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1 **Generation of avian influenza virus (AIV) contaminated fecal fine particulate**
2 **matter (PM_{2.5}): genome and infectivity detection and calculation of immission**

3

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12

13 **Abstract**

14

15 As a model for aerosol transmission, chicken feces was spiked with avian influenza
16 virus (AIV) subtype H10N7 and used to generate a fine particulate matter aerosol.
17 For this an innovative aerosol chamber was developed, that collected PM_{2.5} on quartz
18 microfiber filters. With AIV contaminated PM_{2.5}-dust coated filters different incubation
19 times ranging from 0 to 4 days and storage mainly at +4 and +20 °C and at different
20 relative humidity (RH) were performed. Embryonic death in inoculated hen's eggs
21 with filter elute was the AIV infectivity read out. To determine viral genome presence
22 quantitative real time RT-PCR was applied.

23 The filter elutes contained AIV genome as well as viable virus whereby +20 °C
24 indicated a borderline temperature for infectious virus stability. In addition, high
25 relative humidity was critical for AIV viability in PM_{2.5}. The results allowed a
26 dispersion calculation of infectious AIV in aerosols assuming a worst case scenario

27 for an AIV outbreak in poultry farms. Thus exposure to AIV associated with PM_{2.5} is
28 possible near to infected farms and may be a serious risk for fatal influenza disease
29 in both man and animals. Airborne transmission should be effectively preventable by
30 dispersion of water combined with disinfection into the inside air as well as the
31 exhaust air stream of AIV infected farms.

32
33 *Keywords:* Avian influenza virus; Chicken feces; Fine particulate matter (PM_{2.5});
34 Airborne transmission; Quartz microfiber filters

36 **1. Introduction**

37
38 Some of the most highly contagious viruses of human and veterinary concern are
39 airborne pathogens, e. g. human and avian influenza virus (AIV) and foot-and-mouth
40 disease virus (FMDV). The highly pathogenic avian influenza viruses (HPAI) have
41 been a threat to the poultry industry for many years. However, when the Asian H5N1
42 subtype emerged, a trans-boundary spread became evident and a new dimension of
43 zoonotic danger and fear for evolution of a pandemic virus was raised (Belshe, 2005;
44 Peiris et al., 2007; WHO, 2007). After the first localized Asian H5N1 epidemic in
45 Germany, the virus was spread rapidly and caused many small epidemics in wild
46 birds. In 2007, the virus also was detected on duck raising farms (Rinder et al., 2007;
47 Starik et al., 2007; Weber et al., 2007; Harder et al., 2008). The routes of
48 transmission of Asian H5N1 and other HPAI subtypes among susceptible mammals
49 and birds is still a matter of debate. Air borne transmission of infection cannot be
50 excluded and might be an effective way of infection transmission when virus is
51 inhaled directly in the deeper respiratory tract (Shinya et al., 2006; Tellier, 2006;
52 Brankston et al., 2007; Nicholls et al., 2007). Fine particulate matter from AIV

53 contaminated fecal dust can play an important role in the transmission and onset of
54 infection since particles as small as $PM_{2.5}$ (particle size $< 2,5 \mu m$) can directly invade
55 the lower part of the respiratory tract (DFG, 2008). Rapid and direct transport of AIV
56 into the lungs will be in favor for onset of infection (Shinya et al., 2006, van Riel et al.,
57 2006). The indoor exposure to aerosolized HPAI-AIV is equally given for humans
58 working in poultry stables and susceptible birds. As workers can prevent virus
59 inhalation by using appropriate masks birds are most endangered by aerosolized AIV
60 within the flocks and possibly in nearby holdings.

61 Recently the stability and transmission of aerosolized Newcastle Disease Virus has
62 been tested under practical conditions with chickens (Li et al., 2009). The results
63 indicated that virus shed from infected animals readily aerosolized and airborne
64 transmission to susceptible chickens was very efficient. We therefore wanted to
65 know, i) whether AIV in contaminated fecal dust can be found in the $PM_{2.5}$ fraction
66 and, ii) if this is the case, how long viable virus is present under environmental
67 conditions.

68

69 **2. Materials and Methods**

70

71 *2.1. AIV stock solution*

72 The low pathogenic avian influenza virus (LPAI) subtype H10N7 has been isolated
73 and multiplied in 10 day old embryonated chicken eggs as described (Rinder et al.,
74 2007). Allantoic fluid was collected at the time of embryonic death, clarified by
75 centrifugation at $800 \times g$, the supernatants were pooled and the virus was pelleted by
76 ultra centrifugation in a Beckman Coulter Optima L-90K ultra centrifuge. The pellets
77 were pooled, re-suspended in phosphate buffer and stored at $-70 \text{ }^\circ\text{C}$. The
78 concentrated AIV stock solution contained 10^9 EID₅₀/ml and showed a

79 hemagglutination (HA) titer of 1:2048 in allantoic fluid from infected eggs, the titer in
80 MDCK-cells was 3×10^5 /ml TCID₅₀.

81

82 *2.2. Generation of a storable powder of chicken feces (PCF)*

83 About 8 kg of fresh feces were sampled in a layer farm and analyzed by RT-qPCR to
84 be free of AIV genomes. To avoid bacterial or fungal infection of embryos careful
85 removal of these germs was necessary. Since filtration of feces suspensions can
86 cause an incalculable loss of spiked AIV by adsorption to micro filter membranes , it
87 was steam sterilized (20 min; +121 °C). Subsequently the material was dried at +70
88 °C, ground and sieved very fine to a storable powder of chicken feces (PCF) with a
89 final particle size of $< 63 \mu\text{m}$. Approximately 51 % of PCF dry mass were water
90 soluble.

