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1 # Factors influencing the accuracy of the plating method used to
2 # enumerate low numbers of viable micro-organisms in food

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14

15

16 **Key words**

17 Plate counts; colony counts; measurement uncertainty; Poisson; heterogeneity; powdered food;

18 duplicate plating;

19 **Abstract**

20 This study aims to assess several factors that influence the accuracy of the plate count technique
21 to estimate low numbers of micro-organisms in liquid and solid food. Concentrations around 10
22 CFU/ml or 100 CFU/g in the original sample, which can still be enumerated with the plate count
23 technique, are considered as low numbers. The impact of low plate counts, technical errors,
24 heterogeneity of contamination and singular versus duplicate plating were studied. Batches of
25 liquid and powdered milk were artificially contaminated with various amounts of *Cronobacter*
26 *sakazakii* strain ATCC 29544 to create batches with accurately known levels of contamination.
27 After thoroughly mixing, these batches were extensively sampled and plated in duplicate. The
28 coefficient of variation (*CV*) was calculated for samples from both batches of liquid and
29 powdered product as a measure of the dispersion within the samples. The impact of technical
30 errors and low plate counts were determined theoretically, experimentally, as well as with Monte
31 Carlo simulations. *CV*-values for samples of liquid milk batches were found to be similar to their
32 theoretical *CV*-values established by assuming Poisson distribution of the plate counts. However,
33 *CV*-values of samples of powdered milk batches were approximately five times higher than their
34 theoretical *CV*-values. In particular, powdered milk samples with low numbers of *Cronobacter*
35 spp. showed much more dispersion than expected which was likely due to heterogeneity. The
36 impact of technical errors was found to be less prominent than that of low plate counts or of
37 heterogeneity. Considering the impact of low plate counts on accuracy, it would be advisable to
38 keep to a lower limit for plate counts of 25 colonies/plate rather than to the currently advocated
39 10 colonies/plate. For a powdered product with a heterogeneous contamination, it is more
40 accurate to use 10 plates for 10 individual samples than to use the same 10 plates for 5 samples
41 plated in duplicate.

42

43 **1. Introduction**

44 In food microbiology, plate counting is a longstanding and widely used enumeration method
45 to estimate the number of viable micro-organisms in food samples based on the assumption that
46 the micro-organisms are homogeneously distributed within foods. Assuming that all cells are
47 spatially separated, each viable micro-organism is expected to form one colony on an agar plate
48 provided that the medium, the temperature, the oxygen conditions and the incubation period are
49 suitable for potential recovery and growth. The number of colony forming units (CFU) per gram
50 or milliliter of sample is calculated from the plate counts, the dilution factor and the plated
51 volume.

52 The counting range of the acceptable number of colonies per plate has been reported early on
53 as a factor affecting the accuracy of the plate counting method and recommendations for suitable
54 counting ranges have been published accordingly. A range of 30-500 colonies per plate has been
55 recommended by Breed and Dotterer (1916) in their proposal to revise the standard methods of
56 milk analysis. This original recommendation has later been amended to a range of 30-300
57 colonies per plate, which has found wide acceptance (Adams and Moss, 2008 ; Sutton, 2006). An
58 optimum counting range of 25-250 colonies per plate for a 10-fold dilution series of raw milk has
59 been recommended by Tomasiewicz et al. (1980). A range of 15-300 for non-selective plates has
60 been prescribed in ISO standard 4833 (ISO, 2003). Most recently, the lower limit of the
61 acceptable counting range was decreased to 10 in ISO standard 7218 (ISO, 2007). Over the years,
62 the number of replicate plates advised for enumeration reduced from triplicate (Breed and
63 Dotterrer,1916; Tomasiewicz et al., 1980), over duplicate (ISO, 2003), to singular plating for at
64 least two successive dilutions (ISO, 2007). As the number of replicate plates directly affects the
65 volume and the total number counted, this factor also impacts the accuracy of the plating method.

66 Regarding the dilution factor and the plated volume used to calculate the number of
67 micro-organisms in a sample (expressed as CFU/g or CFU/mL), pipet volume and sample weight
68 can both be assumed to be normally distributed and to be characterised by a mean and standard
69 deviation. However, plate counts vary according to a Poisson distribution as Fischer et al. (1922)
70 showed for replicate plates of soil samples and Wilson (1935) showed for plate counts of milk
71 samples. Because the standard deviation of a Poisson distribution is equal to the square root of
72 the mean of the distribution, the count itself is a measure of the precision of the method. Plate
73 count data will always be more variable than the variability resulting only from sampling
74 homogeneously distributed micro-organisms (Cowell and Morisetti,1969)**.** Therefore, variability
75 in the colony count on plates enables one to calculate the limiting precision of counts. The
76 limiting precision caused by the Poisson distribution error can be expressed by the coefficient of
77 variation (*CV*). *CV*-values have been shown to increase for lower plate counts (Cowell and
78 Morisetti, 1969; Jarvis, 2008). Additionally to the Poisson distribution error, the error in
79 counting the actual colonies on plates can be assumed to be normally distributed.
80 Understanding the various factors that impact on accuracy of the plating method is
81 important to confidently assess numbers of micro-organisms in foods. Since the microbial
82 distribution in foods is inherently heterogeneous (Corry et al., 2007; ICMSF, 2002), and
83 hazardous micro-organisms generally are present in low numbers, both heterogeneity and low
84 numbers will influence the enumeration of micro-organisms. Plate counts from rather
85 homogeneous products have been studied in quite good detail. However, plate counts from
86 heterogeneous products such as solid and powdered foods have received less attention.
87 Therefore, this study systematically determined the impact of three factors on the
88 accuracy of the plating method when estimating low numbers of *Cronobacter sakazakii* strain
89 ATCC 29544 in liquid milk as compared to powdered milk: 1) the number of colonies on plates,

90 2) heterogeneity of the food product and 3) technical errors caused by pipetting, weighing and
91 counting. As the overall accuracy of the plate count technique is extensively discussed in the
92 review of Corry et al. (2007), our study expands on this and previous investigations by also
93 taking microbiological heterogeneity into account and determining the impact of technical errors,
94 low numbers of micro-organisms as well as singular versus duplicate plating. The accuracy of the
95 plating was investigated theoretically, experimentally and using Monte Carlo simulations. The
96 impact of low numbers was determined by repeating the experiment for different numbers of the
97 *C. sakazakii* in liquid and powdered milk, taking a large series of samples in each experiment and
98 keeping all other conditions constant.

99

100 **2. Materials and methods**

101 **2.1 Defining accuracy**

102 According to ISO standard 5725-1 (ISO, 1994), the accuracy of measurement methods and results
103 depends on both trueness and precision. Trueness is defined as the closeness of agreement
104 between the average value obtained from a large series of test results and an accepted reference
105 value. If an accepted reference value is not available, the expected measurable quantity may be
106 used as the reference for comparison of test results. Precision is defined as the closeness of
107 agreement between independent test results obtained under stipulated conditions. The precision of
108 a measurement method is indicated by the reading error of a measurement or the standard
109 deviation of a series of measurements. The accuracy in directly measured quantities such as
110 sample weight, dilution volume, and plated volume will propagate in the final enumeration value
111 (the number of micro-organisms in a sample, expressed as CFU/g or CFU/mL).

