syndrome (Liebers, Raulf-Heimsoth, & Bruning, 2008). In horses, research suggests that the inhalation of endotoxins is not the sole cause of heaves, but it is likely that it contributes to airway inflammation in concert with other inhalants in both horses already familiar with Recurrent Airway Obstruction (RAO) and in healthy horses, when they are exposed to high levels in poor stable environments (Pirie, Dixon, Collie, & McGorum, 2003).

Fungi are easily accumulated and aerosolized, acting as indoor air biocontaminants especially in densely horse-populated environments and in enclosed buildings. Continuous exposure may result in respiratory damage, as high environmental fungal burdens have been shown to be involved in the pathogenesis of chronic bronchitis/alveolitis, also called Recurrent Airway Obstruction (RAO) in stabled horses (Beeler-Marfisi, et al., 2010, Vol. 71, No. 6).

For the reduction of dust and endotoxin exposure, horse owners are advised to keep their horses sensitive to RAO outside, to feed the horses with pelleted feed (Reed, Bayly, & Sellon, 2004) and if this is not possible, with soaked or steamed hay (Clements & Pirie, 2007) (James & Moore-Colyer, 2009). In this study, we examined the effect of placing a negative air ionizer on the concentrations of dust, endotoxins and fungi in the air with 2 different types of stable beddings and feeding regimes. Recently, the use of air purifiers has gained new interest, due to their claimed bactericidal effects. These effects can be caused by several mechanisms of electrical origin; the action of ions, the production of ozone or the action of the electric field (Fletcher, et al., 2007). The emission of bipolar ions enhances the agglomeration of smaller particles into larger ones, which then gravitationally settle and thereby purify the air. Ionization may also cause attraction between particles and

grounded surfaces resulting in electrostatic deposition, thereby reducing the levels of airborne dust in the room where the ionizer is placed (Grinshpun, Mainelis, Trunov, Adhikari, Reponen, & Willeke, 2005). Besides this, the differences between 2 types of stable management, i.e. one where the horses were kept on shavings and being fed with haylage, and one where the horses were kept on straw and being fed with dry hay, were investigated, as well as the difference in concentration levels during day and night. Four different but similar stable units were used, and specific locations were sampled in order to determine if there were locations within those units with a higher concentration in dust, endotoxins or fungal spores. This in order to determine where a horse with respiratory problems should be situated. Is this horse best placed close to a door? Or maybe he's best off in the back of the stable where there is the least air movement?

Methods

Study design

All sampling was done in the clinic of the Faculty of Veterinary Medicine, Utrecht. All the samples were collected in an 8 week period. The ambient inhalable dust and viable airborne fungal samples were collected in four identical stable units, each giving room to 6 stalls. Dimensions of these units were 12m by 8m by 4m. In two of the stable units the horses were kept on straw and fed with dry hay. In the other two the horses were kept on wood shavings and fed with haylage. All stalls were occupied by one horse per stall during the whole period of this study, so 6 horses in total in each stable unit. There were no restrictions for the use of the horses during this study. All horses were turned out and trained on a daily basis. The main doors to the stable units were permanently opened to provide the best ventilation possible. In each unit the windows were opened and next to the natural ventilation there was also a lower pressure ventilation mechanism active. The air collected by this ventilation mechanism was directed to the other side of the building and there expelled in the air.



Fig 1: A plan of a single unit used in these studies. The blue circle indicates the standard location for sampling. The letters A - E indicate the locations where the variable samples were taken. The arrows indicate doors, with the middle door being an important inlet for fresh air. The red lines indicate the corona wires, the blue lines indicate the iron wires that are necessary to create sufficient earthed surface.

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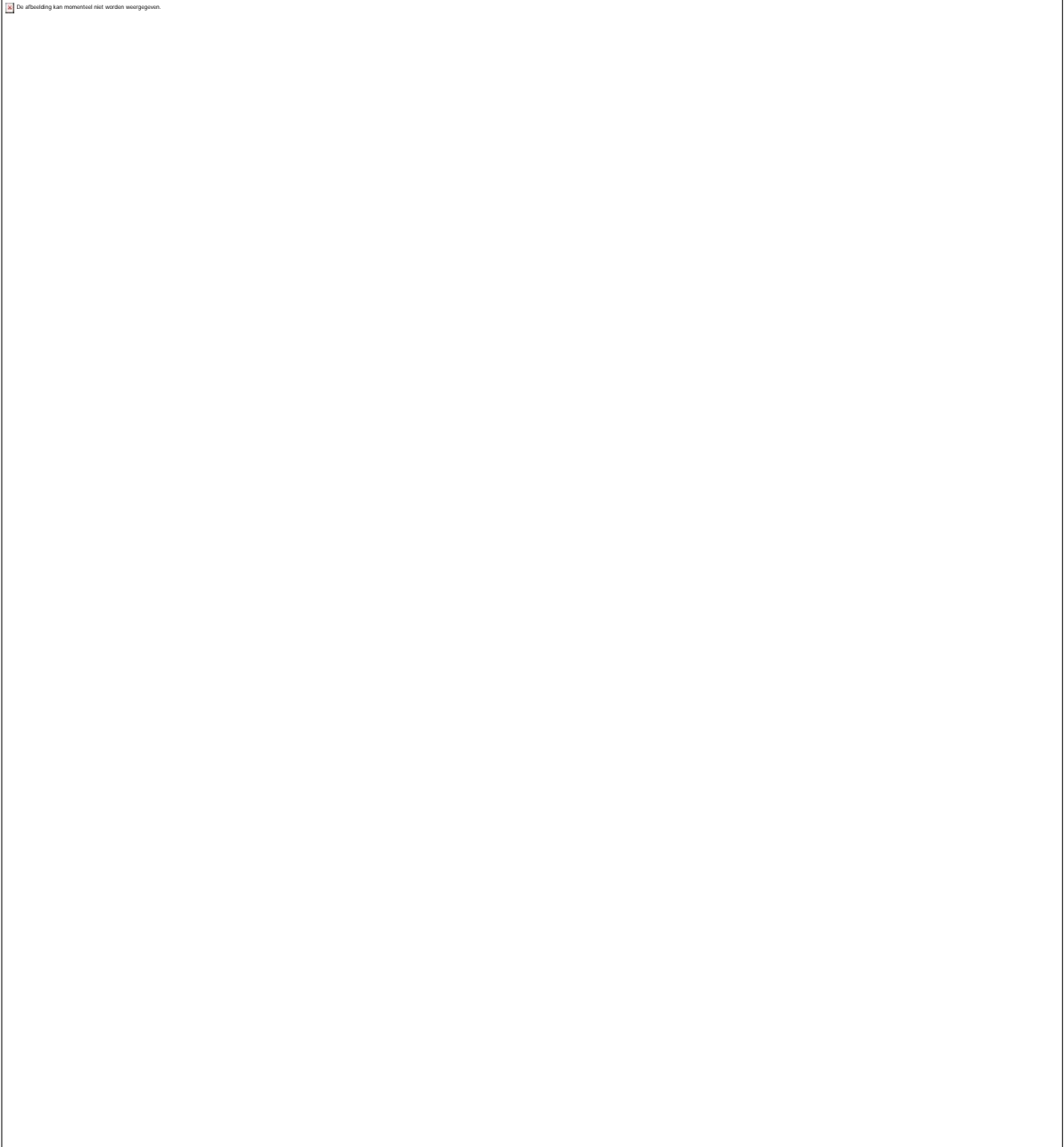


Fig 2: A plan of a part of the stables used for these studies. The blue lines indicate the airflow. Fresh air can enter through the main door at the bottom of the scheme and through the windows on the side of each unit. The blue boxes indicate the location of the active ventilation system. The red lines indicate each unit.