91

92 *2.3. Generation and sampling of AIV contaminated fecal PM_{2,5}*

93 Aliquots of PCF (50 g/l) were re-suspended in distilled water and supplemented with
94 5 ml/l AIV stock solution corresponding 10^8 EID₅₀/g PCF. Comparable AIV
95 concentrations were found in fresh feces of infected birds (WHO, 2007). The
96 AIV/PCF-suspension and compressed air (flow of 5 l/min) were used in an aerosol
97 generator (AGK 2000, Palas GmbH, Germany) for nebulization. Figure 1
98 demonstrates the aerosol generator and the generation of different air streams (A, B,
99 C) leading to the collection of PM_{2,5} (stream A) on quartz microfiber filters. The
100 aerosol generator produced droplets with particle sizes $< 15 \mu\text{m}$ diameter. In the new
101 developed mixing and drying chamber these droplets shrank to PM_{2,5}. For this the
102 aerosol was diluted and dried with compressed air in a ratio of 1/6.6 (v/v). The
103 resulting air stream (38 l/min) was split in 3 parts in an isokinetic air stream splitter:
104 Stream A (84 % or 32 l/min) for the collection of AIV contaminated fecal PM_{2,5} on

105 quartz microfiber filters (Type QF050-25, Pieper Filter GmbH, Germany; Average: 2
106 mg PM_{2,5}/Filter in 3 min). Stream B (13 % or 5 l/min) for monitoring particle size
107 distribution and concentration inside the chamber. For this purpose, Stream B was
108 diluted by a factor of 10 in a dilution chamber (VKL-10E, Palas GmbH, Germany) and
109 analyzed with an aerosol spectrometer (welas 2000, Palas GmbH, Germany).
110 Stream C (3 % or 1 l/min) for exhaust air stream which finally collected all other air
111 streams. Exhaust air was heat treated (250 °C; > 10 s) and HEPA filtered before
112 release to the atmosphere to assure complete disinfection. PM_{2,5}-dust coated filters
113 were stored at -70 °C (control temperature) before elution.

114

115 *2.4. Incubation of AIV contaminated fecal PM_{2,5} deposited on filters*

116 AIV contaminated fecal PM_{2,5} deposited on filters was exposed to various
117 combinations of incubation periods (0, 15 h, 48 h, 96 h), temperatures (-70, -20, +4,
118 +20, +37 and +50 °C) and humidity (“water-saturated”, “normal atmosphere”, and
119 “exsiccated dry”). “Water-saturated” means: dust-coated filters were exposed to
120 water-saturated air (RH_{4°} 90 %, RH_{20°} 60 %); “normal atmosphere” means: dust-
121 coated filters were exposed to unchanged indoor air (RH_{22°} 30%) and “exsiccated
122 dry” means: dust coated filters were exposed in silica gel dried air in an airtight jar
123 (RH_{4°} 20 %, RH_{20°} 19 %). At the end of the different incubation procedures, filters
124 were stored at -70 °C.

125

126 *2.5. Inoculation of embryonated eggs and quantitative AIV detection*

127 Ten or eleven day old embryonated hen’s eggs (n = 2 per experiment) were
128 inoculated with 200 µl of filter elutes into the allantoic cavity. With selected filter
129 elutes (3 ml) titration in embryonated eggs (n = 4 per dilution) was performed to
130 determine the content of viable virus. Allantoic fluid was pooled from the 4 eggs for

131 real time RT-PCR analysis and hemagglutination. From each egg, at least 4 ml of
132 allantoic fluid was harvested either when the embryo was found dead or at the end of
133 the incubation period after 4 days. According to end point dilutions indicating
134 productive AIV replication in the eggs and the inoculation volume (0.2 ml) utilized, the
135 virus content in the filter elutes was determined and calculated as infectious AIV
136 present in the fine particulate matter eluted from the filters.

137

138 *2.6. Hemagglutination test*

139 Filter elutes as well as allantoic fluids were analyzed for hemagglutinating activity.
140 Hemagglutination assays were carried out in V-bottomed microtiter plates using 50 µl
141 of 2.5 % suspensions of chicken red blood cells in PBS as described (O.I.E.
142 Terrestrial Manual, 2008). Fresh chicken blood was supplemented with 1.6 % sodium
143 citrate in sterile H₂O and was centrifuged at 800 x g for 10 min at room temperature
144 to separate red blood cells. Thereafter, the cells were washed 3 times with PBS
145 before addition to the assay.

146

147 *2.7. Reverse transcription quantitative real-time PCR (RT-qPCR)*

148 Filters coated with AIV contaminated fecal PM_{2.5} were eluted with 3 ml sterile PBS
149 solution (8.0 g/l NaCl; 0.2 g/l KCl; 1.44 g/l Na₂HPO₄; 0.24 g/l KH₂PO₄; pH 7.4) and
150 placed in a shaker for 30 min at 4 °C. Isolation of total RNA from elutes or allantoic
151 fluid was performed with 200 µl using the TriZol isolation method (Invitrogen, de
152 Shep, Netherlands). The resulting 50 µl RNA extracts were tested by real time RT-
153 qPCR protocol for influenza A viruses (Spackman et al., 2002) as recommended by
154 the German National Reference Laboratory. Triplicates of 10 µl of every RNA extract
155 were analyzed in a Mx3000P instrument (Stratagene, La Jolla, CA). Dilutions of AIV
156 positive control standards were used for the quantification of PCR results. RNA

157 extracts from elutes of AIV negative fecal $PM_{2.5}$ as well as allantoic fluid of non
158 inoculated eggs served as negative controls. In accordance to the findings of
159 Spackman et al. (2002) the RT-qPCR was able to detect 10^{-1} EID₅₀ of the virus.

160

161 *2.8. Dispersion calculation*

162 Dust from poultry farms is mainly composed of the abrasion from feces and litter
163 material. As measured in the exhaust air stream of a 120,000 animal broiler farm and
164 of a 40,000 animal layer farm in Bavaria (TÜV, 2000) the total dust emissions were
165 1.3 kg/h and 0.5 kg/h, respectively. About 10 % of the broilers total dust emissions
166 were particulate matter $PM_{2.5}$ and about 60 % PM_{10} while the layers total dust
167 emission contained only 3 % $PM_{2.5}$ and 33 % PM_{10} . Thus the main part of inhalable
168 fine particulate matter has been detected in the particle size range from 2.5 to 10 μm .
169 Based on these $PM_{2.5}$ and PM_{10} emissions a dispersion calculation was performed
170 according to the "Lagrangian dispersion model – LASAT" following conventional
171 assumptions (very stable boundary layers, very low and constant wind velocity as 1
172 m/s over 24 hours and a wind direction straight towards the next adjacent
173 settlement). In order not to underestimate the inhalable amount of viable AIV the
174 dispersion calculation was performed only for the PM_{10} particulate matter fraction.

