Project Number 289706
Start date of the project: 01/12/2011, duration: 48 months

Deliverable 9.2b
Protocol for difference and equivalence testing for ERA

Environmental Risk Assessment of Genetically Modified Organisms:

Simulation study to investigate properties of difference and equivalence tests

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March 2014

Dissemination Level: Public, http://edepot.wur.nl/455505
with appendices http://edepot.wur.nl/455504

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1 Introduction

1.1 Statistical analysis and design for environmental risk assessment

A basic statistical approach to environmental risk assessment (ERA) has been outlined in the EFSA Guidance Document (EFSA, 2010b) and in Perry et al. (2009). However, this approach is not specified in great detail. The aim of the statistics work package is to make the EFSA guidelines workable, practical and to fill in the gaps. This will result in a protocol which will provide risk assessors with a step-by-step approach for both design and statistical analysis of field trials. Statistical consideration of the EFSA for the safety evaluation of genetically modified organisms (EFSA, 2010a) will be incorporated in this protocol. Work package 9 will develop statistical concepts, methods, software and protocols for environmental risk assessment (ERA) and post-market environmental monitoring (PMEM). Main objectives are:

- to develop appropriate statistical methods to handle Genotype by Environment interaction in studies over multiple bio-geographic regions and under varying agronomical conditions. This is expected to be a major issue in the context of European ERA;
- to introduce equivalence testing as a main approach for ERA in addition to difference testing, and to establish protocols for experimental design based on acceptable test characteristics;
- to develop statistical approaches for handling data sets with many low counts and presence/absence data, as often encountered in ERA. Current practice is to use models based on normal distributions but this may not be appropriate;
- to implement methods in software for practical use;
- to provide protocols and draft texts for guidelines. The protocol will provide risk assessors with a set of evaluated, standardized and harmonized sampling and testing methods for environmental risk assessment;
- to provide guidelines for multivariate statistical approaches appropriate for PMEM.

Existing datasets will be studied to characterise baseline conditions found in different bio-geographic regions, and to typify the variation of genotypes and environments (Task 9.1). Based on these results a simulation model will be built (Task 9.2), which will be used to test various statistical approaches for data analysis in relation to the possible design of experiments (e.g. sample size). Statistical approaches will use both difference and equivalence testing, and a graphical display of assessment results will be developed (Task 9.3). Also for multi-environment studies appropriate statistical methodology will be developed, including the consideration of genotype by environment interaction (Task 9.4). The statistical methods for analysis and design of field trials for Environmental Risk Assessment that give the best performance will be described in protocols for both single-environment (Task 9.3) and multi-environment studies (Task 9.6).

Tasks 9.1 (overview of existing ERA datasets) and 9.2 (simulation model for ERA data) are described in Goedhart et al (2013, 2014). This report describes results of a simulation study to investigate properties of various statistical models, which are used to perform difference and equivalence testing, for analysing count data.

1.2 A protocol for the design and analysis of single-environment field trials – Task 9.3.

This task is preparatory for Tasks 9.4 and 9.5 where multi-environment trials are addressed. Several statistical issues regarding data types, difference testing, equivalence testing and test characteristics can however be better researched in the relatively simple situation of a single-environment trial. This is also relevant because of the emphasis of the EFSA guidance document on single-environment trials. The EFSA guidance document states that "For field trials, since each field trial at a site on a particular occasion should have sufficient replication to be able to yield a stand-alone analysis if required, this power analysis should relate to a single site". Therefore protocols for power analysis and statistical analysis of a single field trial will be developed in this task. To develop such protocols it is important to known the statistical properties of various tests which are used in practice, for example the power and robustness of a test and whether the test has the correct significance level. This can best be researched by means of a simulation model. This reports describes such a simulation study.

1.3 Overview of this report

The simulation model developed in Task 9.2 was used to generate count data for the simple, but important, situation in which a field study is conducted to compare a GM plant with its conventional counterpart . It is assumed that a completely randomized experiment is used and that a single count, without excess zeros, of a non-target organism is available for each experimental unit. Chapter 2 describes the setup of the simulation study. Four different count distributions were used to simulate count data for a mean count ranging from 0.5 (for rare species) to 100 (for more common species). Different coefficients of variation and different levels of replication, ranging from 4 to 100, were used to simulate data. The ratio of the means of the GM plant and its comparator was set to 1, 0.75, 0.50 and 0.25. A ratio of 1 implies no difference between the GM plant and its comparator. The simulated data were analysed by means of eight different models, such that the most robust model could be selected. Chapter 3 describes the results obtained for difference testing; this includes the simulated size and power of the difference test as well as coverage of confidence intervals. It also compares an approximate fast method to obtain the power of a difference test. Finally a recommendation is given about which difference test is to be preferred. Chapter 4 deals with one-sided equivalence testing and describes the simulated significance level of various methods, the simulated power and a fast way of calculating the power. This also results in a recommendation about which equivalence test is to be preferred. Chapter 5 shortly deals with the problem of zero inflation, i.e. more zeros than predicted by the count distribution

2 Setup of simulation study

2.1 Basic setup and simulation distributions

The most simple trial in which a *GM* plant is compared to its conventional counterpart is a completely randomized field trial with level of replication N. In that simple case there are only two parameters: the mean count of the non-target organism for the *GM* plant (μ_G) and the mean count (μ_C) for the comparator. In practice there might be repeated counts on the same plots, but this is ignored in this simulation study. Goedhart et al (2013, 2014) describe five statistical distributions commonly used to simulate counts: the Poisson distribution, the overdispersed Poisson distribution, the negative binomial distribution, the Poisson-Lognormal distribution and a distribution which follows Taylor's power law. The Poisson distribution was not used in this simulation study because it is generally believed (Perry et al 2003, Duan et al, 2006) that counts of non-target organisms (NTOs) typically have larger variance than according to the Poisson distribution. Table 1 summarizes the four distributions which are used to simulate data, with the dispersion parameter σ^2 as a function of the mean μ and the variation coefficient CV in the last column. There is no statistical distribution associated with Taylor's power law, as it only specifies a relationship between the variance and the mean. Perry et al (2003) used the negative binomial distribution to simulate according to Taylor's power law employing a negative binomial dispersion parameter which follows from equating the variance of the negative binomial to the power law. The same approach is followed here. Using the negative binomial is however somewhat arbitrary, as e.g. the Poisson-Lognormal has the same variance to mean relationship, but has a different distribution.

Table 1: Distributions and values for the dispersion parameter used to simulate data.

Distribution	Abbreviation	Mean	Variance	Dispersion parameter σ^2 as a function of CV
Overdispersed Poisson	*OP*	μ	$\sigma^2\mu$	$\mu\,(CV/100)^2$
Negative Binomial	*NB*	μ	$\mu+\sigma^2\mu^2$	$(CV/100)^2-1/\mu$
Poisson-Lognormal	*PL*	μ	$\mu+\sigma^2\mu^2$	$(CV/100)^2-1/\mu$
Power model (p=1.5)	*P1*	μ	$\sigma^2\mu^{1.5}$	$\mu^{0.5}\,(CV/100)^2$

The variance function of the Power model is more generally given by $Var=\sigma^2\mu^p$ in which p is some power. In this simulation study p=1.5 was chosen because this results in a variance function nicely in between the variance function for the overdispersed Poisson on the one hand and the negative binomial and Poisson-Lognormal on the other hand.

The assumed variability in field testing of NTOs is mostly defined in terms of the coefficient of variation (CV), for example Duan et al (2006), and this convention is also used here. The mean μ_C of the comparator and the coefficient of variation CV define the dispersion parameter σ^2, see Table 1. This same dispersion parameter is then used to generate counts for the comparator and also for the *GM* plant. So for example with μ_C=10 and CV=100%, the negative binomial dispersion parameter equals σ^2=0.9. In case the *GM* plant, in the same simulation, has a mean μ_G=2.5, the corresponding CV value equals $\sqrt{2.5+0.9\times2.5^2}/2.5$ = 114%. Moreover, a mean μ_G=1 has a corresponding CV=138% in this setting. This somewhat

higher CV value than for the comparator reflects the general believe that smaller means are associated with larger CV values. The quotient of the CV value for the *GM* plant and the comparator for each distribution is given below as a function of $Q = \mu_G/\mu_C$.

Overdispersed Poisson simulation distribution

The overdispersed Poisson distribution requires a dispersion parameter σ^2 which is larger than or equal to 1, where the limiting value of 1 results in an ordinary Poisson distribution. The quotient of the variation coefficients is given by

$$\frac{CV_G}{CV_C} = \sqrt{\frac{\sigma^2/\mu_G}{\sigma^2/\mu_C}} = \sqrt{\frac{\mu_C}{\mu_G}} = \sqrt{\frac{1}{Q}}$$

This implies that with Q = 0.25 the *GM* plant has a CV value which is twice as large as the CV of the comparator, irrespective of the value of μ_C.

Negative binomial and Poisson-Lognormal simulation distributions

The negative binomial and Poisson-Lognormal distributions both require a dispersion parameter σ^2 which is larger than 0. The quotient of the variation coefficients is given by a more complicated formula:

$$\frac{CV_G}{CV_C} = \sqrt{\frac{(\mu_G + \sigma^2\mu_G^2)/\mu_G^2}{(\mu_C + \sigma^2\mu_C^2)/\mu_C^2}} = \sqrt{1 + \frac{1-Q}{Q\ \mu_C\ (CV/100)^2}}$$

This will be close to 1 for large CV values and for large values of μ_C.

Power law simulation distribution

For simulating according to the Power model, first the following equation is solved for τ: $\sigma^2\mu^p = \mu + \tau\mu^2$; subsequently data are simulated according to a negative binomial distribution with dispersion parameter τ. Note that the equation is separately solved for the comparator, with mean μ_C, and for the GMO with mean $\mu_G = Q\mu_C$. This might results in a combination of parameter values which is not allowed. Suppose, as an example, μ_C=9, μ_G=1 and CV=50%. The dispersion parameter of the Power model with p=1.5 is then given by σ^2=0.75. However the equation for μ_C: $1+\tau 1^2 = 0.75*1^{1.5}$ cannot be solved for positive τ.

The quotient of the coefficients of variation is given by

$$\frac{CV_G}{CV_C} = \sqrt{\frac{\sigma^2\mu_G^p/\mu_G^2}{\sigma^2\mu_C^p/\mu_C^2}} = Q^{0.5p-1}$$

This implies that with Q = 0.25 and p=1.5 the *GM* plant has a CV value which is $\sqrt{2}$ as large as the CV of the comparator.

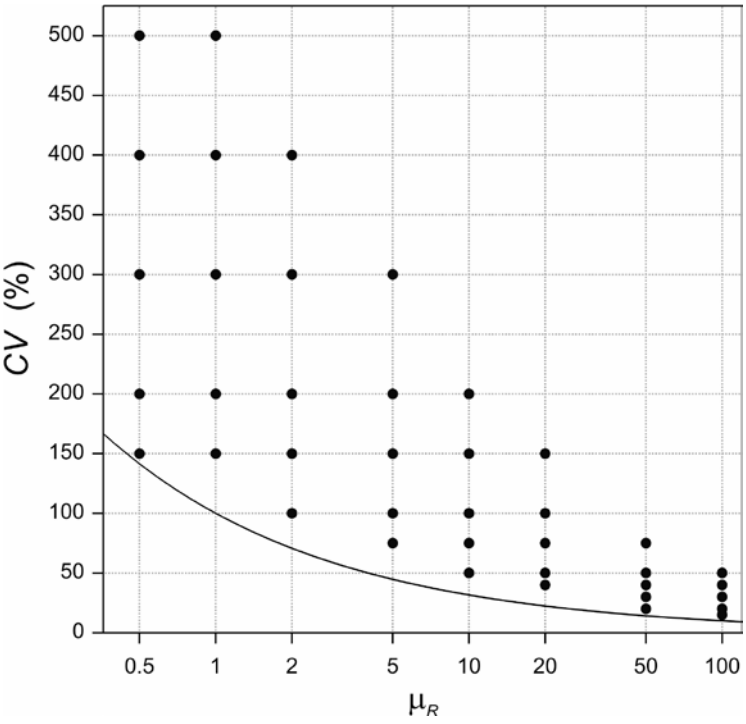
2.2 Parameter values used in the simulation

Depending on the NTO at hand, mean counts can be very small but can also be quite large. A range of 0.5 to 100 for the mean μ_C of the comparator is therefore employed.

Rather than focusing on the difference between μ_C and μ_G, it is more natural to focus on the ratio $Q = \mu_G/\mu_C$ of the two means. Generally accepted values in field testing for Q are between 0.5 and 0.25 (Comas et al, 2012). We used values 1, 0.75, 0.5 and 0.25. The value of 1, i.e. no difference between the comparator and the *GM* plant, is specifically meant to examine whether the difference test attains its nominal α-level.The other values of Q assume that the *GM* plant has a negative effect on the mean count.

The assumed variability in field testing of NTOs is mostly defined in terms of the coefficient of variation (CV). Duan et al (2006) present graphs with CV values ranging from 25% to 200% with generally low CV values for means larger than 5 and CV values up to 200% for means close to zero. In this study, five different values of CV are used for different values of μ_C as given in Figure 1 and Table 2. Compared to Duan et al (2006) the larger CV values used in this simulation study seem to be at the upper end of what can be expected in practical field trials.

Figure 1: Combinations of comparator means μ_C and coefficients of variation CV. The solid line denotes the coefficient of variation of a Poisson distribution.



Finally the level of replication N must be specified. Environmental risk assessment of *GM* plants is typically performed in experiments with a small number of plots. This is (partly) due to the fact that relatively large plots and large guard rows are required in order to measure effects on NTOs without bias, see Perry et al (2003). It is therefore that such experiments are frequently repeated in different years and different locations such that larger levels of

replication are obtained. A range of 4 to 100 for the level of replication N is employed in this study with some emphasis on lower values.

Table 2 summarizes the parameter values which are used in the simulation study. These values result in 1600 parameter combinations. For each combination of the simulation distribution (*OP*, *NB*, *PL* and *P1*) and parameter values 1000 datasets were simulated. Each dataset was analysed using the models given in the next session and an appropriate difference test at the 5% level was performed (details are given below). The proportion of datasets for which the difference test is rejected then gives an estimate of the true significance level (α) of the test when there is no difference, i.e. Q=1, and the power (β) of the test when there is a difference, i.e. $Q \neq 1$. These are only estimates of the true size of the test. Suppose that the size of the test is indeed exactly 5%, then with 1000 simulations a 99% prediction interval for the number of times the null hypothesis will be rejected is given by (33, 67) resulting in an interval of 3.3% – 6.7% for the true size. So only when the simulated significance level is outside this interval there is an indication that the true level of the test does not equal 5%.

Table 2: Parameters used in the simulation study.

Parameter		Values used in simulation				
Mean μ_C of comparator		0.5, 1, 2, 5, 10, 20, 50, 100				
Ratio $Q = \mu_G/\mu_C$		1, 0.75, 0.5, 0.25				
Number of replication N		4, 6, 8, 10, 15, 20, 30, 40, 60, 100				
μ_C	Coefficient of variation CV for comparator					
	CV-1	CV-2	CV-3	CV-4	CV-5	
0.5	150	200	300	400	500	
1	150	200	300	400	500	
2	100	150	200	300	400	
5	75	100	150	200	300	
10	50	75	100	150	200	
20	40	50	75	100	150	
50	20	30	40	50	75	
100	15	20	30	40	50	

Data were simulated using the statistical package GenStat (VSN international, 2013).

2.3 Statistical models for analysis

Fitting the Poisson-Lognormal model by means of maximum likelihood requires (adaptive) Gauss-Hermite integration within an iterative weighted least squares algorithm. This algorithm turned out to fail too frequently for data with small means, small levels of replication and/or small coefficients of variation. Therefor the Poisson-Lognormal model was not used to analyse simulated data. The other models with which each dataset was analysed are summarized in Table 3. All models were fitted using standard facilities in the statistical package GenStat (VSN international, 2013). Details for each analysis model are given below. A difference test for all models can be obtained by comparison of the fit of the model, more specifically the deviance, under the null-hypothesis H_0: $Q = 1$ and the fit of the model under the alternative hypothesis H_1: $Q \neq 1$.

Table 3: Statistical models used to analyse the simulated data.

Analysis model	Abbreviation	Type of difference test
Log transformation	*LN*	t-test
Squared-root transformation	*SQ*	t-test
Overdispersed-Poisson	*OP*	scaled deviance difference
Negative binomial	*NB*	deviance difference
Power model p=1.5	*P1*	scaled deviance difference
Power model p=1.7	*P2*	scaled deviance difference
Power model p=1.99	*P3*	scaled deviance difference
Gamma model	*GM*	scaled deviance difference

LN: Log transformation followed by a t-test

The count data are log-transformed after the addition of 1 to prevent taking the logarithm of zero. The simple two-sample t-test is then applied to the log transformed counts. The log transformation stabilizes the variance for distributions with a standard deviation which is proportional to the mean, or $Var(Y) \propto \mu^2$. This transformation therefore seems appropriate for the negative binomial and the Poisson-lognormal distribution with means that are not too small.

