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VALIDATION REPORT: VALIDATION OF EFFICACY METHODS FOR ANTIMICROBIALS USED ON HARD SURFACES: PART 2

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VALIDATION REPORT: VALIDATION OF EFFICACY METHODS FOR ANTIMICROBIALS USED ON HARD SURFACES – PART 2

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

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ABOUT THE OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 34 industrialised countries in North and South America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in ten different series: Testing and Assessment; Good Laboratory Practice and Compliance Monitoring; Pesticides and Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; and Safety of Manufactured Nanomaterials. More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (www.oecd.org/ehs/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organisations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. UNDP is an observer. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

FOREWORD

This document contains additional ring test data of the test method for evaluating fungicidal activity of antimicrobials used on hard surfaces. It was a complementary study conducted in 2010 with *Aspergillus niger* and glutaraldehyde. This validation report is intended to support the Test Guideline "*Quantitative method for evaluating fungicidal activity of antimicrobials used on hard non-porous surfaces*".

The first part of the Validation Report contains the ring test data of four test methods for evaluating bactericidal, fungicidal, mycobactericidal and virucidal activity of antimicrobials used on hard surfaces; it is published under No. 154 in the series on Testing and Assessment. Six test micro-organisms – *Adenovirus type 5, Aspergillus niger, Bacillus subtilis, Mycobacterium terrae, Pseudomona aeruginosa* and *Staphylococcus aureus* – and four test substances – peracetic acid, glutaraldehyde, phenol and benzalkonium chloride – were used in this ring test conducted in 2008-2009.

The validation report was endorsed by the WNT at its meeting held on 12-14 April 2011. The Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 5th August 2011.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

Completion of Validation of Efficacy Methods for Antimicrobials used on Hard Surfaces

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Contents

Summary

Within the validation process of efficacy methods for antimicrobials used on hard surfaces seven test organisms, *Adenovirus type 5*, *Aspergillus niger*, *Bacillus subtilis*, *Enterococcus hirae, Mycobacterium terrae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were tested with several active substances such as peracetic acid, glutaraldehyde, phenol and benzalkonium chloride. Within the main study from 2008 to 2009 not for all test organisms and substances enough data for the validation procedure were available: This was especially true for tests of fungicidal activity. Therefore in 2010, a complementary study was carried out with *Aspergillus niger* and glutaraldehyde. Altogether five laboratories stated their participation and four laboratories provided data. These data were combined with the results of three further data sets obtained in an initial study in 2008. In total, seven independent data sets of seven technicians in six different laboratories were available.

These data sets were used to calculate standard and normalized inactivation curves and both intra- and inter-laboratory reproducibility standard deviations. According to these results, the efficacy method can be considered validated with regard to the fungicidal activity of glutaraldehyde to *Aspergillus niger: t*he absolute reproducibility standard deviation of the normalized inactivation curve is in line with results of the main study for other combinations of microbes and active substances.

Introduction

1. The development of antimicrobials (chemical technologies that can kill microbes) is an important element in combating infectious diseases; however determining the effectiveness of certain antimicrobials is difficult. Unlike insecticides where the user can visually determine whether a product is effective, biocidal efficacy against microbial pathogens can only be determined in the laboratory. Inaccurate measurement of efficacy could have severe adverse impacts on public health. Over the years, simple yet robust carrier tests have been developed to assess antimicrobials against all major classes of pathogens, and protocols based on them are now used in various countries; however, no universally accepted test method for efficacy testing exists.
2. The aim of the study “Validation of Efficacy Methods for Antimicrobials used on Hard Surfaces” was to establish and validate universally accepted test methods and to develop test protocols that allow laboratories an accurate measurement of efficacy.
3. The general purpose of the validation process is to determine the performance characteristics, usefulness, and limitations of a test method that is under consideration for use in a regulatory context, and to determine the extent that results from the test can be used for hazard identification, and to support risk assessments or other health and safety decisions (Guidance Document 34).
4. In 2006, after a detailed examination of existing methods, OECD’s Task Force on Biocides had developed draft harmonized quantitative efficacy test methods for antimicrobials used on hard surfaces for use against bacteria, mycobacteria, viruses, fungi and spores; the modifications necessary for the various organism types have been worked into five separate test protocols.
5. In spring and summer of 2007 pre-validation work had been carried out. The analysis of the pre-validation data resulted in providing criteria for establishing an appropriate ring trial design and in establishing statistical methods for the analysis of the results of the ring trial. Also sources of errors, which may be relevant for the ring trial, had been identified.
6. After pre-validation had successfully been completed – between autumn 2007 and spring 2008 – the formal inter-laboratory validation process had been initiated. Laboratories were acquired, test protocols were refined and workbooks for data entry were developed. In order to reduce cost and efforts, results of the pre-validation had been used in the design of the formal inter-laboratory validation study.
7. Between March 2008 and August 2008 the initial ring trial (referred to as “Phase 1”) was conducted. Thereby 27 laboratories carried out the efficacy test independently with several organisms and substances using identical test protocols, and reported the results. However, not all laboratories tested all thirteen combinations of organism and active substances. For each of the combinations, results from five to thirteen laboratories were available.
8. The results from this work are compiled in the final validation report of 2009 [1] including reproducibility of the test methods within and amongst laboratories. However reproducibility is highly varying throughout organisms and substances, and not for all test organisms and

substances enough data for the validation process were available. Thus, the test methods could only considered as being validated with restrictions.

9. In order to correct this weakness, the whole study was extended. Between October 2008 and February 2009 test protocols were refined again and a second phase of the validation study was planned. This phase 2 was meant (1) to identify and correct shortcomings in test protocols, (2) to examine systematic deviations, (3) to test the new quantification principles established during initial validation and (4) to extend the validation database.
10. Tests focused on *Staphylococcus aureus, Enterococcus hirae*, *Pseudomonas aeruginosa* and *Adenovirus type 5.* The results of the statistical analyses of both phase 1 and phase 2 are summarized in the final validation report of 2009 [1]. With regard to fungicidal activity, the efficacy method was tested in three laboratories only and was not considered validated.
11. The aim of the fungal trials in 2010 was to extend the small data base of the initial ring trial for *Aspergillus niger* with glutaraldehyde. Altogether seven independent data sets of seven technicians in six laboratories were used for the analysis of precision figures
12. The correspondence with laboratories and submission of data to quo data GmbH was carried out by Enterprise Innovation & Technology, Worldwide RD&E and Lysoform, Dr. Hans Rosemann GmbH. Data analysis and reporting of statistical results were carried out by quo data GmbH.

Fungal trials 2010

Participating laboratories

13. Of the five laboratories that had stated their participation in the validation study, two laboratories from the U.S. and three laboratories from Europe were selected.

14. From one U.S. laboratory only results of the water control were provided so that this laboratory is not included into the following analysis.

Test organism and active substance

15. *Aspergillus niger* was tested with glutaraldehyde.

Experimental design

16. Each laboratory had to perform three runs. Each test run included at least four control carriers. Six concentration levels with three measurement replicates per concentration level were tested. The following concentration levels were used: 5000 ppm, 10000 ppm, 12500 ppm, 15000 ppm, 20000 ppm and 25000 ppm.

Preparation of data

Data selection

17. Data sets of four laboratories were available from the initial study in 2008, and four laboratories submitted data of the fungal trial in 2010. Two of the latter had conducted the same trial with the same active substance and the same microbial already in 2008. As the technician had changed in one of the two laboratories, it was decided to consider its two datasets as independent ones (they were actually very different). As the two data sets of the other laboratory had been obtained by the same technician, the second data set of this laboratory was eliminated for the calculation of reproducibility figures.

18. The statistical analysis was based on seven data sets.

Calculation of results for statistical analysis

19. The calculation of log reductions was carried out by quo data according to the agreed rules described in the final validation report [1] (paragraphs 39 – 58).

20. Originally – in the workbooks and also in the first statistical analysis – the calculation of the CFU/carrier was based on a weighted mean of the plate counts. In further analyses, however, all dilutions are equally weighted, i.e. the arithmetic mean of the respective dilution corrected CFU's is used. This applies to the test carriers as well as to the water controls. For the test carriers, it has to be noted that according to rule II only at most 3 dilutions are taken into account for the statistical analyses.

Description of statistical procedure

General proceeding

21. Calculated log10 CFU values of each carrier are examined with regard to single outliers, and then run-specific inactivation curves are determined on the basis of the outlier-corrected data. Three laboratory-comprehensive outlier tests are applied in order to identify runs and laboratories that differ significantly from the runs of the remaining laboratories. Calculation of the overall (laboratory-comprehensive) inactivation curve and the intra- and inter-laboratory reproducibility is based on the outlier-corrected data. In addition, the 95%-confidence interval for the overall inactivation curve and the 95%-prediction interval for the run-specific inactivation curves are determined. In the following sub-sections, each of these steps is explained in detail.