175

176 **3. Results**

177

178 *3.1. Generation of filter samples coated with AIV contaminated fecal $PM_{2.5}$*

179 Particle size distributions of resulting aerosols showed - with high conformity -
180 maxima of particle numbers at 0.4 to 0.6 μm particle diameter (Fig. 2a) and maxima
181 of particle volumes (or masses) at 2 to 4 μm particle diameter (Fig. 2b). More than 90
182 % of the generated particles had sizes $< 1.5 \mu m$, and therefore represent particulate

183 matter with a size range less than 2.5 μm ($\text{PM}_{2.5}$) which can directly enter the
184 alveolar part of the lungs (DFG, 2008).

185 The aerosol generator produced very high concentrations of $\text{PM}_{2.5}$: a flow of 38 l/min
186 aerosol with particle concentrations up to 30 mg/m^3 could be maintained for several
187 hours. Up to 84 % of the aerosol particles could be collected on quartz microfiber
188 filters. Therefore sampling times of 3 min were sufficient to collect 2.1 mg (median
189 matter) of $\text{PM}_{2.5}$ dust on filters. Comparable loads of fine particulate matter were
190 obtained in routine atmosphere air quality monitoring programs within sampling times
191 of at least 24 hours or more. The filter loads showed good reproducibility within every
192 dust generation series and from one experiment to the next.

193

194 *3.2. Influence of temperature and humidity on stability of AIV genome in fecal $\text{PM}_{2.5}$*

195 Filters coated with AIV contaminated fecal $\text{PM}_{2.5}$ were incubated up to 4 days under
196 conditions typical of European summer and winter temperatures at normal and
197 extreme humidity. Even extreme incubation temperatures ranging from -20 $^{\circ}\text{C}$ to +50
198 $^{\circ}\text{C}$ and an incubation time up to 4 days did not cause any significant change of the
199 number of AIV genome copies detected by quantitative real time RT-PCR, when
200 filters were incubated in a normal indoor atmosphere. The number of the AIV
201 genome fragment copies also remained unchanged when the dust-coated filters were
202 incubated in a water-saturated atmosphere or in exsiccated dry air (data not shown).
203 However, only the infectious stability of AIV particles in fine particulate matter forms
204 the basis for a risk assessment under environmental conditions. We therefore
205 determined the lethal effect of AIV genome containing $\text{PM}_{2.5}$ in embryonated eggs.

206

207 *3.3. Infectivity of AIV in fecal $\text{PM}_{2.5}$ after incubation at different temperature and* 208 *atmosphere conditions*

209 Immediate testing (0 h) and after the 15 h incubation period of the filters always
210 produced positive results (data not shown). Table 1a, b shows the results of the egg
211 inoculation experiments after a 2 and 4 day incubation period of quartz microfiber
212 filters coated with AIV genome positive PM_{2.5}. Hemagglutination titers were only
213 obtained from eggs containing dead embryos from which the majority died on day 2
214 after inoculation. Hemagglutination correlated well with a dramatic raise of AIV
215 genome copy numbers represented by low ct values ranging from 11 to 20 in
216 allantoic fluid from dead embryos indicating a productive virus replication in the eggs.
217 In contrast, before egg inoculation, there were relatively high real time RT-qPCR ct
218 values in the filter elutes ranging from ct 25 to 36 which in no case resulted in a
219 positive hemagglutination (not shown). This demonstrates that a lack of
220 hemagglutination activity is not decisive for the absence of infectious AIV particles. It
221 became evident that normal atmosphere and exsiccated dry air each supported long
222 term viral infectivity in contrast to a water-saturated atmosphere. A 4 day incubation
223 at +4 °C under exsiccated air conditions did not destroy AIV infectivity (Table 1b grey
224 shaded). In one experiment, even after a 4 day incubation at +20 °C, the filter elute
225 still contained infective AIV that killed the embryos and their allantoic fluid showed a
226 low ct in real time RT-qPCR as well as hemagglutinating activity (Table 1b upper
227 section). The same result was obtained after filter incubation under normal
228 atmosphere conditions in two independent experiments (Table 1a middle section). In
229 all experiments filter incubation at a temperature of +37 °C did not result in infective
230 elutes after 2 days (Table 1a, b). Since filter incubation in normal atmosphere
231 revealed long term survival (2 and 4 days) of infectious AIV we performed titration
232 experiments with filter elutes using 4 embryonated eggs per dilution. The embryonic
233 death as well as hemagglutination titers of pooled allantoic fluid from eggs containing
234 dead embryos were taken as an indicator of infectious AIV. In addition in all allantoic

235 fluid samples from embryos that had died before day 4 post inoculation, a significant
236 decline of real time RT-qPCR ct values was measured compared to the ct values in
237 filter elutes before egg inoculation. After a 2 day incubation, the 1/100 dilution
238 marked the limit of infectivity in parallel to the storage control temperature of -70 °C
239 (Table 1a). At an elute dilution of 1/10, three embryos from 4 eggs had died from the
240 infection with elutes stored at +4 °C as well as from the infection with -70 °C storage
241 control samples. The egg inoculation with filter elutes after an incubation time of 4
242 days caused the death of 1 out of 4 embryos and reached the detection limit in
243 hemagglutination at the dilution of 1/50 of the allantoic fluid pool. As demonstrated in
244 five independent experiments (Table 1a), filter elutes which were incubated for 4
245 days at +20 °C in normal atmosphere indicated AIV infectivity in 2 experiments.
246 Another 3 experiments did not result in embryonic death and hemagglutination of the
247 allantoic fluid. The same conditions were chosen in a titration experiment that did not
248 result in embryonic death nor in hemagglutination and AIV genome detection by real
249 time RT-qPCR.