112

113 **2.2 Calculating the number of micro-organisms in the original sample (*N*) from plate**
114 **counts.**

115 The number of micro-organisms in the original sample (*N*) can be calculated from the plate count,
116 the volume plated, and the dilution factor (ISO, 2007):

117 $$N = \frac{\sum C}{V_{\text{plate}} \cdot 1.1 \cdot d} \qquad (1)$$

118 with N: number of colony forming units per milliliter (CFU/mL) or gram (CFU/g), ΣC: sum of
119 the colonies counted on two plates retained from two successive dilutions, at least one of which
120 contains a minimum of 10 colonies, V_{plate}: plated volume (mL), and d: dilution factor
121 corresponding to the first dilution retained; d is 1 when an undiluted liquid sample is plated.
122 For low numbers of micro-organisms in a solid or powdered sample, the 10-1 dilution will be
123 used instead of successive dilutions. Based on this one dilution, Equation 1 results in

124 $$N = \frac{C}{V_{\text{plate}} \cdot d} \qquad (2)$$

125 with C: counted colonies on a plate.
126 Assuming 1 g = 1 mL for a solid or powdered sample, the dilution factor is the ratio between the
127 sample volume and the sample volume plus the dilution volume:

128 $$d = \frac{S}{S + V_{\text{dil}}} \qquad (3)$$

129 with V_{dil}: dilution volume (mL) and S: sample volume (mL) or weight (g). For low numbers of
130 micro-organisms in the original sample, combining equation 2 and 3 results in:

131 $$N = \frac{C}{V_{\text{plate}}} \cdot \frac{(S + V_{\text{dil}})}{S} \qquad (4)$$

132

133 **2.3 Using error propagation to assess the impact of technical errors on *N***

134 The precision errors in the directly measured quantities C, V_{plate}, V_{dil}, and S, will propagate to an

135 error in the resulting N. For each measured quantity, the precision error is expressed in the

136 standard deviation: σ_C , $\sigma_{V_{\text{plate}}}$, $\sigma_{V_{\text{dil}}}$ and σ_S . The standard deviation in the plated volume ($\sigma_{V_{\text{plate}}}$)

137 has been determined by weighing 30 plated volumes with an analytical balance (Sartorius,

138 Göttingen, Germany). The standard deviations in the dilution volume ($\sigma_{V_{\text{dil}}}$) and in the sample S

139 from liquid milk ($\sigma_{S\,\text{liquid}}$) or powdered milk ($\sigma_{S\,\text{powder}}$) were determined in the same way. If the

140 error in C is only determined by counting, the standard deviation σ_C can be derived from a count

141 error of 5% (Peeler et al., 1982). Assuming normally distributed count data, and given a mean

142 value of μ, a maximal count error of 5% results in σ_C = 5/3 % of μ as 99% of normally

143 distributed data are within the interval $\mu \pm 3\sigma$.

144 For independent random errors, the propagation of the precision error was calculated

145 using two rules (Taylor, 1982): the error *(δq)* in the result of an addition or subtraction (Eq. 5)

146 and the relative error ($\frac{\delta q}{|q|}$) in the result of a multiplication or division (Eq. 6).

147 Rule 1: If $q = x + y$ or $q = x - y$ then $\delta q = \sqrt{\delta x^2 + \delta y^2}$ (5)

148 Rule 2: If $q = x \cdot y$ or $q = \frac{x}{y}$ then $\frac{\delta q}{|q|} = \sqrt{\left(\frac{\delta x}{x}\right)^2 + \left(\frac{\delta y}{y}\right)^2}$ (6)

149 Using these two rules and N from Eq. 4, the relative error of N can be described as:

150
$$\frac{\sigma_N}{N} = \sqrt{\left(\frac{\sigma_C}{C}\right)^2 + \left(\frac{\sigma_{V_{\text{plate}}}}{V_{\text{plate}}}\right)^2 + \left(\frac{\sigma_S}{S}\right)^2 + \left(\frac{\sqrt{(\sigma_{V_{\text{dil}}})^2 + (\sigma_S)^2}}{V_{\text{dil}} + S}\right)^2} \quad (7)$$

151

152 **2.4 Simulating the error in *N* with Monte Carlo analysis**

153 The distribution of *N* was simulated using Monte Carlo analysis using @Risk 5.0 (Palisade
154 Corporation) performing 10,000 iterations by Latin Hypercube sampling with random seed
155 generation. *N* was simulated in three different distribution scenarios for *C* using Eq. 4, in which
156 V_{plate}, V_{dil}, and *S* were assumed to be normally distributed with standard deviations as determined
157 experimentally. The error in *C* varied in the three scenarios as follows: 1) *C* normally distributed
158 with a count error of 5%, 2) *C* Poisson distributed, and 3) *C* Poisson distributed and having an
159 additional normally distributed count error of 5%. The sensitivity of the output variable *N* to the
160 input variables *C*, V_{plate}, V_{dil}, and *S* was analysed with a tornado chart.

161

162 **2.5 Enumerating the micro-organism in liquid milk**

163 **2.5.1 Preparing the bacterial suspension to inoculate the milk**

164 A full grown culture of *C. sakazakii* strain ATCC 29544 in 100 mL brain heart infusion (BHI)
165 broth (Beckton Dickinson and Co., Le Point du Claix, France) was stored frozen (-80 ºC) with
166 30% glycerol (87%, Fluka-Analytical GmbH, Buchs, Switzerland). A loopful (1 µL) of this
167 culture was inoculated into 100 mL BHI and grown for 22 h at 37°C. From the resulting BHI
168 suspension containing $1.1x10^{10}$ CFU/mL, 10-2, 10-3 and 10-4 dilutions were made using peptone
169 physiological salt (PPS; 8.5 g NaCl/L and 1 g peptone/L; Oxoid, Basingstoke, England).

170 **2.5.2 Inoculating, sampling, and plating**

171 Commercially sterilised milk obtained from local retail was inoculated with different volumes to
172 obtain 1 L batches of milk with different numbers of *C. sakazakii* aiming at $4x10^2$, $7x10^2$, $1x10^3$,
173 $3x10^3$, $5x10^3$, $1x10^4$, $2.x10^4$ CFU/mL. While each batch was being thoroughly stirred, 30 samples
174 of 0.5 mL were taken with a pipette. Each sample was diluted in 4.5 mL PPS and 0.1 mL was

175 plated in duplicate on Trypton Soy Agar plates (TSA; Oxoid, Basingstoke, England) with a spiral
176 plater (Eddy Jet; IUL Instruments, I.K.S., Leerdam, The Netherlands). The TSA plates were
177 incubated overnight at 37°C and the numbers of colonies on each plate counted manually. The
178 detection limit of the enumeration method was 1.7 log CFU/mL (50 CFU/mL). A concentration
179 of 50 CFU/mL in a sample can be detected by plating 0.2 mL of a 10-1 dilution.
180

181 **2.6 Enumerating the micro-organism in powdered milk**

182 **2.6.1 Preparing the bacterial suspension to spike the powder**

183 A loopful (1 µL) of the *C. sakazakii* strain ATCC 29544 culture stored frozen was inoculated into
184 100 mL BHI and grown for 22 h at 37 °C. To harvest the cells, the BHI suspension was
185 centrifuged 10 min at 20 °C at 1725 g (Eppendorf AG, Hamburg, Germany). *C. sakazakii* cells
186 were washed in 40 ml PPS and centrifuged 10 min at 20 °C at 1725 g twice and subsequently
187 suspended in 10 mL PPS.

