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

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Key words

Plate counts; colony counts; measurement uncertainty; Poisson; heterogeneity; powdered food; duplicate plating;

Abstract

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

1. Introduction

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

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

Regarding the dilution factor and the plated volume used to calculate the number of micro-organisms in a sample (expressed as CFU/g or CFU/mL), pipet volume and sample weight can both be assumed to be normally distributed and to be characterised by a mean and standard deviation. However, plate counts vary according to a Poisson distribution as Fischer et al. (1922) showed for replicate plates of soil samples and Wilson (1935) showed for plate counts of milk samples. Because the standard deviation of a Poisson distribution is equal to the square root of the mean of the distribution, the count itself is a measure of the precision of the method. Plate count data will always be more variable than the variability resulting only from sampling homogeneously distributed micro-organisms (Cowell and Morisetti,1969)**.** Therefore, variability in the colony count on plates enables one to calculate the limiting precision of counts. The limiting precision caused by the Poisson distribution error can be expressed by the coefficient of variation (*CV*). *CV*-values have been shown to increase for lower plate counts (Cowell and Morisetti, 1969; Jarvis, 2008). Additionally to the Poisson distribution error, the error in counting the actual colonies on plates can be assumed to be normally distributed.

Understanding the various factors that impact on accuracy of the plating method is important to confidently assess numbers of micro-organisms in foods. Since the microbial distribution in foods is inherently heterogeneous (Corry et al., 2007; ICMSF, 2002), and hazardous micro-organisms generally are present in low numbers, both heterogeneity and low numbers will influence the enumeration of micro-organisms. Plate counts from rather homogeneous products have been studied in quite good detail. However, plate counts from heterogeneous products such as solid and powdered foods have received less attention.

Therefore, this study systematically determined the impact of three factors on the accuracy of the plating method when estimating low numbers of *Cronobacter sakazakii* strain ATCC 29544 in liquid milk as compared to powdered milk: 1) the number of colonies on plates,

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

2. Materials and methods

2.1 Defining accuracy

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

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

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

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

with *N*: number of colony forming units per milliliter (CFU/mL) or gram (CFU/g), Σ*C*: sum of the colonies counted on two plates retained from two successive dilutions, at least one of which contains a minimum of 10 colonies, V_{plate}: plated volume (mL), and *d*: dilution factor corresponding to the first dilution retained; *d* is 1 when an undiluted liquid sample is plated.

For low numbers of micro-organisms in a solid or powdered sample, the 10-1 dilution will be used instead of successive dilutions. Based on this one dilution, Equation 1 results in

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

with C: counted colonies on a plate.

Assuming 1 g = 1 mL for a solid or powdered sample, the dilution factor is the ratio between the sample volume and the sample volume plus the dilution volume:

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

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

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

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

The precision errors in the directly measured quantities *C*, V_{plate}, V_{dil}, and *S*, will propagate to an error in the resulting *N*. For each measured quantity, the precision error is expressed in the standard deviation: σ_C, $\sigma_{V_{plate}}$, $\sigma_{V_{dil}}$ and σ_S. The standard deviation in the plated volume ($\sigma_{V_{plate}}$) has been determined by weighing 30 plated volumes with an analytical balance (Sartorius, Göttingen, Germany). The standard deviations in the dilution volume ($\sigma_{V_{dil}}$) and in the sample *S* from liquid milk ($\sigma_{S\,liquid}$) or powdered milk ($\sigma_{S\,powder}$) were determined in the same way. If the error in *C* is only determined by counting, the standard deviation σ_C can be derived from a count error of 5% (Peeler et al., 1982). Assuming normally distributed count data, and given a mean value of μ, a maximal count error of 5% results in σ_C = 5/3 % of μ as 99% of normally distributed data are within the interval $\mu \pm 3\sigma$.