All stable units were equipped with an negative ionization installation composed of an ionizationbox fixed to the wall and ionization wires (corona wires) that were strained from one side of the stable unit to the other side (Appendix 1). The power on the corona wires was 5000 Volts. At a 30cm distance from the corona, iron wires were strained trough the stable unit to create the necessary earthed surface. The ionization installation was switched on and off by plugging the plug in the power socket. After 4 weeks of sampling, an extra iron gauze was installed to increase the earthed surface. Two units (one with shavings and one with straw bedding) were with the activated

ionization, whereas the other two had no ionization. This was altered per week, in order to prevent possible influences of the location of the unit in the building.

Eight hour ambient inhalable dust samples were taken repeatedly during daytime (8.00 a.m. to 4 p.m.) and during nighttime (12a.m. to 8.00 a.m.). Airborne fungal samples were collected on 3 different moments, 12.30 a.m., 9a.m and 4.30p.m. Regular work pattern for the stable workers had 3 shifts. The first shift was from 7a.m. to 3p.m., the second from 3 p.m. to 11p.m., and the third from 11p.m. to 7a.m. During the first shift most work is done in the stables. The stalls are being mucked out, fresh bedding is supplied, floors are being swept, horses go to the horse walker and if necessary, medication is administered. The third shift consists of mainly observational tasks. We have chosen to take samples during the first and third shift, due to the biggest difference in activity and the biggest expected difference in inhalable dust and airborne fungal levels.

After 4 weeks of sampling, a iron gauze was installed in the stable units with straw. This was to see if increasing the earthed surface would enhance the positive effects of ionization on the air quality. After installation of the gauze we sampled another 2 weeks, during daytime.

Week number	Sampling	Unit 1	Unit 2	Unit 3	Unit 4
1	Day	On	Off	On	Off
2	Day	Off	On	Off	On
3	Night	On	Off	On	Off
4	Night	Off	On	Off	On
5	Day	Off	On	Off	On
6	Day	On	Off	On	Off

Fig 3. Scheme showing when ionization was turned on/off in which unit and which time of the day samples were taken. Unit 1 and 2 are the units where horses are being kept on shavings, being fed haylage. Units 3 and 4 are the units where horses are being kept on straw, being fed dry hay.

Exposure measurements

Stationary inhalable dust samples were collected on 37mm glass fiber filters (Whatmann, GFA) using a Gillian Gil-Air 5® constant flow pump and Gesamtstaubprobenahme an der Person (GSP) sampling heads (Appendix 1). All filters were weighed before use. The pump flow rate (3.5 l min⁻¹) was calibrated using a rotameter at the beginning and the end of each sampling period. Each sampling period lasted for 8 hours. The volume of air sampled was calculated, based on the flow rate and duration of sampling. Calibrating the pump at the end of each sampling period provided information about the potential loss in flow during the sampling period. If the decrease in flow is below 5%, the loss is acceptable. The average air flow in liters per minute was multiplied by the amount of minutes the Gil-Air 5® pump had been running. The dust samples were stored until further processing at -20°C immediately after the sampling period.

The ambient inhalable dust samples were collected at a fixed position, which was the same for all 4 stable units, at approximately 150 cm above the floor level. In addition, we collected dust samples at 5 different predetermined spots, also identical in the 4 stable units to see if there was a location within the units with higher levels of dust. These spots were labeled with the letters A, B, C, D and E (See figure 1 for the location of these spots). During the preparation of the GSP sampling heads for

each sampling day, blank samples were also run to make sure there was no contamination during the preparation phase.

The amount of dust on the filters was calculated by pre- and postsampling weighing of the filters on a Mettler AX 105[®] analytic scale in an acclimatized room. The filters were conditioned to room temperature and humidity for 24 hours prior to weighing. This was transferred into a dust concentration by taking into account the samples volume of air.

For endotoxin determination, endotoxin was extracted as described previously (Spaan et al). Briefly, filters were immersed in 4ml 0.05% Tween20 in pyrogen-free water and rocked vigorously by an end-to-end roller, for one hour at room temperature. After 15 minutes of centrifugation at 1000 G (=2094 rpm), supernatant was harvested and stored in 0.1 ml aliquots at -20°C until analysis. All extracts were analysed with the kinetic chromogenic Limulus amoebocyte lysate (LAL) method 1:500, without using Tween during the assay as described by Spaan et al. (Spaan, et al., 2008) .

Cultivable fungi were collected with an Andersen one-stage 400 hole impactor, with a flow rate of 28.3 l min⁻¹ for 30 seconds. Each time 5 samples were collected, in each unit one, and one control sample that was only placed inside the impactor, without the Andersen pump being switched on. For the collection of fungi dichloran-glycerol agar 18 (DG-18) plates were used. Samples were collected at 3 different moments. The first moment was at 9p.m. The second moment at 5a.m. and the third one at 1p.m. In between the samplings, the impactor was cleaned by wiping it with ethanol. Before taking another sample, some time was given to let the ethanol vaporize completely. At the end of the day, the samples were incubated for 4 days at 24°C. The samples that were collected in the morning had been stored at 4°C until the end of the day.

Colonies were counted in duplicate, and the counts were adjusted for multiple hits in a jet, using the positive hole correction factor (Andersen, 1958). The average of two counts was divided by the volume of air sampled to express the bioaerosol concentrations as colony-forming units per cubic meter of air (CFU m⁻³). The numbers of counted colonies were corrected for blank values (Samadi, Wouters, Houben, Jamshidifard, van Eerdenburg, & Heederik, 2009).

It was determined if the use of a ionizator decreased the amount of colony forming units per cubic meter air, if there was a difference in CFU's between straw and shavings and if there was a difference in the CFU per cubic meter air during day- or nighttime. The amount of colonies that grew on the DG-18 Agar plate when the ionizator was switched on was compared with when it was switched off.

Based on blank filters, the lower limit of detection (LOD) for dust weight was 0.01 mg. This corresponds to a dust concentration of 0,059 mg m⁻³. The limit of detection of the endotoxin assay was on average 0.015 EU ml⁻¹ for undiluted samples but since the samples here were diluted 500 times, the LOD low was 7.5 EU per sample. Since the samples were extracted in 4 mls the the LOD low per filter is 30 EU, which corresponds to an endotoxin concentration of 17,820 EU m⁻³ ; two (1.25%) of the successful samples were below this LOD.

Computational and statistical analysis

A general linear model was carried out with as main effects bedding (straw/shavings), moment (day/night) and ionization (on/off). Stall is nested within bedding and week is nested within moment. The interaction between bedding x moment and bedding x ionization was viewed. The

residuals were checked for normality. Data analysis was carried out with 'R' software version 3.1.0. (www.r-statistics.com). Later data analysis have been done on the log-normally transformed data for total dust and endotoxins for straw and shavings to see if installation of the gauze had beneficial effects (Wilcoxon signed rank test). This analyses was carried out in SPSS.

Results

Dust filters and endotoxins

A total of 159 dust and endotoxin samples were collected. Of the 159 collected samples, 3 dust and 5 endotoxin samples were lost during analysis and extraction. Thus, 156 samples were examined for dust levels, 154 samples were examined for endotoxin levels. Of these samples none were below the limit of detection (LOD) for dust and 30 tested below the LOD for endotoxin. All but 2 were blank samples. During the collection of one of the 2 samples that tested below the LOD for endotoxin the GilAir 5[®] pump stopped working during the 8 hours sampling time. There was no information about how long the GilAir 5[®] pump kept working during this period of sampling, so this sample was excluded from the results. The outcome that all control filters but one tested below the limit of detection shows that no contamination during installation of the filters had occurred. The single control filter that was not tested below the LOD had been exposed to the air during the whole sampling period of 8 hours, without being attached to a GilAir 5[®] pump, so no air ran through this filter.