The two-sample t-test employs an estimate of the difference between the *GM* plant and the comparator on the transformed logarithmic scale. This difference is however a quantity that is not easy to interpret, especially when the underlying means μ_G and μ_C are small. Instead interest is in the ratio $Q = \mu_G/\mu_C$. The so-called generalized confidence interval approach can be applied to provide an interval for the ratio of two lognormal means, see Krishnamoorthy & Mathew (2003) and Chen and Zou (2006). According to these authors such an interval has excellent coverage probabilities. This approach uses the fact that, assuming that the log-transformed counts follow a normal distribution, the residual mean square follows a scaled Chi-squared distribution and that the two sample means follow a normal distribution which is independent of the Chi-squared distribution. A simulation approach is then used to generate a large sample for the ratio of the two lognormal means, accounting for the addition of 1. Percentiles of this large sample then define a confidence interval. More specifically, with X_C and X_G the two sample means on the log-transformed scale, S^2 the estimate of the variance on the transformed scale and $2N$-2 the number of degrees of freedom for S^2, a large sample for the ratio Q is generated in the following way

1. A random draw Chi is generated by means of Chi = ($2N$-2) S^2/χ_{2N-2} where χ_{2N-2} is a random draw from a Chi-squared distribution with $2N$-2 degrees of freedom;
2. N_C is a random draw from a normal distribution with mean X_C and variance Chi/N;
3. N_G is a random draw from a normal distribution with mean X_G and variance Chi/N;
4. Back-transform N_C by means of $N_C = \exp(N_C + Chi/2)$ and similarly N_G. Note that the back-transformation uses the equation for the mean of the lognormal distribution;
5. Subtract 1 from N_C and N_G; this accounts for the addition of 1 before log-transforming the count. This might sometimes result in a negative value for N_C or N_G. Such values are replaced by a small positive value, i.e. by 0.0001.
6. Calculate the ratio N_G/N_C

7. Repeat steps 1-6 many times, e.g. 10.000 or when more precise results need to be obtained 100.000 times. Calculate appropriate percentiles of the large sample which is the generalized confidence interval.

The generalized confidence interval can be used for difference testing as well as for equivalence testing.

SQ: Squared root transformation followed by a t-test

The squared root transformation is frequently used as an alternative for the log transform, and a simple t-test is also performed on squared root transformed counts. This transformation stabilizes the variance when the variance is proportional to the mean, or $Var(Y) \propto \mu$. This transformation is therefore especially appropriate for the overdispersed Poisson distribution.

The generalized confidence interval approach can also be employed to obtain an interval for the ratio on the original scale. The only modification to the seven steps described for the LN analysis is the back-transformation in step 4. For the squared root transform this is given by $N_C = N_C^2 + Chi$ which employs the well-known relation $Var(X) = \mathbb{E}X^2 - (\mathbb{E}X)^2$ where $\mathbb{E}$ denoted taking the expectation. Step 5 has to be skipped.

OP: Overdispersed Poisson by a GLM-like analysis

There does not seem to be standard software to fit the overdispersed Poisson distribution by means of maximum likelihood. However, a common way to analyse overdispersed counts is to use the quasi-likelihood approach of McCullagh and Nelder (1989). This amounts to fitting the ordinary log-linear model, which employs the Poisson distribution and a log-link, and to scale standard errors of parameter estimates by means of the squared root of an estimate of the dispersion parameter. This is the approach which is followed here. A scaled likelihood ratio statistic is obtained by calculating the scaled deviance difference of the model under H_0 and H_1. Scaling can be done by the mean deviance or by Pearson's Chi-squared statistic, both under H_1, and both methods are compared. The scaled likelihood ratio statistic is compared with a F distribution with 1 and $2N$-2 degrees of freedom to obtain a p-value.

In this model the underlying mean is log-transformed, rather than taking logs of the observed counts. This implies that the logarithm of the ratio of the two means, i.e. $\log(Q)$, is directly estimated in this model. A so-called Wald test statistic (Buse, 1982) can then be used for difference testing. This equals the quotient of the estimate of $\log(Q)$ and its standard error, and this is usually compared to a t-distribution to compensate for the estimation of the dispersion parameter. However it is generally believed that the likelihood ratio statistic has better statistical properties (McCullagh and Nelder, 1989). Moreover the Wald statistics breaks down when either sample only contains zero's since the estimate of $\log(Q)$, and its standard error, then becomes plus or minus infinity. So difference testing is based on the scaled likelihood ratio test. Equivalence testing under this model is however based on the estimate of $\log(Q)$ and its standard error, scaled by Pearson's statistic, which can be used to generate a confidence interval and thus to perform equivalence testing for arbitrary limits of concern. An alternative would have been to calculate a so-called profile likelihood interval but this requires a search algorithm which was considered to be too computer intensive in this simulation study.

NB: Negative binomial model by a GLM-like analysis

The negative binomial regression model, with logarithmic link, is fitted to the counts by means of maximum likelihood. The likelihood ratio test is calculated and compared to a Chi-squared(1) distribution. The dispersion parameter of the negative binomial distribution was bounded to the interval [0.001, 1000] to avoid numerical problems.

The estimate of $\log(Q)$ and its standard error is used for equivalence testing.

P1, P2 and P3: Power Law model by a GLM-like analysis

The Power model is defined by a variance-to-mean relationship and there is no true statistical distribution associated with it. Therefore, like the overdispersed Poisson model, quasi likelihood is used to fit the model. The quasi deviance D can be obtained by employing its definition, see McCullagh & Nelder (1989):

$$D(y;\mu) = 2\int_{\mu}^{y} \frac{y-t}{Var(t)}dt$$

For Taylors Power Law, i.e. $Var(t) = t^{\beta}$, the quasi deviance becomes

$$D(y;\mu) = 2\int_{\mu}^{y} \frac{y-t}{t^{\beta}}dt = 2\left[\frac{y\,t^{1-\beta}}{1-\beta} - \frac{t^{2-\beta}}{2-\beta}\right]_{\mu}^{y} = 2\left[\frac{y^{2-\beta}}{1-\beta} - \frac{y^{2-\beta}}{2-\beta}\right] - 2\left[\frac{y\,\mu^{1-\beta}}{1-\beta} - \frac{\mu^{2-\beta}}{2-\beta}\right]$$
$$= 2\left[\frac{y^{2-\beta}}{(1-\beta)(2-\beta)} - \frac{y\,\mu^{1-\beta}}{1-\beta} + \frac{\mu^{2-\beta}}{2-\beta}\right] = 2(z1 - z2 + z3)$$

The model is fitted using GenStats facilities for generalized linear models with non-standard variance functions. The GenStat directives for defining the model are as follows, where 'response' is the observed count, 'power' is the value of p in the variance function and 'z1', 'z2' and 'z3' are the three terms between squared brackets in the equation above.

```
CALCULATE b1,b2 = 1,2 - power
EXPRESSIO dcalc[1] ; VALUE=!e(vfunction = mu**power)
EXPRESSIO dcalc[2] ; VALUE=!e(z1 = response**b2/(b1*b2))
EXPRESSIO dcalc[3] ; VALUE=!e(z2 = response*mu**b1/b1)
EXPRESSIO dcalc[4] ; VALUE=!e(z3 = mu**b2/b2)
EXPRESSIO dcalc[5] ; VALUE=!e(deviance = 2*(z1-z2+z3))
MODEL     [DISTRIBUTION=calculated ; DCALCULATION=dcalc[] ; \
          LINK=log ; DMETHOD=pearson ; DISPERSION=*] response ; \
          FITTED=fitted ; VFUNCTION=vfunction ; DEVIANCE=deviance
```

To obtain a test-statistic the deviance difference can be scaled by the mean deviance or Pearson's test statistic, both under H_1. The test statistic was compared to a F distribution with 1 and $2N$-2 degrees of freedom. The power model was fitted with a fixed power p of 1.5, of 1.7 and of 1.99, and these are denoted by *P1*, *P2* and *P3* respectively. Note that a power p=2 is not allowed by the model as this implies division by zero.

A confidence interval is obtained for the estimate of $\log(Q)$ and its standard error, scaled by Pearson's statistic and using a t-distribution, and this is used for equivalence testing.

GM: Gamma model using a GLM-like analysis

The final analysis is by means of the Gamma distribution employing a log-link. Since the gamma distribution cannot handle zero observations, zeroes were replaced by 0.001. Again the deviance difference was scaled by the mean deviance or Pearson's chi-squared and compared with a F distribution with 1 and N-2 degrees of freedom to obtain a p-value. Also a confidence interval is obtained for the estimate of $\log(Q)$ and its standard error, scaled by Pearson's statistic and using a t-distribution, and this is used for equivalence testing.

Special cases

For small means and small levels of replication sample means can easily become zero for a simulated dataset. When both sample means equal zero, or more generally when both variances within samples equal zero, the analysis according to the log-transformation cannot be performed because the residual mean square equals zero. Some decision has to be taken to deal with such situations. Consider therefore the case with 4 observations of the comparator and 4 observations for the *GM* plant, with obvious generalizations to more observations. The four cases below are then special.

A. Sample 1 equals {0, 0, 0, 0} and sample 2 equals {0, 0, 0, 0}. In this case there is no information and the deviance under the null model and under the alternative model are both zero for all models. The p-value for the difference test is set to 1 for all analysis models as there is no indication of a difference between the two samples. For the most extreme parameter combination μ_R=0.5, CV=500, Q=0.25, N=4 and the overdispersed Poisson distribution this situation occurs for 570 of the 1000 simulated datasets. For negative binomial, Poisson-LogNormal and Power models these numbers are respectively 511, 287 and 565. Clearly there is also no information for calculating a confidence interval and thus formal equivalence testing cannot be performed. Graphical results for equivalence testing present the proportion of these cases separately. Note that this case can be considered as "equivalent more likely than not".
B. Sample 1 equals {0, 0, 0, 0} and sample 2 equals {c, c, c, c} where c is some positive value. The deviance under the alternative model equals zero and so no test statistic can be calculated. However this situation is very rare. For the Poisson-LogNormal distribution there are 28 parameter combinations for which this situation occurs with a maximum of 5 out of 1000 such datasets at most. For the other distributions this situation occurs even less. These situations are therefore discarded, i.e. the corresponding p-value is set to missing.
C. Sample 1 equals {0, 0, 0, 0} and sample 2 has different values with a positive variance. In this case all the p-values can be calculated in the usual way.
D. The mean of both samples are positive with a zero variance, e.g. {1, 1, 1, 1} and {3, 3, 3, 3}. This is essentially the same as case B although it will occur even rarely. There are only 2 simulated datasets for which this occurs and these are discarded.

3 Results for difference testing

3.1 General remarks on difference testing

A key element in environmental risk assessment it to test whether the *GM* plant is different from its conventional counterpart. The aim of a statistical difference test is to reject the null hypothesis of no difference between the *GM* plant and its comparator. A significant difference test is then a "proof of difference", but this does not state that the difference is biologically relevant and constitutes a true hazard to the environment. Poorly designed experiments with low levels of replication may have low statistical power of finding a true difference. So the absence of a significant difference is not a proof that there is no difference, or "absence of evidence is not evidence of absence" (Altman and Bland, 1995). There are two possible types of errors for a difference test. A type I error occurs when the null hypothesis of no difference is falsely rejected when it is actually true. In that case the incorrect conclusion is drawn that the *GM* plant is different from its comparator. A type II error on the other hand occurs when the null hypothesis is not rejected although it is untrue. Typically the probability of a type I error, also known as the size of the test or α, is set to some pre-described small value such as 5%, implying that in 5% of all tests the null hypothesis of no difference is falsely rejected. Given the size of the test, the probability of a type II error depends on the true difference, the level of variation and the level of replication. Note that the power of a test, frequently denoted by β, equals one minus the probability of a type II error.

The size of tests based on the normal distribution, such as the t-test, is exact. However tests based on other distributions, like the Poisson and the negative binomial, depend on asymptotic (meaning large levels of replication) arguments and are therefore not exact. This implies that a test, which is supposed to have a size of say 5%, might in practice have a different size. When the actual size of the test is larger than α the test is said to be progressive, when it is smaller the test is said to be conservative. Progressive tests are considered to be specifically bad because the null hypothesis of no difference is falsely rejected more often than the pre-described α level. Frequently the true underlying distribution of counts is not known. We might for instance falsely analyse data according to the Poisson distribution while in practice the data follow the negative binomial distribution or vice versa. This is particularly likely to happen when counts are small, as encountered frequently in ERA experiments, because then it is hard to discriminate between probability models. This ignorance about the true underlying distribution might result in difference tests to become even more progressive or conservative.

The power of a difference test based on the normal distribution can be calculated exactly. For non-normal distributions, small sample properties of difference tests are not straightforward. A simulation approach for sample size calculations for a difference test is employed by many authors, e.g. Shieh (2001) and Hrdličková (2006) for the Poisson distribution, Shieh (2001) and Demidenko (2008) for the binomial distribution, Aban et al (2009) and Friede and Schmidli (2010) for the negative binomial distribution. A general practical approach to computing power for non-normal distributions is given by Lyles et al (2007).

In the remainder of this chapter simulation results of various properties of the difference tests are presented. All results presented are for a two-sided test of no difference with a significance level α=5%. Detailed results are given in a separate document with Appendices.

3.2 Scaling of the deviance difference for *OP*, *P1*, *P2*, *P3* and *GM*

When data are analysed by means of the overdispersed Poisson, Power or Gamma model the likelihood ratio statistic can be scaled by means of the mean deviance or by means of Pearson's chi-squared, both for the full model. The simulated significance level of these two variants of the test statistic for specific parameter combinations is given in Figure 2 and Figure 3 when data are simulated by means of the negative binomial distribution with coefficients of variation as given by CV-1 and CV-3, and in Figure 4 and Figure 5 when data are simulated by means of the Poisson-Lognormal distribution. Each small plot has a range of 0 to 0.1 along the y-axis. The green line is halfway each small plot and denotes the assumed α=0.05. The red lines denote values 0.033 and 0.067 which provide a range that could be expected when 1000 datasets are simulated. So simulated sizes within the red lines are OK and such values are denoted by open circles. Values outside this range are denoted by filled circles, while values larger than 0.096 are given by triangles. Results for all parameter combinations are given in Appendix 1 A-D.

Overdispersed Poisson (OP) as analysis model

For small CV values (Figure 2 and Figure 4) and the overdispersed Poisson distribution as analysis model the size of both test statistics is good for values of $\mu \geq 2$. For smaller values of μ more replications are needed to attain the correct size. Scaling by means of Pearson's chi-squared seems to have the edge over scaling by means of the mean deviance. For larger CV values (Figure 3 and Figure 5) the size of the both test statistics is generally bad for $\mu \leq 2$. For larger replication levels and larger μ scaling by means of Pearson's chi-squared results in a better size than scaling by means of the mean deviance.

Power(1.5) (P1) as analysis model

For small CV values (Figure 2 and Figure 4) and the Power(1.5) analysis model, scaling by means of the mean deviance generally gives a conservative test for smaller values of μ, while scaling by means of Pearson's chi-squared has correct size, except for small values of μ and low level of replication N. For larger CV values (Figure 3 and Figure 5) both test statistics are progressive for small values of μ even for large replication levels N. For larger μ and simulating according to the negative binomial scaling by means of the mean deviance has better size than scaling by means of Pearson's chi-squared. However when data are simulated by means of the Poisson-LogNormal this is the other way around

Gamma (GM) as analysis model

For small CV values (Figure 2 and Figure 4) and the Gamma analysis model, scaling by means of the mean deviance is very conservative, while scaling by means of Pearson's chi-squared generally has the correct size. For larger CV values (Figure 3 and Figure 5) both test statistics perform badly for values $\mu \leq 5$. For larger means scaling by means of Pearson does have the edge especially when simulating according to the negative binomial distribution.

Figure 2: Size of the test when the deviance difference is scaled by means of the mean deviance and by means of Pearson's chi-squared. Data are simulated by the negative binomial distribution with CV-1 values.