For independent random errors, the propagation of the precision error was calculated using two rules (Taylor, 1982): the error (δq) in the result of an addition or subtraction (Eq. 5) and the relative error ($\frac{\delta q}{|q|}$) in the result of a multiplication or division (Eq. 6).

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

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)

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

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

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

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

2.6 Enumerating the micro-organism in powdered milk

2.6.1 Preparing the bacterial suspension to spike the powder

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

2.6.2 Spiking the powdered milk

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

2.6.3 Mixing, sampling and plating

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

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

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

Since the amount of spiked powder (with a *C. sakazakii* concentration of $1.93x10^6$ CFU/g) mixed into the batch of PIF is known, the expected number of micro-organisms in a batch can be calculated. For instance, mixing 3g of spiked powder into 1 kg PIF will result in an expected concentration of 3.76 log CFU/g This expected number can be used as a reference. In the same way, the expected number of micro-organisms in milk can be calculated as the number of micro-organisms in the suspension (with a *C. sakazakii* concentration of $1.1x10^{10}$ CFU/mL), the dilution factor and the volume mixed into 1 L milk are known. The expected concentration for the highest level of contaminant in liquid milk is 4.34 log CFU/mL.

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

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

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 the samples.

2.8 Preparing representations of variability between sample results

Since the location in the box of the samples drawn from the powdered milk was known, the sampling data for the powdered milk can be represented as a function of the sampling location using MATLAB® 7.8.0 , R2009a (The MathWorks™, Natick, Massachusetts). The sampling data for both liquid and powdered milk were displayed as an empirical cumulative distribution function (ecdf). Calculations were performed in Microsoft Excel 2003.

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

The dispersion of data points around the mean in data series is commonly quantified by variance, standard deviation, or coefficient of variation (*CV*). Since the *CV* is the standard deviation divided by the mean, this scaled measure compares the degree of variation in situations where means differ. For plate counts, *CV* is:

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

with $\overline{C}$ being the mean colony count per plate of a sample. If the number of colonies on a plate follows a Poisson distribution, the standard deviation will be equal to the square root of the mean of the counts ($\sigma_C = \sqrt{\overline{C}}$), which leads to:

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

3. Results

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

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

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

For powdered milk the relative error is:

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

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

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

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

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

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

In scenario 3, *C* was assumed to be Poisson distributed with an additional count error of 5%, which also resulted in a strong relationship between *N* and *C*. The error in *N* was slightly higher than if *C* was only Poisson distributed.

3.3 The sampling data of liquid milk

Using the experimental ecdf-curve established at the highest inoculum level ($2x10^4$ CFU/mL) as the reference and assuming an identical variability at lower inoculum levels, predictions were made of the ecdf-curves for the lower inoculum levels evaluated (i.e. $4x10^2$, $7x10^2$, $1x10^3$, $3x10^3$, $5x10^3$, and $1x10^4$ CFU/mL). Predicted ecdf-curves are displayed as lines in Figure 2a and can be compared with the experimental ecdf-curves for the individual batches which are displayed as symbols. Although for low concentrations the variability is slightly higher than the predicted lines, experimental and predicted ecdf-curves match well.

3.4 The sampling data of powdered milk

Also for the contaminated milk powder, ecdf-curves were predicted for various levels of the micro-organism evaluated using the ecdf-curve derived from experimental data for the most highly contaminated batch as the reference and assuming the same variability for all levels. The reference batch contained 3 g of spiked powder, while the other four batches contained 0.15, 0.30, 1, and 2 g of spiked powder. Figure 2b shows the various predicted ecdf-curves as lines, while the experimental ecdf-curves are displayed as symbols. Because all batches were very thoroughly mixed using 3-D mixing equipment, it was expected that the contaminant would have been well distributed throughout the sample and that even for low contamination levels samples would mostly be above the detection limit (1.7 log CFU/g). However, as can be seen from Fig 2b, for the lowest three contamination levels there were rather many samples below detection limit.