188 **2.6.2 Spiking the powdered milk**

189 Powdered infant formula (PIF) obtained from local retail was artificially contaminated as follows.
190 *C. sakazakii* cells suspended in PPS were sprayed three times with a perfume sprayer (designed
191 by Gérard Brinard, DA Drogisterij, Leusden, The Netherlands) over a flat layer of 20g PIF. The
192 powder was stirred well and again sprayed three times. The contaminated powder was stored in a
193 desiccator with saturated lithium chloride (VWR international, Fontenay sous Bois, France) at
194 20°C to maintain a water activity of 0.11. After 3 days, the contaminated powder contained
195 between 10^6 and 10^7 CFU/g (data not shown).

196 **2.6.3 Mixing, sampling and plating**

197 Small amounts (0.15, 0.3, 1, 2 and 3 g) of the contaminated powder (1.93×10^6 CFU/g, measured
198 at the day of mixing and sampling) were mixed into batches of 1 kg PIF for 1 h with a 3-

199 dimensional powder mixer (Willy A. Bachofen AG Maschinenfabrik, Basel, Switzerland) with a
200 rotational speed of 56 rpm. After thorough mixing, each batch of PIF was separately poured into
201 a stainless steel box (60 cm x 30 cm x 10 cm). A plasticized grid (Gamma, Leusden, The
202 Netherlands) was placed on top of the box to visually divide the box into 72 square sections of 5
203 x 5 cm^2 allowing for systematic sampling of the powder. Two samples of 0.5 g were drawn from
204 each section, resulting in 144 samples. Each sample was suspended in 4.5 mL PPS and 0.1 mL of
205 the suspension was plated in duplicate onto TSA plates. After overnight incubation at 37 ˚C, the
206 number of colonies per plate was counted. The lower detection limit was 1.7 log CFU/g.
207

208 **2.7 Assessing the expected number of micro-organisms in a batch of powdered or liquid**
209 **milk as the reference number.**

210 Since the amount of spiked powder (with a *C. sakazakii* concentration of 1.93×10^6 CFU/g) mixed
211 into the batch of PIF is known, the expected number of micro-organisms in a batch can be
212 calculated. For instance, mixing 3g of spiked powder into 1 kg PIF will result in an expected
213 concentration of 3.76 log CFU/g This expected number can be used as a reference. In the same
214 way, the expected number of micro-organisms in milk can be calculated as the number of micro-
215 organisms in the suspension (with a *C. sakazakii* concentration of 1.1×10^{10} CFU/mL), the dilution
216 factor and the volume mixed into 1 L milk are known. The expected concentration for the highest
217 level of contaminant in liquid milk is 4.34 log CFU/mL.

218 If the micro-organisms are log-normally distributed within a batch, the log counts of the
219 samples and the variance between the log counts will also give an estimation of the number of
220 micro-organisms in the batch. According to Rahman (1968), the arithmetic mean $\overline{C}$ is related to
221 the geometric mean $\overline{\log C}$ as follows:

222 $$\log(\overline{C}) = \overline{\log C} + 0.5 \cdot \ln 10 \cdot \sigma^2_{\log C} \quad (8)$$

223 with: $\overline{\log C}$ the mean of the log counts of the samples, and $\sigma^2_{\log C}$ the variance of the log counts of

224 the samples.

225

226 **2.8 Preparing representations of variability between sample results**

227 Since the location in the box of the samples drawn from the powdered milk was known, the

228 sampling data for the powdered milk can be represented as a function of the sampling location

229 using MATLAB® 7.8.0 , R2009a (The MathWorks™, Natick, Massachusetts). The sampling data

230 for both liquid and powdered milk were displayed as an empirical cumulative distribution

231 function (ecdf). Calculations were performed in Microsoft Excel 2003.

232

233 **2.9 Using the coefficient of variation (*CV*) to assess the Poisson distribution error**

234 The dispersion of data points around the mean in data series is commonly quantified by variance,

235 standard deviation, or coefficient of variation (*CV*). Since the *CV* is the standard deviation

236 divided by the mean, this scaled measure compares the degree of variation in situations where

237 means differ. For plate counts, *CV* is:

238 $$CV = \frac{\sigma_C}{\overline{C}} \cdot 100\% \quad (9)$$

239 with $\overline{C}$ being the mean colony count per plate of a sample. If the number of colonies on a plate

240 follows a Poisson distribution, the standard deviation will be equal to the square root of the mean

241 of the counts ($\sigma_C = \sqrt{\overline{C}}$), which leads to:

242 $$CV = \frac{1}{\sqrt{\overline{C}}} \cdot 100\% \quad (10)$$

243 **3. Results**

244 **3.1 The relative error $\frac{\sigma_N}{N}$ calculated with error propagation**

245 The various measured quantities (i.e. plated volume, dilution volume, and sample weight/volume)
246 that affect the error in the final enumeration value *N* (the number of micro-organisms in a sample,
247 expressed as CFU/g or CFU/mL) were determined individually and are shown in Table 1 in terms
248 of mean ($\bar{x}$) measure values, standard deviations (*s*) and precision errors ($s/\bar{x}$) . The
249 theoretical relative error $\frac{\sigma_N}{N}$ for liquid and powdered milk can then be calculated with Eq. 7
250 using the individual standard deviations $\sigma_{V_{\text{plate}}}$, $\sigma_{V_{\text{dil}}}$ and σ_S from Table 1 and assuming a normally
251 distributed count error (scenario 1) with σ_C = 5/3 %. From this it follows that the relative error
252 $\frac{\sigma_N}{N}$ for liquid milk is:

253 $$\frac{\sigma_N}{N} = \sqrt{(1.67\%)^2 + (1.77\%)^2 + (1.55\%)^2 + (0.915\%)^2} = 3.03\% \qquad (11)$$

254 For powdered milk the relative error is:

255 $$\frac{\sigma_N}{N} = \sqrt{(1.67\%)^2 + (1.77\%)^2 + (2.83\%)^2 + (0.944\%)^2} = 3.85\% \qquad (12)$$

256 In these equations, every precision error contributes to the relative error $\frac{\sigma_N}{N}$. Since the precision
257 errors are squared, the larger precision errors have a proportionally large impact on the relative
258 error in the final enumeration value. As proposed by Taylor (1982), if one of the errors is 5 times
259 any of the other errors, then its square is 25 times that of the others and the other errors can be
260 ignored. Assuming that the counts on plates are Poisson distributed (scenario 2), the relative error

261 in the counted number of colonies on plates $\frac{\sigma_C}{C}$ will increase for lower counts. For example, for

262 a colony count of 300, the relative error is 5.77% ($\sqrt{300}/300$); for liquid milk, this will result in:

263 $$\frac{\sigma_N}{N} = \sqrt{(5.77\%)^2 + (1.77\%)^2 + (1.55\%)^2 + (0.915\%)^2} = 6.30\% \qquad (13)$$

264 If the count is 25, the relative error $\frac{\sigma_C}{C}$ is 20.0%, which will result in:

265 $$\frac{\sigma_N}{N} = \sqrt{(20.0\%)^2 + (1.77\%)^2 + (1.55\%)^2 + (0.915\%)^2} = 20.2\% \qquad (14)$$

266 If the count is 10, the relative error $\frac{\sigma_C}{C}$ is 31.6%, which will result in:

267 $$\frac{\sigma_N}{N} = \sqrt{(31.6\%)^2 + (1.77\%)^2 + (1.55\%)^2 + (0.915\%)^2} = 31.7\% \qquad (15)$$

268 The relative errors $\frac{\sigma_{V_{\text{plate}}}}{V_{\text{plate}}}$, $\frac{\sigma_{V_{\text{dil}}}}{V_{\text{dil}}}$ and $\frac{\sigma_S}{S}$ are independent of the colony counts on plates, but the

269 relative error $\frac{\sigma_C}{C}$ increases greatly for lower colony counts. Using the error propagation

270 approach therefore shows that the Poisson distributed count error greatly determines $\frac{\sigma_N}{N}$. Even

271 for high plate counts (Eq.13), precision errors contribute little to the error in the enumeration

272 value and thus the precision errors do not need to be considered in establishing the higher limit of

273 the counting range. Comparing equations 14 and 15 shows that changing from a lower limit of

274 the counting range of 10 to 25 colonies/plate, would reduce the Poisson distribution error from

275 32% to 20% and thus improve accuracy of the plating method.