Response considered

22. The responses of the statistical analysis are the mean log10 reductions at a specific concentration level, separately for each run of a laboratory. After recalculating all mean CFU values per carrier – for the water controls and the test carriers – they are logarithmized. Thereby the so-called log10 CFU values per carrier are obtained. For the water controls the arithmetic mean of these log10 CFU over all carriers is calculated. This coincides with the logarithmized geometric mean of the CFU values per carrier. In the following, this mean value will be referred to as the average log10 of the water controls or AWC. Then for the test carriers – separately for each replication at one concentration level – the log10 reduction is calculated by subtracting the log10 of the CFU of the test carrier from the average log10 of the water controls. Finally, the mean log10 reduction for one concentration level is determined over all carriers of this concentration level.

23. The normalized approach has been considered during the analyses, where the log10 reduction has been normalized. For more details it is referred to the final validation report of 2009 [1] (paragraph 64)

Analysis of water controls

24. A statistical analysis of the water controls has been carried out according to ISO 5725-2 based on the values of average log10 of the water controls per run of each laboratory.

The inactivation curve

25. The relationship between the mean normalized log10 reductions and the concentration of the active substance is described by the inactivation curve. In pre-validation examinations as well as in the first statistical analysis it has been confirmed that the following non-linear model provides realistic fit of the negative (normalized) log10 survival rate in dependence on the concentration of the active substance for all microorganisms tested:

$$log10\ reduction = R = D - \frac{D}{1 + \left(\frac{conc}{C}\right)^{B}},$$

where

D is a theoretical value. It describes the mean (normalized) log10 reduction (the negative (normalized) log10 survival rate) one would measure at the most effective concentration under the conditions described in the test method (e.g. soil load, nature of the test surface, temperature, contact time).

C denotes the mean concentration at which the inactivation curve is changing from being convex to concave (on log scale of concentration). C would have practical relevance only if **D** could be measured empirically (which is to be expected only if the microorganism is very resistant). Otherwise C represents that concentration at which mean (normalized) log10 reduction = D/2.

B is proportional to the steepness of the inactivation curve at concentration C.

Parameters B, C and D are depending on laboratory and run, i.e. for different laboratories and different runs the inactivation curve is changing randomly.

For the normalization of the presentation of the inactivation curves, the average log10 of the water controls is set to 100%. Thus, the parameter D of the statistical model of the inactivation curves can be derived from the standard inactivation curve by dividing it by the average log10 of the water controls.

26. The statistical analysis of inactivation curves offers the possibility to detect and assess systematic and random sources of errors separately.

Statistical outlier identification with regard to the single log10 CFU values of each carrier

27. For details it is referred to the final validation report of 2009 [1] (paragraphs 71 and 72).

Determination of run-specific inactivation curves

28. For details it is referred to the final validation report of 2009 [1] (paragraphs 73 – 79).

Statistical outlier identification with regard to the run-specific inactivation curves

29. For details it is referred to the final validation report of 2009 [1] (paragraphs 80 – 83).

Determination of overall (laboratory-comprehensive) inactivation curves

30. The overall or laboratory-comprehensive inactivation curves are derived via bootstrap calculation. It is assumed that the residuals of all laboratories and all runs behave in the same way and furthermore it is assumed that there are 3 runs per laboratory. Thereby, within 250 bootstrap cycles 750 run-specific inactivation curves are calculated.

31. The overall inactivation curve equals the average over all 750 bootstrap curves at each concentration. It cannot be described by the formula described in paragraph 25.

Intra- and inter-laboratory reproducibility

32. The general model for the mean normalized log10 reduction for one laboratory at a specific concentration level c is given by

$$\textbf{mean normalized log10 reduction} = R_{ij}(c) = \mu(c) + \alpha_i(c) + \beta_{ij}(c) + e_{ij}$$

where

- $\mu(c)$ denotes the overall inactivation curve at concentration level c, i.e. the probability of a randomly selected microorganism to survive can be estimated $10^{-\mu(c)}$;
- $\alpha_i(c)$ denotes the laboratory specific deviation of the inactivation curve of laboratory i from the overall inactivation curve $\mu(c)$, $\alpha_i(c)$ is assumed to be random variable with mean 0 and variance $\sigma_\alpha^2(c)$;
- $\beta_{ij}(c)$ denotes the run specific deviation of the inactivation curve of laboratory i at run j from the laboratory specific inactivation curve $\mu(c) + \alpha_i(c)$ of laboratory i, $\beta_{ij}(c)$ is assumed to be random variable with mean 0 and variance $\sigma_\beta^2(c)$;
- $e_{ij}(c)$ denotes the random deviation of laboratory i at run j and concentration c from the respective inactivation curve $\mu(c) + \alpha_i(c) + \beta_{ij}(c)$, e_{ij} are assumed to be independent and identically distributed random variables with mean 0 and repeatability variance σ_r^2.

Note that the variance $\sigma_\alpha^2(c)$ and run variance $\sigma_\beta^2(c)$ are depending on the concentration c of the active substance, whereas the repeatability variance σ_r^2 is assumed to be constant. The latter assumption can be considered approximately fulfilled: the calculation of the actual dependency on the concentration c would require considerably more data than available.

33. Note that only the run-specific inactivation curves are modeled, the laboratory-specific inactivation curves are derived from the run-specific inactivation curves of one laboratory by taking the mean value at the concentration level c.

34. The variances between the laboratories $\sigma_L^2(c)$ as well as the variances between the runs $\sigma_{run}^2(c)$ can be derived directly from $\sigma_\alpha^2(c)$, $\sigma_\beta^2(c)$ and the standard error of the runs.

35. Generally speaking, the estimation of variances is carried out according to the standard procedure of analysis of variance of nested random effects. However, the estimations are corrected for the effect of the standard error. The standard error of the run-specific inactivation curves is estimated by the bootstrap method. The same applies to the intra-laboratory variability within one run (repeatability standard deviation) s_r. With these estimations and the method of moments, the following variability parameters can be estimated:

- Inter-laboratory reproducibility: s_R (ISO 5725: Reproducibility standard deviation)
- Intra-laboratory reproducibility over time: s_I (ISO 5725: Intermediate standard

deviation)

- Intra-laboratory reproducibility within one run: s_r (ISO 5725: Repeatability standard deviation)

36. Finally, on the basis of $\hat{\sigma}_L^2(c)$, $\hat{\sigma}_{run}^2(c)$ and the standard error, the 95%-confidence interval for the overall inactivation curve is determined.

Run-specific sensitivity

37. The shape of inactivation curves of different laboratories and different runs is often very similar; quite often they can be derived from the overall inactivation curve by a horizontal shift – on a log concentration scale. This horizontal shift represents deviations in sensitivity: the de-logarithmized shift is referred to as the sensitivity λ of the respective run depending on the laboratory. If λ equals about 1, the sensitivity of the respective run is about the same as the sensitivity over all runs of all laboratories. If λ is larger than 1, the inactivation curve of the laboratory is shifted to the right, i.e. the sensitivity of the respective laboratory is smaller than the average sensitivity; a run with a λ smaller than 1 exhibits a higher sensitivity than the average sensitivity. The sensitivity λ is changing from run to run and from laboratory to laboratory.

Results of statistical analyses

Raw data

38. Of the five laboratories that stated their participation, four laboratories submitted data for the required organism/substance combination. No data – only results of the water control – were submitted by the U.S. Laboratory LC0019.

39. In the initial ring trial, data sets of five laboratories are available.

40. In total, seven independent data sets of six different laboratories were available. The following Table 1 offers an overview of the available data of the initial ring tratial and the fungal trials 2010.

Table 1: Available data for *Aspergillus niger* and glutaraldehyde

Laboratory	Trial	Number of runs	Included into the analysis	Note
LC0011a	initial ring trial	3	yes	-
LC0011b	fungal trials 2010	3	no	same technician as in initial ring trial
LC0019a	initial ring trial	1	no	only two values available and both equal AWC
LC0019b	fungal trials 2010	0	no	only results of water control available
LC0020	fungal trials 2010	2	yes	-
LC0021a	initial ring trial	3	yes	-
LC0021b	fungal trials 2010	3	yes	not the same technician as in the initial ring trial
LC0022	initial ring trial	2	yes	-
LC0023	initial ring trial	3	yes	-
LC0032	fungal trials 2010	3	yes	-

Data evaluation

Analysis of water controls

41. All available data of the seven independent data sets and the water control data of laboratory LC0019a as a further independent data set are included in the analysis according to ISO 5725-2.