The mean sampling time was 481 minutes with a range of 455 to 524 minutes, this is taken into account when results are expressed per cubic meter of air that passed through the filters.

Table 3 gives a summary of the observed exposure levels for dust and endotoxins.

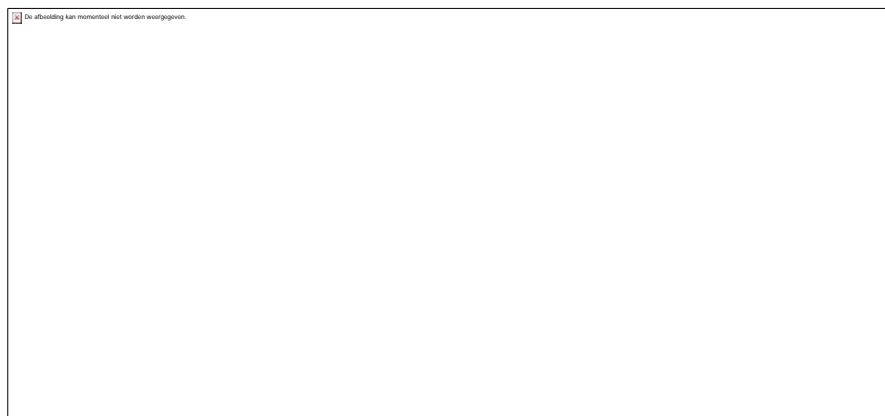


Fig. 3; Left: a glassfiber filter used in a unit filled with shavings. Right: a filter used in a unit filled with straw.

The general linear model for dust showed that there was a P-value of 2.25×10^{-7} for bedding, with an estimate of $1.630(\pm 0,274)$. Therefore the type of bedding had a substantial effect in the amount of inhalable dust in the horse stable. The analysis of ionization for dust showed a P-value of 0,35, with an estimate of $0,172(\pm 0,183)$. There was an interaction of straw to daytime sampling with a P-value of 7.0×10^{-4} , with an estimate of $0,952(\pm 0,266)$, and the interaction of straw related to stable unit showed a P-value of 3.0×10^{-3} , with an estimate of $0,557(\pm 0,179)$ so both are significant, which suggests that there is a convincing difference in the amount of dust contained by straw compared to that of wood shavings, and that there is less dust during night than during day. There was no significant P-value for ionization. So there was no major difference in dust level with the ionization activated, compared to when the ionization was not activated.

The general linear model for endotoxins showed that the effect of the type of bedding on endotoxins is even more significant than the effect on dust. There was a P-value of 2×10^{-16} , with an estimate of 2,80(±0,18) for bedding. In this analysis also the moment of sampling (day or night) showed significance. For moment there was a P-value of 3.7×10^{-4} , with an estimate of 0,69(±0,18).v This means that during day time, there are significantly more endotoxins than during nighttime. Finally there was a P-value of $0,046 \times 10^{-2}$, with an estimate of 0,32(±0,16) for the interaction between daytime sampling and week 2. This means that there is a higher level of dust in the second week, compared to week 1.

Table 1. Results for daytime sampling. Dust in mg m⁻³, endotoxins in EU m⁻³.

	Shavings				Straw			
	Unit 1		Unit 2		Unit 3		Unit 4	
	Dust	Endotox.	Dust	Endotox.	Dust	Endotox.	Dust	Endotox.
Week 1 Ionization activated in 1 and 3	0,113	181	0,112	168	1,152	2.232	0,666	1.525
	0,107	190	0,101	107	0,694	2.568	0,911	2.610
	0,058	109	0,113	178	0,543	1.445	0,996	2.514
	0,102	120	0,067	72	2,823	5.074	1,631	2.801
	0,178	210	0,016	131	0,742	3.072	1,694	4.152
Week 2 Ionization activated in 2 and 4	0,120	193	0,092	185	0,848	2.303	0,467	1.433
	0,071	189	0,121	136	0,939	3.179	0,776	2.001
	0,094	200	0,082	142	1,277	3.994	0,109	3.381
	0,070	139	0,100	165	0,899	4.331	0,976	4.482
	0,104	267	0,115	225	2,183	11.236	2,288	12.121

Table 2. Results for nighttime sampling. Dust in mg m⁻³, endotoxins in EU m⁻³,

	Shavings				Straw			
	Unit 1		Unit 2		Unit 3		Unit 4	
	Dust	Endotox.	Dust	Endotox.	Dust	Endotox.	Dust	Endotox.
Week 3 Ionization activated in 1 and 3	0,102	99	0,081	116	0,518	1.042	0,545	840
	0,109	110	0,334	149	0,334	863	0,413	1.089
	0,137	104	0,174	129	0,0192	528	0,280	598
Week 4 Ionization activated in 2 and 4	0,125	119	0,170	93	0,512	1,075	0,773	1.922
	0,082	54	0,093	46	0,914	1.862	0,783	1.852
	0,091	148	- ^a	- ^a	1,252	4.519	1,612	4.574

^a Gil Air 5 pump stopped working during sampling period

			Dust					Endotoxins				
			n	NM	AM	GM	GSD	n	ND	AM	GM	GSD
Wood shavings	Day	+	10	-	0,107	-2,276	0,287	10	-	166	5,09	0,24
		-	10	-	0,087	-2,558	0,604	10	-	164	5,05	0,37
	Night	+	5	1	0,122	-2,125	0,245	5	1	90	4,46	0,36
		-	6	-	0,148	-2,058	0,556	6	-	119	4,73	0,38
	Total	+	15	1	0,112	-2,226	0,275	15	1	141	4,88	0,14
		-	16	-	0,110	-2,371	0,620	16	-	147	4,93	0,39
Straw	Day	+	10	-	1,157	-0,020	0,580	10	-	3.790	8,02	0,65
		-	10	-	1,204	-0,120	0,374	10	-	3.864	8,11	0,53
	Night	+	6	-	0,703	-0,568	0,736	6	-	1.798	7,25	0,71
		-	6	-	0,653	-0,549	0,541	6	-	1.664	7,17	0,76
	Total	+	16	-	0,987	-0,226	0,676	16	1	3.043	7,73	0,77
		-	16	-	0,998	-0,131	0,542	16	-	3.039	7,76	0,75

N, number of samples; NM, number of missing samples; AM, arithmetic mean; GM, geometric mean; GSD, geometric standard deviation

		Dust			Endotoxins		
		AM	GM	GSD	AM	GM	GSD
Wood Shavings	Day	0,097	-2,296	0,308	165	5,07	0,31
	Night	0,136	-2,120	0,433	106	4,61	0,38
	Total	0,111	0,178	0,605	144	7,75	0,75
Straw	Day	1,181	0,042	0,519	3.827	8,07	0,58
	Night	0,678	-0,559	0,616	1.730	7,21	0,70
	Total	0,992	-2,300	0,482	3.041	4,90	0,39

N, number of samples; NM, number of missing samples; AM, arithmetic mean; GM, geometric mean; GSD, geometric standard deviation.