276

277 **3.2 The relative error $\frac{\sigma_N}{N}$, simulated with Monte Carlo**

278 The relative error $\frac{\sigma_N}{N}$ was simulated using Monte Carlo analysis for colony counts between 5
279 and 300 for three different scenarios as compared to the theoretical *CV,* shown as the solid line in
280 Figure 1. From this it is evident that the dispersion of the plate count data (also called Poisson
281 distribution error) increases very significantly for the lower counts. The colony counts 10, 15, 25,
282 and 30 were chosen because they were previously advocated as possible lower plate count
283 boundaries. For both liquid and powdered milk, the relative errors $\frac{\sigma_N}{N}$ are presented as *CV*-
284 values in Table 2. For liquid milk, the relative errors are presented as *CV*-values in Figure 1.

285 In scenario 1, all input variables $V_{plate,}$ V_{dil}, *S*, and *C* were assumed to be normally
286 distributed. For all colony counts, this resulted in a normally distributed *N* with a *CV*-value of 2.9
287 for liquid milk. For powdered milk, the *CV*-value was 3.6. These *CV*-values correspond well to
288 the relative errors in $\frac{\sigma_N}{N}$ (liquid milk 3.03, powdered milk 3.85) calculated with the error
289 propagation. According to sensitivity analysis, the input variables ranked as $V_{plate,}$ *C,* *S* and V_{dil}
290 determined *N* (data not shown).

291 In scenario 2, the input variables $V_{plate,}$ V_{dil}, and *S* were assumed to be normally
292 distributed while *C* was Poisson distributed. The input variable *C* significantly determined *N* as
293 shown in Table 2 and according to the sensitivity analysis (data not shown). The relative error
294 $\frac{\sigma_N}{N}$ was slightly higher than the theoretical Poisson distribution error.

295 In scenario 3, *C* was assumed to be Poisson distributed with an additional count error of
296 5%, which also resulted in a strong relationship between *N* and *C*. The error in *N* was slightly
297 higher than if *C* was only Poisson distributed.
298
299 **3.3 The sampling data of liquid milk**
300 Using the experimental ecdf-curve established at the highest inoculum level ($2x10^4$ CFU/mL) as
301 the reference and assuming an identical variability at lower inoculum levels, predictions were
302 made of the ecdf-curves for the lower inoculum levels evaluated (i.e. $4x10^2$, $7x10^2$, $1x10^3$, $3x10^3$,
303 $5x10^3$, and $1x10^4$ CFU/mL). Predicted ecdf-curves are displayed as lines in Figure 2a and can be
304 compared with the experimental ecdf-curves for the individual batches which are displayed as
305 symbols. Although for low concentrations the variability is slightly higher than the predicted
306 lines, experimental and predicted ecdf-curves match well.
307
308 **3.4 The sampling data of powdered milk**
309 Also for the contaminated milk powder, ecdf-curves were predicted for various levels of the
310 micro-organism evaluated using the ecdf-curve derived from experimental data for the most
311 highly contaminated batch as the reference and assuming the same variability for all levels. The
312 reference batch contained 3 g of spiked powder, while the other four batches contained 0.15,
313 0.30, 1, and 2 g of spiked powder. Figure 2b shows the various predicted ecdf-curves as lines,
314 while the experimental ecdf-curves are displayed as symbols. Because all batches were very
315 thoroughly mixed using 3-D mixing equipment, it was expected that the contaminant would have
316 been well distributed throughout the sample and that even for low contamination levels samples
317 would mostly be above the detection limit (1.7 log CFU/g). However, as can be seen from Fig 2b,
318 for the lowest three contamination levels there were rather many samples below detection limit.

319 The percentages of samples below the detection limit were 39%, 50%, 14% and 2% for the

320 batches mixed with 0.15 g, 0.30 g, 1 g and 2 g, respectively.

321 The ecdf-curves derived from the reference at the highest concentration level run

322 comparably steep, but less steep than the ecdf-curves found for liquid milk. It can be clearly seen

323 that experimental ecdf data deviate very considerably from the predicted ecdf-curves for all

324 contamination levels and mostly so for the lowest levels of contamination.

325 The experimental ecdf-curve for the batch spiked with 0.15 g contaminated milk powder

326 showed two outliers, namely at 4.6 and 5.2 log CFU/g. For both outliers, one of the plate counts

327 was above 100 colonies whereas the other had a colony count of zero. Such a large difference in

328 colony count may have been caused by clumping of cells in the 10-1 dilution, with clumps not

329 dissolving after vortexing. These two outliers have not been taken into account in further

330 calculations.

331 The samples of the batch mixed with 3 g of spiked powder had a mean ($\overline{\log C}$) of 3.57 log

332 CFU/g and a standard deviation ($s_{\log C}$) of 0.36 log CFU/g. Assuming log-normally distributed

333 micro-organisms and using Eq. 8, this resulted in an arithmetic mean ($\log(\overline{C})$) of 3.73 log

334 CFU/g, which is close to the reference concentration of 3.76 log CFU/g.

335 In Figure 3 the sampling data of powdered milk for the 5 levels of contamination

336 investigated are displayed as 3-dimensional graphs. The mean concentration of the duplicate

337 samples drawn from each section in the box with milk powder is displayed. Comparing the

338 graphs, it can be seen that the surface plot is positioned higher in terms of mean concentration

339 with increasing contamination level but also that there is an apparent relationship between the

340 level of contamination of the powdered milk batch and the smoothness of the surface plot. The

341 higher the contamination level (going from Graph 3a to 3d) the smoother the surface plot, which

342 indicates that there is an increasingly smaller variability between the samples. The experimental
343 data for batches spiked with 0.15 g and 0.30 g contaminated powder in particular resulted in
344 very erratic surface plots, with some sections characterised by very high counts, whereas in others
345 no contamination could be detected at all.
346
347 **3.5 The Poisson distribution error of liquid and powdered milk samples**
348 Figure 4 shows the Poisson distribution error of the liquid and powdered milk samples expressed
349 as the coefficient of variation and its relationship to the mean colony count of the samples per
350 batch. The *CV*-values of the samples from liquid milk are very well in line with the curve of
351 theoretical *CV*-value that has been established assuming a Poisson distribution. Moreover, fitting
352 the plate counts of the samples per batch to a Poisson distribution with χ^2 as a criterion, also
353 confirms that plate counts are Poisson distributed. As compared to the curve of theoretical *CV*-
354 values for liquid milk, *CV*-values of samples from powdered milk were always much higher.
355 They coincided relatively well with a curve of theoretical *CV*-values established by multiplying
356 values five times.
357 For both liquid and powdered milk samples the coefficient of variation increases for low
358 plate counts. Increasing the lower limit of the counting range from 10 to 25 will reduce the *CV*
359 for liquid milk from 32% to 20% (reduction of the Poisson distribution error) and for powdered
360 milk from 160% to 100% (reduction of the Poisson distribution error times five).
361
362 **3.6 The difference in concentration based on singular or duplicate plating**
363 Two methods, singular and duplicate plating, to enumerate the contaminating micro-organisms
364 were evaluated. Figure 5 shows the concentration of the same sample singular plated versus
365 duplicate plated assessed for liquid milk (Fig. 5a) and powdered milk (Fig. 5b). All plate counts