42. Please note that the average log10 of the water controls have been recalculated due to the revised determination of the mean CFU/carrier.

43. The variability of the average log10 reductions of the water controls of laboratory LC0020 proved to be significantly higher than the variability of the other laboratories (Cochran test at level 1%). The respective values have been eliminated from the further analysis of the average log10 of the water controls.

44. The following Table 2 summarizes the statistical parameters obtained in the analysis.

Table 2: Parameters of the statistical analysis of the average log10 of the water controls

Organism	Substance	number of participating laboratories	number of laboratories after outlier elimination	overall mean	reproducibility s.d.	repeatability s.d.
Aspergillus niger	glutaraldehyde	7	6	4.97	0.51	0.16

Outlier tests

Statistical outlier identification with regard to the single values

45. Only one single log10 CFU value of laboratory LC0032 was identified as an outlier and deleted from the data set, see Table 3.

Table 3: Outliers with regard to the single log10 CFU values of each carrier

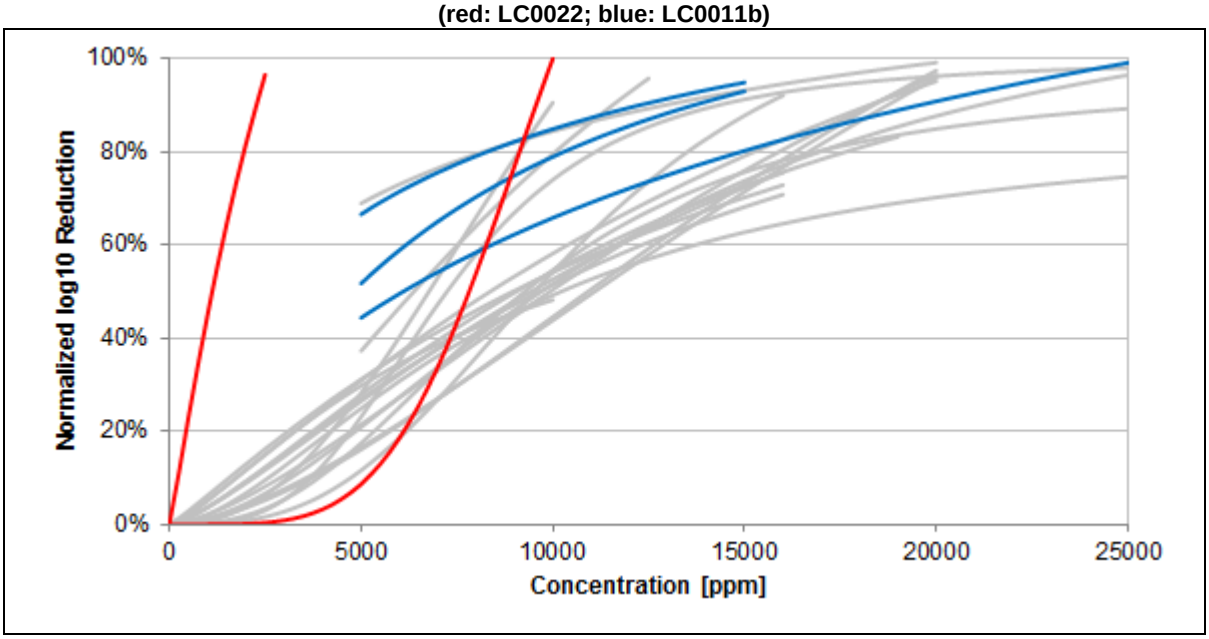
Trial	Lab Code	Run	Outlier Carrier	Concentration
Fungal trials 2010	LC0032	3	5	10000 ppm

Statistical outlier identification with regard to the run-specific inactivation curves

46. The residual standard deviation due to the run-specific inactivation curves of laboratory LC0022 significantly exceeded the residual standard deviation of the other laboratories (Cochran test). Therefore the data of laboratory LC0022 were eliminated from the analysis.

47. The following Figure 1 shows the run-specific inactivation curves for the normalized approach for *Aspergillus niger* and glutaraldehyde. The outlier inactivation curves of laboratory LC0022 that has been eliminated from further statistical analyses are marked in red, while the inactivation curves of laboratory LC0011b regarding the fungal trials (same technician as in initial ring trial) are marked in blue. The remaining inactivation curves of all six independent data sets, i.e. non-outliers on which all further statistical analyses are based on, are shown in a light-grey.

Figure 1: Run-specific inactivation curves for *Aspergillus niger* and glutaraldehyde (red: LC0022; blue: LC0011b)



Run-specific inactivation curves

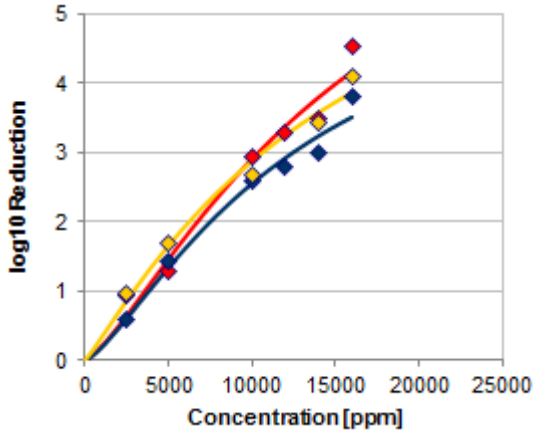
48. In

Figure 3 and

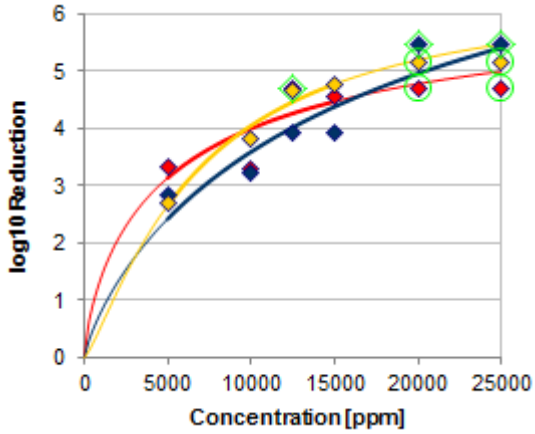
Figure 3, the run-specific standard inactivation curves and the run-specific normalized inactivation curves and the mean log10 reductions at each concentration level are shown for each laboratory/independent data set, respectively. All available data are being displayed, including those data that are omitted in the statistical analysis. Extrapolated curves are shown as thin continuations of the non-extrapolated curve. This applies to cases where the mean log10 reduction of the available runs at the smallest concentration level is larger than 2 and for concentrations larger than the last concentration included in the estimation of the curve. Values of the average log10 of the water controls that have been included in the final estimation of the run-specific curve are marked by green diamonds, values that have not been included in the final estimation of the run-specific curve are marked by green circles.

Figure 2: Standard run-specific inactivation curves for *Aspergillus niger* and glutaraldehyde (red = 1st run, blue = 2nd run, yellow = 3rd run)

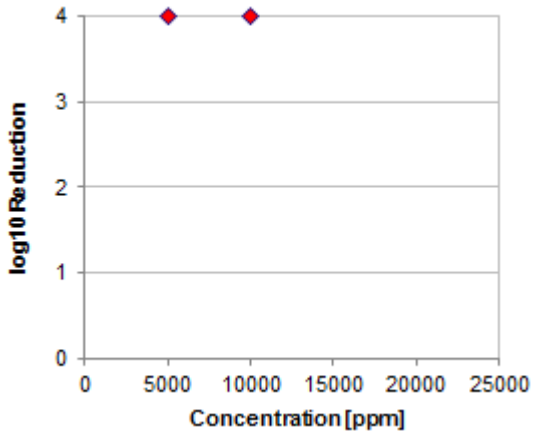
LC0011a
Average log10 of water controls:
run 1: 5.51, run 2: 4.96, run 3: 5.31



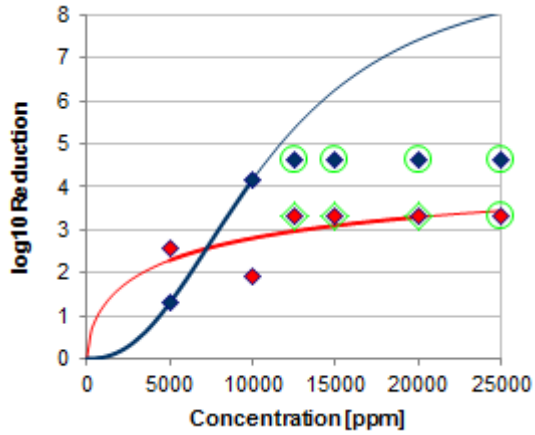
LC0011b
Average log10 of water controls:
run 1: 4.71, run 2: 5.46, run 3: 5.14