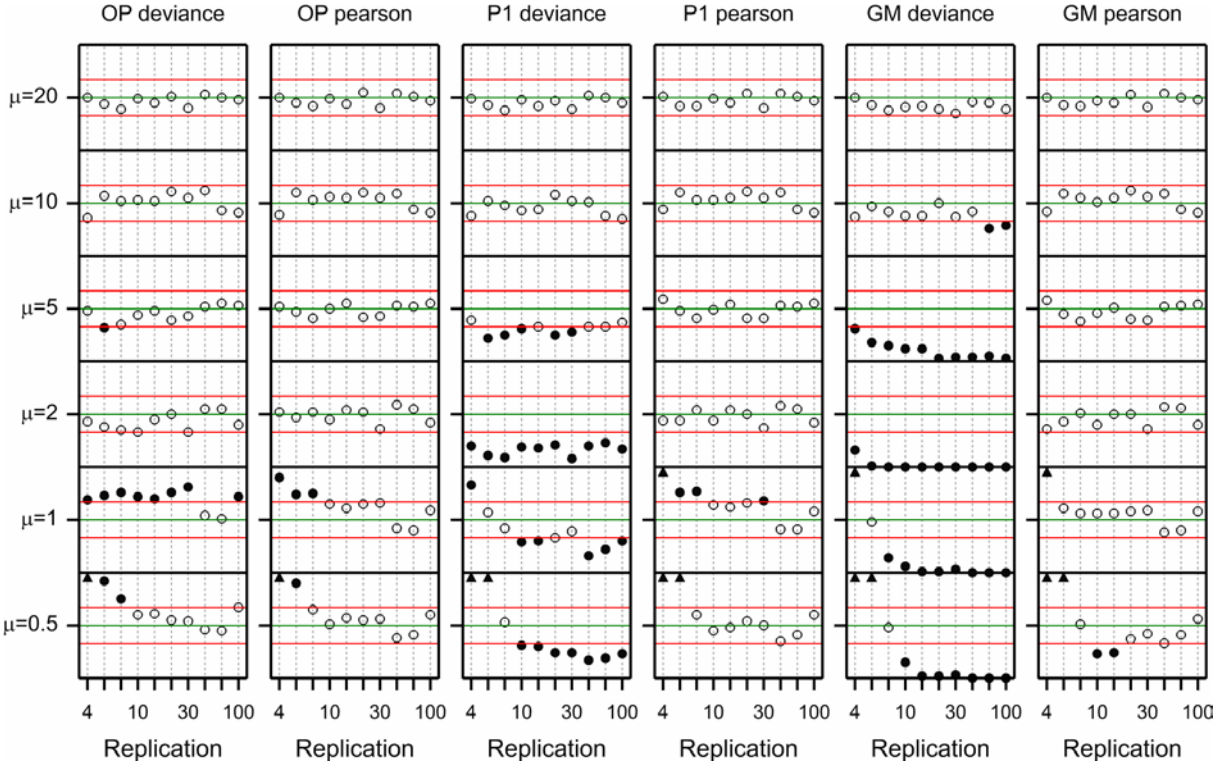


Figure 3: Size of the test when the deviance difference is scaled by means of the mean deviance and by means of Pearson's chi-squared. Data are simulated by the negative binomial distribution with CV-3 values.

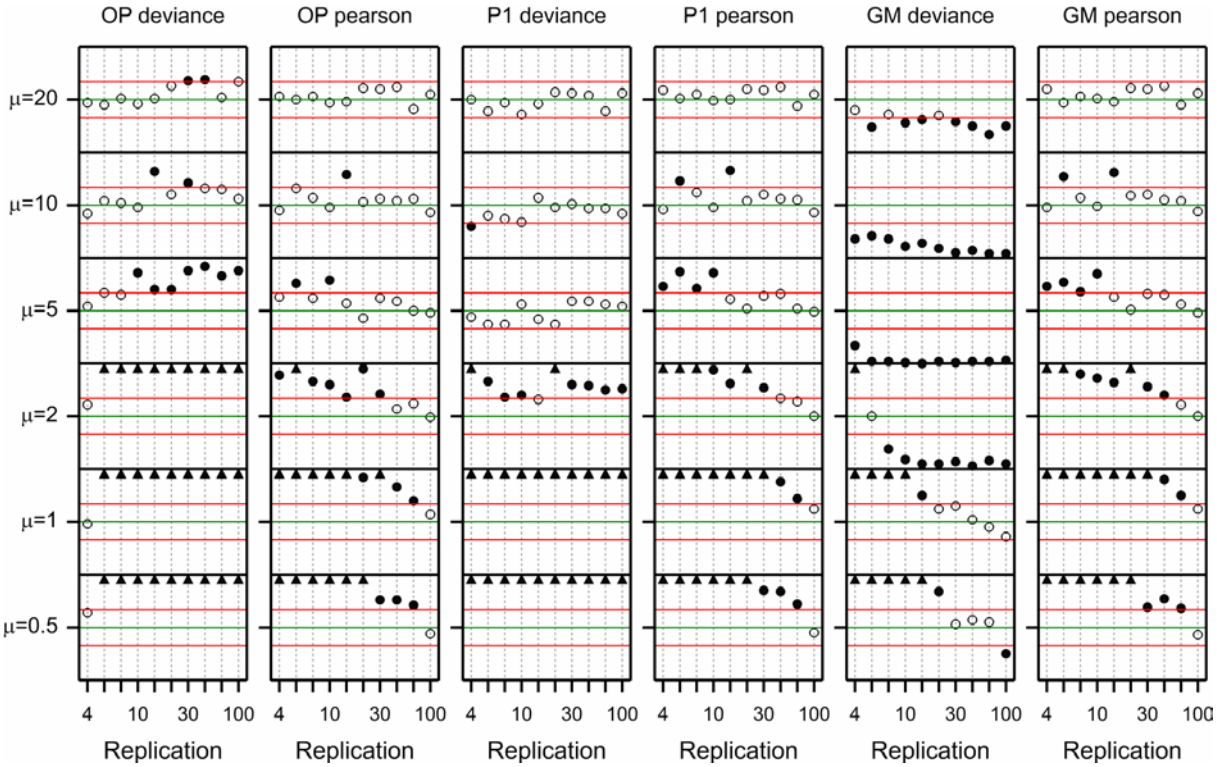


Figure 4: Size of the test when the deviance difference is scaled by means of the mean deviance and by means of Pearson's chi-squared. Data are simulated by the Poisson-LogNormal distribution with CV-1 values.

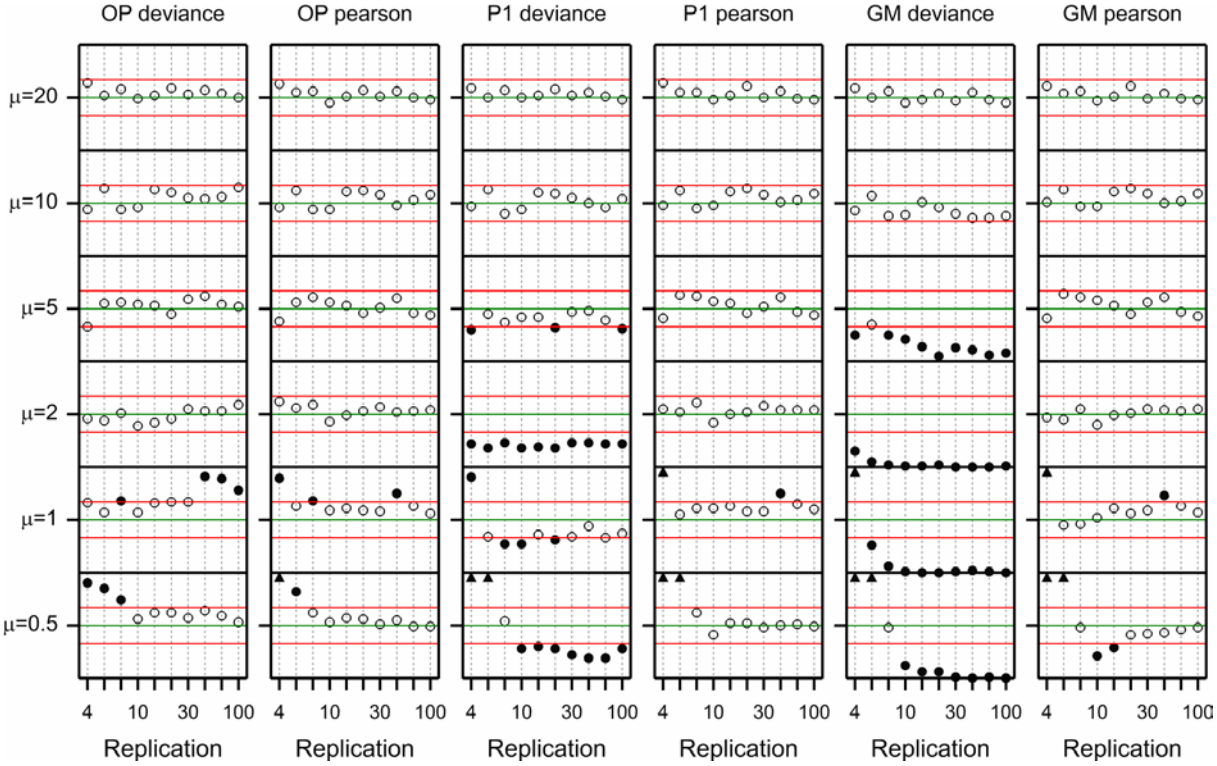
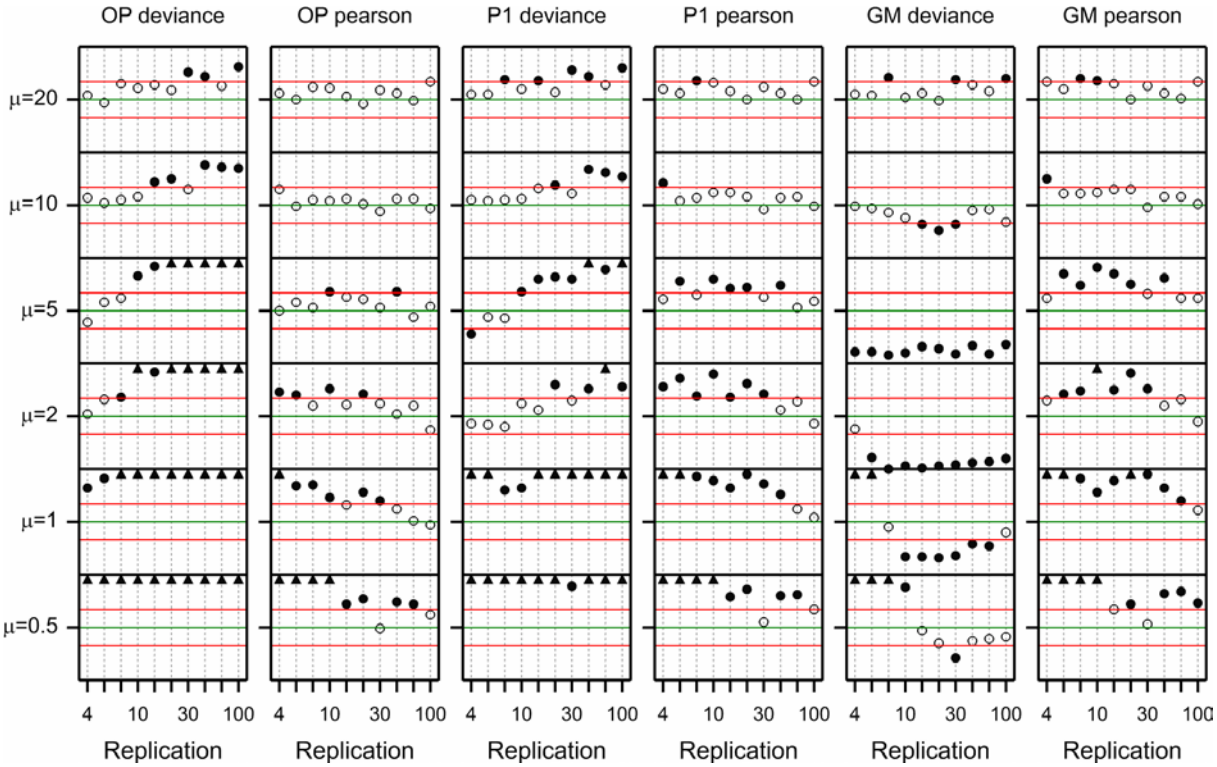


Figure 5: Size of the test when the deviance difference is scaled by means of the mean deviance and by means of Pearson's chi-squared. Data are simulated by the Poisson-LogNormal distribution with CV-3 values.



Conclusion

Scaling of the deviance difference by means of Pearson statistic seems to have somewhat better properties especially when the coefficient of variation is small. This conclusion is not only based on Figure 2 to Figure 5 but also on the results presented in Appendix 1 A-D. Therefore in subsequent comparisons the deviance difference will be scaled by means of Pearson's chi-squared for analysis according to the overdispersed-Poisson, the Power models and the Gamma model.

3.3 Simulated significance level of difference test

Having decided that scaling of the deviance difference by means of Pearson's statistic for *OP*, *P1*, *P2*, *P3* and *GM* generally has better properties than scaling by means of the mean deviance, the size of all analysis methods can be compared. Full details of the size of the difference test for all parameter combinations and simulation distributions are given in Appendix 1 E-H. Results for the *P3* model, with power 1.99, are not displayed since they are very similar to the results for the Gamma (*GM*) model. Results for specific combinations are given in Figure 6 to Figure 9.

Figure 6: Size of the difference test under various analysis models for data simulated by the negative binomial distribution with CV-1 values.

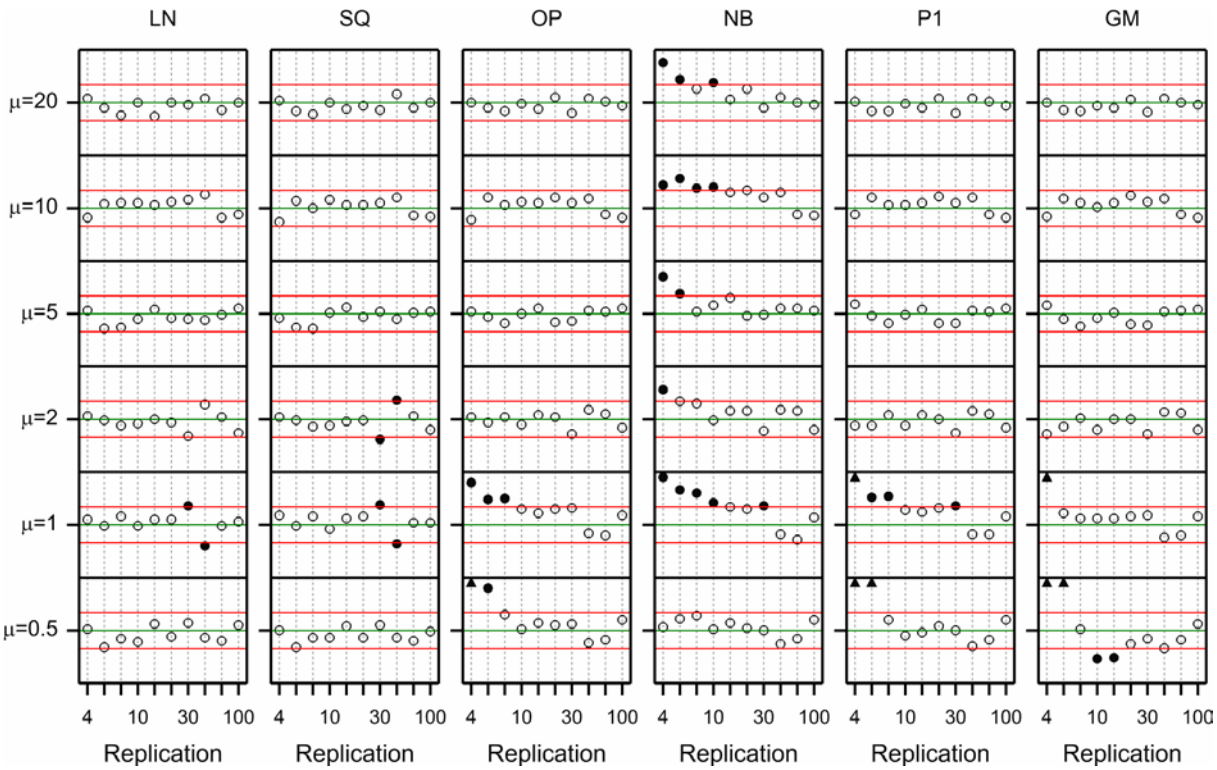


Figure 7: Size of the difference test under various analysis models for data simulated by the negative binomial distribution with CV-3 values.