366 of liquid milk contained more than 1 colony per plate. For powdered milk, at the lowest
367 contamination levels one of the duplicate plates contained zero colonies, resulting in series of
368 data points laying in horizontal lines. In both figures, the vertical line at a reference concentration
369 of 3 log CFU/mL (or 3 log CFU/g) corresponds to 10 colonies per plate, which is the currently
370 advocated lower limit of the plate counting range (ISO, 2007). From the reference level upward,
371 for both liquid and powdered milk, concentrations determined by both methods coincided well;
372 the data points were close to the line of equality ($y = x$), which is according to Bland and Altman
373 (1986) the criterion for a perfect agreement between two methods. Below the reference
374 concentration, however, the distance of data points to the line of equality increased, which
375 resulted in a clear difference between the two methods especially in the case of powdered milk.
376
377 **3.7 The impact of samples taken and singular or duplicate plating related to heterogeneity**
378 The impact of samples taken and singular or duplicate plating in relation to heterogeneity was
379 investigated. Using Monte Carlo simulations, it was evaluated whether it would be better to take
380 10 samples and plate them singularly, or to take 5 samples and plate them in duplicate. Two
381 powdered milk batches characterised by a different level of heterogeneous distribution of the
382 contaminant were investigated. The levels of the contaminant were either 0.15 or 3 g of spiked
383 milk powder per 1 kg batch of milk powder. The spiked powder was mixed into each batch, with
384 the lower contamination level representing the more heterogeneous distribution (Fig 3a) and the
385 higher contamination level representing the more homogeneous distribution (Fig. 3e).
386 The data of the homogeneous and heterogeneous powder were re-sampled in silico
387 (Bootstrap @Risk, 10.000 simulations) by drawing 5 samples plated in duplicate and 10 samples
388 plated singularly. Figure 6 represents the distribution of the mean concentrations of the log counts
389 calculated from 5 samples (duplicate) and 10 samples (singular) drawn from homogeneous data

390 (Fig. 6a) and heterogeneous data (Fig. 6b). Re-sampling the data of the homogeneous powder
391 resulted in no significant difference between the means of the log counts from 5 samples plated in
392 duplicate or 10 samples plated singularly. The mean values as well as the standard deviation
393 values matched closely. However, re-sampling the data of the heterogeneous powder resulted for
394 5 samples plated in duplicate in a significantly smaller mean and a larger standard deviation, than
395 for 10 samples plated singularly.
396
397 **4. Discussion**
398 This study sets out to determine the relative importance of low plate counts, technical errors,
399 heterogeneity in the distribution of micro-organisms, and singular or duplicate plating as factors
400 influencing accuracy of the plating method for microbiological contaminants in liquid and solid
401 food.
402 Using an error propagation approach, Monte Carlo analysis simulation, as well as
403 generation of experimental data, it was consistently found that low plate counts largely determine
404 the plate count accuracy for samples of liquid and powdered milk. It was furthermore observed
405 that, as compared to the Poisson distributed error in the number of colonies counted on plates,
406 technical errors can be neglected as factors influencing accuracy of the plating method when
407 technical practices are under control. The experimentally determined technical errors were found
408 to be comparable with the errors (1.1% for pipetting sample or diluent fluid) as quantified by
409 Voss et al. (2000), who concluded that counting errors had a much larger effect than pipetting
410 errors. The impact of colony counts has also been indicated by Augustin and Carlier (2006),
411 whereas Forster (2009) has emphasised that low plate counts (i.e. counts < 20) are a major
412 contributor to uncertainty.

413 The impact of heterogeneity in the distribution of a contaminant on accuracy of the plate
414 count technique has not been studied before and forms a specific aspect of the current work.
415 Heterogeneity was investigated by comparing this accuracy for known contamination levels in
416 liquid (with micro-organisms assumed to be rather homogeneously distributed and Poisson
417 distributed) and in powdered milk (with micro-organisms being rather heterogeneously
418 distributed). By comparing the data obtained for liquid and powdered milk, it was observed that
419 heterogeneity greatly impacts the accuracy of the plating method. That micro-organisms are
420 indeed homogeneously distributed in liquid milk, was confirmed experimentally by the steep
421 ecdf-curves obtained. These showed only a small variation between the samples and the *CV*-
422 values for mean colony counts of the samples per batch. The *CV*-values found through sampling
423 furthermore matched the theoretical *CV*-values assuming a Poisson distribution. Since the plate
424 count of the samples from liquid milk fitted the Poisson distribution, and *CV*-values were
425 consistent with Poisson distribution, distribution of the contaminant was homogeneous in liquid
426 milk. However, the investigations with powdered milk showed a much larger variation in
427 enumeration outcomes due to heterogeneity. It was found that *CV*-values generated
428 experimentally aligned well to a theoretical *CV*-values curve positioned five times higher than the
429 theoretical *CV*-values curve that has been established assuming a Poisson distribution.
430 As the number of replicate plates affects the total number of colonies counted, this factor
431 may also impact accuracy of the plating method. Therefore, the difference between singular and
432 duplicate plating was investigated experimentally. Since the concentration in each sample was
433 calculated using both methods, the difference between singular and duplicate plating could be
434 visualized. Above 10 colonies per plate, both methods showed a strong agreement. These
435 findings are in line with the ISO 7218 (2007), which prescribes to count plates with at least 10
436 colonies per plate of two successive dilutions that are singularly plated. This was also supported

437 by Wille et al. (1996), who showed that duplicate or triplicate plating is not more accurate than
438 singular plating provided that there are 10-50 colonies per plate. By doubling the plated volume,
439 however, duplicate plating will increase the detection limit. By doubling the total number of
440 colonies duplicate plating will lower the Poisson distribution error. As Wille et al. (1996)
441 concluded, duplicate plating will heighten the confidence in the reliability of bacterial counts
442 from single plates.

443 The impact of heterogeneity on the possible benefits of duplicate plating over singular
444 plating was investigated by drawing 5 samples plated in duplicate or 10 samples plated singular.
445 In both approaches, the same sample volume was plated. The experimental data generated for the
446 most homogeneously contaminated milk powder (that with the highest level of spiked powder)
447 and the most heterogeneous powder (with the lowest level of spiked powder) were re-sampled
448 using Monte Carlo simulations. Re-sampling the homogeneous powder showed no significant
449 difference between the means of the 5 or 10 samples. However, re-sampling the heterogeneous
450 powder showed a significantly smaller mean and a larger standard deviation between the means.
451 Drawing 5 samples plated in duplicate resulted in a probability of 1.1% that in all 5 samples no *C.*
452 *sakazakii* was detected. Although a relatively small probability, such an incorrect enumeration
453 could have hazardous consequences for consumers in case of severe pathogens. In case of 10
454 samples plated singularly, *C. sakazakii* was detected in all cases, even though the same amounts
455 of plates and dilution fluid was used.