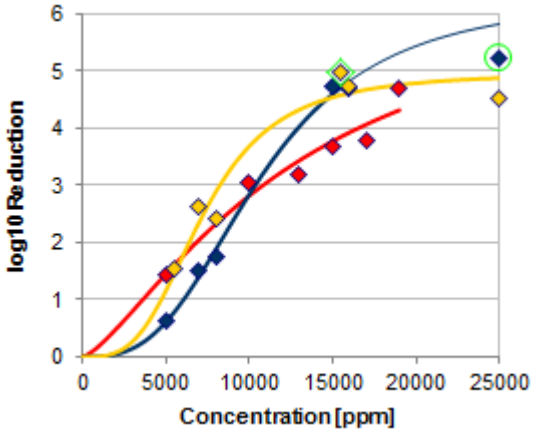
LC0019a
Average log10 of water controls:
run 1: 4.00



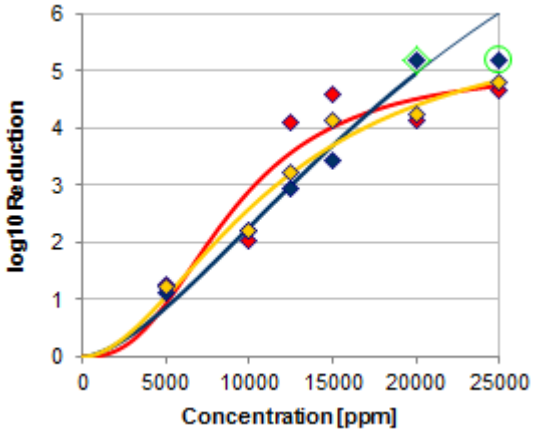
LC0020
Average log10 of water controls:
run 1: 3.33, run 2: 4.60



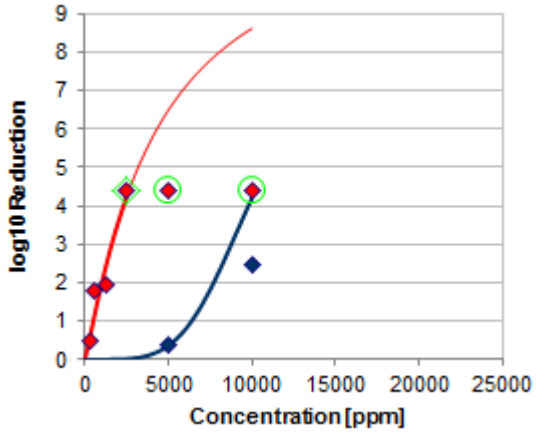
LC0021a
Average log10 of water controls:
run 1: 5.19, run 2: 5.24, run 3: 4.98



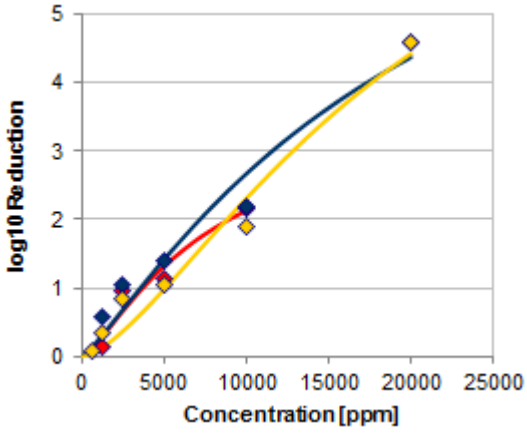
LC0021b
Average log10 of water controls:
run 1: 5.31, run 2: 5.17, run 3: 5.02



LC0022
Average log10 of water controls:
run 1: 4.39, run 2: 4.20



LC0023
Average log10 of water controls:
run 1: 4.42, run 2: 4.59, run 3: 4.59



LC0032
Average log10 of water controls:
run 1: 5.57, run 2: 5.39, run 3: 5.56

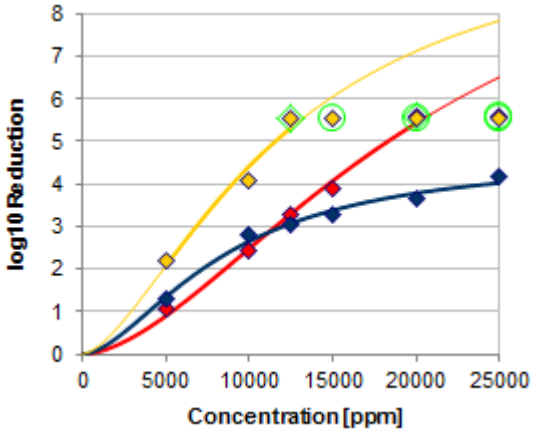
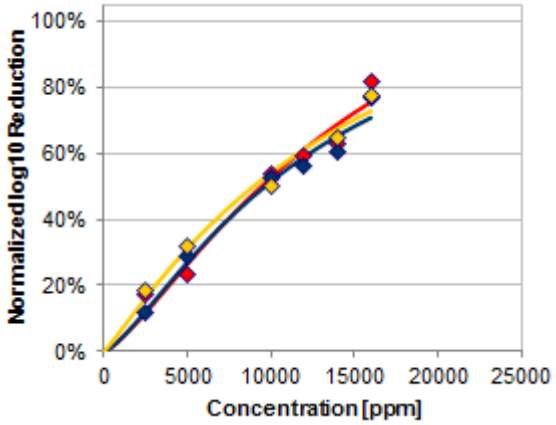
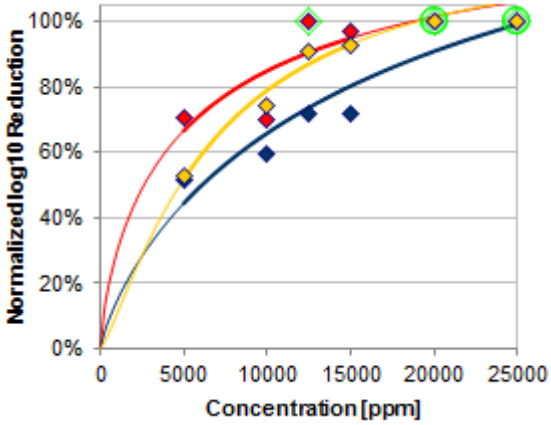


Figure 3: Normalized run-specific inactivation curves for *Aspergillus niger* and glutaraldehyde (red = 1st run, blue = 2nd run, yellow = 3rd run)

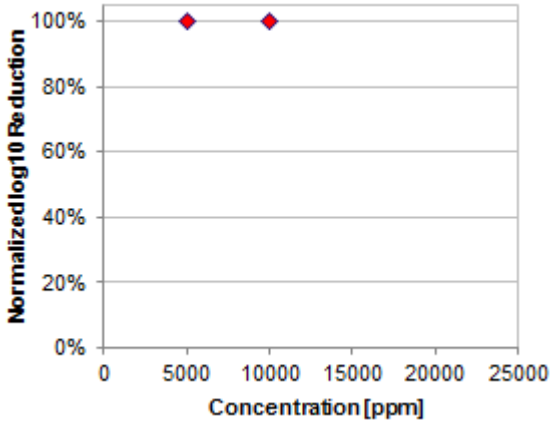
LC0011a
Average log10 of water controls:
run 1: 5.51, run 2: 4.96, run 3: 5.31



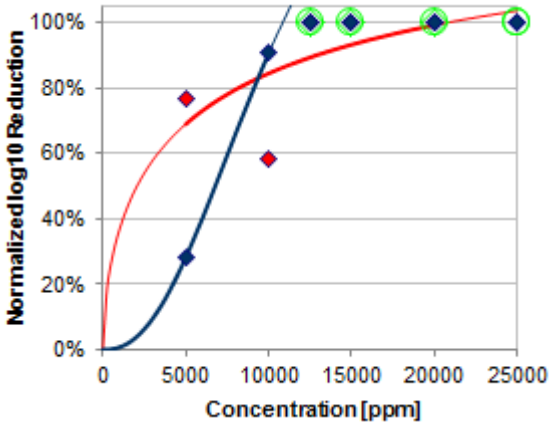
LC0011b
Average log10 of water controls:
run 1: 4.71, run 2: 5.46, run 3: 5.14