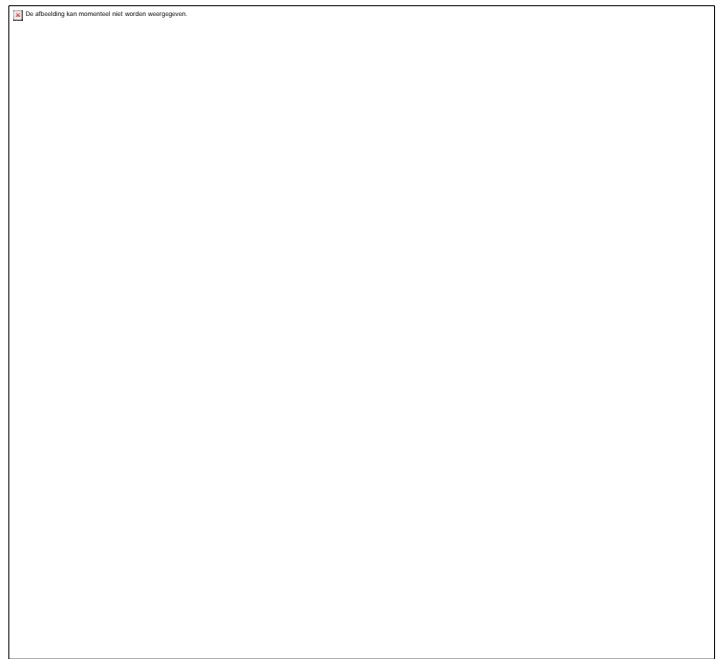
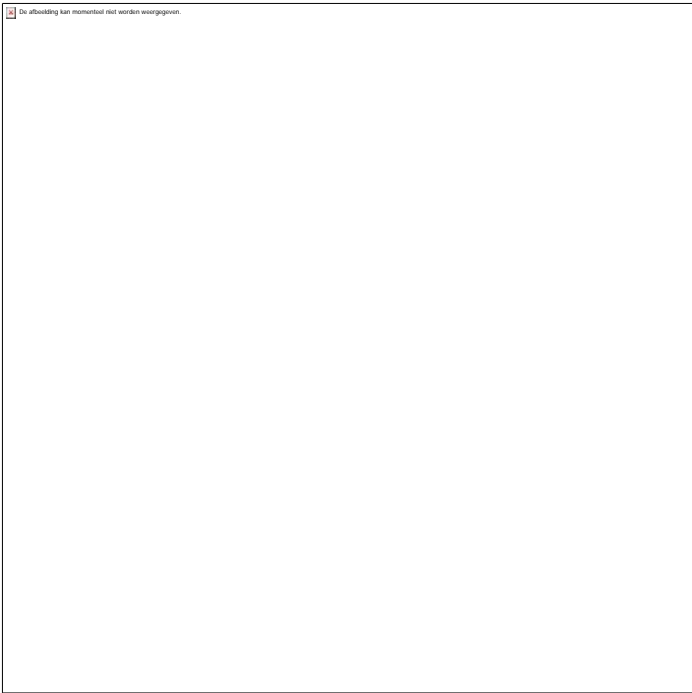


Fig. 3; amount of dust during day and during night. Differences in dust levels are bigger during the day, but in both day and night the level of dust is higher in the stable units where horses are kept on straw instead of shavings. Unit 1 and 2; horses are kept on shavings, being fed haylage. Unit 3 and 4; horses are kept on straw, being fed dry hay.

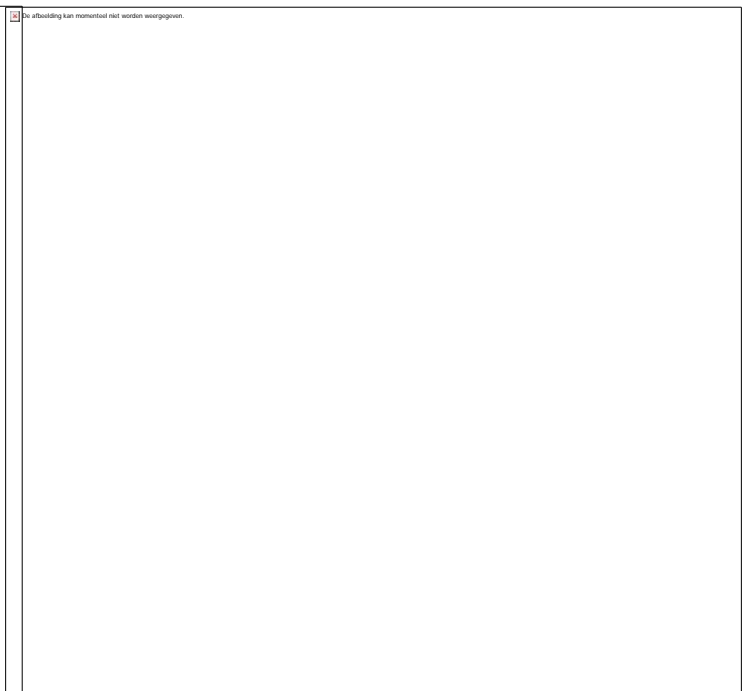
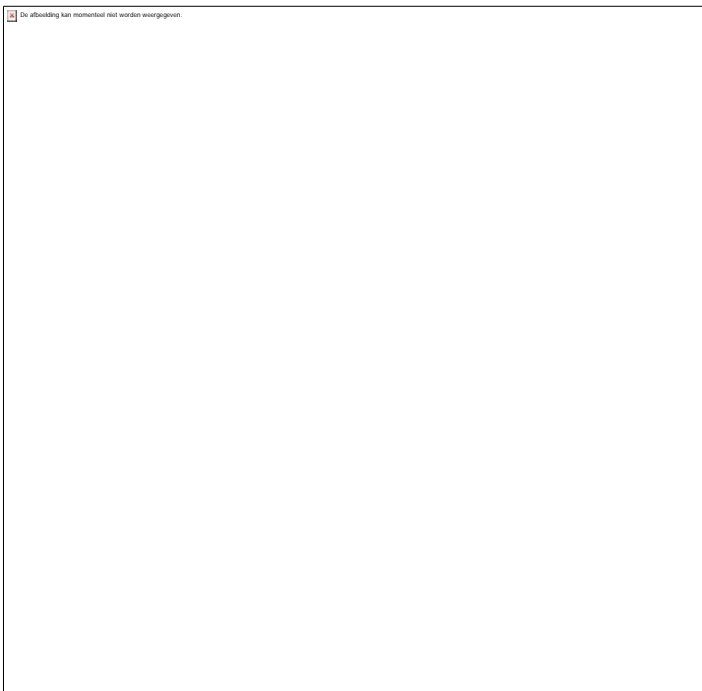


Fig. 4: levels of endotoxins during day and during night. The levels are higher during daytime and higher in the stable units where the horses are kept on straw. The levels of endotoxin fluctuate more in the straw units than they do in the units where the horses are kept on shavings. Unit 1 and 2; horses are kept on shavings, being fed haylage. Unit 3 and 4; horses are kept on straw, being fed dry hay.