250 As determined from filter elute titration results in embryonated eggs, the content of
251 infectious virus particles per mg PM_{2.5} was calculated to be 3,950 viable AIV/mg
252 PM_{2.5} in the -70 °C storage control sample. For the +4 °C incubation sample 3,750
253 and 710 viable AIV/mg PM_{2.5} were calculated after a 2 day and 4 day incubation at
254 normal atmosphere, respectively. Thus, related to the virus content of the -70 °C
255 storage control sample, the number of viable AIV/mg PM_{2.5} decreased within 4 days
256 at +4 °C in normal atmosphere by more than 80 %. Compared to the content of
257 viable AIV in the PCF/AIV suspension before nebulization (10^8 EID₅₀/g PCF) the
258 deposited PM_{2.5} of the -70 °C storage control sample contained viable AIV in an
259 amount of 4×10^6 EID₅₀/g PM_{2.5}.

260

261 *3.4. Dispersion calculation*

262 Figure 4 a and b shows the calculation results of PM₁₀ as maximum immission
263 concentration values per hour. The PM₁₀ immission concentration at the next
264 adjacent settlement at a distance of 350 m to the broiler farm and 150 m to the layer
265 farm was about 140 µg/m³ and 95 µg/m³, respectively. Using the virus titration results
266 of the samples stored at +4 °C for 2 days (3,750 viable AIV/mg PM_{2.5}, fictively taken
267 as PM₁₀), the PM₁₀ immission concentrations at the next adjacent settlements would
268 correspond to 530 viable AIV/m³ at the broiler farm and 360 viable AIV/m³ at the layer
269 farm.

270

271 **4. Discussion**

272

273 The most common methods for viral aerosol sampling are liquid impactors, solid
274 impactors combined with petri dishes and different filter types such as cellulose,
275 polycarbonate, gelatine and PTFE (Verreault et al., 2008). So far, influenza virus in
276 bioaerosol has most frequently been collected by liquid impactors. Recently, Pyankov
277 et al. (2007) also performed the collection of airborne AIV (LPAI H11N9) in an open
278 channel type aerosol chamber using liquid impactors in combination with real-time
279 PCR and the hemagglutination assay. Recently, Fabian et al. (2008) collected human
280 influenza virus from exhaled breath on Teflon filters and detected viral genome using
281 real-time PCR and in addition performed viral particle count. They found that 87 % of
282 the exhaled particles were under 1 µm in diameter, and therefore suggested that fine
283 particle aerosols may play a role in human influenza transmission.

284 In this study, we collected avian influenza A virus (LPAI H10N7) from the air stream
285 in a new aerosol chamber by deposition of the fine particulate matter generated from
286 aerosolized spiked chicken feces on quartz microfiber filters. For the first time, a

287 particle size of PM_{2.5} from fractionated AIV spiked chicken feces aerosol has been
288 used in infection experiments with embryonated eggs as a highly sensitive system for
289 viable AIV. For inoculation of living chicken embryos it was necessary to avoid non-
290 specific toxic effects due to viable bacterial content of feces and filtration was not an
291 option due to incalculable losses of spiked virus. Therefore, a sterilized storable
292 powder of chicken feces (PCF) with a final particle size of < 63 µm was produced. In
293 contrast to other detection methods for AIV in untreated bioaerosol, e.g.
294 hemagglutination or genome tracing by real time PCR (Pyankov et al., 2007), virus
295 replication could be determined in vivo. Since in virus contaminated aerosols larger
296 particles are less hazardous than smaller ones (Verreault et al., 2008), the intention
297 was to exclusively analyze the < 2.5 µm bioaerosol fraction (fine particulate matter –
298 PM_{2.5}) trapped on quartz microfiber filters which are used in routine atmosphere air
299 quality monitoring programs. It became evident that the 3 ml elute of the filters
300 contained viral genome as well as infectious AIV when 10⁸ EID₅₀/g feces were
301 present in chicken feces powder before nebulization. Compared to this the -70 °C
302 storage control sample contained viable AIV in PM_{2.5} dust deposited on filters in an
303 amount of 4 x 10⁶ EID₅₀/g PM_{2.5}. The lack of hemagglutinating activity in filter elutes
304 could be a result of the relatively large elute volume (3 ml). Severe damage of the
305 hemagglutinin protein can be excluded since infectivity of AIV in filter elutes was not
306 affected. A homogenous size distribution of particle numbers and particle volumes
307 was achieved in fine particulate matter with the aerosol generator (Fig. 2 a and b). It
308 was obvious that quartz microfiber filters had no destructive effect neither on AIV
309 genome stability nor on infectivity within the calculated number of genome copies
310 and viable AIV/mg PM_{2.5} settled on the filters. However, it should be noted that the
311 RT-qPCR assay detects only a small fragment of the entire AIV genome which might
312 be much more robust than the entire genome. In addition, the amount of viable

313 spiked virus was relatively high and the recovery rate of the system has not been
314 tested for a low virus dose spiked in feces.

315 Relative humidity (RH) has a great impact on droplet size and structural integrity of
316 particles in aerosol (Lowen et al., 2007; Verreault et al., 2008). We therefore tested
317 three different RH conditions – normal indoor atmosphere, exsiccated dry air and
318 water-saturated atmosphere. The relative higher stability of AIV in exsiccated dry air
319 and normal indoor atmosphere was confirmed as described for *orthomyxo-* and
320 *paramyxoviridae* (Schaffer et al., 1976; Ijaz et al., 1985; Lowen et al., 2007).
321 Temperatures above +4 °C are well known to have a negative impact on virus
322 infectivity (Ijaz et al., 1985; 1987). Temperatures of +37 °C and +50 °C completely
323 abolished AIV infectivity in fine particulate matter after a 2 day filter incubation.
324 However, the AIV subtype H10N7 used in this study remained remarkably stable for
325 a period of even 4 days at +20 °C in exsiccated dry air and under normal indoor
326 humidity conditions. Differences in stability of aerosolized influenza A virus have
327 been reported indicating higher decay rates for human and swine strains compared
328 to avian viruses (Mitchell et al., 1968; 1972). The presence of infectious AIV in fine
329 particulate matter originating from contaminated feces and its relative stability at +20
330 °C might explain the H5N1 infection of people in subtropical and tropical regions.
331 Short-term exposure of feces dust to temperatures up to +37 °C has not been proven
332 to immediately destroy infectivity of contaminating AIV if the virus protective effect of
333 protein and lipid is considered (EFSA, 2008; Verreault, 2008). Close contact with
334 virus shedding animals or aerosol from dried animal feces and low relative humidity
335 would be the most important conditions for effective virus transmission (Brankston et
336 al., 2007; Lowen et al., 2007).