The percentages of samples below the detection limit were 39%, 50%, 14% and 2% for the batches mixed with 0.15 g, 0.30 g, 1 g and 2 g, respectively.

The ecdf-curves derived from the reference at the highest concentration level run comparably steep, but less steep than the ecdf-curves found for liquid milk. It can be clearly seen that experimental ecdf data deviate very considerably from the predicted ecdf-curves for all contamination levels and mostly so for the lowest levels of contamination.

The experimental ecdf-curve for the batch spiked with 0.15 g contaminated milk powder showed two outliers, namely at 4.6 and 5.2 log CFU/g. For both outliers, one of the plate counts was above 100 colonies whereas the other had a colony count of zero. Such a large difference in colony count may have been caused by clumping of cells in the 10-1 dilution, with clumps not dissolving after vortexing. These two outliers have not been taken into account in further calculations.

The samples of the batch mixed with 3 g of spiked powder had a mean ($\overline{\log C}$) of 3.57 log CFU/g and a standard deviation ($s_{\log C}$) of 0.36 log CFU/g. Assuming log-normally distributed micro-organisms and using Eq. 8, this resulted in an arithmetic mean ($\log(\overline{C})$) of 3.73 log CFU/g, which is close to the reference concentration of 3.76 log CFU/g.

In Figure 3 the sampling data of powdered milk for the 5 levels of contamination investigated are displayed as 3-dimensional graphs. The mean concentration of the duplicate samples drawn from each section in the box with milk powder is displayed. Comparing the graphs, it can be seen that the surface plot is positioned higher in terms of mean concentration with increasing contamination level but also that there is an apparent relationship between the level of contamination of the powdered milk batch and the smoothness of the surface plot. The 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

of liquid milk contained more than 1 colony per plate. For powdered milk, at the lowest contamination levels one of the duplicate plates contained zero colonies, resulting in series of data points laying in horizontal lines. In both figures, the vertical line at a reference concentration of 3 log CFU/mL (or 3 log CFU/g) corresponds to 10 colonies per plate, which is the currently advocated lower limit of the plate counting range (ISO, 2007). From the reference level upward, for both liquid and powdered milk, concentrations determined by both methods coincided well; the data points were close to the line of equality ($y = x$), which is according to Bland and Altman (1986) the criterion for a perfect agreement between two methods. Below the reference concentration, however, the distance of data points to the line of equality increased, which resulted in a clear difference between the two methods especially in the case of powdered milk.

3.7 The impact of samples taken and singular or duplicate plating related to heterogeneity

The impact of samples taken and singular or duplicate plating in relation to heterogeneity was investigated. Using Monte Carlo simulations, it was evaluated whether it would be better to take 10 samples and plate them singularly, or to take 5 samples and plate them in duplicate. Two powdered milk batches characterised by a different level of heterogeneous distribution of the contaminant were investigated. The levels of the contaminant were either 0.15 or 3 g of spiked milk powder per 1 kg batch of milk powder. The spiked powder was mixed into each batch, with the lower contamination level representing the more heterogeneous distribution (Fig 3a) and the higher contamination level representing the more homogeneous distribution (Fig. 3e).

The data of the homogeneous and heterogeneous powder were re-sampled in silico (Bootstrap @Risk, 10.000 simulations) by drawing 5 samples plated in duplicate and 10 samples plated singularly. Figure 6 represents the distribution of the mean concentrations of the log counts calculated from 5 samples (duplicate) and 10 samples (singular) drawn from homogeneous data

(Fig. 6a) and heterogeneous data (Fig. 6b). Re-sampling the data of the homogeneous powder resulted in no significant difference between the means of the log counts from 5 samples plated in duplicate or 10 samples plated singularly. The mean values as well as the standard deviation values matched closely. However, re-sampling the data of the heterogeneous powder resulted for 5 samples plated in duplicate in a significantly smaller mean and a larger standard deviation, than for 10 samples plated singularly.