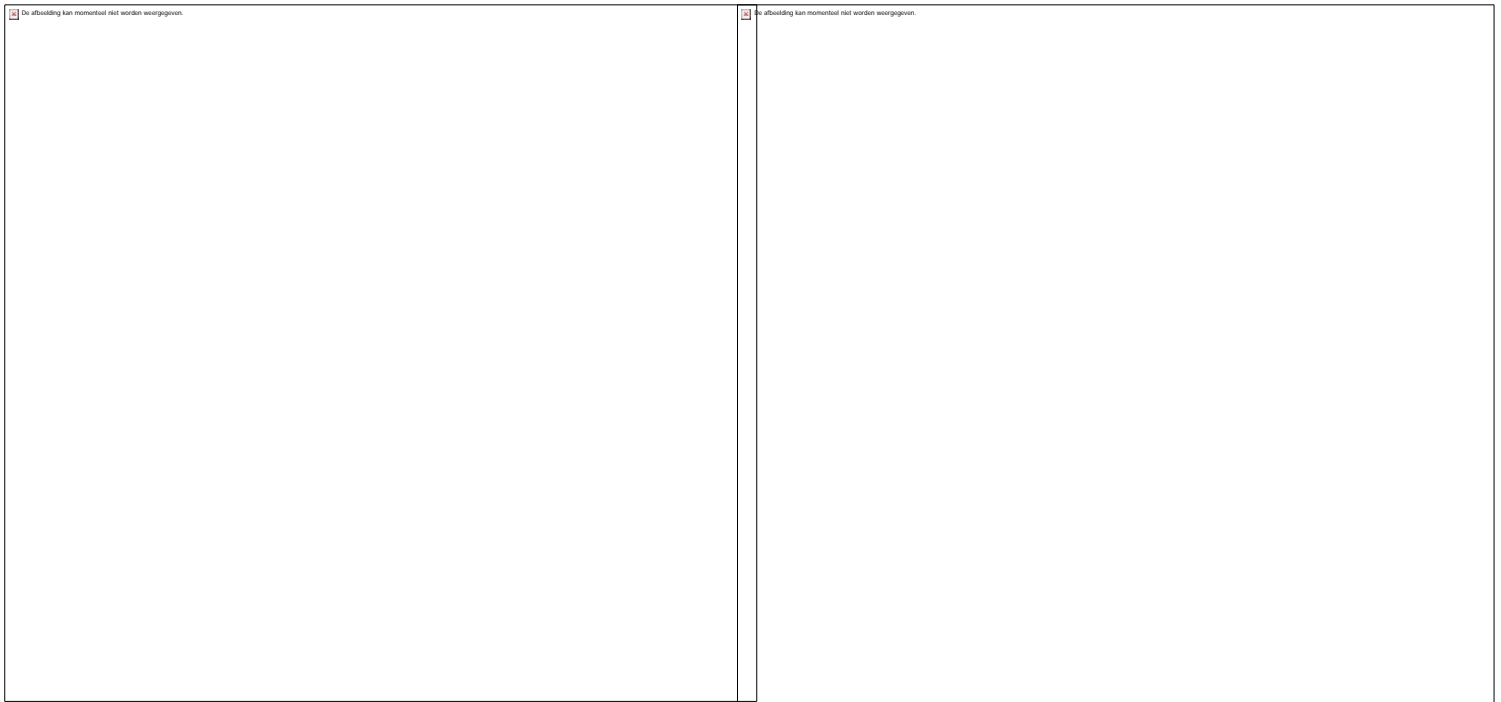


Fig. 5; representation of the additional samples taken on 5 different locations within each stable unit for dust and endotoxins. Unit 1 and 2; horses are kept on shavings, being fed haylage. Unit 3 and 4; horses are kept on straw, being fed dry hay.

The additional samples taken on the variable spots showed no consistency. There was no specific location within the stable units where a higher level of dust or endotoxins was measured in every unit. It does again show clearly that in general the levels of dust and endotoxins are higher in units where the horses are kept on straw.

After installation of the gauze in the stable units where horses were kept on straw, samples were repeatedly taken during daytime for the period of 2 weeks. A Wilcoxon signed rank test was used for analysis. For dust a Z-value of 0,560, and for endotoxins 0,523 was found with a P-value of 0,575 and 0,601. Therefore, installation of the gauze had no significant effect on dust and endotoxin levels. The Wilcoxon signed rank test also showed a significant correlation between dust and endotoxin ($R=0,81$, $0,01 < P < 0,001$).

Table 5. Results after installation of the gauze. Dust in mg m^{-3} , endotoxins in EU m^{-3} .

	Straw			
	Unit 3		Unit 4	
	Dust	Endotoxins	Dust	Endotoxins
Week 5 Ionization activated in 4	0,736	1.895	1,251	3150
	1,345	4.073	2,950	5.513
	1,299	3.590	0,908	2.047
	0,686	2.380	0,672	1.599
	1,314	2.053	1,055	2.284
Week 6 Ionization activated in 3	2,281	11.028	0,771	2.057
	2,322	6.063	- ^a	- ^a
	2,712	4.013	2,153	4.243
	0,843	1.449	0,525	995
	1,404	2.121	0,820	1.535

^a Gil Air 5 pump stopped working during sampling period

		Dust					Endotoxins				
		N	NM	AM	GM	GSD	N	NM	AM	GM	GSD
before	+	10	-	1,157	-0,020	0,580	10	-	3.790	8,02	0,65
	-	10	-	1,204	-0,120	0,374	10	-	3.864	8,11	0,53
after	+	10	-	1,640	0,370	0,532	10	-	3.927	8,07	0,66
	-	9	1	1,072	-0,021	0,443	9	1	2.536	7,74	0,48

N, number of samples; NM, number of missing samples; AM, arithmetic mean; GM, geometric mean; GSD, geometric standard deviation.

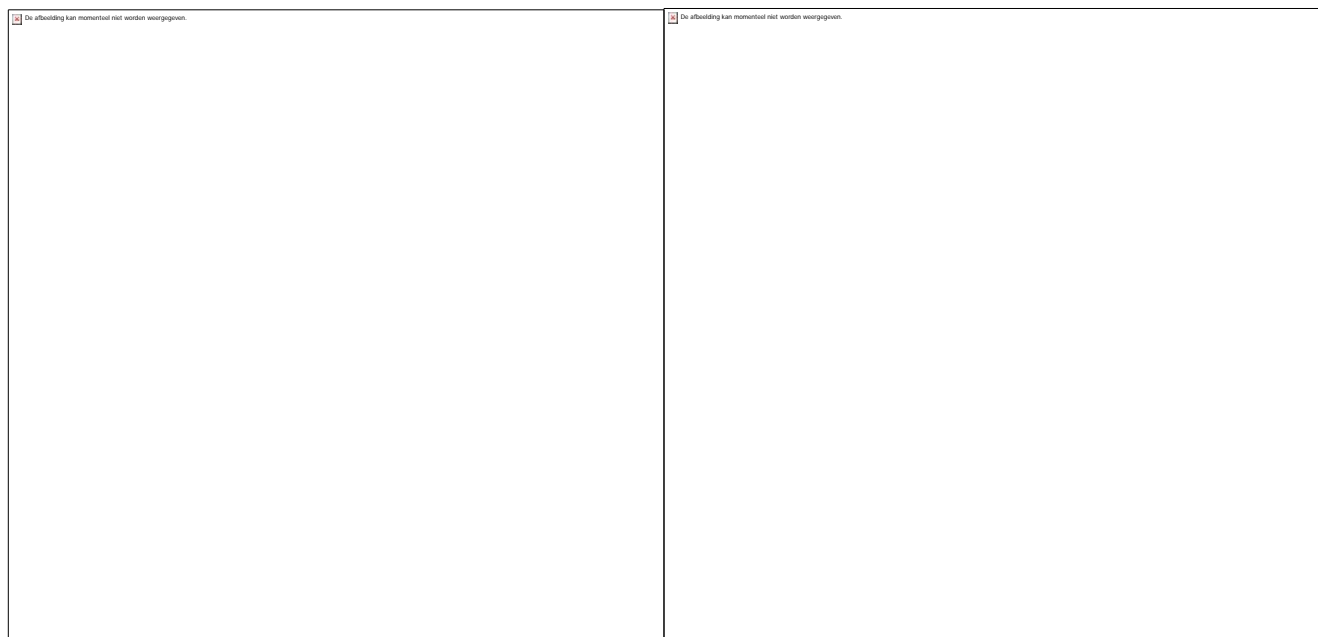


Fig. 6; Comparison in dust and endotoxin levels. The continuous line shows the results before installation of the gauze, the in continuous line shows the results after installation of the gauze.