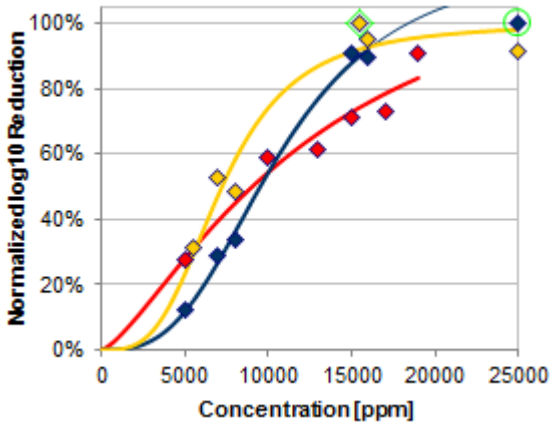
LC0019a
Average log10 of water controls:
run 1: 4.00



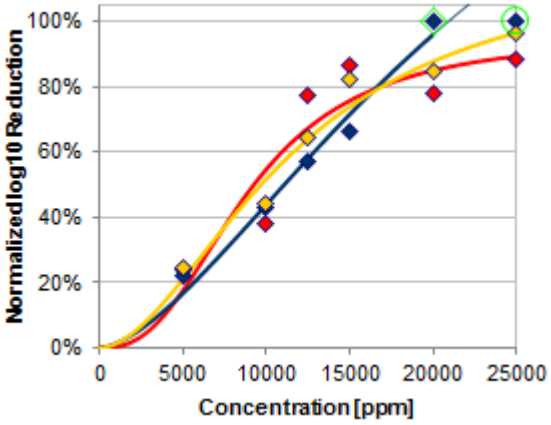
LC0020
Average log10 of water controls:
run 1: 3.33, run 2: 4.60



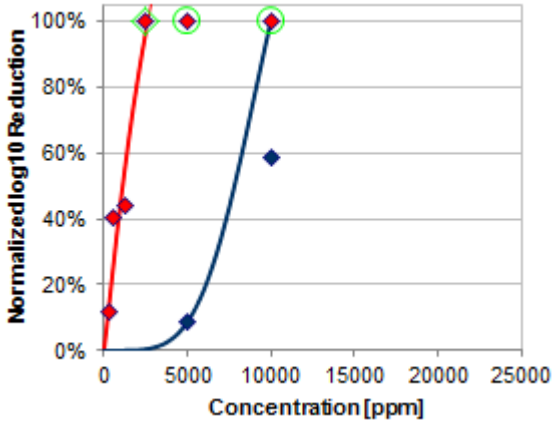
LC0021a
Average log10 of water controls:
run 1: 5.19, run 2: 5.24, run 3: 4.98



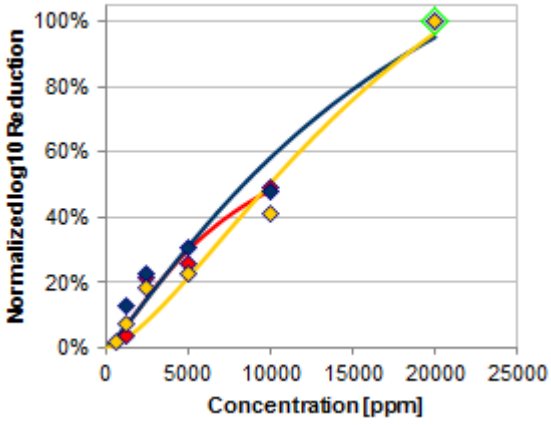
LC0021b
Average log10 of water controls:
run 1: 5.31, run 2: 5.17, run 3: 5.02



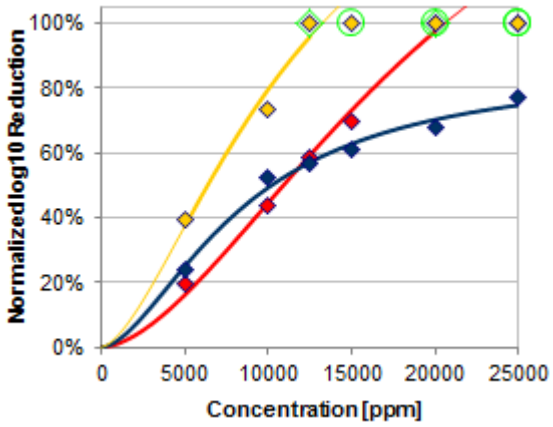
LC0022
Average log10 of water controls:
run 1: 4.39, run 2: 4.20



LC0023
Average log10 of water controls:
run 1: 4.42, run 2: 4.59, run 3: 4.59



LC0032
Average log10 of water controls:
run 1: 5.57, run 2: 5.39, run 3: 5.56



49. In Figure 4 and Figure 5, the run-specific standard inactivation curves and the run-specific normalized inactivation curves of all six independent data sets are shown in one figure, respectively. Mean log10 reductions at each concentration level are displayed as well. The inactivation curves in blue/green shades belong to the tests carried out in 2008, whereas the inactivation curves in red/yellow shades belong to the fungal trials 2010. This chart contains only those runs which are used for the statistical analysis, without outliers.

Figure 4: Single log10 reductions and run-specific standard inactivation curves for *Aspergillus niger* and glutaraldehyde

(blue/green shades: initial ring trial; red/yellow shades: fungal trials 2010)

