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**VALIDATION REPORT: VALIDATION OF EFFICACY METHODS FOR ANTIMICROBIALS USED  
ON HARD SURFACES: PART 1**

**Series on Testing and Assessment**

**No. 154**

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**VALIDATION REPORT: VALIDATION OF EFFICACY METHODS FOR ANTIMICROBIALS  
USED ON HARD SURFACES: PART 1**

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**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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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/](http://www.oecd.org/ehs/)).

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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.

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## FOREWORD

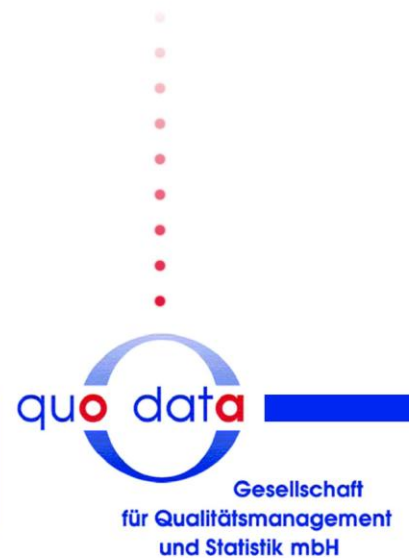
This document contains the ring test data of four test methods for evaluating bactericidal, fungicidal, mycobactericidal and virucidal activity of antimicrobials used on hard surfaces. Six test micro-organisms – *Adenovirus type 5*, *Aspergillus niger*, *Bacillus subtilis*, *Mycobacterium terrae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* – and four test substances – peracetic acid, glutaraldehyde, phenol and benzalkonium chloride – were used in the ring test conducted in 2008-2009. This validation report is intended to support the four Test Guidelines "*Quantitative method for evaluating – bactericidal, fungicidal, mycobactericidal, and virucidal – activity of antimicrobials used on hard non-porous surfaces*".

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 5<sup>th</sup> 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.



# Validation of Efficacy Methods for Antimicrobials used on Hard Surfaces



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**2009-11-20**

**On behalf of OECD**

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## 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. Altogether 29 laboratories from Australia, Canada, Czech Republic, France, Germany, Italy, USA and UK participated in the several rounds of this validation process.

The validation process consisted of three phases, pre-validation phase, initial validation phase and confirmatory validation phase.

Results of initial validation suggested the principal applicability of the tests, although reproducibility varied throughout organisms and substances, and not for all test organisms and substances enough data for the validation procedure were available: This was especially true for tests of fungicidal activity (*Aspergillus niger*) and tests of sporicidal activity (*Bacillus subtilis*).

A confirmatory validation phase was conducted which focused on bacteria. This phase was aiming (1) to identify shortcomings in test procedures, (2) to examine systematic deviations, (3) to test new quantification principles established during initial validation and (4) to extend the validation database. Some shortcomings in test procedures were corrected, quantification principles were successfully tested, and deviations of results between laboratories could be identified as being random, i.e. no persistent systematic deviations of results between laboratories were revealed.

Validation results suggest that a major source of imprecision is the biological variability of microorganisms. This inherent source of variability causes a relatively high level of imprecision. For a true normalized logarithmic reduction of 80 %, inter-laboratory reproducibility standard deviation is in the range of 20 % - 35 % for bacteria and of 15 % - 25 % for *Adenovirus type 5* and *Mycobacterium terrae*.

This can be reduced by parallel testing of several test organisms or several cultures of the same test organism in several laboratories. If three laboratories conduct three tests each, the reproducibility standard deviation of the average normalized log<sub>10</sub> reduction is expected to be in the range of 8 % - 15 % for the bacteria and 5 % - 11 % for *Adenovirus type 5* and *Mycobacterium terrae*. This might be considered a reasonable basis for making comparisons between different products.