	Dust			Endotoxins		
	AM	GM	GSD	AM	GM	GSD
Wood Shavings	0,110	-2,30	0,087	144	4,90	0,07
Straw	0,992	-0,178	0,107	3.041	7,75	0,13

AM, arithmetic mean; GM, geometric mean; GSD, geometric standard deviation.

Airborne cultivatable fungal colonies

Eighty six samples were examined for airborne cultivatable fungal colonies. All DG-18 Agar plates showed fungal growth after being incubated for 4 days at 24°C, but for unit 1 and 2, two night samples were excluded due to the fact the stables just were mucked and provided with fresh straw, while other nights were without any mucking activities. There was a visibility in color of the colonies that grew on agar plates that were sampled in the units with a bedding of shavings and in the units with a bedding of straw. The color of the fungal colonies collected in the units with shavings were predominantly yellow or white, while the samples collected in the units with straw were predominantly green or black. Microscopical examination of the colonies showed that the main fungal types that grew on the DG-18 Agar plates were *Penicillium spp.* and *Aspergillus spp.*, but also *Mucor spp.* and other black pathogenic fungal colonies that might belong to the *Exophiala* type were cultivated.

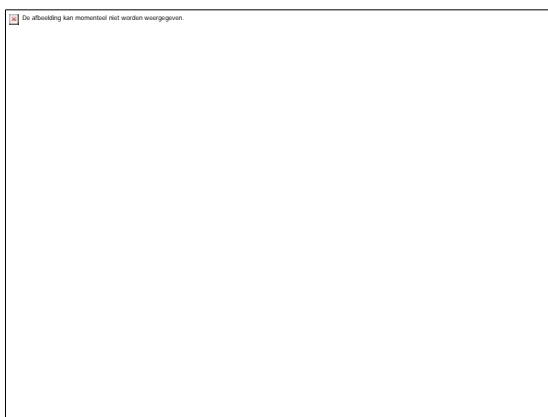


Fig. 7; Agar plate collected in a unit with shavings

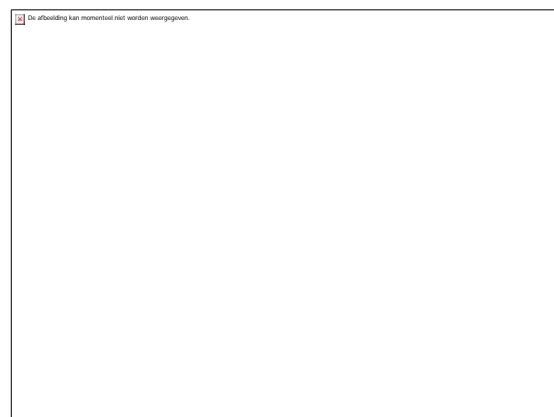


Fig. 8; Agar plate collected in a unit with straw

Besides the difference in color, there was a substantial difference in the amount of colonies that grew on samples collected in units with shavings and collected in units with straw as bedding. The viable fungal concentrations varied greatly, ranging from 0,32 CFU m⁻³ to 40,25 CFU m⁻³ for shavings and 6,28 CFU m⁻³ to 184,98 CFU m⁻³ for straw. The arithmetical mean (AM) of colony forming units per cubic meter of air (CFU m⁻³) for the units on shavings was 9,21 CFU m⁻³, with a geometrical mean (GM) of 1,25(±0,18) and for straw the AM was 148,03 CFU m⁻³, with a GM of 4,78(±0,87). The DG-18 Agar plates that were sampled in the units with straw bedding showed many times an excessive growth of fungal colonies. We couldn't really specify the amount of colonies on these plates, but since a 400 hole impactor was used we knew that there were at least 400 colonies. Using the positive whole correction for colonies (Macher, 1989). this resulted in an outcome of 2628 colonies per Agarplate.

Table 8. amount of CFU m ⁻³ per type of bedding				
		AM	GM	GSD
Wood shavings	+	3,58	1,03	0,73
	-	14,83	1,48	1,46
	Total	9,21	1,25	0,18
Straw	+	147,80	4,83	0,73
	-	148,26	4,74	0,99
	Total	148,03	4,78	0,87

AM, arithmetic mean; GM, geometric mean; GSD, geometric standard deviation.

The outcome of the linear model analysis for ionization was a P-value of 0,63, with an estimate of -0,1087 ($\pm 0,230$) so there was no significant difference in CFU's per cubic meter of air when the ionization was activated compared to when it was not activated. The outcome of the analysis for type of bedding was a P-value of 1.88×10^{-7} , with an estimate of 2,87 ($\pm 0,44$) and therefore significant. Since the difference between the 2 types of bedding was so great we could not really count the number of colonies on the samples taken from the straw units, regardless is ionization was switched on or off, we decided to take a closer look to the differences in shavings. The AM for CFU m⁻³ for shavings with deactivated ionization was 14,83CFU m⁻³ with a SD of 21,04, and with activated ionization it was 3,58 CFU m⁻³ with a SD of 2,66. A paired t-test was performed to see whether there was a significant difference between activated ionization and deactivated ionization for CFU for shavings. The activation of ionization elicited a statistically significant decrease in colony forming units in shavings compared to when ionization was not activated of 11,245 ($\pm 19,63$), $t(19)=2,562$, $p=0,019$. So we can state that horses kept on shavings and being fed with haylage are less exposed to fungal spores than horses kept on straw and being fed with dry hay.

		AM	GM	GSD
Wood Shavings	Morning	9,67	1,72	0,32
	Afternoon	6,74	1,06	0,28
	Midnight	2,56	1,21	0,51
Straw	Morning	175,37	5,04	0,18
	Afternoon	139,91	4,71	0,21
	Midnight	107,44	4,39	0,28

AM, arithmetic mean; GM, geometric mean; GSD, geometric standard deviation.

Discussion

The present study demonstrated the effect of negative ionization on the inhalable dust, endotoxin in a horse stable. Solely for fungal colonies, we saw a significant reduction in CFU m⁻³ for the use of ionization in shavings, not in straw. So far, this is the first study about the use of an air purifier in a horse stable. Other studies have focused on the use of air purifiers in poultry houses and hatcheries (Mitchell & Baumgartner, 2007) and universal confined indoor spaces (Grinshpun, Mainelis, Trunov, Adhikari, Reponen, & Willeke, 2005).

In the present study no convincing proof was observed for the installation of a negative air ionizer in the horse stables. Different explanations can be given why there was no significant difference in dust, endotoxin with the use of our air ionizer. The main doors to the stable units that have been used were opened for the whole 8 weeks. This is according to the standards in this specific stable. This to improve the ventilation in the stable units. Of course, this also greatly expands the cubic meters of air that can go in and out the stable units. Possibly, the air ionizer has a greater effect in a confined space with active ventilation.

Another possible reason for the ionizer not to work as expected, can be found in a shortage of earthed surface compared to the amount of cubic meters of air in the stable unit. The Dutch Government has stated some rules for the use of air ionization in the reduction of dust emission in poultry houses. Per square meter of surface, 0.45m of corona wire needs to be strung on a height of at least 2,5m from the ground surface (Rijksoverheid, 2009). Since no previous studies have been done about the use of air ionization in horse stables, we have extrapolated the rules stated for the poultry houses for the use in our horse stables. The stable units used in the present study have a surface of 96m², so 43ms of corona wire had to be installed. In our stable units only 36m of corona wires were strung. This could be a reason why the effects of the negative ionization in this study were so low. Initially, there was 48 meter of earthed surface (other wires strung trough the stable unit). The Dutch Government doesn't say how much earthed surface should be provided, but if this was the reason why there is no significant difference with or without ionization, the effect should have become evident after the installation of the gauze. This greatly expanded the earthed surface in the stable units. Unfortunately, expansion of the earthed surface after some weeks of sampling, did not resulted in a significant change in inhalable dust, endotoxin and CFU levels.