LC0011a	LC0020	LC0021a	LC0021b	LC0023	LC0032

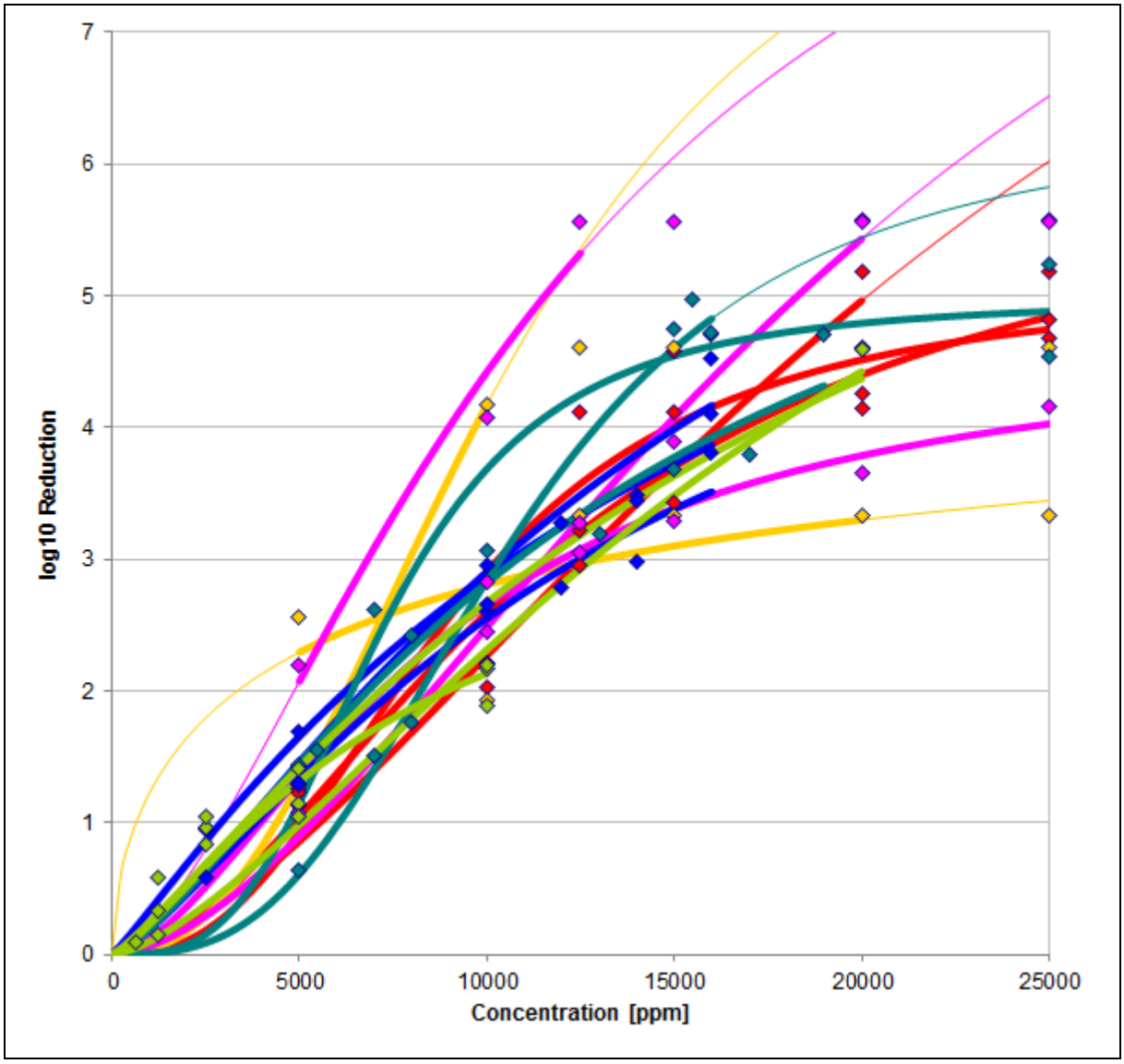
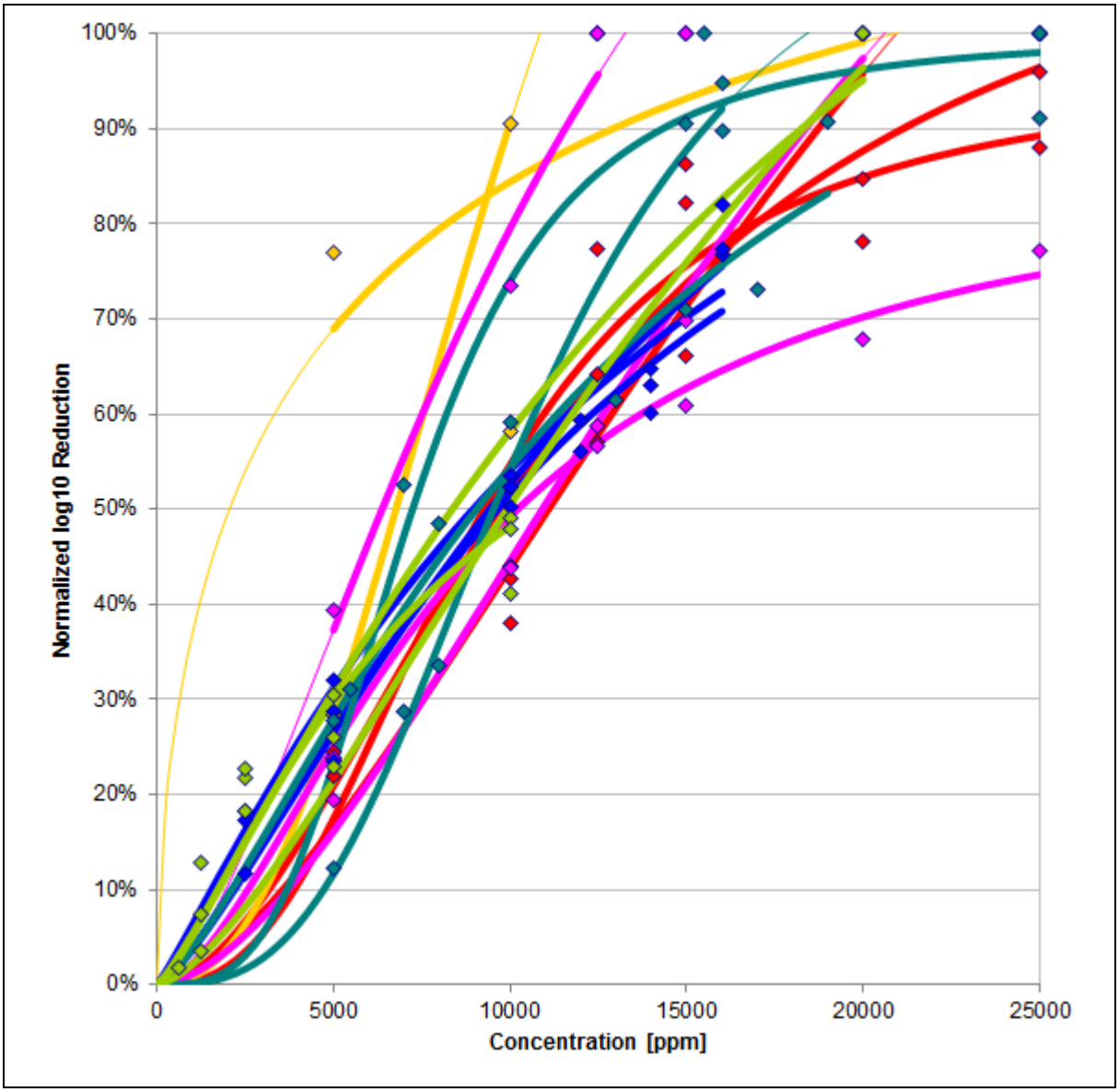


Figure 5: Single normalized log10 reductions and run-specific normalized inactivation curves for *Aspergillus niger* and glutaraldehyde
(blue/green shades: initial ring trial; red/yellow shades: fungal trials 2010)

LC0011a	LC0020	LC0021a	LC0021b	LC0023	LC0032



Overall (laboratory-comprehensive) inactivation curves and intra- and inter-laboratory reproducibility

50. In the following Figure 6, the overall (laboratory-comprehensive) inactivation curves (black) are shown. Furthermore, the 95%-confidence interval for the overall inactivation curve (blue) and the run-specific inactivation curves of all laboratories (light-grey) with the 95%-prediction interval (purple) are shown in the figures as well. The concentration range of the overall inactivation curve is defined by the median of the smallest and the median of the largest concentration levels of the 5 mainly used levels in the ring trial.

51. The overall inactivation curve is followed by Figure 7 showing the inter-laboratory reproducibility s_R (blue) and the intra-laboratory reproducibility within one run s_r (red) in dependence of the concentration. By definition the intra-laboratory reproducibility within one run s_r (repeatability) is smaller than the inter-laboratory reproducibility $s_{R.}$

52. In Table 4 the values of the overall inactivation curve as well as the absolute values of the inter-laboratory reproducibility s_R, intra-laboratory reproducibility over time s_I and intra-laboratory reproducibility within one run s_r are summarized for the six concentration levels that were originally suggested to the laboratories. No values are given if the respective concentration lies not within the concentration range considered for the bootstrap.

53. Please be aware that due to the normalized response expressed in percent, the standard deviations are expressed in percent as well although they are absolute values.

Figure 6: Overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Aspergillus niger* and glutaraldehyde

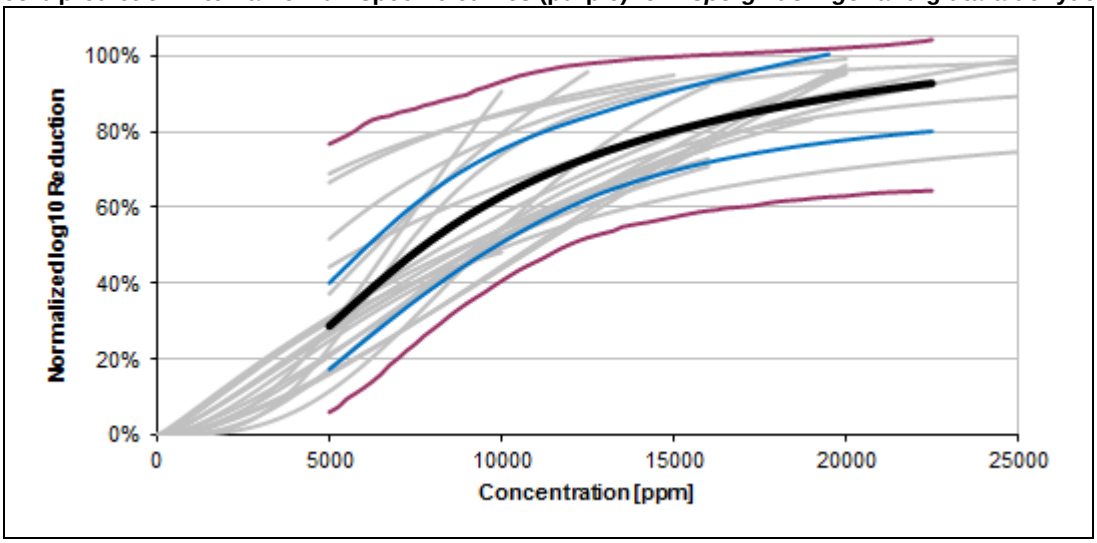


Figure 7: Inter-laboratory reproducibility s_R (blue) and intra-laboratory reproducibility within one run s_r (red) depending on concentration for *Aspergillus niger* and glutaraldehyde