4. Discussion

This study sets out to determine the relative importance of low plate counts, technical errors, heterogeneity in the distribution of micro-organisms, and singular or duplicate plating as factors influencing accuracy of the plating method for microbiological contaminants in liquid and solid food.

Using an error propagation approach, Monte Carlo analysis simulation, as well as generation of experimental data, it was consistently found that low plate counts largely determine the plate count accuracy for samples of liquid and powdered milk. It was furthermore observed that, as compared to the Poisson distributed error in the number of colonies counted on plates, technical errors can be neglected as factors influencing accuracy of the plating method when technical practices are under control. The experimentally determined technical errors were found to be comparable with the errors (1.1% for pipetting sample or diluent fluid) as quantified by Voss et al. (2000), who concluded that counting errors had a much larger effect than pipetting errors. The impact of colony counts has also been indicated by Augustin and Carlier (2006), whereas Forster (2009) has emphasised that low plate counts (i.e. counts < 20) are a major contributor to uncertainty.

The impact of heterogeneity in the distribution of a contaminant on accuracy of the plate count technique has not been studied before and forms a specific aspect of the current work. Heterogeneity was investigated by comparing this accuracy for known contamination levels in liquid (with micro-organisms assumed to be rather homogeneously distributed and Poisson distributed) and in powdered milk (with micro-organisms being rather heterogeneously distributed). By comparing the data obtained for liquid and powdered milk, it was observed that heterogeneity greatly impacts the accuracy of the plating method. That micro-organisms are indeed homogeneously distributed in liquid milk, was confirmed experimentally by the steep ecdf-curves obtained. These showed only a small variation between the samples and the *CV*-values for mean colony counts of the samples per batch. The *CV*-values found through sampling furthermore matched the theoretical *CV*-values assuming a Poisson distribution. Since the plate count of the samples from liquid milk fitted the Poisson distribution, and *CV*-values were consistent with Poisson distribution, distribution of the contaminant was homogeneous in liquid milk. However, the investigations with powdered milk showed a much larger variation in enumeration outcomes due to heterogeneity. It was found that *CV*-values generated experimentally aligned well to a theoretical *CV*-values curve positioned five times higher than the theoretical *CV*-values curve that has been established assuming a Poisson distribution.

As the number of replicate plates affects the total number of colonies counted, this factor may also impact accuracy of the plating method. Therefore, the difference between singular and duplicate plating was investigated experimentally. Since the concentration in each sample was calculated using both methods, the difference between singular and duplicate plating could be visualized. Above 10 colonies per plate, both methods showed a strong agreement. These findings are in line with the ISO 7218 (2007), which prescribes to count plates with at least 10 colonies per plate of two successive dilutions that are singularly plated. This was also supported

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

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

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

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

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Figure captions:

Fig. 1. The coefficient of variation (*CV*) as a function of the number of colonies on a plate. The dark line represents the theoretical *CV* assuming that the colonies per plate are Poisson distributed. The relative error $\frac{\sigma_N}{N}$ for samples of liquid milk was simulated for three scenarios regarding the error in colony count on plate (*C*) namely: 1) normally distributed with a count error of 5%, (●), 2) Poisson distributed (♦), and 3) Poisson distributed and having an additional normally distributed count error of 5% (□).

Fig. 2. Comparison between predicted and experimental ecdf-curves for (a) liquid milk and (b) powdered milk. The broken vertical line represents the detection limit of 1.7 (log CFU/mL or log CFU/g). For liquid milk, six predicted ecdf-curves are shown as lines with an indication of the *Cronobacter sakazakii* contamination level they were derived for from the reference (the experimental ecdf of $2x10^4$ CFU/mL); the symbols depict the experimental ecdf-curves for the following contamination levels: (×) $4x10^2$, (○) $7x10^2$, (●)$1x10^3$, (□) $3x10^3$, (Δ) $5x10^3$, (■) $1x10^4$, and (▲) $2x10^4$ CFU/mL. For powdered milk, the reference experimental ecdf was established for a contamination level of 3g spiked powder per 1 batch of 1 kg (Δ) 3 g; the lines show ecdf-curves derived for the various contamination levels indicated in the figure; experimental ecdf (symbols) were generated with the amount of spiked powder being: (×), 0.15 g; (○), 0.3 g (●); 1 g, (□); 2 g, or (Δ) 3 g.