Pirie et al (2003). described a synergistic effect on airway inflammation in horses, between endotoxins and other components of organic dust. Dust that was free from endotoxins induced no respiratory effects. When solely endotoxins were inhaled, effects similar to those seen in RAO were observed but to a milder extent than when both organic dust and endotoxins were inhaled. Endotoxins are one of the few biologic agents where a value of maximal accepted concentration (MAC value) has been described, for humans. In the Netherlands the MAC value was 200 EU m⁻³ for endotoxins (Spaan, Smit, Visser, Arts, Wouters, & Heederik, 2004). At the moment, there are no defined MAC values for endotoxins but there are maximum advised values. The maximum advised value in the Netherlands is 90 EU m⁻³ over an eight-hour period of endotoxin exposure. This level is described as a Non Observed Effect Level (NOEL), and so it can be assumed to be safe (DECOS, 2010). We can see that the means of endotoxins for straw exceed the old MAC value for endotoxin substantially. With a arithmetic mean of 3041 EU m⁻³, straw bedding exceeded the MAC value for endotoxin more than 15 times. This MAC value is set for humans, but most probably this amount of exposure also is not healthy for horses. Besides, the animal care takers are exposed to these

enormous amounts of endotoxins too. When the arithmetical means were compared, shavings were a lot safer, with a mean of 144 EU m⁻³, this did not exceed the MAC value, though it is still higher than the maximum advised exposure of 90 EU m⁻³ (DECOS, 2010), and horses are, especially during winter, exposed to these levels for more than 8 hours a day.

There was a week effect for endotoxins in straw. The cause for this remains uncertain, but there was one thing that could have contributed to a great difference from week to week. Of course, different bales of straw were used. Supposedly, all bales of straw were coming from the same batch, but this is not certain. Even when from the same batch, the composition of the individual bales of straw can be different. Maybe the straw used in week 2 was coming from a wet part of the field where it was harvested, enhancing the likelihood of bacteria and mold to flourish. If the bales of straw were not even from the same batch, the differences could be even greater from bale to bale. Unfortunately, the real cause of this significant difference between the weeks remains unknown.

In the present study, the levels of dust concentrations were relatively low, compared to other studies. Woods et al (1993) found dust concentrations of 0,218 mg m⁻³ for horses kept on wood shavings and being fed with haylage, compared to 0,111 mg m⁻³ in this study and 4,018 mg m⁻³ for horses kept on straw and being fed with dry hay, compared to our 0,992 mg m⁻³. A major difference in study design in our study compared to other studies (Vandenput, Istasse, Nicks, & Lekeux, 1997) (Clemens & Pirie, 2007), is that we collected the samples on a fixed spot within the different, but similar stable units, while other studies collected the samples close to the horses nostrils. These samples show obviously a higher results for dust since horses are constantly exploring their feeding bins and stalls looking for food.

Fungal samples taken in units with straw show a more than 16 times higher growth of colonies than samples taken in units with wood shavings (9 CFU m⁻³ versus 148 CFU m⁻³ for straw). The finding of higher levels of CFU's in straw compared to (wood) shavings is consistent with the findings of other studies (Tanner, et al., 1998, Vo. 18-7), (Airaksinen, 2006). The exposure level of fungal burden was the highest during the morning (AM of 104,6 CFU m⁻³), and lowest during the night (69,7 CFU m⁻³), though the results showed no significant difference. These lower exposures during night are probably due to reduced horse- and personnel activity. Beeler-Marfisi et. al (2010) has consistently identified *Lichtheimia corymbifera*, *Aspergillus fumigatus*, and *Eurotium amstelodami* in moldy hay. Horses susceptible to heaves showed clinical signs of RAO after exposure with the moldy hay. Due to the difference in color of the agarplates after incubation we expect more pathogen fungal spores in straw bedding, but there was no quantitative determination of the fungal colonies done. What was clear after microscopical examination of the agarplates, was that there were high levels of *Penicillium spp.* and *Aspergillus spp.* in both types of stable bedding. The fact that there was a significant reduction in CFU's with ionization in the units where shavings were used might indicate that the ionizing device in this study design is not powerful enough to reduce the high amounts of fungal spores in the straw units.

Different studies have been done on sources of feed and types of bedding in relation to air quality. Vandenput et. al(1997), described the contrary to what we found in this study. They found the highest levels of dust and viable fungal colonies in wood shavings(31492±12910 particles/litre of air), and the lowest in straw(11571±4897 particles/litre of air). Despite the fact that both studies used wood shavings designed for the use in horse stables, a difference in type of shavings might be held

responsible for the difference in outcome. Clemens and Pirie (2007), found results that were similar to our results. The dust levels in their study for straw in combination with a feeding regime of dry hay were over 18 times higher than the levels they found for shavings in combination with haylage. In our study, dust levels were 9 times higher in the units with straw compared to the units with wood shavings. Clemens and Pirie (2007) found the highest levels of dust in stables where horses were kept on straw and were fed with hay ($4,018 \text{ mg m}^{-3}$). Units where horses were fed with haylage on a straw bedding showed lower dust values ($0,267 \text{ mg m}^{-3}$). This was even lower than when horses were kept on shavings, but fed with dry hay ($0,916 \text{ mg m}^{-3}$). So a change in feeding regime might even be more important than the type of bedding to improve the air quality in a horse stable.

Overall we can state that the installation of an air purifier in the form of a negative ionizator in the horse stable, under the conditions used in this study, does not contribute to a healthier climate in the stable, since it has no effect on the reduction of dust, endotoxins and viable fungal spores.