337 To estimate the possibility of a relevant exposure of humans and animals near to
338 infected farms we performed a dispersion calculation. For this it was assumed that all

339 animals in the farm have been infected simultaneously, and therefore, the complete
340 PM₁₀ emission of each farm would be AIV contaminated without a decay of virus
341 infectivity during aerosol transmission, for example by UV radiation. As described by
342 De Benedictis et al. (2007) UV radiation light is not able to inactivate AIV in a timely
343 manner, as data have shown that 45 min exposure to a UV source is not sufficient for
344 complete inactivation of HPAI strain A/chicken/Pakistan/94 (H7N3). Another example
345 for long term AIV stability in feces (more than 20 days) is that of subtype H7N2
346 (EFSA, 2008). However, AIV stability under environmental conditions may be greatly
347 influenced by subtype peculiarities. According to our worst case scenario, a virus
348 immission concentration of 530 viable AIV/m³ means that an adult person with an
349 average respiratory volume of 20 m³/24 h (AGS, 2008) could inhale up to 10⁴
350 infectious virus particles per day at a distance of 350 m to an infected farm. Since
351 chicken have an average respiratory volume of 1.2 m³/24 h (Flindt, 2000), they could
352 inhale up to 300 infectious virus particles per day by breathing an 70 µg/m³ PM₁₀
353 aerosol containing 260 viable AIV/m³ coming from a neighbouring farm at a distance
354 of 550 m.

355

356 **5. Conclusion**

357

358 As poultry farms are known to emit fine particulate matter (PM_{2.5} and PM₁₀) which is
359 composed of the abrasions from feces and litter material, they are a potential risk for
360 airborne neighborhood infection. According to the results from a dispersion
361 calculation for a 120,000 animal broiler farm, assuming a worst case scenario for an
362 adult person, she/he could inhale up to 10⁴ infectious AIV per day living in a nearby
363 settlement. The 50 % human infective dose by aerosol inoculation of influenza virus
364 A2/Bethesda/10/63 has been determined to be only 0.6 – 3 TCID₅₀ (Tellier, 2006). So

365 far the minimal infective dose of H5N1 for man has not been determined, however, it
366 is clear that inhalation of AIV contaminated dust aerosols should be avoided. It also
367 can be concluded that animals in a nearby poultry farm can become exposed and
368 potentially infected by inhalation of about 300 infectious AIV per day. This risk
369 assessment relies on the aerosol 50 % infective dose for hens which has been
370 estimated to be only 2 TCID₅₀ (Savatov et al., 2007).

371 As recently demonstrated in the guinea pig model the formation of droplet nuclei is
372 important for virus transmission, and that high RH (80%) leads to an increase in size
373 of exhaled small droplets (< 5 µm) and also a rapid settling of large droplets limits the
374 spread of virus (Lowen et al., 2007). In AIV affected poultry farms, culling actions and
375 subsequent cleaning of the stables can cause very high dust production and the
376 generation of bioaerosol including fine particulate matter. Thus airborne AIV
377 transmission might be effectively prevented by fine dispersion of water combined with
378 environmentally tolerable disinfection (Heckert et al., 1997) into the inside air of
379 stables as well as the exhaust air stream of AIV infected farms.

380

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516 Table 1

517 Infectivity of AIV in fine particulate matter (PM_{2.5}) generated from spiked avian feces

518 after storage for two and four days at different temperatures and humidity conditions

519 a) Incubation under “normal atmosphere” conditions

520 b) Incubation under “exsiccated dry air” and “water saturated air” conditions