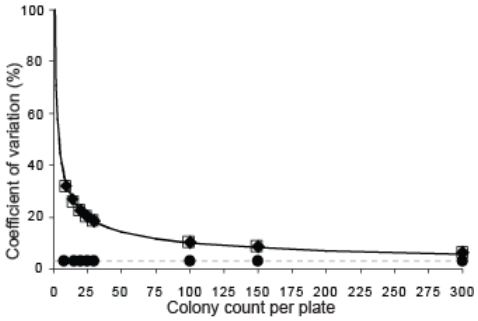
Fig. 3. The mean concentration of *C. sakazakii* in two samples (log CFU/g) powdered milk as a function of their location in the box (x and y axes). 1 kg batches of powdered milk were thoroughly mixed with (a) 0.15, (b) 0.30, (c) 1, (d) 2, or (e) 3 g of spiked powder.

Fig. 4. Coefficient of variation (*CV*) as a function of the mean number of colonies of the samples per batch. The symbols represent the *CV*–values based on experimental values from batches of liquid milk (●) and powdered milk powder (■).The solid line represents the curve of theoretical *CV*-values assuming that the mean colony count of the samples per batch are Poisson distributed. The broken line represents the curve of theoretical *CV*-values times 5.

Fig. 5. Relationship between the concentration (log CFU/mL or log CFU/g) in the samples of (a) liquid milk and (b) powdered milk, based on enumeration using one plate per sample versus two plates per sample. Solid line: $y = x$. The vertical broken line indicates the concentration of 3 log CFU/mL or 3 log CFU/g, which equates to the currently advocated lower limit of the enumeration range (10 colonies per plate).

Fig. 6. Comparison of two sampling strategies by re-sampling using the bootstrap method of the powdered milk sampling data (a) homogenously distributed *C. sakazakii* (3 g spiked powder/kg powdered milk) and (b) heterogeneously distributed *C. sakazakii* (0.15 g of spiked powder/kg powdered milk). Probability distributions of the mean concentration (log CFU/g) were established by a scenario of taking 10 samples plated singularly (black bars) or the mean of 5 samples plated in duplicate (grey bars). Parameters μ and σ represent mean and standard deviation of the 10,000 simulations drawing 5 (duplicate) or 10 samples (singular)