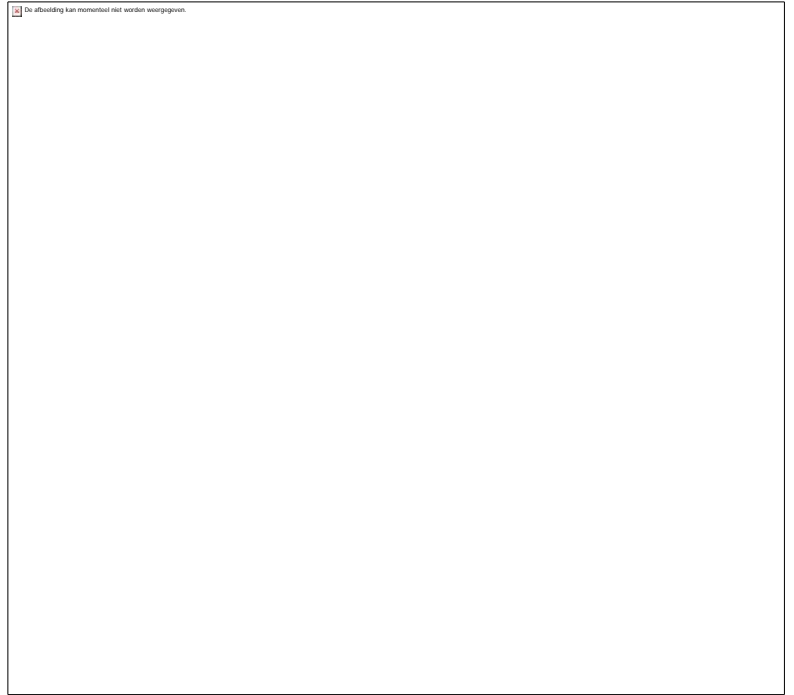
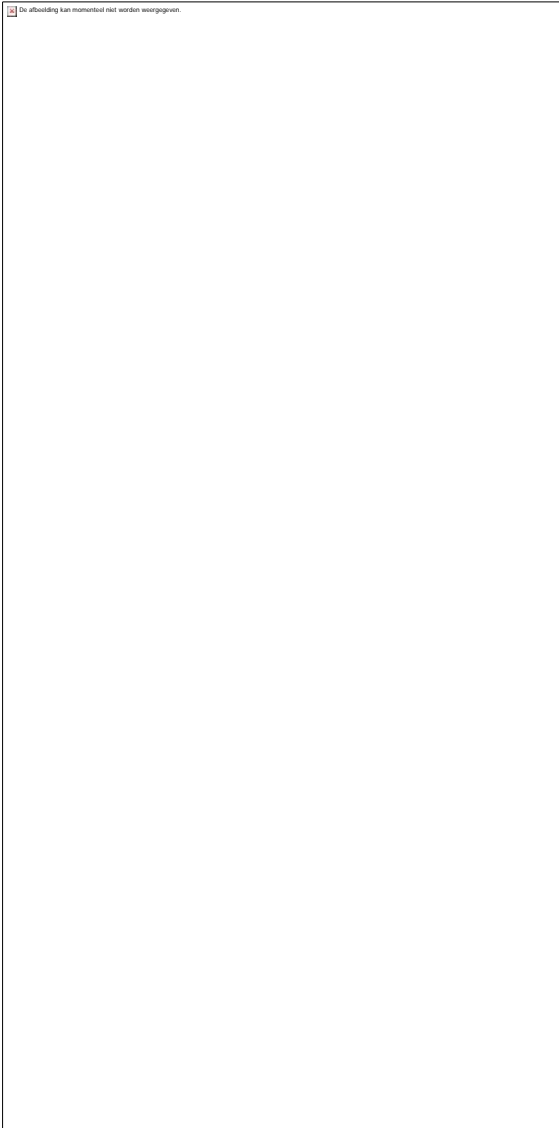
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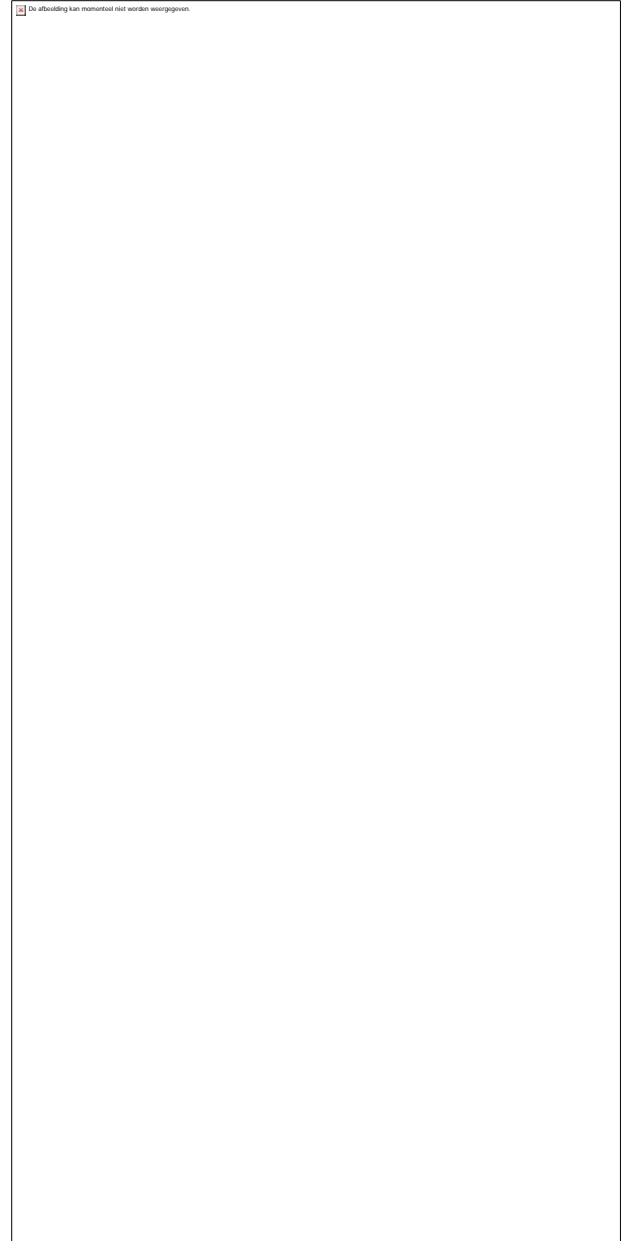
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Appendix 1.



Left; the fixed position sampling. Right top; close up of the GilAir 5 pump. Right bottom; close up of the GSP sampling head



Left top; overview of the ionization installation, after installation of the gauze. The 2 iron wires that provided the earthed surface before is used to place the gauze. Left bottom; a close up of a corona wire. Right; photo of when ionization was not active, since the plug is not placed in the socket. At this point, it had been turned on for some weeks. Note the dust deposit on the wall.

Appendix 2. Distribution of dust and endotoxin per stable unit												
			Dust					Endotoxins				
			n	ND	AM	GM	GSD	n	ND	AM	GM	GSD
Unit 1	Day	+	5	-	0,111	-2,255	0,235	5	-	162,118	5,055	0,293
		-	5	-	0,092	-2,411	0,398	5	-	197,523	5,264	0,232
	Night	+	3	-	0,116	-2,161	0,157	3	-	104,329	4,646	0,055
		-	3	-	0,099	-2,329	0,222	3	-	106,882	4,588	0,527
	Total	+	8	-	0,113	-2,220	0,316	8	-	140,447	4,902	0,308
		-	8	-	0,094	-2,381	0,218	8	-	163,532	5,011	0,482
Unit 2	Day	+	5	-	0,102	-2,296	0,159	5	-	170,515	5,121	0,206
		-	5	-	0,082	-2,705	0,841	5	-	131,288	4,826	0,371
	Night	+	2	1	0,132	-2,073	0,427	2	1	69,687	4,183	0,499
		-	3	-	0,196	-1,786	0,708	3	-	131,487	4,874	0,125
	Total	+	7	1	0,110	-2,232	0,243	7	1	141,707	4,853	0,529
		-	8	-	0,125	-2,360	0,881	8	-	131,363	4,844	0,289
Unit 3	Day	+	5	-	1,191	-0,019	0,650	5	-	2896,441	7,887	0,458
		-	5	-	1,229	0,138	0,392	5	-	5008,648	8,360	0,593
	Night	+	3	-	0,349	-1,128	0,487	3	-	811,006	6,659	0,351
		-	3	-	0,893	-0,178	0,454	3	-	2485,508	7,642	0,724
	Total	+	8	-	0,875	-0,435	0,799	8	-	2114,403	7,427	0,748
		-	8	-	1,103	0,020	0,417	8	-	4062,471	8,091	0,699
Unit 4	Day	+	5	-	1,123	-0,022	0,578	5	-	4683,588	8,161	0,824
		-	5	-	1,179	0,102	0,399	5	-	2720,317	7,859	0,357
	Night	+	3	-	1,056	-0,008	0,421	3	-	2782,750	7,838	0,511
		-	3	-	0,413	-0,921	0,334	3	-	842,297	6,707	0,301
	Total	+	8	-	1,098	-0,017	0,492	8	-	3970,774	8,040	0,700
		-	8	-	0,892	-0,281	0,635	8	-	2016,059	7,427	0,674

N, number of samples; NM, number of missing samples; AM, arithmetic mean; GM, geometric mean; GSD, geometric standard deviation.

Appendix 3. Results of the GLM models, statistical analysis in 'R'

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