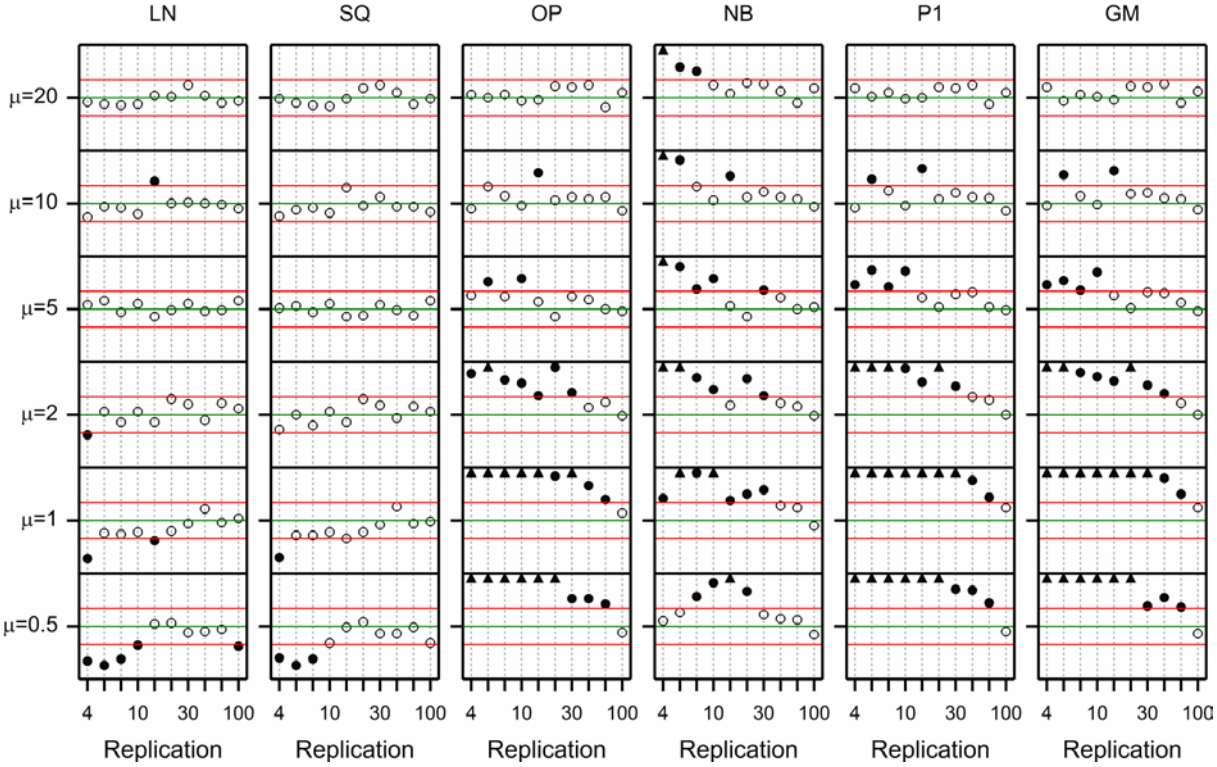


Figure 8: Size of the difference test under various analysis models for data simulated by the Poisson-LogNormal distribution with CV-1 values.

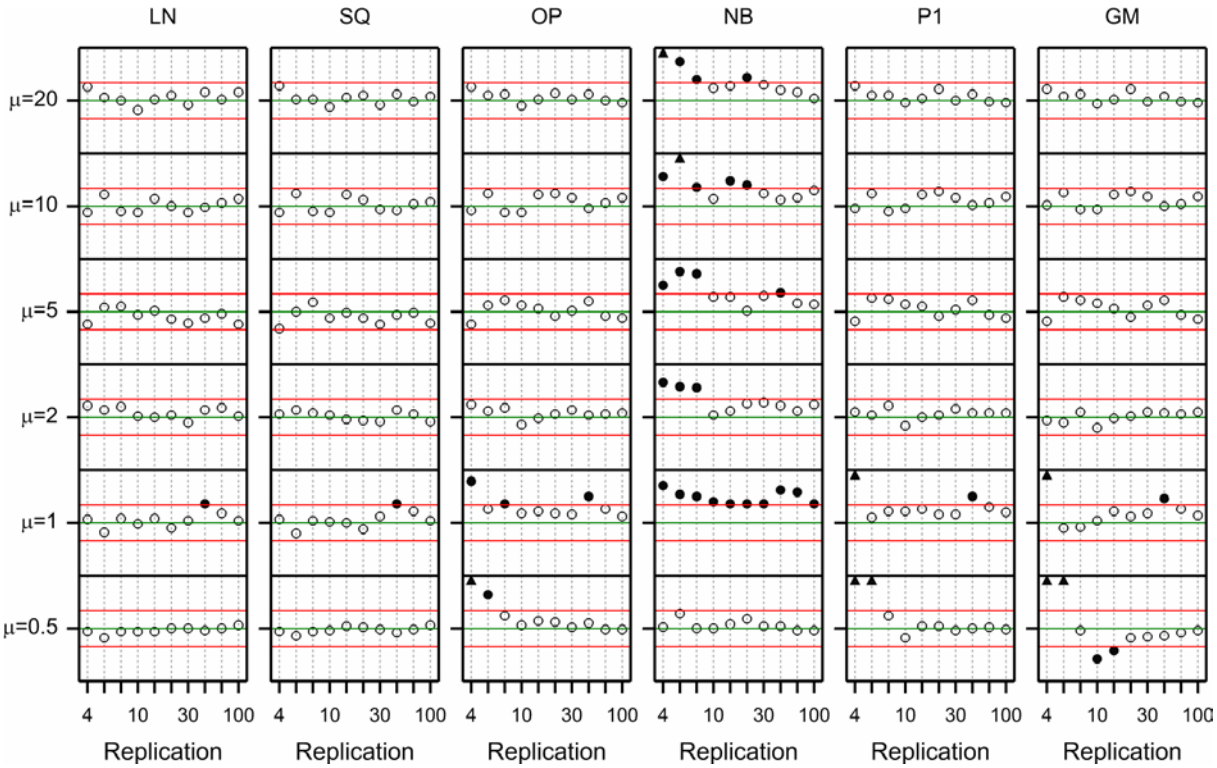
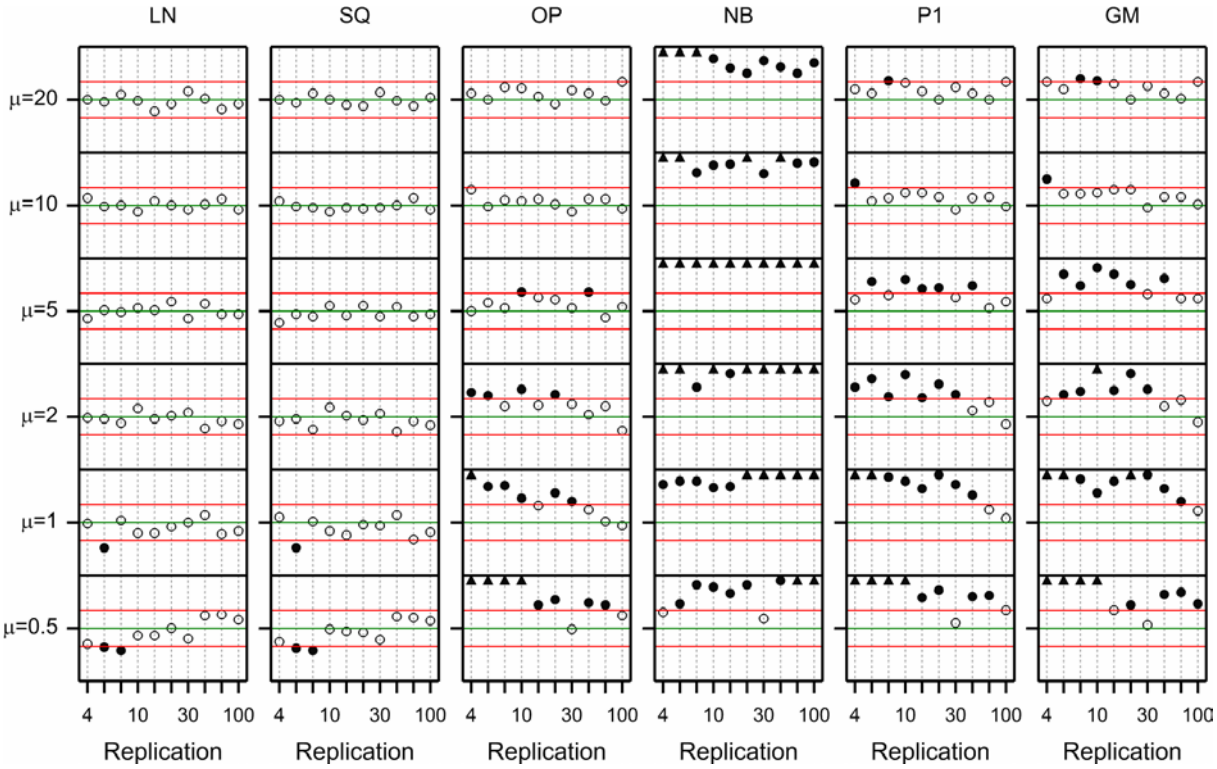


Figure 9: Size of the difference test under various analysis models for data simulated by the Poisson-LogNormal distribution with CV-3 values.



The size of the *LN* and *SQ* analysis is extremely good for all parameter combinations, except for small values of μ combined with large coefficients of variations CV and low levels of replication N. In such cases the *LN* and *SQ* tests are conservative. The GLM-like models result in sometimes progressive test especially for small means in combination with a large coefficient of variation. Among the GLM-like models there is no clear winner although the *OP* analysis seems to outperform the other GLM models somewhat, especially when data are simulated according to the Poisson-Lognormal distribution.

The simulated significance level for all parameter combinations and simulation distributions is summarized in Figure 10 to Figure 13. The symbols in Figure 10 to Figure 13 have the following meaning: *open circle* denotes that the test is conservative for lower levels of replication and has the correct size for larger replication; *closed circle* denotes that the test has correct size for all replication levels; *cross* means that the test is mainly progressive; *number* denotes that the test has correct size for levels of replication larger than the plotted number. These plots can be used to quickly check for which parameter combination, and for which level of replication, the difference test has correct size. These plots clearly indicate, once again, that the *LN* and *SQ* analysis models have superior size. The best alternative, especially for for larger means and smaller coefficients of variation is the *OP* analysis model.

Figure 10: Summary of size of difference test; data simulated by Overdispersed Poisson (see text for explanation of symbols)

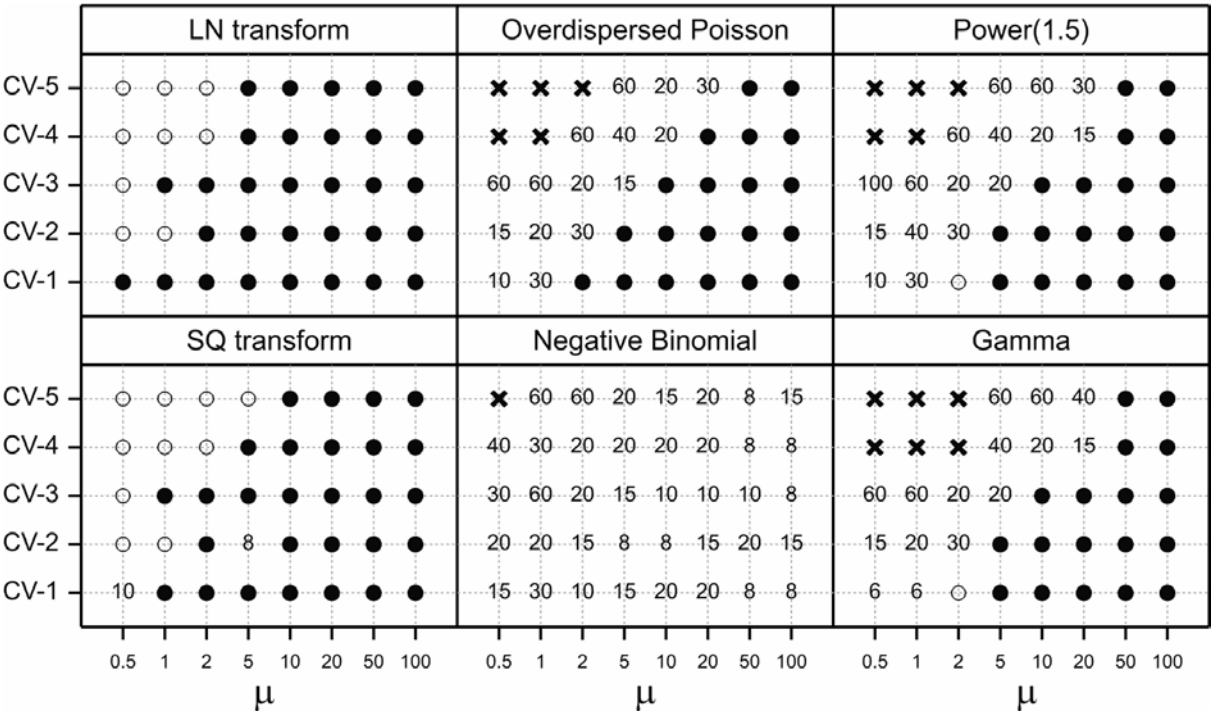


Figure 11: Summary of size of difference test; data simulated by Negative Binomial (see text for explanation of symbols)

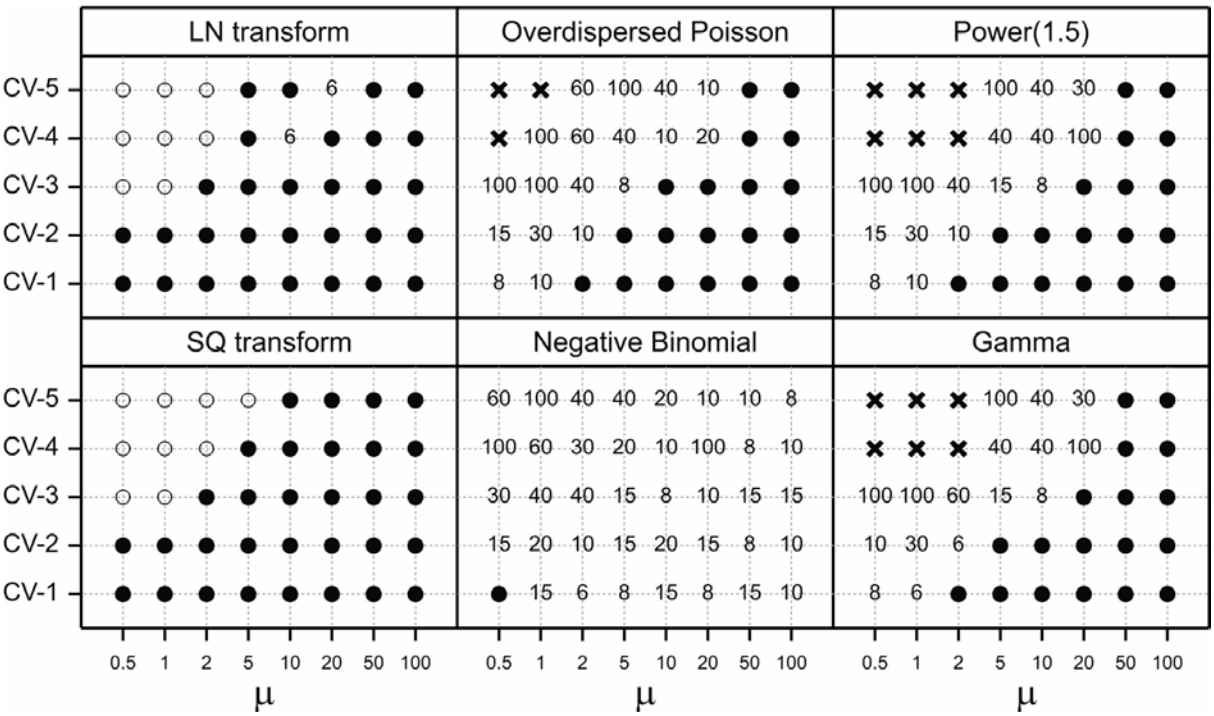


Figure 12: Summary of size of difference test; data simulated by Poisson-Lognormal (see text for explanation of symbols)