573 Figure 1



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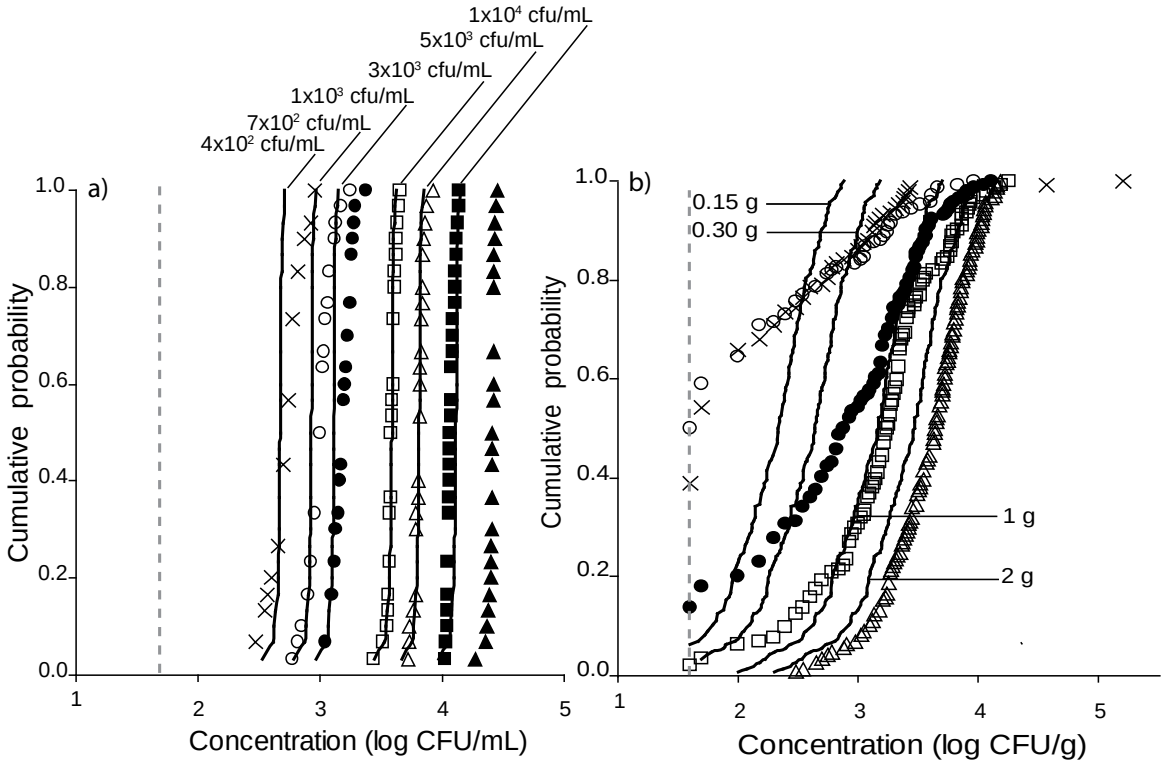
575 Figure 2



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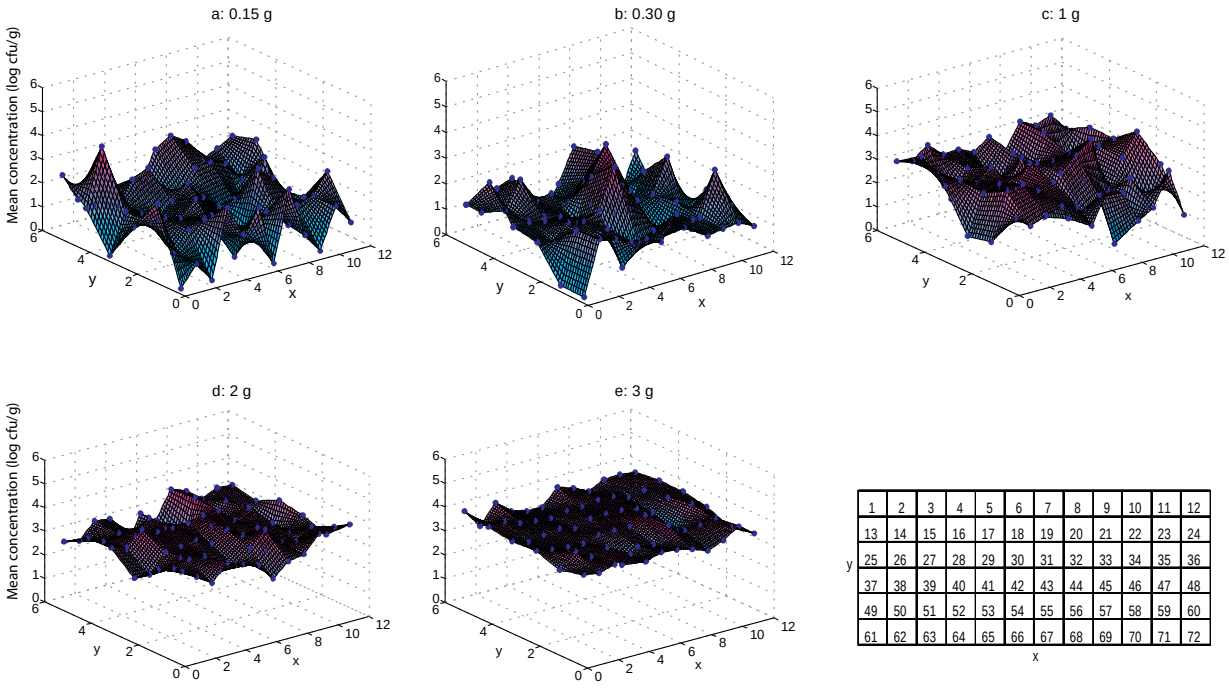