521

522 Figure 1

523 Experimental set up and flowchart of air streams A, B, C

524 Stream A leads to the deposit of fine particulate matter

525 Stream B was diluted by a factor of 10 and analyzed with an aerosol spectrometer

526 Stream C was directly drained off in an exhaust air stream

527

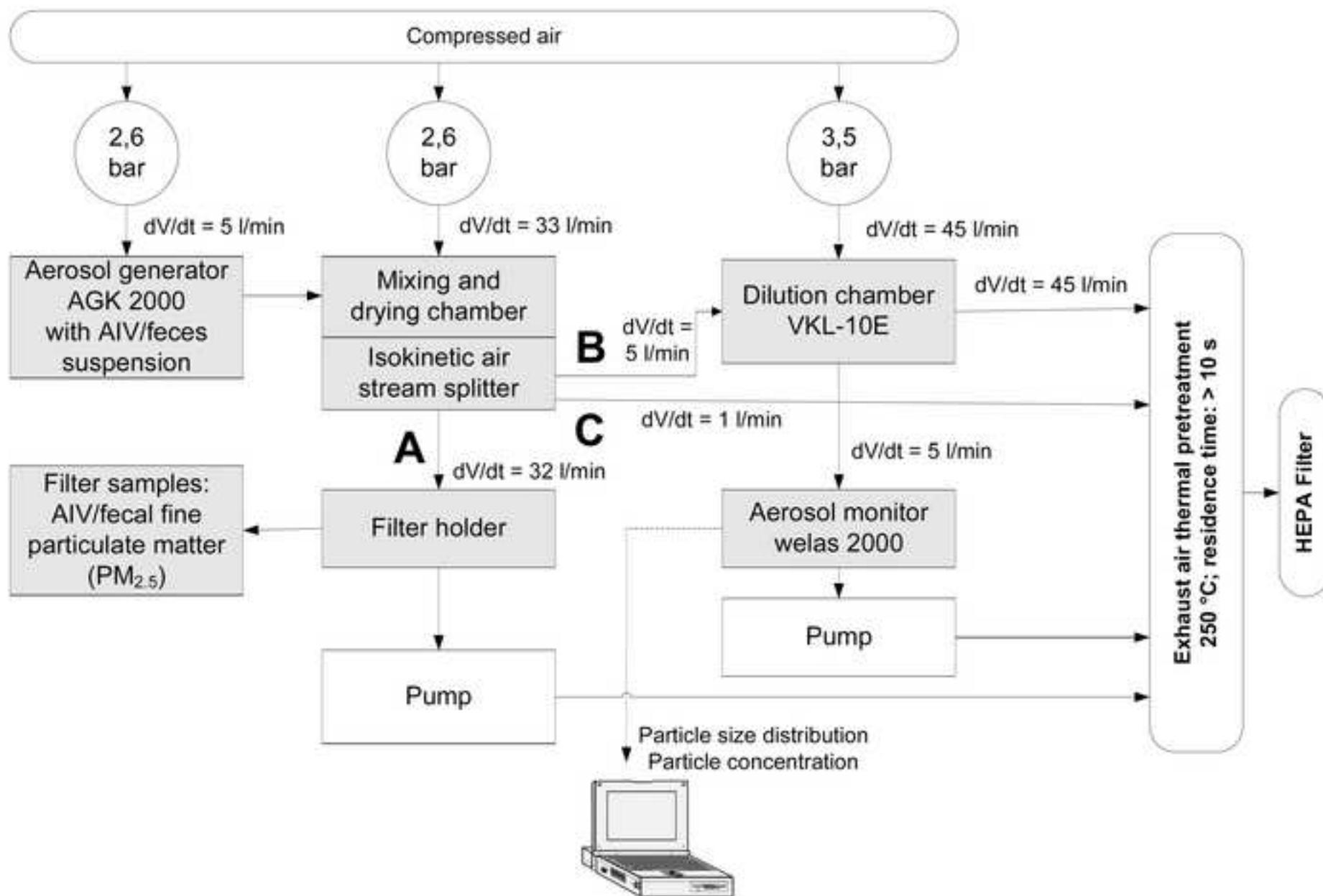
528 Figure 2

529 Comparison of size distribution of particle numbers (a) and particle volumes (b) of

530 fine particulate matter aerosols (PM_{2.5}) generated from suspensions of AIV spiked

531 chicken feces samples (data of four filter generation runs)