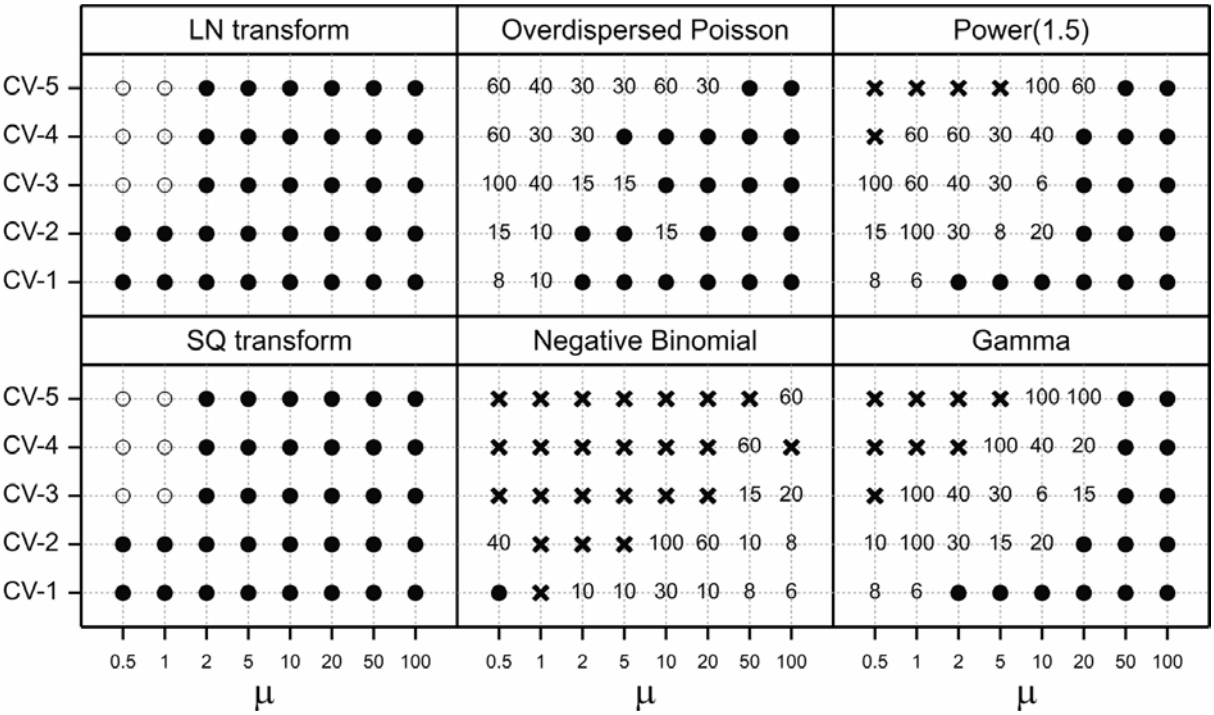
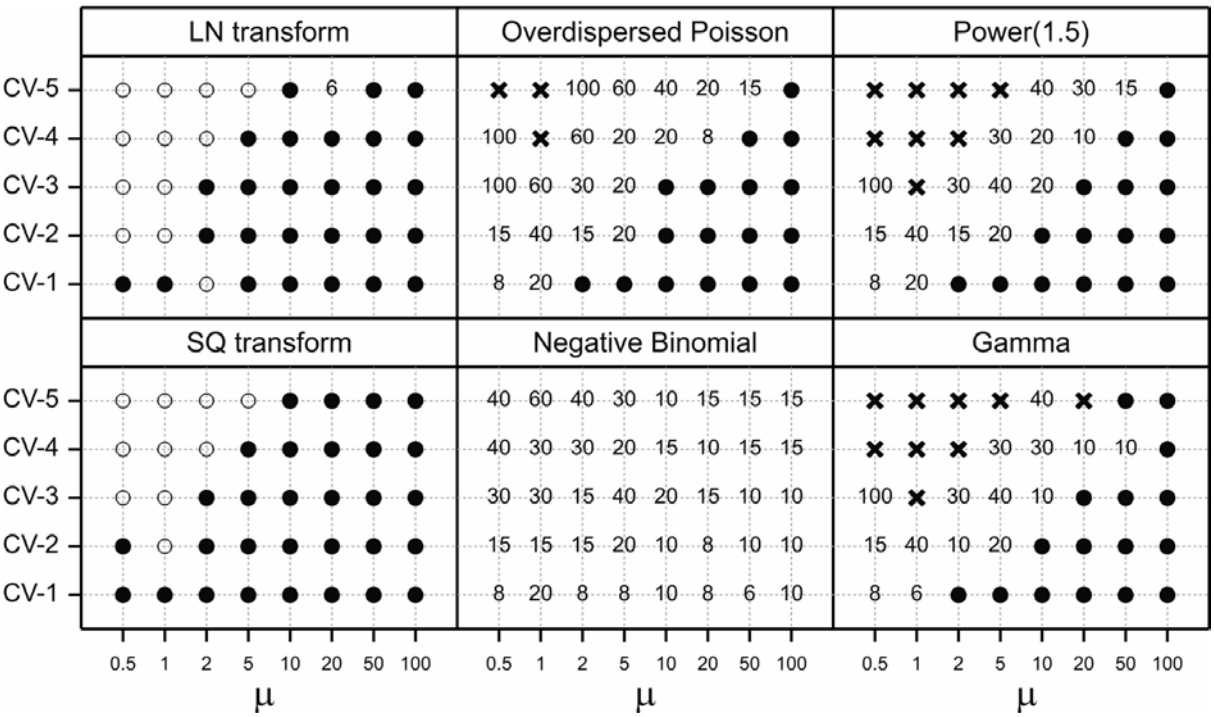


Figure 13: Summary of size of difference test; data simulated by Power(1.5) model (see text for explanation of symbols)



There is a large body of literature about the robustness of the two-sample t-test against departures from normality, two early references are Pearson and Adyanthāya (1929) and Gayen (1950). Miller (1986) summarizes the literature by noting that in case the skewness of the two samples is equal and so is the kurtosis then “the kurtosis parameters have little effect

on the t statistic and when the sample sizes are approximately equal the skewness parameters cancel each other approximately". So assuming that the two samples follow an identical distribution, the t-test is very robust against departures from normality. This does not imply that the t-test can also be applied to the counts themselves. Taking logs or a squared root makes the count distribution more symmetric giving less departures from normality which results in better properties of the t-test.

Conclusion

The simulated size of the t-test after a log or squared-root transformation, models *LN* and *SQ* respectively, is close to its nominal level, except for small means and large CV values. The other analysis models are progressive for small means and larger coefficients of variation. In other cases the *OP* analysis seems to outperform the other GLM-like models.

3.4 Power of difference test

The power of the difference test for all parameter combinations is given in Appendices I-L for effects size Q=0.75 (black), Q=0.50 (red) and Q=0.25 (green). Results for specific combinations are given in Figure 14 to Figure 17. Each small plot has a range of 0 to 1 along the y-axis. The grey horizontal lines denote power values of 0.25, 0.50 and 0.75. Values for progressive tests, i.e. when the simulated size of the test is larger than 0.067, are not displayed. This once again shows that the *LN* and *SQ* tests are never progressive.

When data are simulated according to the overdispersed Poisson distribution (Appendix 1 I) there is very little difference between the power of the various analysis models. However the *LN* and *SQ* method seem to have a somewhat larger power for larger values of μ.

When data are simulated according to the negative binomial distribution (Appendix 1 J, Figure 14 and Figure 15) the *LN* test occasionally has slightly smaller power than the other tests. An example is given in Figure 15 for μ=10 and μ=20. The GLM-like models have very similar power.

When data are simulated according to the Poisson-Lognormal distribution (Appendix 1 K, Figure 16 and Figure 17) the power of the *LN* and *SQ* tests is as least as good as for the other models.

When data are simulated according to the Power *P1* model (Appendix 1 L), once again the power of the *LN* and *SQ* tests is as least as good as for the other models.

Conclusion

The power of the *LN* and *SQ* approach is generally very similar to the power of the other analysis methods. In some cases the power of *LN* and *SQ* is marginally larger, in other cases it is marginally lower. Because there is very little difference between the power of the various models, other properties, like the size of tests, of the various models should be decisive as to which method is to be preferred.

Figure 14: Power of the difference test for effects Q=0.75 (black), 0.50 (red) and 0.25 (green) under various analysis models for data simulated by the negative binomial distribution with CV-1 values

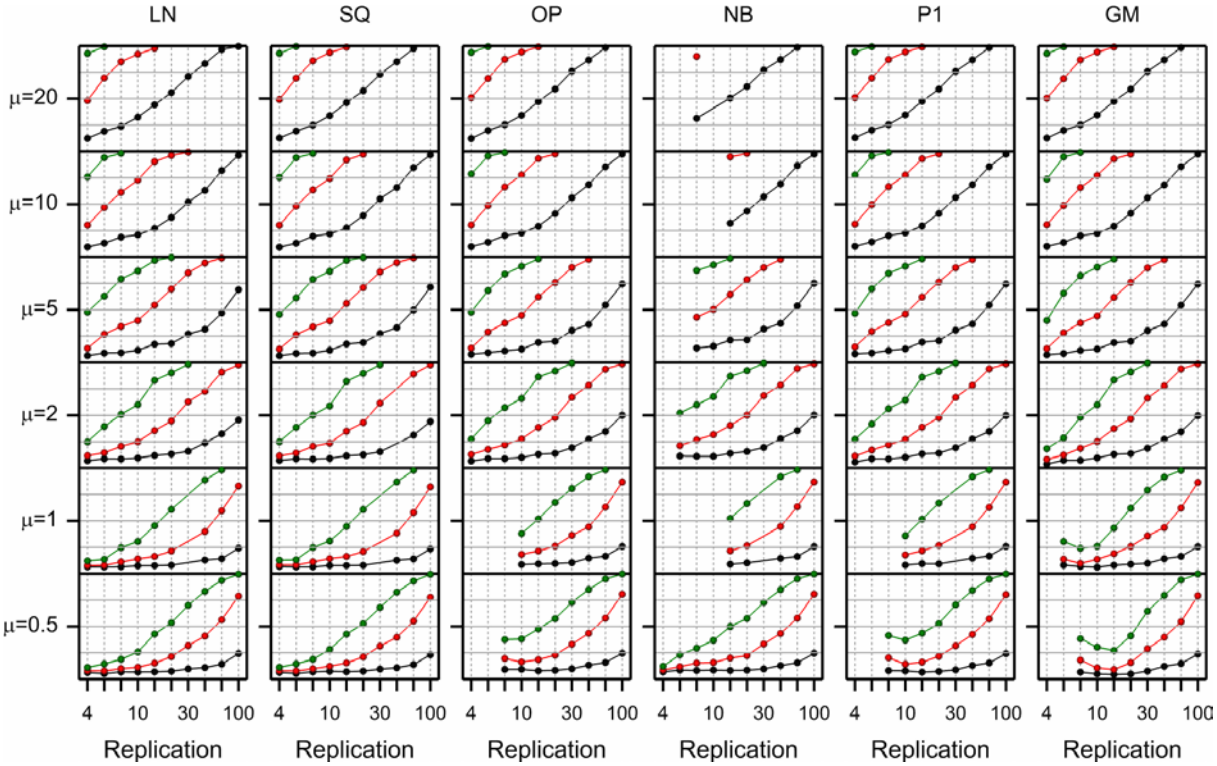


Figure 15: Power of the difference test for effects Q=0.75 (black), 0.50 (red) and 0.25 (green) under various analysis models for data simulated by the negative binomial distribution with CV-3 values

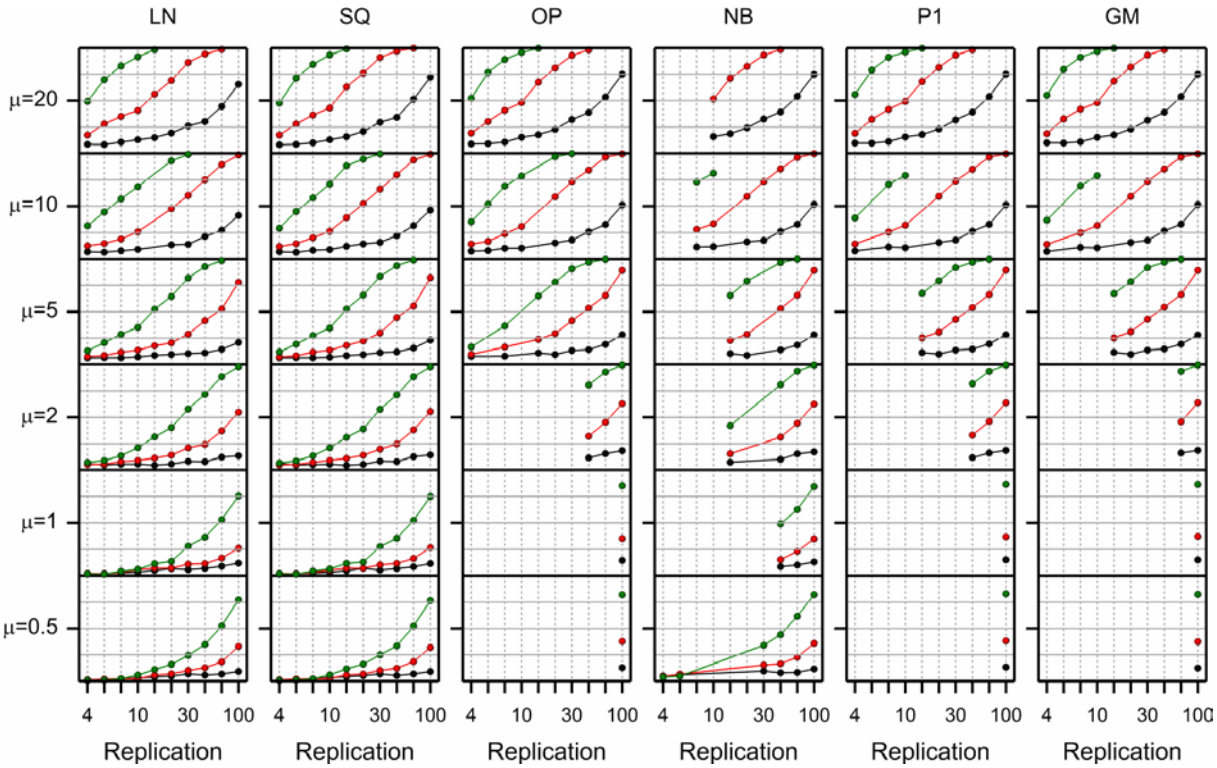


Figure 16: Power of the difference test for effects Q=0.75 (black), 0.50 (red) and 0.25 (green) under various analysis models for data simulated by the Poisson-Lognormal distribution with CV-1 values

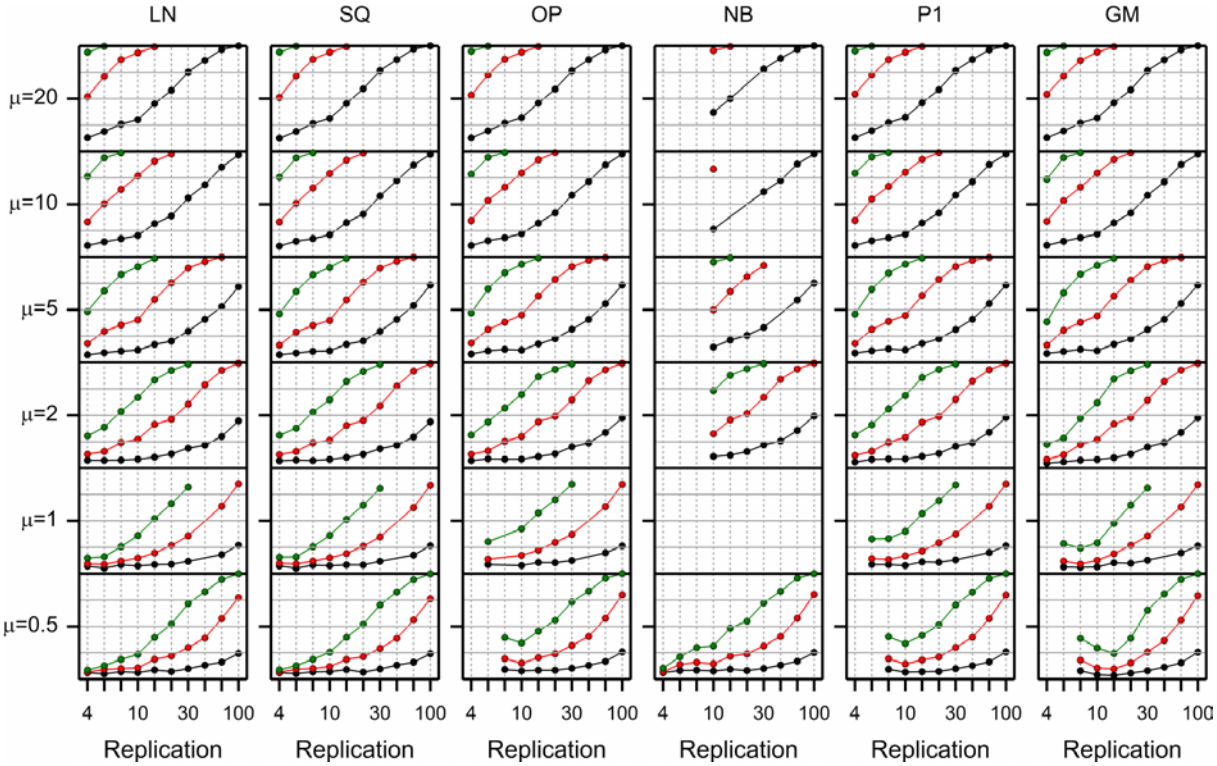
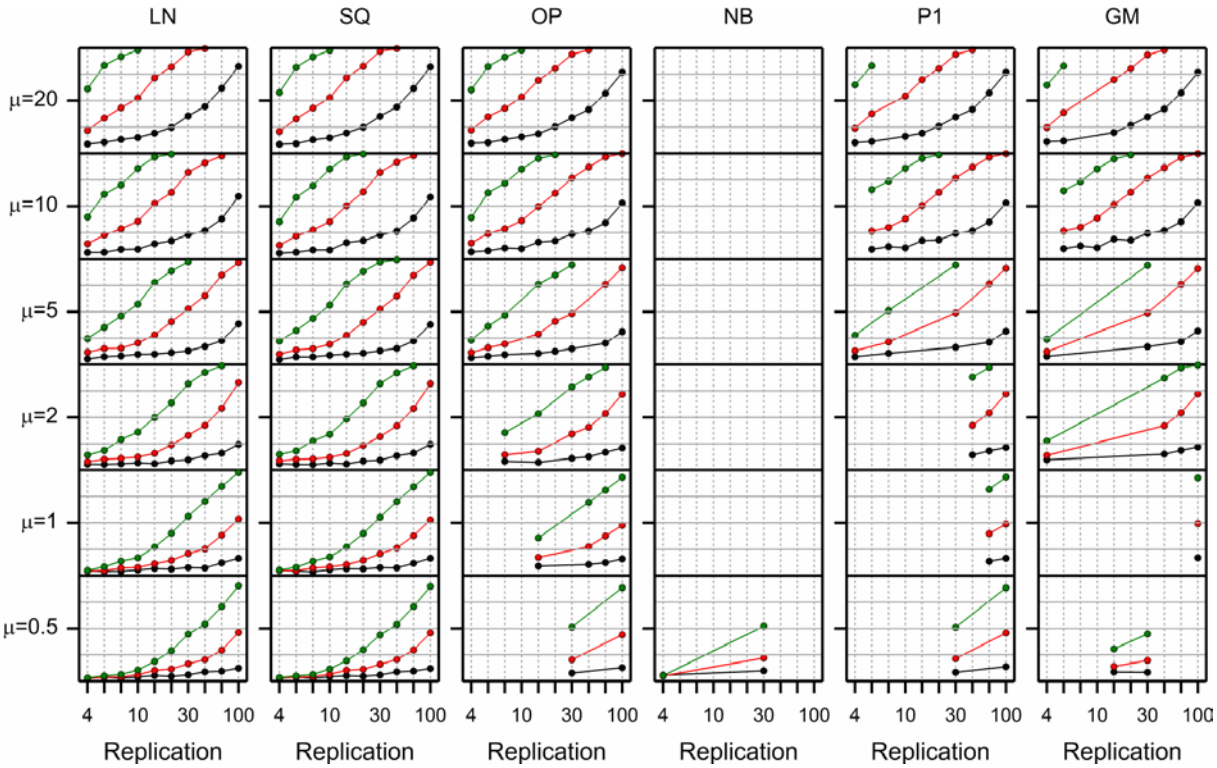