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	25	26	27	28	29	30	31	32	33	34	35	36
	37	38	39	40	41	42	43	44	45	46	47	48
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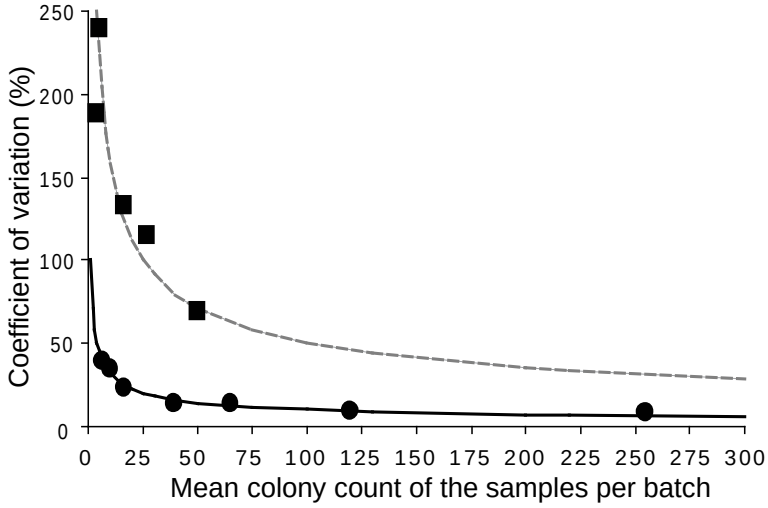
579 Figure 4



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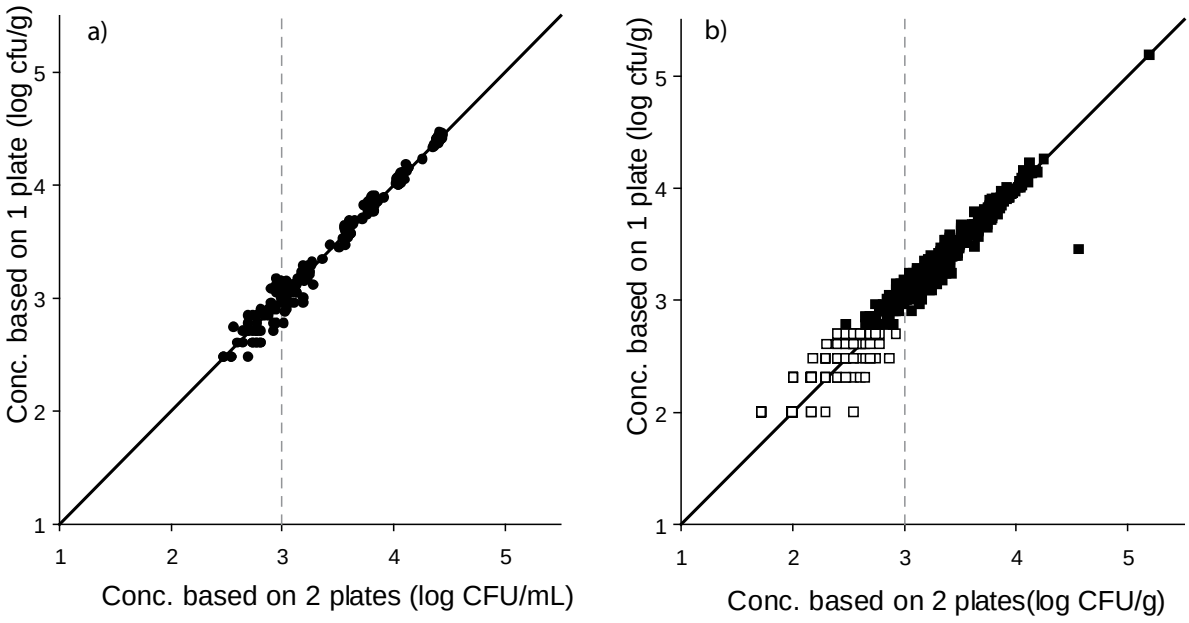
581 Figure 5