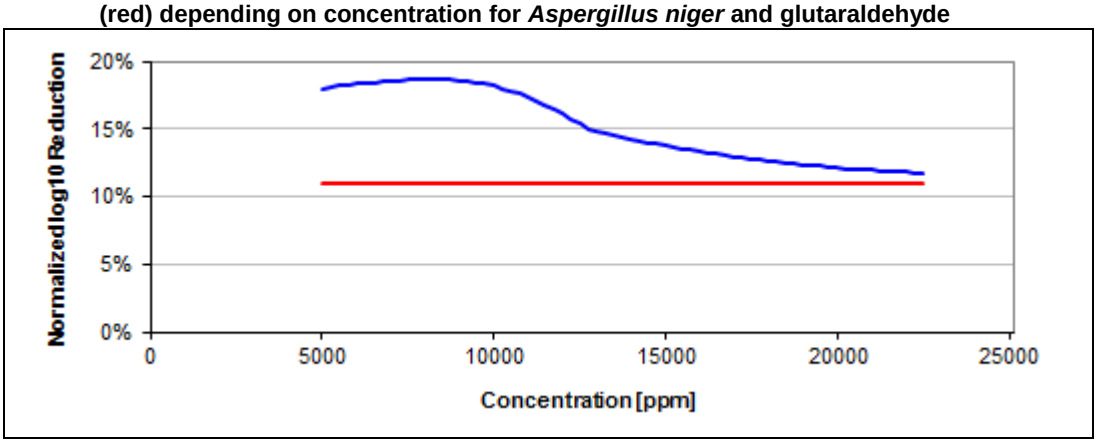


Table 4: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Aspergillus niger* and glutaraldehyde

Concentration [ppm]	Overall inactivation curve (normalized log10 reduction)	Absolute inter-laboratory reproducibility s_R	Absolute intra-laboratory reproducibility within one run s_r	Absolute intra-laboratory reproducibility over time s_I
5000	29%	18%	11%	15%
10000	63%	18%	11%	13%
12500	73%	15%	11%	11%
15000	80%	14%	11%	11%
20000	90%	12%	11%	11%
25000				

Comparison of intra-laboratory results: initial ring trial vs. fungal trials 2010

54. Two laboratories – LC0011 and LC0021 – carried out the tests for *Aspergillus niger* with glutaraldehyde in both trials, i.e. in the initial ring trial as well in the fungal trial 2010. The following two figures show the run-specific inactivation curves of both trials in different colors – for each laboratory separately. Although the technician in laboratory LC0011 was the same in both trials, the run-specific inactivation curves differ clearly: In the 2010 trial, the log10 reductions exhibit both higher sensitivity and higher variability than in the initial ring trial. The test results of laboratory LC0021 were obtained by different technicians; differences between technicians are in line with the calculated inter-laboratory reproducibility.

Figure 8: Normalized run-specific inactivation curves for *Aspergillus niger* with glutaraldehyde realized in initial ring trial (cyan) as well as realized in the fungal trials 2010 (red) of laboratory LC0011 (same technician in both trials)

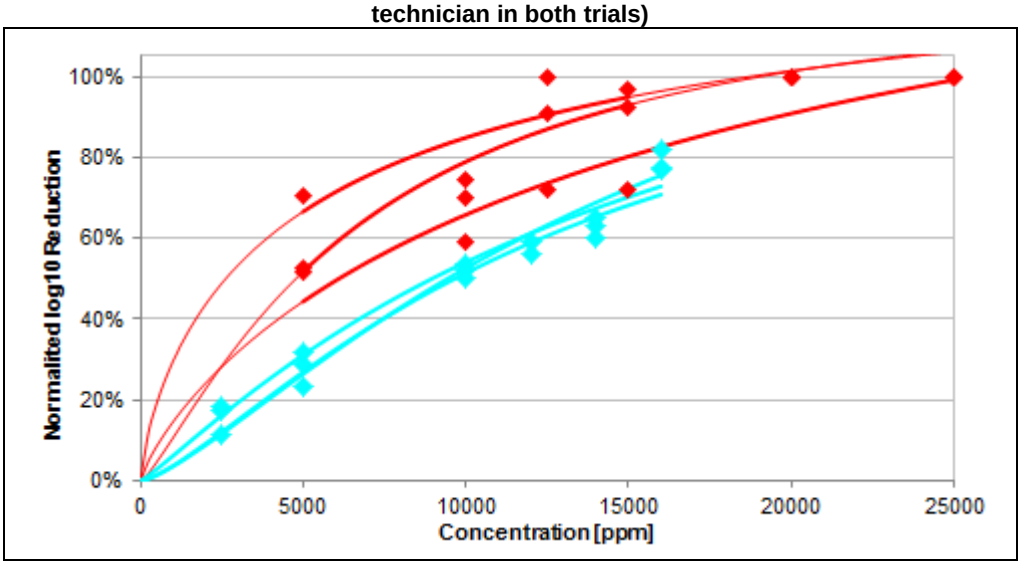
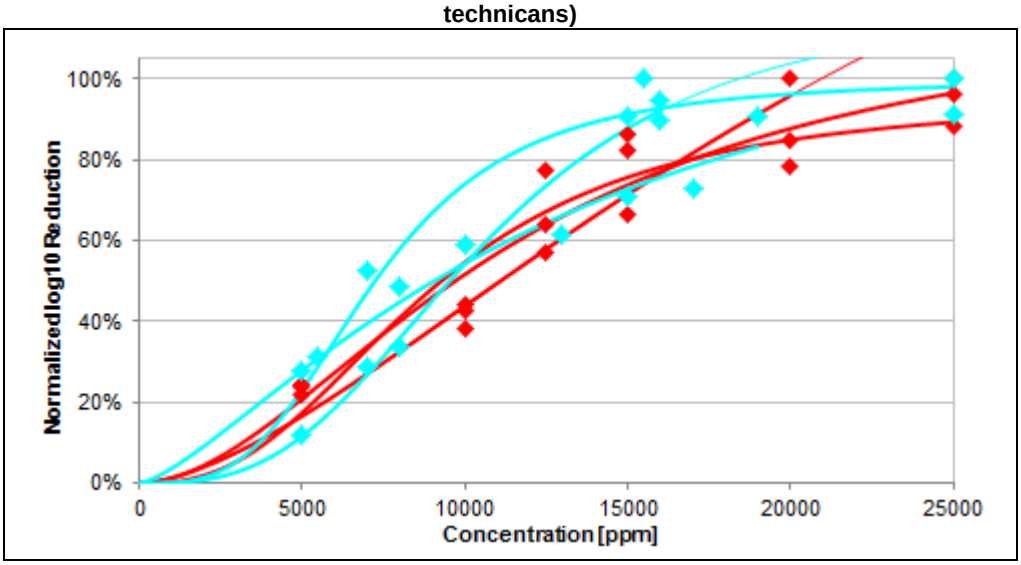


Figure 9: Normalized run-specific inactivation curves for *Aspergillus niger* with glutaraldehyde realized in initial ring trial (cyan) as well as realized in the fungal trials 2010 (red) of laboratory LC0021 (different technicans)



Conclusions

55. The result of the fungal trials shows that the preliminary prediction interval obtained in the initial ring trial (based on test results of three laboratories) was rather optimistic. The new results are based on six independent data sets.

56. For assessing whether test methods can be considered validated, the maximum absolute interlaboratory reproducibility standard deviation within the concentration range of the bootstrap may be consulted. The maximum absolute reproducibility standard deviation for *Aspergillus niger* with glutaraldehyde is equal to 18 % and hence not exceeding the limit of 30 % so that the method can be considered validated.

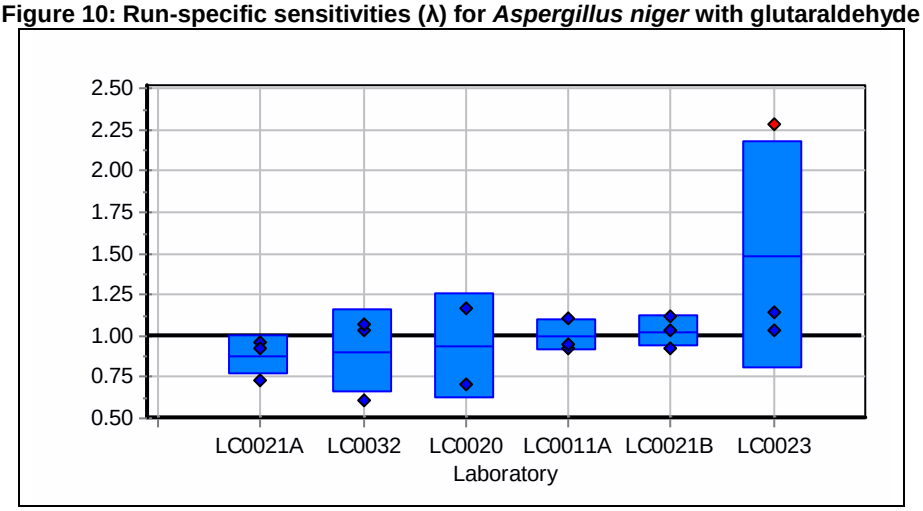
57. Of special interest is the performance of the methods for concentrations with high log10 reduction factors to be expected, e.g. 66.7 % for the normalized approach which is close to a log10 reduction of 3. The precision figures for this level are presented in the following Table 5. Intermediate and repeatability standard deviations are equal, i.e. the variation between runs is not larger than the variation within runs. Also the variation between laboratories is only slightly exceeding the variation within laboratories, i.e. systematic errors between laboratories are relatively small compared with the error under repeatability conditions.