Figure 1



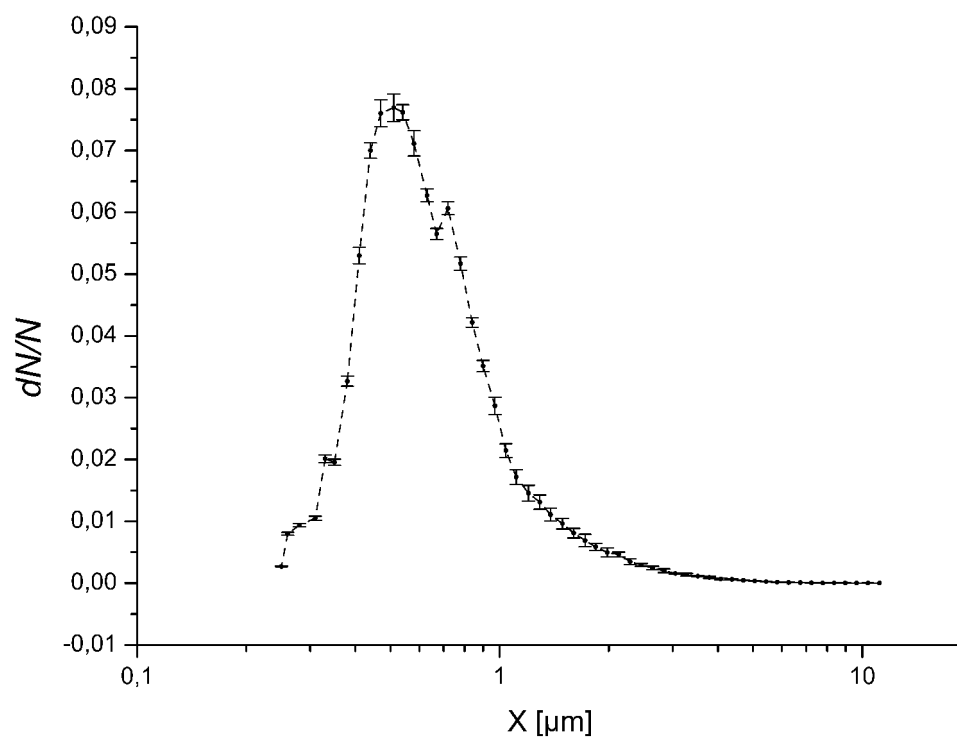


Figure 2a: Average of 4 filter samples and standard deviation

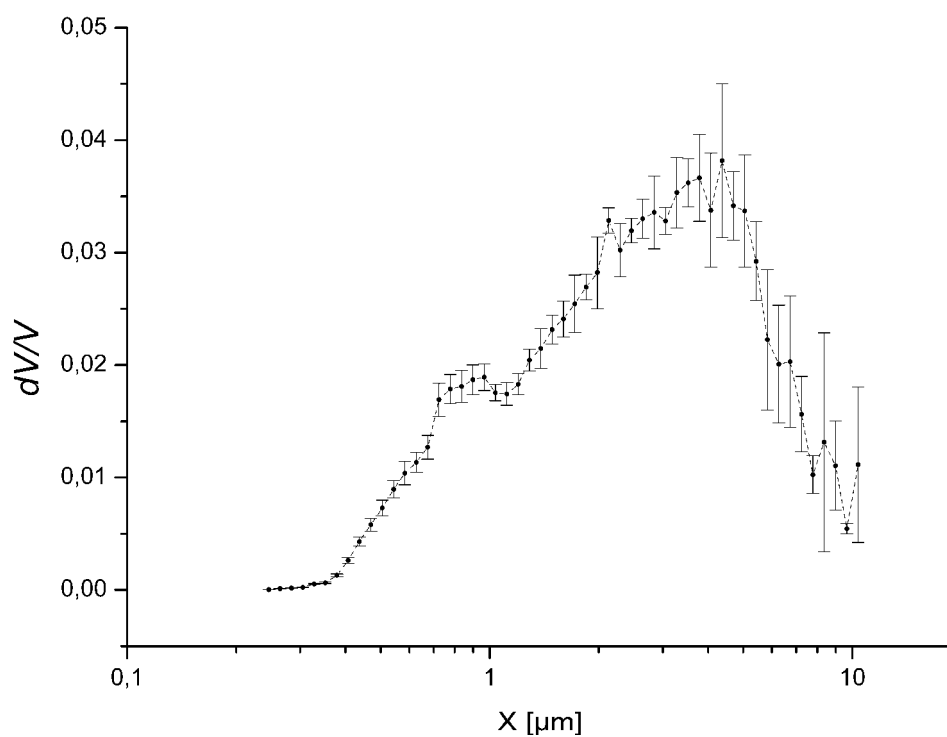


Figure 2b: Average of 4 filter samples plus standard

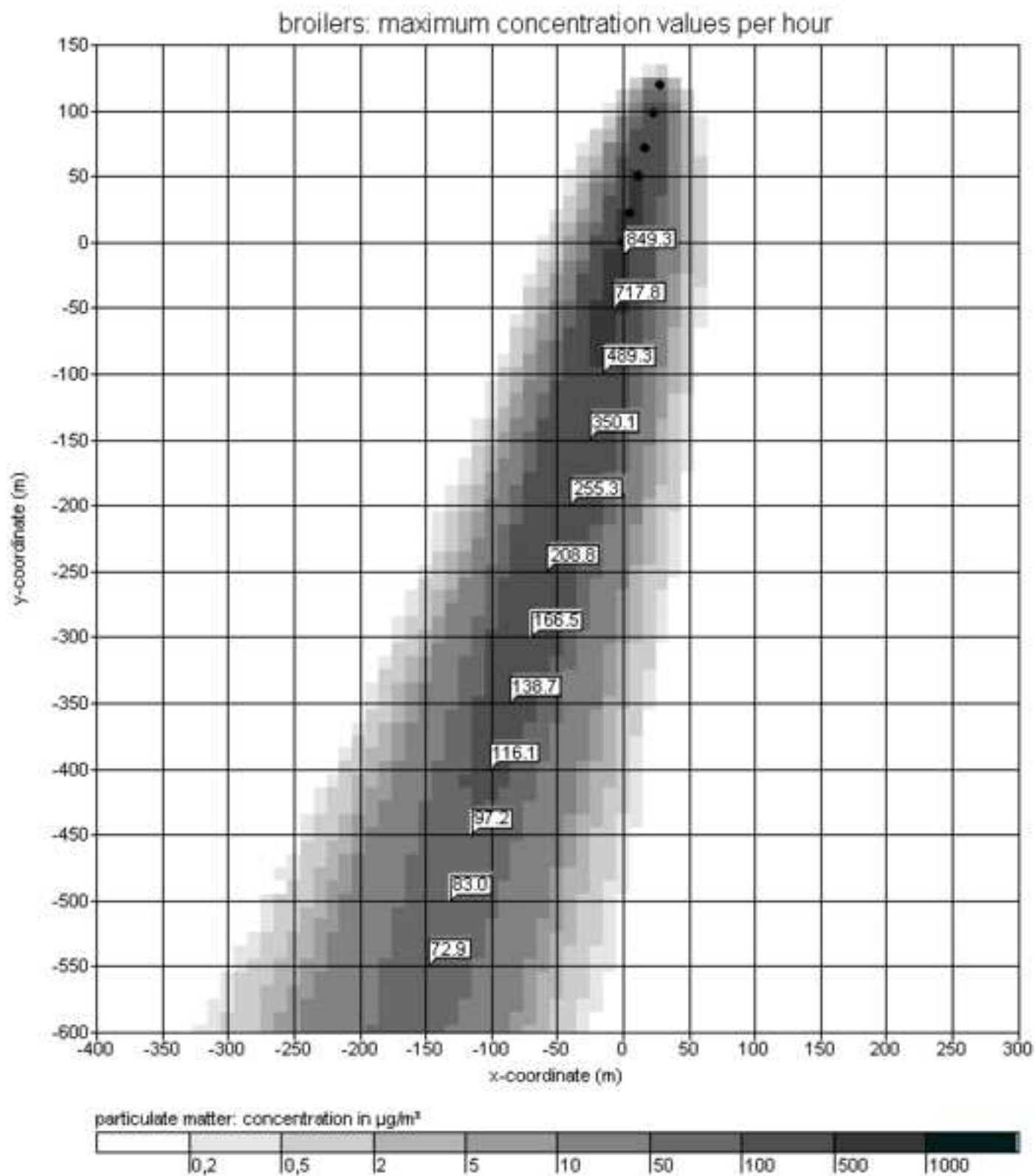


Figure 3B

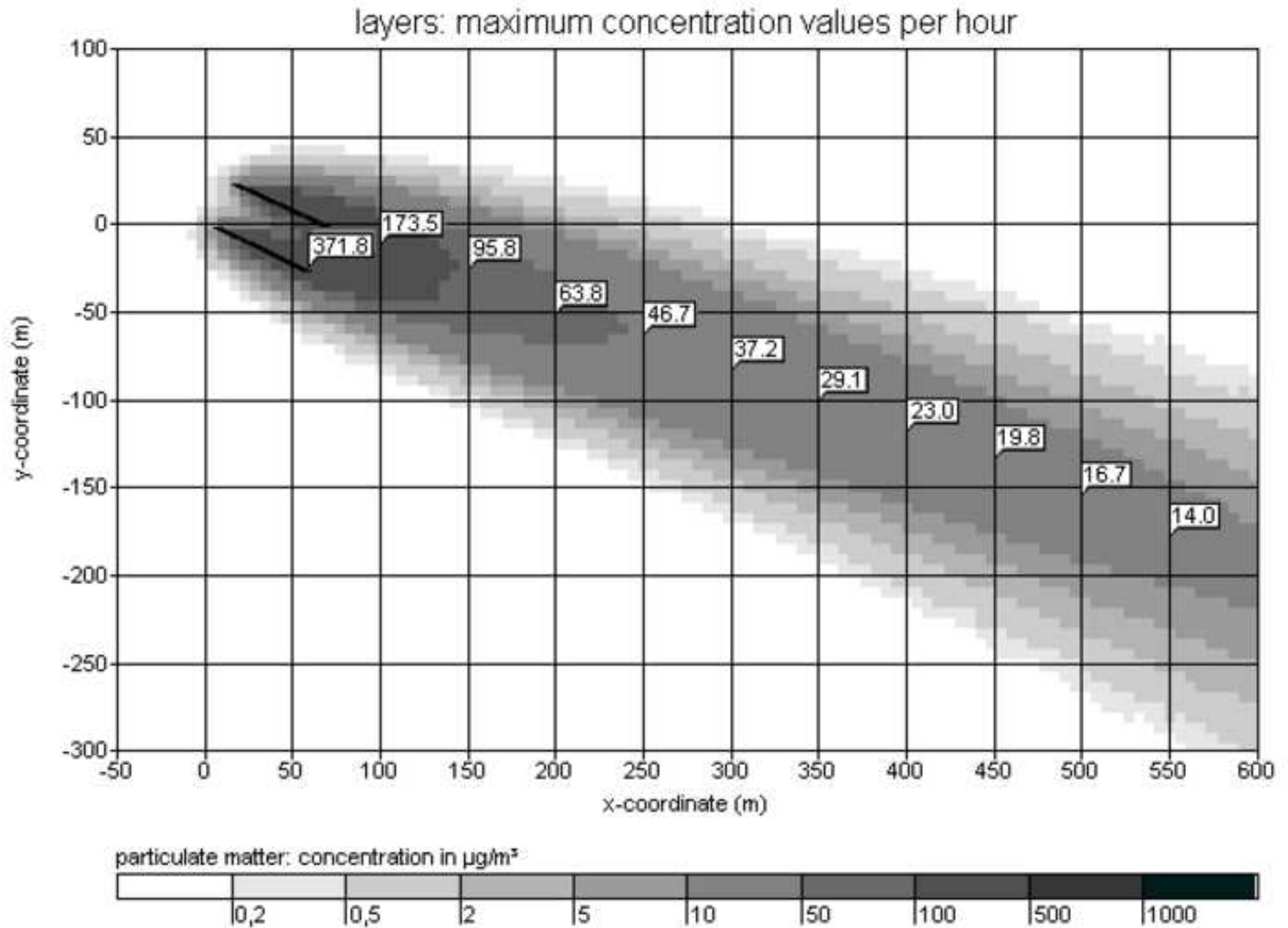


Table 1a Incubation under “normal atmosphere” conditions

no.	incu- bation temp. °C, dilution	incubation time: 2 days			incubation time: 4 days		
		<u>RT-qPCR ct</u>		<u>HA titer</u>	<u>RT-qPCR ct</u>		<u>HA titer</u>
		filter elute	all. fluid	after egg passage	filter elute	all. fluid	after egg passage
Storage control sample – 70 °C		not exposed					
	undiluted	26,0	18,5	1:16			
	1:2	27,0	12,5	1:16			
	1:10	34,2	11,8	1:32			
	1:50	32,8	12,9	1:8			
	1:100	34,9	-	-			
1	-20	26.5	20.6	1:8	27.0	19.0	1:8
2	-20	26.5	11.9	1:16	26.9	11.2	1:16
1	4	27.9	19.7	1:8	33.5	19.2	1:8
2	4	27.7	11.9	1:8	34.8	19.4	1:8
3	4	-	12.5	1:4	36.0	16.3	1:4
4	4	34.4	13.5	1:16	35.2	12.9	1:16
5	4 undiluted	29.4	12.7	1:16	26.1	12.1	1:64
	1:2	26,6	16,5	1:16	37,1	-	-
	1:10	33,2	12,5	1:16	34,0	18,7	1:32
	1:50	33,2	12,2	1:16	34,5	-	-
	1:100	33,3	38,9	-	35,5	-	-
1	20	33.8	17.0	1:16	-	19.7	1:8
2	20	27.8	19.5	1:16	26.7	13.2	1:8
3	20	34.1	-	-	35.1	-	-
4	20	25.6	30.6	-	25.2	36.1	-
5	20 undiluted	26.0	40.8	-	25.9	-	-