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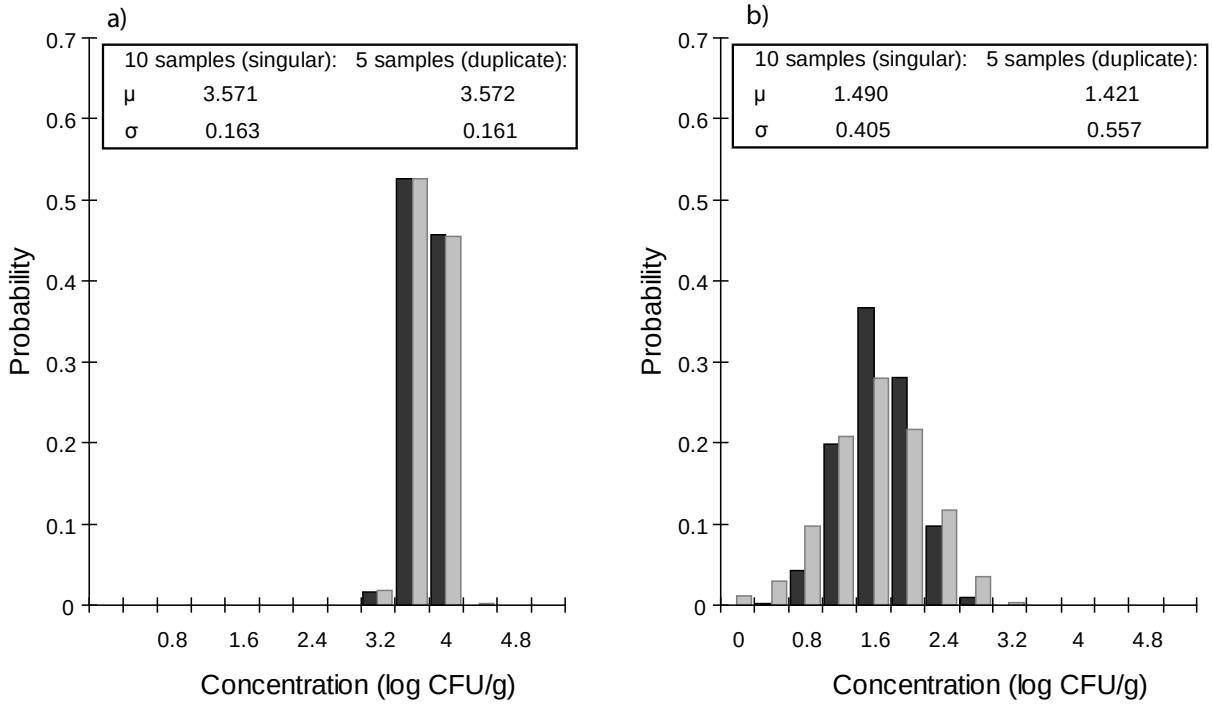
583 Figure 6



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