The number of laboratories can be reduced if the outcome of the assessment is only “compliant” or “not compliant”: With a qualitative statistical decision rule, also referred to as hypothesis testing, it can be examined whether the true, unknown log reduction (which is not equivalent with the “measured” log reduction) is below or above a given limit. If the measured log reduction is significantly exceeding this limit, it can be concluded that the log reduction is higher than the given limit (i.e. the product tested is compliant). This decision is depending on the number of laboratories and on the reproducibility standard deviation. The higher the number of laboratories and the lower the reproducibility standard deviation, the lower will be the hurdle for confirmation of compliance.

High correlation between different substances suggests parallel testing with a reference substance: the reproducibility standard deviation might be substantially reduced by correcting test results for the product under consideration by test results for a reference substance.

Statistical analysis of the data identified also some outliers, and this suggests that not all laboratories carried out the tests in the same way. For this reason it is highly recommended that laboratories participate periodically in a proficiency testing scheme. In order to guarantee worldwide comparability of test results, such a proficiency testing scheme should run on a global basis. The data of such a global scheme could also be used to improve test methods and to lower the above-mentioned hurdle in a stepwise process,

## 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. In September 2008 a preliminary statistical analysis had been presented. The data as well as the statistical methods had been intensively discussed between microbiologists and biostatisticians at several meetings and teleconferences. The discussions eventually resulted in refinements of test protocols, quantification principles and statistical procedures.
9. The refined quantification principles and statistical procedures were again applied to the data of the initial ring trial in phase 1. The results from this work are compiled in the present validation report including reproducibility of the test methods within and amongst laboratories. This second analysis confirmed an earlier assessment that the efficacy test methods are principally applicable; 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 be considered as being validated with restrictions.
10. 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. There were three parts in phase 2 (carried out between May 2009 and September 2009).
11. In part 1, two laboratories from the U.S. and two laboratories from Europe were selected according to specific characteristics. Both pairs of laboratories carried out the tests in parallel in one laboratory only. Thereby it was possible to (1) identify differences between U.S. and Europe, (2) to directly compare laboratory work within Europe and the U.S. and (3) to identify parts of the test protocols that need further refinement.
12. In part 2, with the help of a mini ring trial it was checked if these refinements were successful. In particular, new laboratories – completely unfamiliar with the test protocol so far – took part in order to examine to which extent a laboratory training affects the outcome of the test.
13. After further refinements of the test protocols, eventually, in part 3, a confirmatory ring trial with 7 participants was conducted for a test organism not yet considered in phase 1 or 2.
14. The results of the statistical analyses of both phase 1 and phase 2 are summarized in the present report. Furthermore, results from phase 2 are compared to the results from the initial ring trial in phase 1.

15. 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.

## Description of ring trial and additional tests

### **Phase 1 – Initial ring trial**

#### **Pre-validation**

16. A pre-validation study has been carried out in order to determine the design of the ring trial. The lead laboratories for this pre-validation study were the Centre for Research on Environmental Microbiology (CREM) at the Faculty of Medicine of University of Ottawa, Canada, and Lysoform Dr. Hans Rosemann GmbH, Berlin, Germany. The pre-validation report is attached as an Annex *Pre-validation report (by quo data GmbH, 8th January 2008)*, p. 124.

#### **Participants**

17. In total, thirty-five test laboratories from eight member countries (Australia, Canada, Czech Republic, France, Germany, Italy, the United Kingdom, the United States of America) and the two lead laboratories stated their participation in the study. Of these, twenty-seven laboratories eventually participated in the initial ring trial in phase 1 and submitted data at least for one combination of organism and active substance. Additional details on the participating laboratories and their principal investigators are summarized in the Annex *Participating laboratories*, p. 156. All laboratories participated on a voluntary and self-supporting basis. The laboratories included those that were experienced with the use of antimicrobials on hard surfaces and those that had not used them prior to this study.

#### **Test organisms and active substances**

18. For the bactericidal method, two test organisms were available: *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. For the mycobactericidal, fungicidal, virucidal and sporicidal methods, one test organism each was available: *Mycobacterium terrae*, *Aspergillus niger*, *Adenovirus type 5* and *Bacillus subtilis*, respectively.
19. As active substances were available in the ring trial: phenol, glutaraldehyde, peracetic acid and benzalkonium chloride. The combinations of test organisms and substances tested in the initial ring trial are summarized in Table 1.