Figure 17: Power of the difference test for effects Q=0.75 (black), 0.50 (red) and 0.25 (green) under various analysis models for data simulated by the Poisson-Lognormal distribution with CV-3 values



3.5 Coverage of confidence intervals

After each analysis a 95% two-sided confidence interval can be constructed for the ratio $Q = \mu_G/\mu_C$ of the two means. This uses the generalized confidence interval approach for the *LN* and *SQ* models. For the GLM-like models the confidence interval is constructed in the usual way employing the estimate of $\log(Q)$ and its standard error. One minus the simulated coverage probabilities of these intervals are given in Appendix 1 M-P. Each small plot has a range of 0 to 0.1 along the y-axis. The green line is halfway each small plot and denotes the assumed α=0.05. The red lines denote values 0.033 and 0.067 which provide a range that could be expected when 1000 datasets are simulated. So simulated sizes within the red lines are OK and such values are denoted by open circles. Values outside this range are denoted by filled circles, while values larger than 0.096 are given by triangles. Note that in this case large values denote a confidence interval that is too small (i.e. has smaller coverage probability than 95%), while small values indicate a confidence interval that is too wide (i.e. has larger coverage probability than 95%).

The *LN* and *SQ* generalized confidence interval can be used to test the null hypothesis of equal means. For *LN* both the simulated significance level and the simulated power of the generalized confidence interval are identical to those of the t-test. This can, for the simulated significance level of the *LN* analysis when data are simulated according to the overdispersed Poisson, be seen by comparing the first columns in Appendix 1 E with the top graphs in Appendix 1 M1. Similarly for *SQ* the second columns in Appendix 1 E can be compared with the top graphs in Appendix 1 M3. It then turns out that the generalized confidence interval for *SQ* has a slightly lower size significance level than the corresponding t-test for small means combined with small levels of replication and larger CV values. Similar comparisons can be made when data are simulated by the other three distributions. Results for the simulated power of the difference test employing the generalized confidence interval are not shown, but these are for the *LN* analysis also identical to the power of the t-test.

However the properties of the *LN* and *SQ* generalized confidence interval are only good when testing the null hypothesis of equal means. Coverage of the *LN* interval deteriorates when the ratio $Q = \mu_G/\mu_C$ of the two means becomes smaller, e.g. the bottom graphs in Appendix 1M1 for Q=0.75 and Appendix 1 M2 for Q=0.50 and Q=0.25. Coverage of the *SQ* interval is even worse for values $Q \neq 1$, see Appendix 1 M3 and M4. So it appears that the generalized confidence interval of *LN* and *SQ* can be used for difference testing, but it cannot be used for equivalence testing.

The coverage probability of the *OP* interval, when simulating according to the overdispersed Poisson, is generally better than those of the other GLM-like models (Appendix 1 M5-12). For smaller values of μ and larger values of CV the *OP* interval is too wide indicating that the corresponding test is conservative. Intervals for *NB*, *P1* and *GM* can be too short or too wide depending on the parameter combination.

When data are simulated by means of the negative binomial distribution (Appendix 1 N) the *OP* interval generally has the better properties; the *OP* interval is almost never too small. However for Q=0.25 the *OP* interval is somewhat too wide, while the *NB* and *P1* intervals

then have a better coverage except when the CV is large in which case these intervals can become too small. The *P1* interval seems to have somewhat better coverage than the *NB* interval.

When data are simulated according to the Poisson-Lognormal distribution (Appendix 1 O) the *NB*, *P1* and *GM* interval can be too small especially for smaller μ and larger CV values. For other values the *P1* interval seems to have the edge over the *NB* and *GM* intervals. The *OP* interval is, once again, somewhat too wide for Q=0.25.

Results for the Power model (Appendix 1 P) are similar to those for the Poisson-Lognormal.

Conclusion

The *LN* and *SQ* generalized confidence intervals have the same properties as the t-test for difference testing, although the *SQ* interval has a somewhat lower simulated significance level for some parameter combinations. However these intervals do not have good coverage probability for $Q \neq 1$, especially not for small values of Q. In such cases the *LN* interval has a less worse coverage probability than the *SQ* interval. The *OP* interval is almost never too small (meaning that the coverage is not smaller than 95%). It can be too wide though especially for Q=0.25 in combination with a simulation distribution other than overdispersed Poisson. In such cases the *P1* interval seems to be the method of choice although *P1* has the disadvantage that interval can be too small when simulating according to the overdispersed Poisson, and also for smaller μ and larger CV values for the other simulation distributions.

3.6 Approximate power of the difference test

Lyles et al (2007) describe a general method to approximate the power of a difference test for generalized linear models. Their approach makes use of a single 'expanded' dataset based on the response distribution. This expanded dataset is then analysed using an appropriate model and the power of the test statistic, either Wald or likelihood ratio, can then be calculated employing a Chi-squared distribution with a non-centrality parameter which can be easily calculated.

This approximate method is compared with the simulated power of the *LN* analysis. The approximate method consists of the following steps:

1. Create an 'expanded' dataset for the simulation distribution at hand. First choose the possible outcomes $y_{C1}, \ldots, y_{CK}$ for a mean value μ_C of the distribution and calculate the corresponding probabilities $w_{C1}, \ldots, w_{CK}$. The sum of these probabilities should then be close to one. Do the same for a mean value of μ_G giving possible outcomes $y_{G1}, \ldots, y_{GL}$ with probabilities $w_{G1}, \ldots, w_{GL}$, with again a sum close to one. Then simply stack the two vectors of possible outcomes and also the two vectors of corresponding probabilities, denote these as Y and W. Also create an indicator vector X with a zero for the first set of possible outcomes and a one for the second set. This results in the 'expanded' dataset consisting of Y, W, and X which are of equal length.
2. The mean and variance of both log-transformed samples are calculated employing $Mean_C = \sum_i w_{Ci} \log(y_{Ci} + 1)$ and $Var_C = \sum_i w_{Ci} [\log(y_{Ci} + 1) - Mean_C]^2$ and

similarly for $Mean_G$ and Var_G. An estimate of the residual variance on the transformed scale is then given by $Var = 0.5(Var_C + Var_G)$.

3. The 'expanded' dataset is analysed by means of a weighted regression of $\log(Y + 1)$ on X with weights W/Var and fixed residual variance equal to 1. This results in an estimate of the regression coefficient β for X along with a standard error se.
4. The non-centrality parameter is then given by $\delta = N\ (\beta/se)^2$ where N is the number of replications. The same non-centrality parameter is obtained by calculating N times the difference between the residual sums of squares of the weighted regression model without X and the residual sums of squares of the model with X.
5. The power is calculated in the following way. A critical value F_{crit} is obtained from the F distribution with 1 and 2N-2 degrees of freedom. i.e. $P(F_{1,2N-2} > F_{crit}) = \alpha$. The approximate power is then calculate by means of the non-central χ_1 distribution with non-centrality parameter δ, i.e. by means of $P(\chi_{1\ (\delta)} > F_{crit})$.

A crucial step is the calculation of the residual variance Var on the transformed scale. The non-centrality parameter δ is proportional to the number of replications N, so there is no need for stacking the two vectors N times as is proposed by Lyles et al (2007). This implies that a single 'expanded' dataset can be used for all levels of replication N instead of a separate 'expanded' dataset for each level of replication.

In the implementation of this approach it was found that it might be numerically more stable to use weights $N_0 W/Var$ where N_0 is some fixed large number, e.g. 100. This is because units with very small weights, in this case with very small probabilities, are sometimes discarded when fitting a regression model. The non-centrality parameter is then given by $\delta = (N/N_0)\ (\beta/se)^2$.

The approximate power is calculated for all four distributions and compared with the simulated power. Graphical results are given in Appendix 1 Q1, R1, S1 and T1. Each small plot has a range of 0 to 1 along the y-axis. The grey horizontal lines denote power values of 0.25, 0.50 and 0.75. Simulated powers are given by the dots for Q=0.75 (black), Q=0.50 (red) and Q=0.25 (green), while the approximate power is given by the lines. Across the board there is very good agreement between the two methods. For low power values and smaller numbers of replications the approximate method of Lyles can be somewhat too small, but such low power values are hardly of interest.

The same approach can be followed for the *SQ* analysis, see Appendix 1 Q2, R2, S2 and T2, except that the squared root transformation is used instead of the log transformation. Also in this case there is very good agreement between the simulated power and the approximated power.

The same approximate method can be applied for an analysis according to one of the other models. For parameter combinations with a simulated significance level which is not (too) progressive, the two methods agree closely when analysing with a negative binomial for all four simulation distributions (Appendix 1 Q4, R4, S4 and T4). For an analysis with the power *P1* model (Appendix 1 Q5, R5, S5 and T5) the approximation is good when data are simulated according to the negative binomial or the power model especially for larger power values. When simulating with the overdispersed Poisson or the Poisson-Lognormal

distribution the method of Lyles sometimes gives less good results for the *P1* analysis. For an analysis with the *OP* model ((Appendix 1 Q3, R3, S3 and T3) the approximation is frequently less good, except for larger means with low CV levels.

Conclusion

When two-sample count data are analysed by means of the *LN* or *SQ* model the method of Lyles et al (2007) approximates the power very well for all four simulation distributions. In such a case there is no need to perform a simulation study to approximate the power.

3.7 Method of choice for difference test

The simulated size of the t-test after a log or squared-root transformation, models *LN* and *SQ* respectively, is close to its nominal level, except for small means and large CV values where the test is conservative. The other analysis models are progressive for small means, small levels of replication and larger coefficients of variation. In other cases the *OP* analysis seems to outperform the other GLM-like model.

The power of the *LN* and *SQ* approach is generally very similar to the power of the other analysis methods. In some cases the power of *LN* and *SQ* is marginally larger, in other cases it is marginally lower. In those case where the *LN* and *SQ* analysis are conservative (small means, small levels of replication and larger coefficients of variation), the power is so low that it is hardly worthwhile to perform such experiments. In other words for parameter combinations with sufficient power the size of the *LN* and *SQ* tests is close to its nominal level.

The *LN* generalized confidence interval has the same properties as the t-test for difference testing, with respect to the simulated significance level and with respect to the simulated power. This is also true for the *SQ* interval although the simulated size using this interval is smaller than that of the corresponding t-test for small means combined with low replication and larger CV values. The *LN* and *SQ* intervals do not have good coverage probability for ratios $Q \neq 1$. This is especially the case for the *SQ* interval and for values of Q which are well away from one.

The method of Lyles et al (2007) can be used to approximate the power of the difference test. This approximation is very accurate for the *LN* and *SQ* analysis.

The *LN* or *SQ* analysis therefor seems to be the method of choice for all simulation distributions. They have good size for all relevant parameter combinations, their power is comparable to the other analysis methods, a generalized confidence interval has good properties when it is used for difference testing, and an approximate quick method can be employed for a prospective power analysis. Because the *LN* generalized confidence interval has somewhat better properties than the *SQ* interval, the *LN* analysis method seems to be the method of choice.

4 Results for equivalence testing

4.1 General remarks on equivalence testing

A difference test aims to reject the null hypothesis of no difference, i.e. in the current setting to reject the hypothesis that Q=1. Poorly designed experiments with low levels of replication may have low statistical power of finding a true difference. An equivalence test on the other hand employs a null hypothesis of non-equivalence, i.e. that the ratio Q is smaller, or larger, than some pre-described equivalence limit, also called limit of concern (LOC). Rejection of the non-equivalence hypothesis implies that the ratio is larger than the LOC and this can be regarded as a "proof of safety". The advantage of equivalence testing is therefore that the onus is placed back on to those who wish to demonstrate the safety of GMOs to do high quality, well-replicated experiments with sufficient statistical power (Perry et al, 2009). Note that both the difference and equivalence test can be implemented by constructing a confidence interval for the ratio of the means of the *GM* plant and its comparator. When there is both an lower and an upper Limit of Concern, the two one-sided tests (TOST) approach of Schuirmann (1987) for equivalence testing can be used.

In the sequel results for a one-sided equivalence test, with significance level 5%, are given where the limit of concern is smaller than one. The null hypothesis is thus H_0: $Q \leq LOC$ with alternative hypothesis H_1: $Q > LOC$. Different limits of concern are used in different sections.

All results are based on the generalized confidence interval for *LN* and *SQ* and on the ordinary interval for $\log(Q)$ for the GLM-like models where the standard error is scaled by Pearsons Chi-squared if appropriate. An alternative would be to use a likelihood ratio interval.

4.2 Size of equivalence test

The simulated size of the one-sided equivalence test is available for those LOC values which are equal to the ratio Q. For Q=1 the equivalence test equals the one-sided difference test; results for the simulated size of the two-sided difference test are already given in Section 0. Results for values Q=0.75, Q=0.50 and Q=0.25 are given in Appendix 2 A-D. Each small plot has a range of 0 to 0.1 along the y-axis. The green line is halfway each small plot and denotes the assumed α=0.05. The red lines denote values 0.033 and 0.067 which provide a range that could be expected when 1000 datasets are simulated. So simulated sizes within the red lines are OK and such values are denoted by open circles. Values outside this range are denoted by filled circles, while values larger than 0.096 are given by triangles.

The *LN* and *SQ* generalized confidence interval have a generally bad simulated significance level, especially for smaller values of Q. This is in accordance with findings in section 3.5, and the *LN* and *SQ* interval will further not be discussed. Furthermore the *P1*, *P2* and *GM* interval have very similar simulated sizes; only the *P1* analysis method will therefore be considered in the sequel.

For the *OP*, *NB* and *P1* intervals and Q=0.50, Appendices 2 A-D are summarized in Figure 18 to Figure 21. For Q=0.25 the appendices are summarized in Figure 22 to Figure 25. The

symbols in these figures have the following meaning: *open circle* denotes that the test is conservative for lower levels of replication and has the correct size for larger replication; *closed circle* symbolizes that the test has correct size for all replication levels; *cross* means that the test is mainly progressive; *number* denotes that the test has correct size for levels of replication smaller than or equal to the plotted number; / means that for larger levels of replication the test is progressive or that the test is progressive for some other replications; \ indicates that the test is progressive for small replication and has the correct size for larger replication.