Table 5: Overview of validation parameters at a concentration level at which a log reduction of 80% is expected for *Aspergillus niger* with glutaraldehyde

Mean concentration at 66.7% of the average water control [ppm]	Reproducibility s.d. of log10 reductions	Repeatability s.d. of log10 reductions	Intermediate s.d. of log10 reductions
10750	18 %	11%	13%

58. The following Figure 10 shows the run-specific sensitivities (for more details see paragraph 37 on page 11) at a log10 reduction of 3 (approx. 66.7 % of the overall inactivation curve) for *Aspergillus niger* with glutaraldehyde depending on the laboratory. Here, the red marked value of laboratory LC0023 corresponds to run 1; this value is only an extrapolated one that has to be considered with caution. Disregarding this “outlier”, it is apparent that the variation of sensitivities within laboratories is almost as large as the variation between laboratories. This suggests that the reliability of test results can be improved considerably by conducting several measurement runs, both within and between laboratories.

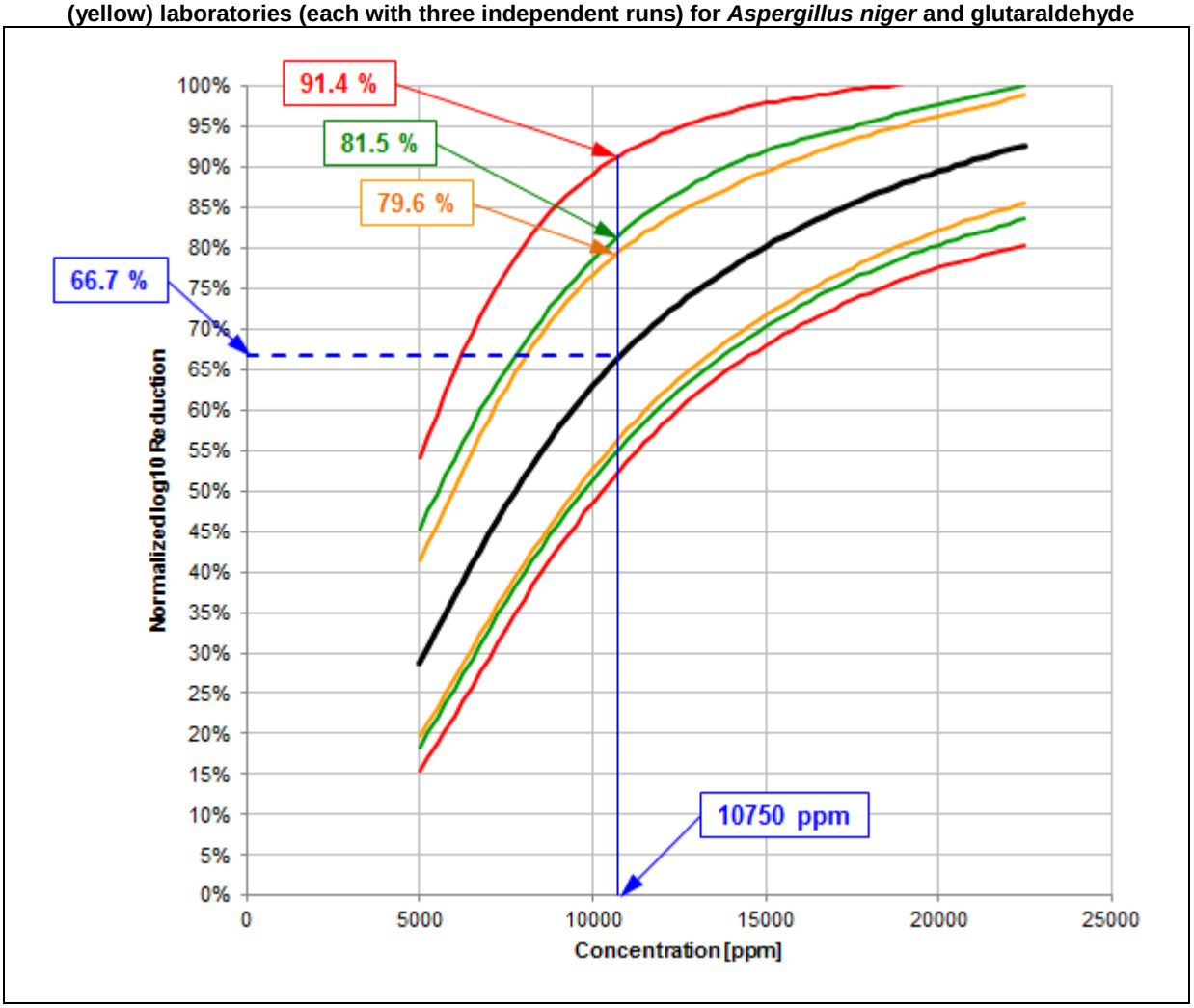
Figure 10: Run-specific sensitivities (λ) for *Aspergillus niger* with glutaraldehyde



59. Decision limits for a qualitative statistical decision rule are always depending on organism and substance. For a qualitative assessment of *Aspergillus niger* and glutaraldehyde the limits can be derived from Figure 11: If the log starting titre equals 4.5 and the limit equals 3, the relative limit is 66.7 %. This limit corresponds to 10750 ppm, and the respective upper 90% prediction interval of the normalized inactivativation curve for three laboratories equals 79.6 % or 3.6 in absolute terms. Only if the measured log reduction is above this upper limit, it can be

concluded that the given limit of four is significantly exceeded. If there is only one laboratory, the respective 90% prediction interval is 91.4 % or 4.1 in absolute terms. This is clearly higher than the upper limit for two or three laboratories.

Figure 11: Overall inactivation curve with 90%-prediction intervals for one (red), two (green) and three (yellow) laboratories (each with three independent runs) for *Aspergillus niger* and glutaraldehyde



60. An overview of decision limits for bacteria, fungi and viruses is given in Table 6. The decision limits for *Aspergillus niger* are derived from the inactivation curve presented in Figure 11. Calculations are depending on the recommended starting titre and the minimum log reduction required. Both figures are displayed in the table. The minimum log reduction is equivalent to the inactivation rate to be reported. For bacteria and fungi an minimum log reduction of 4 is required, whereas for viruses a minimum log reduction of 3 is recommended. It is apparent that the decision limits are the lower, the higher the number of laboratories and the higher the number of runs. Best results can be obtained with 3 laboratories and 3 runs per laboratory. It is also worth noting that 3 laboratories with 1 run each allow lower decision limits than 1 laboratory with 3 runs. The decision limits are depending on the active substance tested.

Apparently, the limits for peracetic acid are somewhat higher than for glutaraldehyde, and for benzalkonium chloride they are lower (for *Staphylococcus* only). As long as no further information on the active substance is available, a worst case approach is recommended, i.e. for viruses and bacteria decision limits should be derived from the limits obtained for peracetic acid.

Table 6: Decision limits derived from 90 % prediction interval for 1-3 laboratories and 1-3 runs

		Bacteria			**Fungi**	**Virus**	
	Organism	Staphyloccocus			Aspergillus niger	Adenovirus	
	Active substance	Peracetic Acid	Glutaraldehyde	Benzalkonium Chloride	Glutaraldehyde	Peracetic Acid	Glutaraldehyde
	Starting titre	6	6	6	6	5	5
	Minimum log reduction	4	4	4	4	3	3
	Inactivation rate	99,99%	99,99%	99,99%	99,99%	99,90%	99,90%
# LABS	# RUNS PER LAB						
1	3	5,92	5,88	4,85	5,48	4,48	4,41
2	2	5,83	5,53	4,70	5,00	4,23	4,02
2	3	5,79	5,51	4,62	4,89	4,17	3,90
3	1	5,78	5,40	4,70	4,87	4,10	4,08
3	2	5,71	5,32	4,60	4,80	4,05	3,82
3	3	5,68	5,28	4,52	4,77	4,00	3,76

References

[1] Uhlig, Steffen; Simon, Kirsten; Antoni, Sabine; Kunath, Kirstin, “Validation of Efficacy Methods for Antimicrobials used on Hard Surfaces”, Final Report, 2009

[2] *ISO 5725-2,* Accuracy (trueness and precision) of measurement methods and results -- Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method (1994).