456 Since the plate count technique is a simple, fast method to quantify levels of micro-
457 organisms, it is an important tool to estimate numbers of micro-organisms in food samples to
458 establish the microbiological quality and or safety of these foods. Many generalizing assumptions
459 are made in the process of establishing what enumeration results would comply with quality or
460 safe foods. A key assumption is that micro-organisms are homogeneously distributed even for

461 foods where this is quite improbable such as structured, semi-solid, solid and powdered foods. It
462 is often acknowledged that the distribution of micro-organisms in food products is inherently
463 heterogeneous (Corry et al., 2007). Nevertheless, the impact of heterogeneity between the
464 samples on accuracy of plating method has not been systematically quantified to the degree as in
465 the current study. To evaluate the accuracy of the plating method, sample taking is important. If
466 the samples do not represent the microbial status of the batch of food, although the plate counts
467 may be accurate, these plate counts will give insufficient information about the microbial status
468 of the batch. As the experiments reported on here have confirmed, low plate counts as well as
469 microbial heterogeneity both have an important influence on the accuracy of the plating method,
470 and are much more prominent than technical errors. For low plate counts, increasing the lower
471 limit of the counting range will notably increase the accuracy of the plate count technique.
472 Because plate counts below 25 are highly dominated by the Poisson distribution error, as shown
473 here, increasing the currently advised lower limit from 10 to at least 25 would reduce the Poisson
474 distribution error from 32% to 20% for liquid milk and from 160% to 100% for powdered milk.
475 For the powdered product with a heterogeneously distributed contamination, taking 10 samples
476 plated singularly provides more accurate information about the product than 5 samples plated in
477 duplicate.
478

479 **Acknowledgements**

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481 mixer at the Laboratory for Zoonoses and Environmental Microbiology, National Institute for
482 Public Health and the Environment (RIVM), Bilthoven, The Netherlands.

483

484 **References**

485 Adams, M.R., Moss, M.O. 2008. Food microbiology. 3rd ed. Royal Society of Chemistry,
486 Cambridge.

487 Augustin, J.-C., Carlier, V. 2006. Lessons from the organization of a proficiency testing program
488 in food microbiology by interlaboratory comparison: Analytical methods in use, impact of
489 methods on bacterial counts and measurement uncertainty of bacterial counts. Food Microbiology
490 23, 1-38.

491 Bland, J.M., Altman, D.G. 1986. Statistical methods for assessing agreement between two
492 methods of clinical measurement. Lancet 327, 307-310.

493 Breed, R.S., Dotterrer, W.D. 1916. The number of colonies allowable on satisfactory agar plates.
494 Journal of Bacteriology 1, 321-331.

495 Corry, J.E.L., Jarvis, B., Passmore, S., Hedges, A. 2007. A critical review of measurement
496 uncertainty in the enumeration of food micro-organisms. Food Microbiology 24, 230-253.

497 Cowell, N.D., Morisetti, M.D. 1969. Microbiological techniques - some statistical aspects.
498 Journal of the Science of Food and Agriculture 20, 573-579.

499 Fisher, R.A., Thornton, H.G., Mackenzie, W.A. 1922. The accuracy of the plating method of
500 estimating the density of bacterial populations. Annals of Applied Biology 9, 325-359.

501 Forster, L.I. 2009. Conclusions on Measurement Uncertainty in Microbiology. Journal of AOAC
502 International 92, 312-319.

503 ICMSF. 2002. Microorganisms in Foods 7: microbiological testing in food safety management.
504 Kluwer Academic/Plenum Publishers, New York.
505 ISO:4833. 2003. Microbiology of food and animal feeding stuffs. Horizontal method for the
506 enumeration of microorganisms. Colony-count technique at 30 °C. International Organization for
507 Standardization, Geneva, Switzerland.
508 ISO:5725-1. 1994. Accuracy (trueness and precision) of measurement methods and results:
509 general principles and definitions. International Organization for Standardization, Geneva,
510 Switzerland.
511 ISO:7218. 2007. Microbiology of food and animal feeding stuffs - General requirements and
512 guidance for microbiological examinations. International Organization for Standardization,
513 Geneva, Switzerland.
514 Jarvis, B. 2008. Statistical aspects of the microbiological examination of foods. 2 ed. Elsevier,
515 Amsterdam, The Netherlands.
516 Peeler, J.T., Leslie, J.E., Danielson, J.W., Messer, J.W. 1982. Replicate counting errors by
517 analysts and bacterial colony counters. Journal of Food Protection 45, 238-240.
518 Rahman, N.A. 1968. A course in theoretical statistics. 298-299 Griffin, London.
519 Sutton, S. 2006. Counting colonies. Pharmaceutical Microbiology Forum Newsletter 12, 2-12.
520 Taylor, J.R. 1982. An introduction to error analysis. The study of uncertainties in physical
521 measurements. Oxford University Press. Mill Valley, Canada
522 Tomasiewicz, D.M., Hotchkiss, D.K., Reinbold, G.W., Read, R.B., Hartman, P.A. 1980. The
523 most suitable number of colonies on plates for counting. Journal of Food Protection 43, 282-286.
524 Voss, B., J., K., Dahms, S., Weiss, H. 2000. A multinomial model for the quality control of
525 colony counting procedures. Biometrical Journal 42, 263-278.

526 Wille, K.K., Vowels, B.R., Foglia, A.N., Berge, C.A., Schnell, B.M., Briese, F.W. 1996.
527 Replicate plating: does it increase reliability? Letters in Applied Microbiology 23, 75-78.
528 Wilson, G.S. 1935. The bacteriological grading of milk, Special report to the Medical Research
529 Council, vol. 206. His Majesty's Stationery Office, London.

530 **Figure captions:**

531 Fig. 1. The coefficient of variation (*CV*) as a function of the number of colonies on a plate. The

532 dark line represents the theoretical *CV* assuming that the colonies per plate are Poisson

533 distributed. The relative error $\frac{\sigma_N}{N}$ for samples of liquid milk was simulated for three scenarios

534 regarding the error in colony count on plate (*C*) namely: 1) normally distributed with a count

535 error of 5%, (●), 2) Poisson distributed (♦), and 3) Poisson distributed and having an additional

536 normally distributed count error of 5% (□).

537

538 Fig. 2. Comparison between predicted and experimental ecdf-curves for (a) liquid milk and (b)

539 powdered milk. The broken vertical line represents the detection limit of 1.7 (log CFU/mL or log

540 CFU/g). For liquid milk, six predicted ecdf-curves are shown as lines with an indication of the

541 *Cronobacter sakazakii* contamination level they were derived for from the reference (the

542 experimental ecdf of $2x10^4$ CFU/mL); the symbols depict the experimental ecdf-curves for the

543 following contamination levels: (×) $4x10^2$, (○) $7x10^2$, (●)$1x10^3$, (□) $3x10^3$, (Δ) $5x10^3$, (■) $1x10^4$

544 , and (▲) $2x10^4$ CFU/mL. For powdered milk, the reference experimental ecdf was established

545 for a contamination level of 3g spiked powder per 1 batch of 1 kg (Δ) 3 g; the lines show ecdf-

546 curves derived for the various contamination levels indicated in the figure; experimental ecdf

547 (symbols) were generated with the amount of spiked powder being: (×), 0.15 g; (○), 0.3 g (●); 1

548 g, (□); 2 g, or (Δ) 3 g.