Figure 18: Summary of size of equivalence test for Q=LOC=0.5; data simulated by Overdispersed Poisson (see text for explanation of symbols)

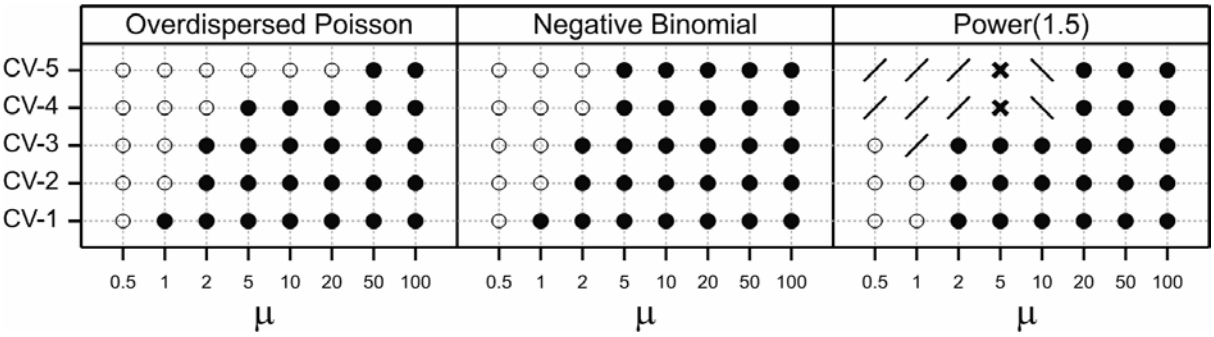


Figure 19: Summary of size of equivalence test for Q=LOC=0.5; data simulated by Negative Binomial (see text for explanation of symbols)

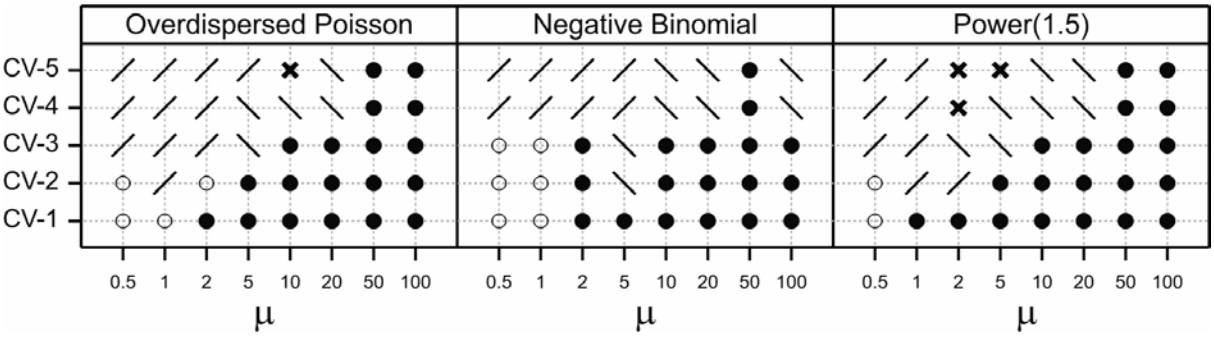


Figure 20: Summary of size of equivalence test for Q=LOC=0.5; data simulated by Poisson-Lognormal (see text for explanation of symbols)

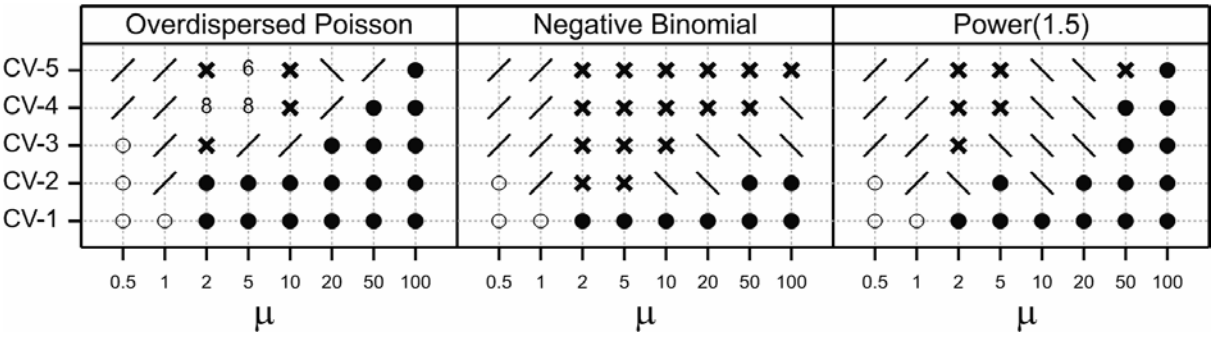


Figure 21: Summary of size of equivalence test for Q=LOC=0.5; data simulated by Power(1.5) model (see text for explanation of symbols)

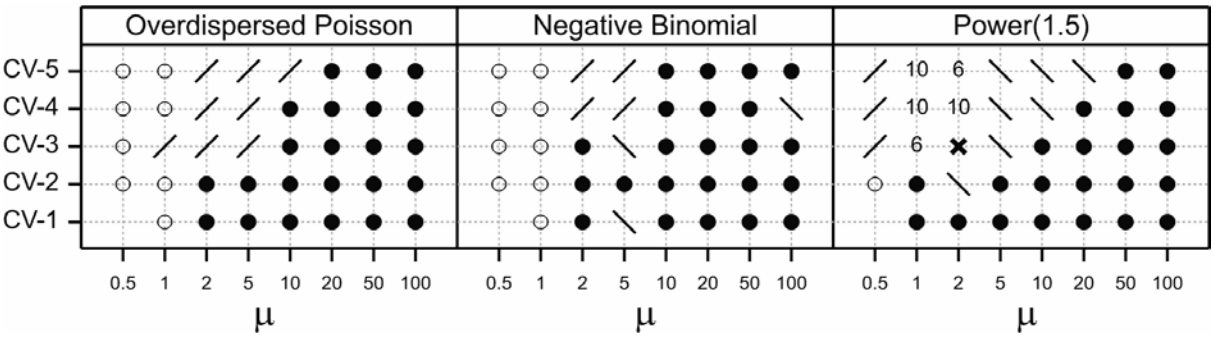


Figure 22: Summary of size of equivalence test for Q=LOC=0.25; data simulated by Overdispersed Poisson (see text for explanation of symbols)

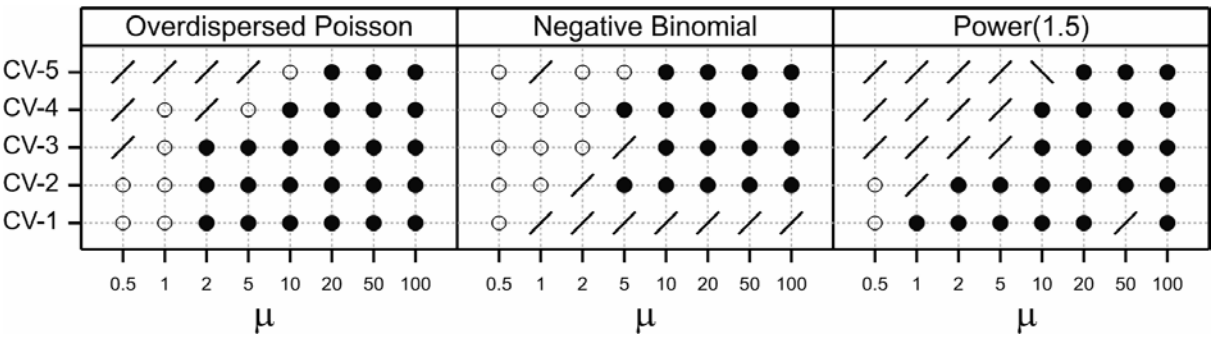


Figure 23: Summary of size of equivalence test for Q=LOC=0.25; data simulated by Negative Binomial (see text for explanation of symbols)

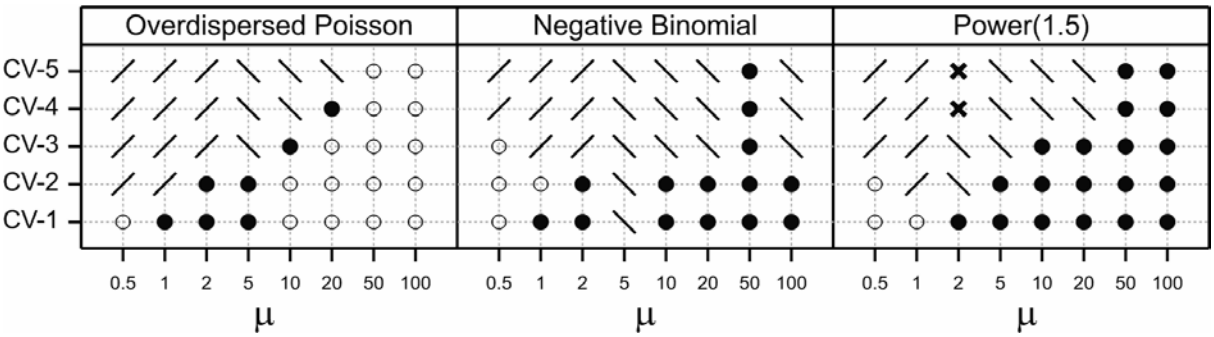


Figure 24: Summary of size of equivalence test for Q=LOC=0.25; data simulated by Poisson-Lognormal (see text for explanation of symbols)

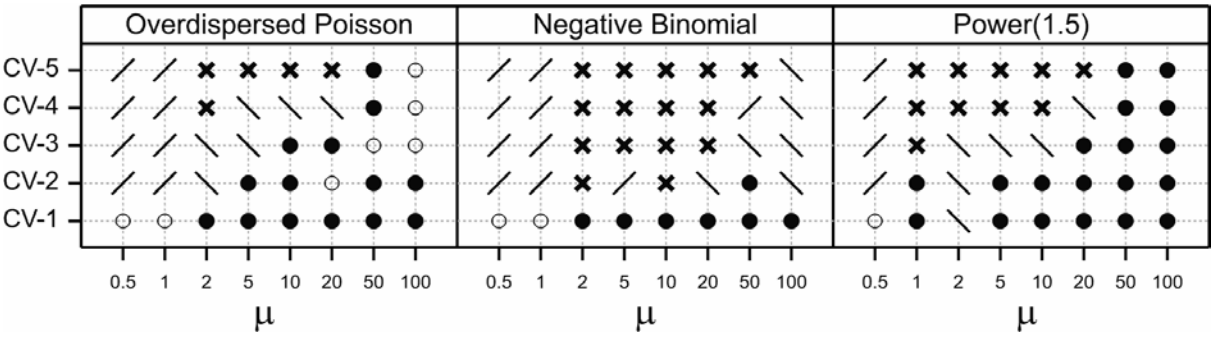
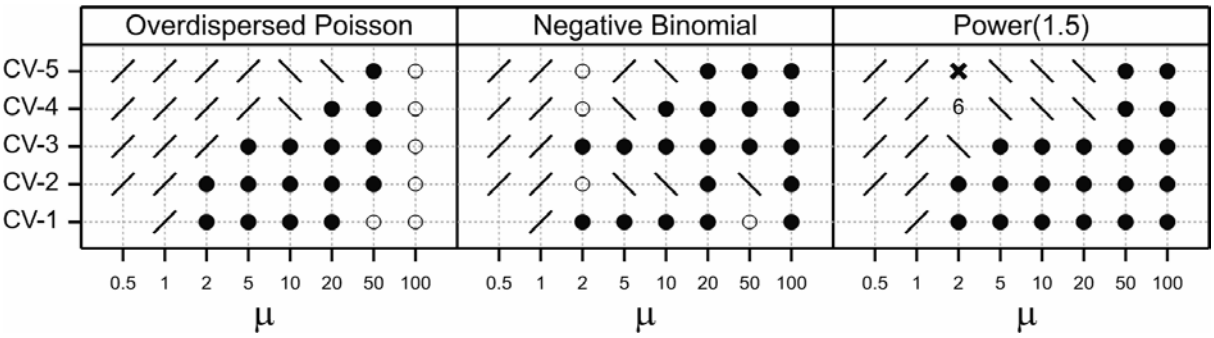


Figure 25: Summary of size of equivalence test for Q=LOC=0.25; data simulated by Power(1.5) model (see text for explanation of symbols)



For Q=0.50 and data simulated by means of the overdispersed Poisson, the intervals according to *OP* and *NB* have a better simulated significance levels than the intervals according to *P1* (Figure 18). For data simulated by means of the negative binomial distribution, there is not much difference between the analysis methods (Figure 19). For Poisson-Lognormal data the *OP* interval has the edge over the other intervals; the *NB* interval is only good for low coefficients of variation (Figure 20). For data simulated according to the Power(1.5) model, the *OP* interval seems to outperform the other analysis models somewhat (Figure 21).

For Q=0.25 and data simulated by means of the overdispersed Poisson, the intervals according to *OP* have a better simulated significance levels than the intervals according to *NB* or *P1* (Figure 22). For data simulated by means of the negative binomial distribution, the *P1* interval performs best while the *OP* interval can be somewhat conservative for larger values of μ (Figure 23). For Poisson-Lognormal data the *P1* interval has the edge over the *OP* interval; the *NB* interval is only good for low coefficients of variation (Figure 24). For data simulated according to the Power(1.5) model, again the *P1* interval has the edge over the *OP* interval (Figure 25).

Conclusion

The *LN* and *SQ* generalized confidence intervals cannot be recommended for equivalence testing. The *P1*, *P2* and *GM* intervals have very similar simulated significance levels. The size of the *NB* interval is particularly worse than that of the other intervals for data simulated according to the Poisson-Lognormal model. The *NB* interval is not better than *OP* and *P1* for the other simulation distributions. For Q=0.5 the *OP* interval seems to have the edge over the *P1* (and this also the case for the *P2* and *GM*) intervals. However for Q=0.25 it is the other way around because the *OP* interval is then somewhat more conservative for certain parameter combinations.

So with respect to size the *P1* (or *P2* or *GM*) and *OP* intervals can be recommended for equivalence testing. The size of both these interval is only problematic for smaller means and larger coefficients of variation.

4.3 Power of equivalence test

Appendices 2 E-H display the power of the one-sided equivalence test for a hypothetical one-sided limit of concern LOC=0.5. Each small plot has a range of 0 to 1 along the y-axis. The red horizontal lines denote power values of 0.25, 0.50 and 0.75. The simulated probability to reject the null-hypothesis of non-equivalence is given by the black dots and the dark grey area in the plot. The probability to decide that "equivalence is more likely than not" is given by the grey area. The red points denote the cumulative probability to reject the null hypothesis or to decide that equivalence is more likely than not. Finally the light grey area denotes the simulated probability that all observations equal zero; these are only present for low values of μ in combination with large CV values. The light grey area can also considered to represent a decision that "equivalence is more likely than not", if that is the case the green dots denote the cumulative probability of equivalence or equivalence more likely than not. In the sequel the "strict test" stands for equivalence while the "liberal tests" stands for the cumulative probability of equivalence and equivalence more likely than not.

There are separate plots for Q=1, Q=0.75, Q=0.5 and Q=0.25. Because the tests based on the *LN* and *SQ* intervals are generally progressive (section 4.2) the power for these tests is larger than for the other models which have a more correct size. The power for the *P1*, *P2* and *GM* tests are very similar. Restricting to those parameter combinations for which the *P1* power is larger than 0.5, the difference for the strict test is maximally 0.01 between *P1*, *P2* and *GM*, while for the liberal test the difference is maximally 0.08. For the same subset the difference between *P1* and *OP* is maximally 0.017 for the strict test of equivalence and 0.027 for the liberal test.

A special case is an effect size Q=0.5 in combination with a limit of concern LOC=0.5. For such cases it is expected that the liberal test will be rejected with a probability of 50%. This is indeed the case, see e.g. Appendix 2 E9-12. Only for small means μ with large CV values there is some deviation from the 50% probability.

It is interesting to see that for an effect size Q=0.75 and small means μ, combined with low replication levels, there is still some probability to reject the liberal hypothesis, i.e. there is a probability of around 25% to decide for "equivalence more likely than not", see e.g. Appendix 2 E13-16.

Conclusion

In the preceding section it was found that the intervals based on *P1* (or *P2* or *GM*) and *OP* have the best simulated significance levels. Here it is shown that these intervals results in very similar power for power values that matter, i.e. values larger than 0.5.

4.4 Approximate power of the equivalence test

The method of Lyles et al (2007), used in section 3.6 for difference testing, can also be used to approximate the power of equivalence tests. The relevant calculation are, in addition to those presented in section 3.6, as follows. A critical value t_{crit} is obtained from Students t-distribution with 2N-2 degrees of freedom. i.e. $P(t_{2N-2} > t_{Crit}) = \alpha$. Furthermore a test

statistic T is calculated by $T = \sqrt{N}\,(\beta - LOC)/se$. The power of the equivalence test is then approximated by means of the upper normal probability $P(u > t_{crit} - T)$.