	1:2	26,9	36,3	-	27,8	-	-
	1:10	34,0	-	-	29,4	-	-
	1:50	34,9	-	-	34,6	-	-
	1:100	34,3	-	-	35,8	-	-
1	37	28.2	-	-	27.4	-	-
2	37	33.7	-	-	26.9	-	-
3	37	27.8	-	-	29.6	-	-

all. = allantoic; HA = hemagglutination; no. = number of independent experiments;

“-“ = not detectable;

grey shaded: viability of AIV after a four day incubation period

Table 1b Incubation under “exsiccated dry air” and “water saturated air” conditions

no.	incu- bation temp. °C	RH	incubation time: 2 days			incubation time: 4 days		
			<u>RT-qPCR ct</u>		<u>HA titer</u>	<u>RT-qPCR ct</u>		<u>HA titer</u>
			filter elute	all. fluid	after egg passage	filter elute	all. fluid	after egg passage
1	4	exs.	26.6	12.3	1:16	26.3	20.3	1:8
2	4	dry	29.4	14.3	1:8	30.8	11.8	1:8
3	4	air	36.8	12.5	1:16	25.8	12.7	1:16
1	20		28.4	14.2	1:4	35.0	-	-
2	20		33.5	12.8	1:16	26.0	13.8	1:8
1	37		28.0	27.9	-	28.0	-	-
2	37		30.8	-	-	29.0	-	-
1	4	wat.	28.2	-	-	-	-	-
2	4	sat.	-	-	-	-	-	-
3	4		26.0	36.3	-	34.3	34.6	-
1	20		26.4	-	-	29.9	-	-
2	20		35.4	37.2	-	26.2	38.0	-
1	37		36.2	37.0	-	-	-	-
2	37		-	-	-	-	-	-

all. = allantoic; exs. = exsiccated; HA = hemagglutination;

no. = number of independent experiments;

RH = relative humidity; wat. sat. = water-saturated;

“-“ = not detectable;

grey shaded: viability of AIV after a four day incubation period