549

550 Fig. 3. The mean concentration of *C. sakazakii* in two samples (log CFU/g) powdered milk as a

551 function of their location in the box (x and y axes). 1 kg batches of powdered milk were

552 thoroughly mixed with (a) 0.15, (b) 0.30, (c) 1, (d) 2, or (e) 3 g of spiked powder.

553

554 Fig. 4. Coefficient of variation (*CV*) as a function of the mean number of colonies of the samples

555 per batch. The symbols represent the *CV*–values based on experimental values from batches of

556 liquid milk (●) and powdered milk powder (■).The solid line represents the curve of theoretical

557 *CV*-values assuming that the mean colony count of the samples per batch are Poisson distributed.

558 The broken line represents the curve of theoretical *CV*-values times 5.

559

560 Fig. 5. Relationship between the concentration (log CFU/mL or log CFU/g) in the samples of (a)

561 liquid milk and (b) powdered milk, based on enumeration using one plate per sample versus two

562 plates per sample. Solid line: $y = x$. The vertical broken line indicates the concentration of 3 log

563 CFU/mL or 3 log CFU/g, which equates to the currently advocated lower limit of the

564 enumeration range (10 colonies per plate).

565

566 Fig. 6. Comparison of two sampling strategies by re-sampling using the bootstrap method of the

567 powdered milk sampling data (a) homogenously distributed *C. sakazakii* (3 g spiked powder/kg

568 powdered milk) and (b) heterogeneously distributed *C. sakazakii* (0.15 g of spiked powder/kg

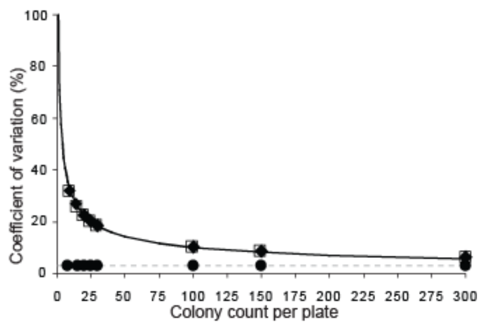
569 powdered milk). Probability distributions of the mean concentration (log CFU/g) were

570 established by a scenario of taking 10 samples plated singularly (black bars) or the mean of 5

571 samples plated in duplicate (grey bars). Parameters μ and σ represent mean and standard

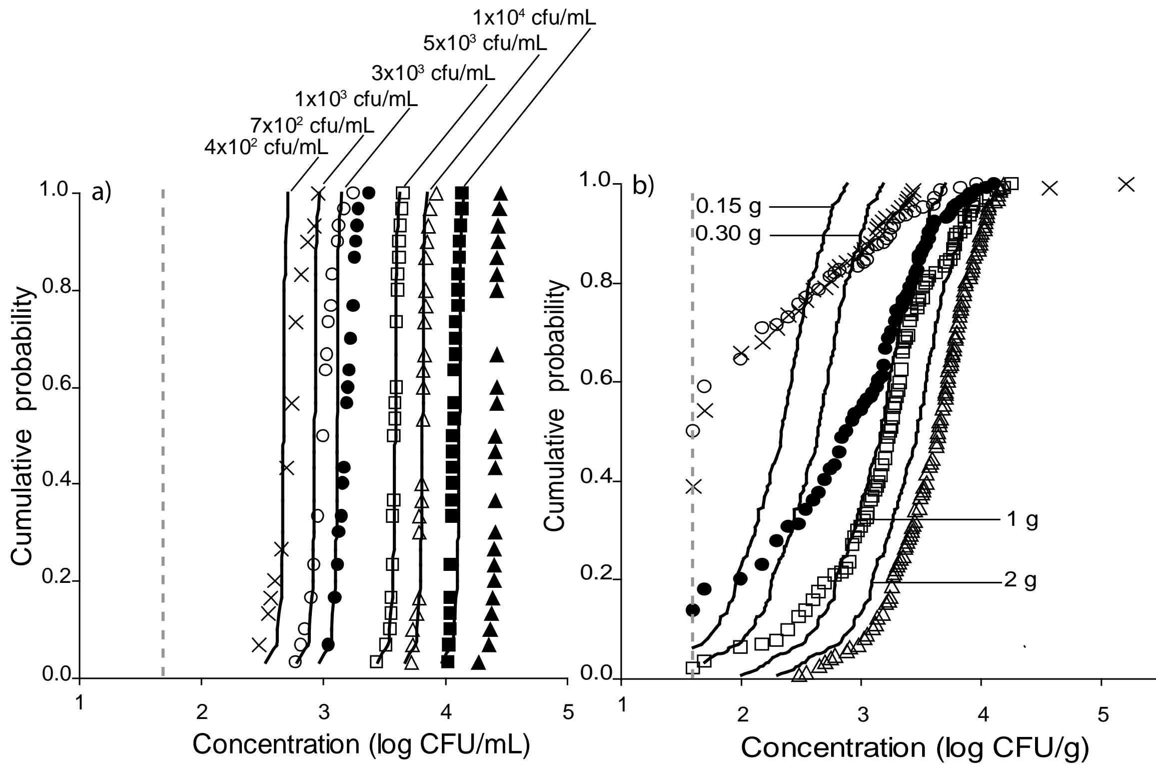
572 deviation of the 10,000 simulations drawing 5 (duplicate) or 10 samples (singular)

573 Figure 1



574

575 Figure 2



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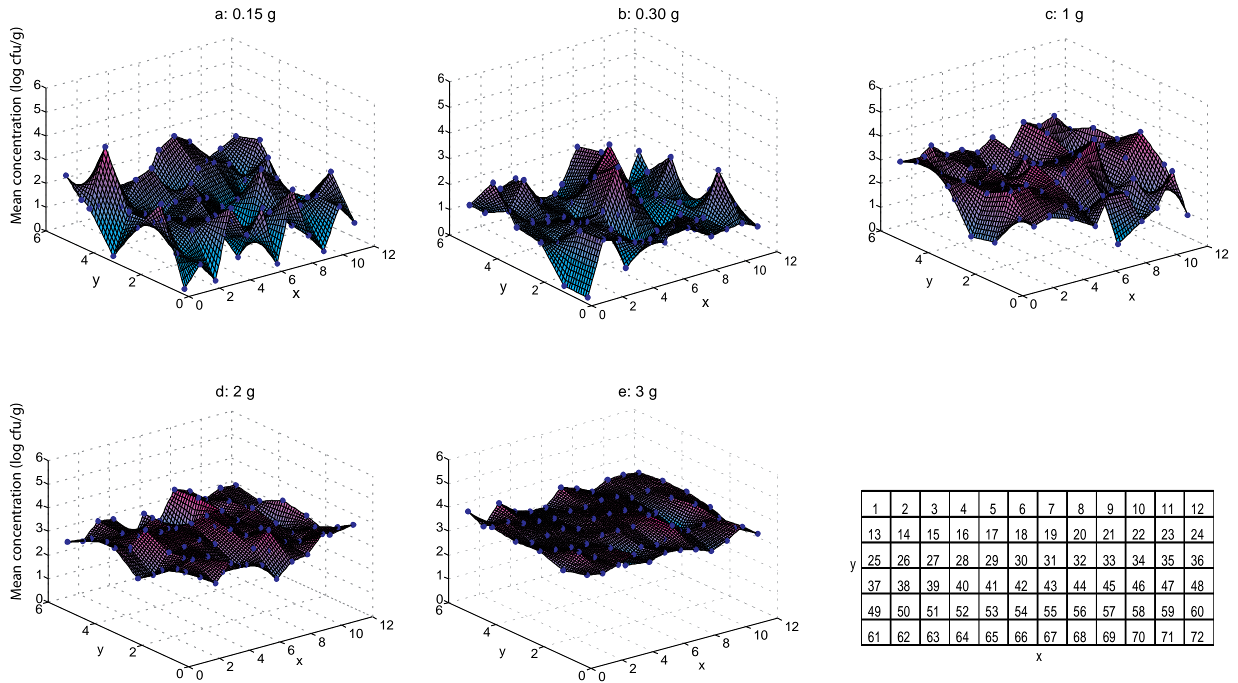
577 Figure 3