Results are presented in Appendices 2 I-L, only for the *OP* interval. The different colours represent different limits of concern: LOC=0.75 (black), LOC=0.50 (red), LOC=0.25 (green), LOC=0.10 (blue). The dots denote the simulated values, while the lines represent the approximate values. The pages are for different values of the effect size Q as given in the title of the page. When data are simulated according to the overdispersed Poisson distribution (Appendix 2 I) the approximation is very good especially for larger power values. The same holds for data simulated by means of the negative binomial distribution (Appendix 2 J) and the Power(1.5) distribution (Appendix 2 L). However for the Poisson-Lognormal distribution the approximation is not good (Appendix 2 K).

Conclusion

The method of Lyles et al (2007) can be used to approximate the power of a one-sided equivalence test when using the *OP* interval, except when the simulation distribution is Poisson-Lognormal. For the other simulation distributions there is no need to perform a simulation study to approximate the power.

4.5 Method of choice for equivalence testing

The *LN* and *SQ* generalized confidence intervals should, in general, not be used for equivalence testing because they are too progressive, i.e. they result in too many rejections of the null hypothesis of non-equivalence.

The simulated significance level of the *OP* and *P1* (or *P2* or *GM*) intervals outperform that of the *NB* interval when data are simulated by the Poisson-Lognormal distribution. There are only small differences between the power of the *OP* and *P1* (or *P2* or *GM*) intervals for power values that matter.

It is thus hard to discriminate between the *OP* and *P1* intervals. Since the *OP* analysis method is more generally used and widely accepted, as opposed to the maybe more esoteric *P1* analysis, the *OP* analysis is recommended.

The method of Lyles et al (2007) can be used to approximate the power of the one-sided equivalence test using the *OP* interval, except when the simulation distribution is Poisson-Lognormal. This approximate method might also work for the *P1* interval but this was not investigated.

5 Zero inflation

5.1 Introduction

In practice the number of zero observations can be larger than predicted by the count distribution. This is termed excess-zeros or zero-inflation. Examples of situations with excess-zeros are given by Cunningham and Lindenmayer (2005), Sileshi (2008) and Lewin et al (2010). Failure to account for zero inflation in a statistical analysis may results in biased estimation of environmental effects of GM plants. Goedhart (2013, 2014) describes the common way to model zero-inflation.

Having a lot of zero observations in itself does not necessarily mean that a zero-inflated model is needed. For instance the negative binomial distribution with a large coefficient of variation and a not too large mean is capable of generating many zeros along with some large observations. As an example, 10 samples of size 10 are given below which are simulated by means of a negative binomial distribution with mean μ=5 and coefficient of variation CV=300. Clearly many zeros can be accompanied by few large observations.

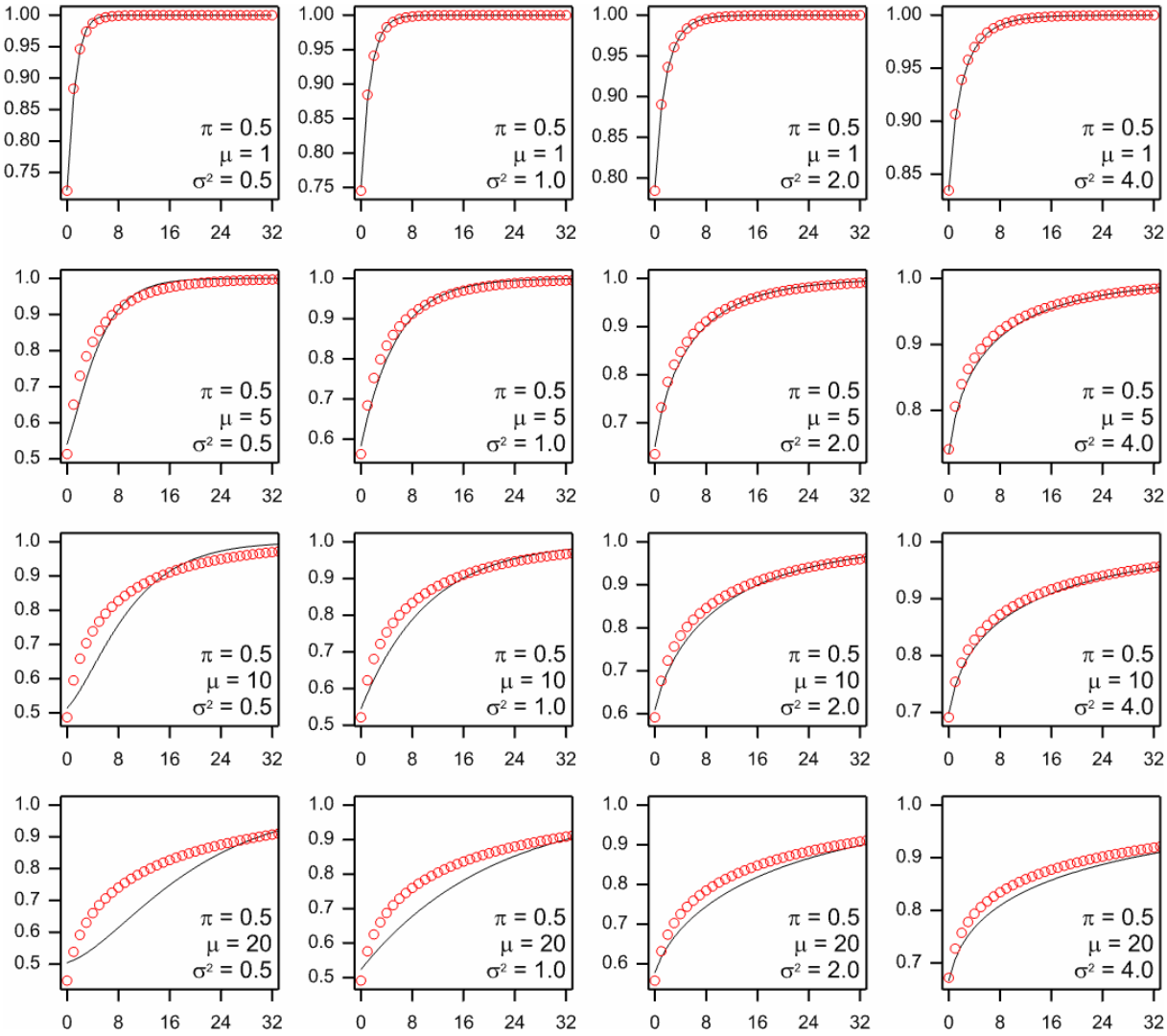
0	0	0	0	0	0	0	0	0	9
0	0	0	0	0	0	6	7	22	39
0	0	0	0	0	0	0	4	13	14
0	0	0	0	0	1	3	13	13	25
0	0	0	0	0	0	1	1	3	9
0	0	0	0	0	1	1	1	5	7
0	0	0	0	0	0	4	7	12	23
0	0	0	0	0	0	0	4	41	61
0	0	0	0	0	0	0	3	4	60
0	0	0	0	0	0	0	0	0	20

Consequently it can be hard, especially for small samples sizes, to distinguish between a zero inflated distribution and an ordinary non-inflated distribution.

5.2 A zero-inflated negative binomial distribution and its non-inflated counterpart.

Consider a zero-inflated negative binomial distribution with parameters π, μ and dispersion parameter σ^2. Note that this distribution has mean $(1-\pi)\mu$. To see whether such a distribution can be distinguished from a non-inflated negative binomial distribution a large number of observations, 10000, are simulated from the zero-inflated distribution. The non-inflated negative binomial distribution was then fitted to this large sample yielding fitted probabilities. This was done for $\pi = 0.5$ and a variety of means μ and dispersion parameters σ^2. Results are given in Figure 26. From this it seems clear that it will only be possible to discriminate between the two distribution for large μ and small dispersion σ^2. For other values an ordinary negative binomial distribution, with the same overall mean, can be used instead.

Figure 26: Theoretical zero-inflated negative binomial cumulative distribution (black line) and fitted non-inflated negative binomial cumulative distribution (red circles) for various means μ and dispersion parameters σ^2.

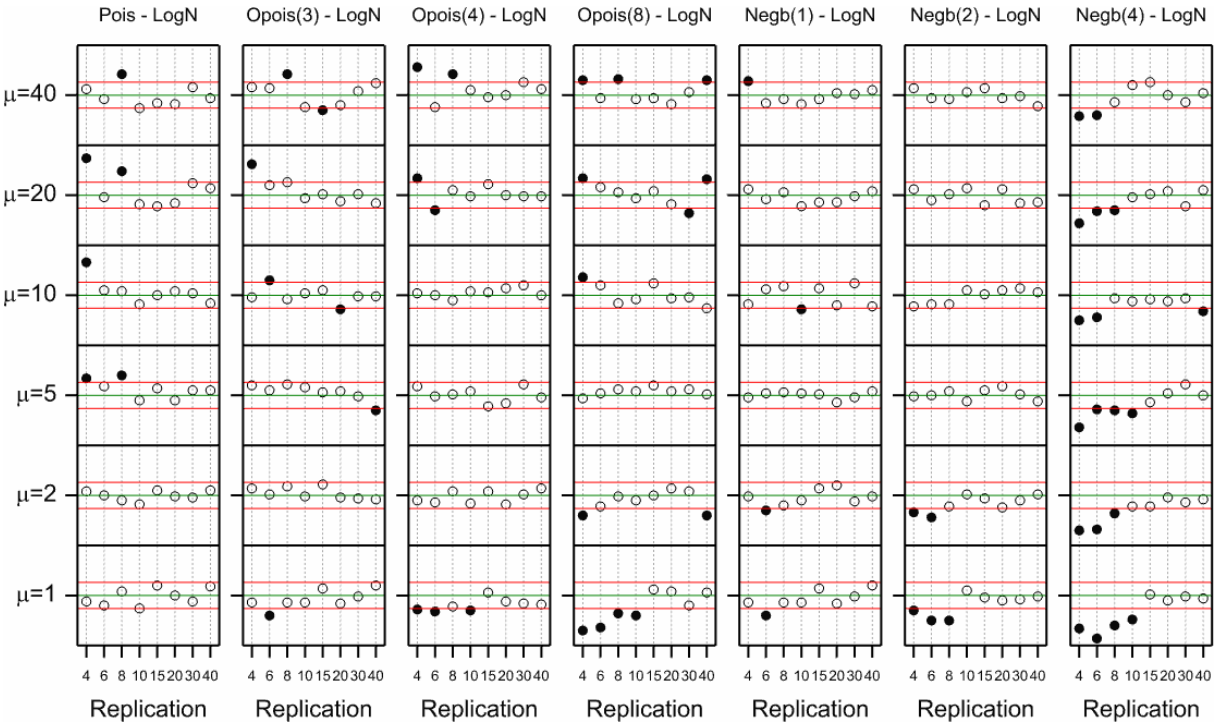


5.3 Size of the *LN* difference test when there is zero inflation

To investigate whether the t-test after a logarithmic transformation is also has correct size when there is zero inflation an additional small simulation study was performed. More specifically it is studied whether the simulated level of the t-test is close to its assumed level when the distribution of the two samples is identical and zero-inflated. This was done by simulating from 7 different count distributions: Poisson, overdispersed Poisson with dispersion parameter 3, 4 and 8, and negative binomial with dispersion parameter 1, 2 and 4. Note that, instead of specifying a coefficient of variation, in this simulation study the dispersion parameter itself is specified. Mean λ values of 2, 4, 10, 20, 40 and 80 were employed with an additional zero-inflation probability of δ=0.5. The mean of a zero-inflated distribution equals $\mu = (1-\delta)\lambda$, implying that mean μ values of 1, 2, 5, 10, 20 and 40 are used here. For each parameter combination 1000 datasets are simulated and a two-sided t-test was performed on the log transformed counts. The simulated significance levels are given in Figure 27. Even in this case the simulated level of the t-test, using the log transformed count,

is good except for small levels of replication in combination with a large overdispersion. In such cases the t-test is generally conservative rather than progressive, with the exception of the overdispersed Poisson distribution with small levels of replication and large means μ. So even in this case the simulated significance level of the *LN* analysis is generally good.

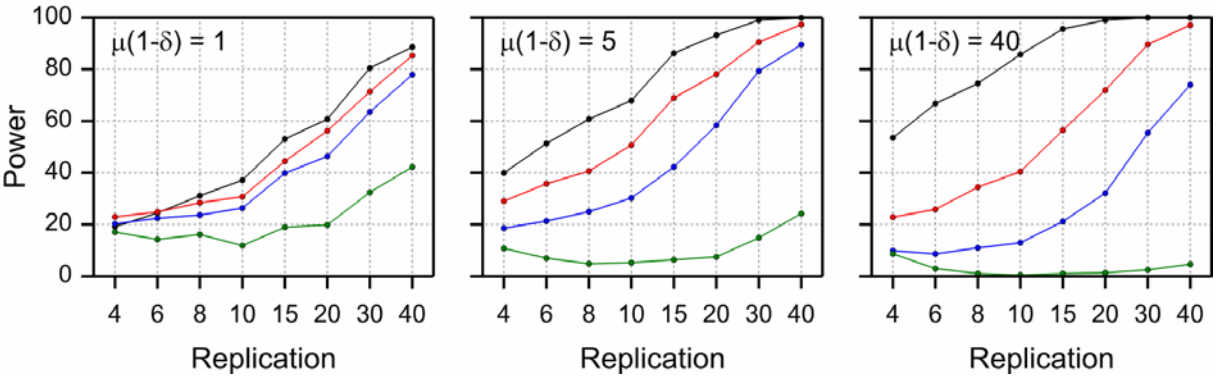
Figure 27: Simulated level of the *LN* test when data are simulated according to a zero-inflated distribution. The green line represent the theoretical 5% level. Open circles when the simulated level is within the expected range as given by the red lines.



5.4 Power of the *NB* difference test for negative binomial data

To evaluate the effect of excess zeros on the power of the ordinary likelihood ratio test a separate simulation with the excess zero negative binomial distribution was executed. Again a single trial without blocking with a single measurement was assumed. Furthermore a multiplicative ratio Q=2 was used between the GM plant and the comparator. The excess zero probability was set to δ = 0, 0.1, 0.2 and 0.5. The mean $(1-\delta)\mu$ of the zero inflated distribution was set to 1, 5 and 40 ensuring that the means of the distributions are identical for different values of δ. The data were analysed with the negative binomial distribution as if there were no excess zeros. The power for different levels of replication are given in Figure 28. This indicates that for small means and small excess zero probabilities the power is not much affected. However for larger means there can be a considerable decline of the power. For an excess probability of δ = 0.5 and larger means the resulting distribution has a spike at zero in combination with larger values with not very much in between. In such a situation the estimate of the dispersion parameter becomes very large so as to "catch" both the zeros and the larger observations. Consequently the distinction between the means of the comparator and the GMO disappears resulting in very low power values. In such a case the data should possibly be analysed by means of an excess zero distribution.

Figure 28: Power of a difference test with α = 0.05 for negative binomial data with overdispersion parameter ω = 0.25 and additional excess zeros with probability δ = 0 (black), 0.1 (red), 0.2 (blue) and 0.5 (green). The comparator has mean $\mu(1-\delta)$ and the GM plant has a mean of $2\mu(1-\delta)$.



6 Conclusion

An important note is that the conclusions below pertain to the situation in which a GM plant is compared with a single counterpart in a completely randomized field experiment with a single count as response. It is however likely that

For difference testing the *LN* or *SQ* method seems to be the method of choice with excellent size for parameter combinations with sufficient power. The power of these tests is generally comparable to that of the other models. So even when data are simulated according to say the overdispersed Poisson distribution, it is still best to perform a difference test on the log or squared root transformed counts. The difference test can probably best be communicated by a confidence interval as this visualizes the result of the difference test. When this is indeed the case, the *LN* method has the advantage over the *SQ* method because the *LN* generalized confidence interval has somewhat better properties. However this interval can and should not be used for equivalence testing as it only has good properties under the null hypothesis of no difference. An approximate method, employing an expanded dataset, is available to quickly calculate the power of the *LN* test making a simulation study superfluous.

For equivalence testing the situation is less clear cut. Two competing methods, *OP* and *P1*, perform equally well with respect to size and power of the one-sided equivalence test. However since the *OP* analysis is more generally used and widely accepted, the *OP* analysis is recommended. It must be considered though that the size of the equivalence test is somewhat problematic for smaller means and lager coefficients of variation. Figure 18 to Figure 25 might be used to provide a guideline for which parameter combinations the *OP* equivalence test has the correct size. Also for the one-sided equivalence test a fast method to calculate the power is available, except when the simulation distribution is Poisson-Lognormal.

Zero inflation, i.e. more zeros than predicted by the count distribution, can be a problem. However for small sample sizes it might be difficult to discriminate between a zero-inflated distribution and a non-inflated distribution. A small simulation study suggests that, for the negative binomial distribution, it is only possible to discriminate between the two for large means and small coefficients of variation. Another simulation study indicates that the power will be heavily affected for larger mean counts combined with a large excess zero probability.

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