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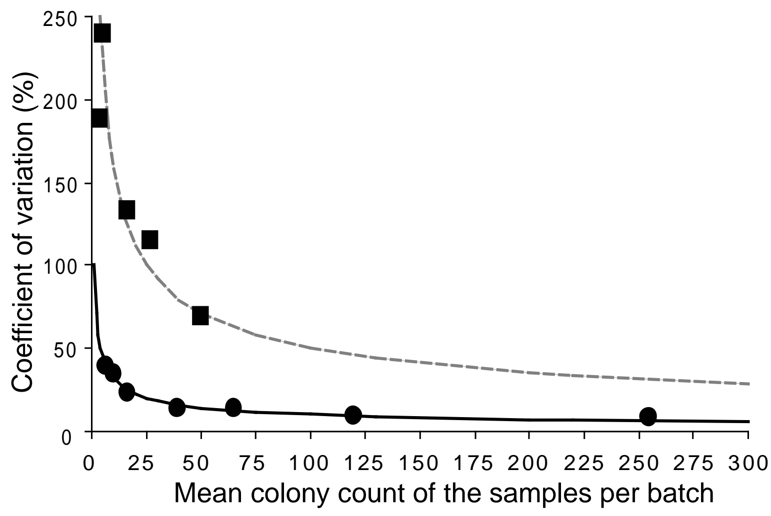
579 Figure 4



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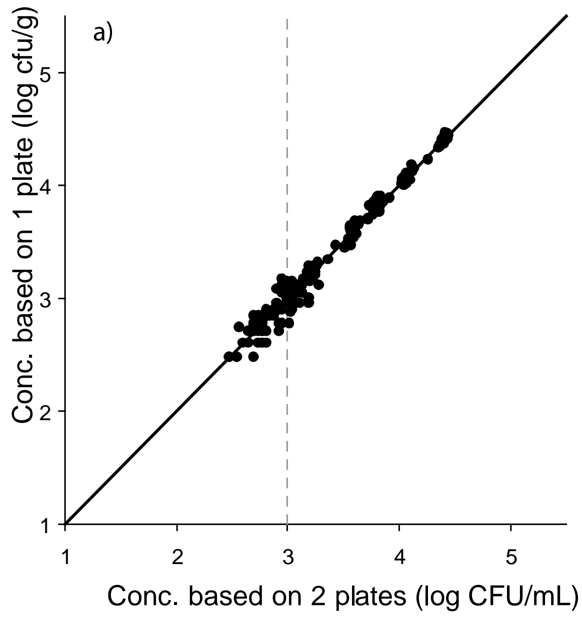
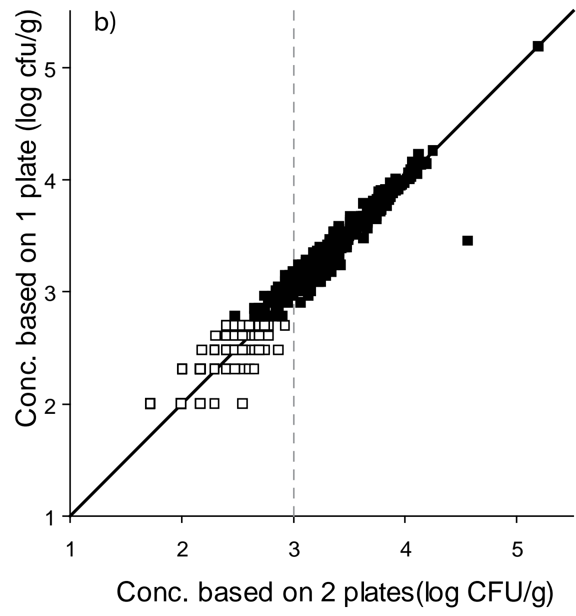
581 Figure 5





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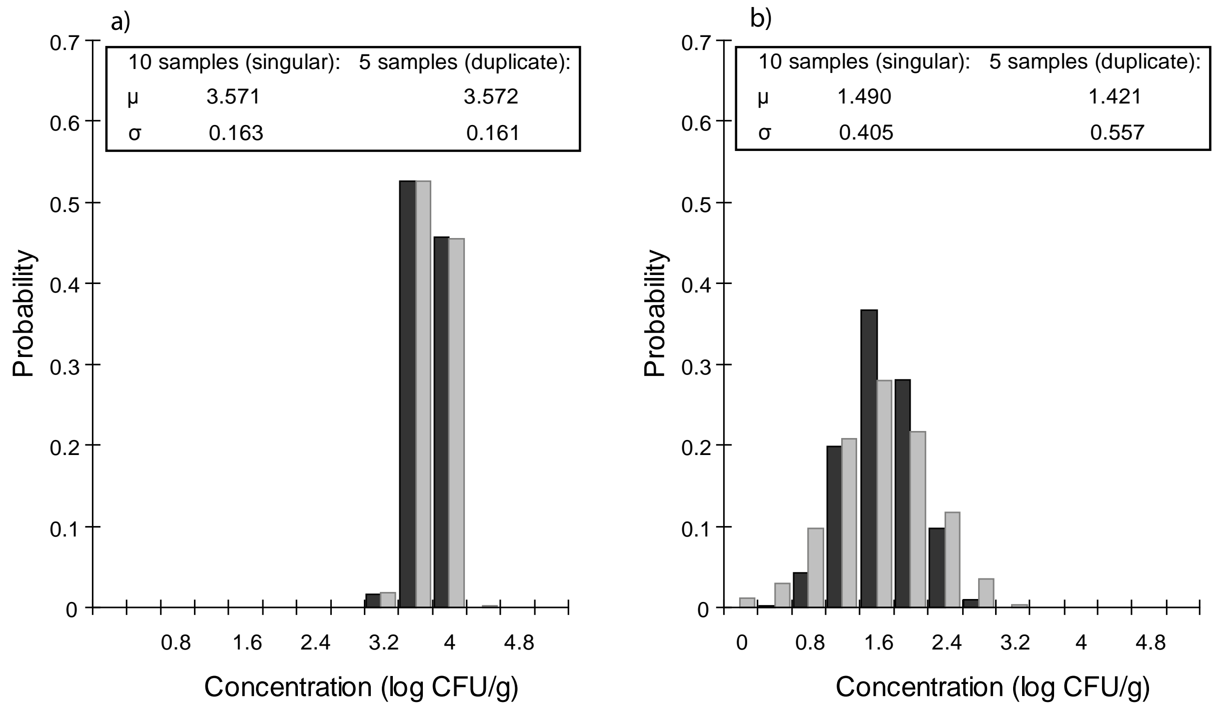
583 Figure 6



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