**Table 1: Combinations of test organisms and active substances tested in the ring trial**

<b>Test organism</b>	<b>Active substance</b>
<i>Adenovirus type 5</i>	glutaraldehyde
	peracetic acid
<i>Aspergillus niger</i>	glutaraldehyde
	peracetic acid
<i>Bacillus subtilis</i>	peracetic acid
<i>Mycobacterium terrae</i>	phenol
	glutaraldehyde
	peracetic acid
<i>Pseudomonas aeruginosa</i>	glutaraldehyde
	peracetic acid
<i>Staphylococcus aureus</i>	glutaraldehyde
	peracetic acid
	benzalkonium chloride

**Experimental design**

20. For each combination of test organism and active substance three test runs had to be performed; the order of these runs was randomized.
21. One test run included at least four control carriers even when two or more tests were being run in parallel; e.g., if two active substances were tested at the same time against one test organism, each one of these actives must include its own four-carrier control.
22. In the first test run, 6 concentration levels with 3 measurement replicates per concentration level had to be tested, whereas in test runs 2 and 3 only 5 concentration levels with 3 measurement replicates each could be tested. The participants were provided with the required concentration levels of the first test run which are summarized in Table 2.
23. The concentration levels in test runs 2 and 3 had to be adapted individually by the participants according to 4 criteria that ensured a reasonable statistical analysis. In order to simplify the procedure for the participants, an Excel workbook has been provided. The original criteria as well as the workbook are shown in the Annex *Original criteria*, p. 160.

**Table 2: Provided concentrations (in ppm) for run 1**

Test organism	Active substance	Suggested concentrations (in ppm)					
		1	2	3	4	5	6
<i>Adenovirus type 5</i>	glutaraldehyde	2000	1000	500	250	125	62.5
	peracetic acid	200	150	100	75	50	25
<i>Aspergillus niger</i>	glutaraldehyde	10000	5000	2500	1250	625	312
	peracetic acid	800	400	200	100	50	25
<i>Bacillus subtilis</i>	peracetic acid	1200	1000	800	600	400	200
<i>Mycobacterium terrae</i>	phenol	20000	15000	14000	13000	10000	5000
	glutaraldehyde	25000	20000	15000	10000	5000	2500
	peracetic acid	1000	700	600	500	300	200
<i>Pseudomonas aeruginosa</i>	glutaraldehyde	600	550	500	450	350	250
	peracetic acid	160	100	80	70	60	40
<i>Staphylococcus aureus</i>	glutaraldehyde	1300	1100	900	800	500	250
	peracetic acid	120	110	100	95	90	75
	benzalkonium chloride	5500	4500	3500	2500	1000	500

**Reporting of data**

24. It was required that the participants should provide all data they have produced including those with invalid results. Not only average values but also single values had to be reported. Furthermore, the participants had to report the sources of microorganisms and media.

**Phase 2****Background**

25. The results from the initial ring trial of phase 1 showed highly varying reproducibility 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 be considered as being validated with restrictions. For this reason a confirmatory validation phase was conducted. This phase was aiming (1) to identify shortcomings in test procedures, (2) to examine systematic deviations, (3) to test new quantification principles established during initial validation and (4) to extend the validation database. This so-called phase 2 was divided into three single parts.
26. *Phase 2 - Part 1:* Two laboratories in the U.S. and two laboratories in Europe, which already had participated in the initial ring trial in phase 1, had been selected. The selection of laboratories was based on the outcome of the initial ring trial. The two U.S. laboratories should have shown (1) internally consistent results but (2) large systematic differences between each other. Then these two laboratories met for one day to carry out the tests in parallel in one laboratory only, in order to find out what the reasons for the differences are. Also the two European laboratories should have shown (1) internally consistent results but (2) large systematic differences between each other. These two laboratories also met for one day

to carry out the tests in parallel in one laboratory only. The findings of each of these meetings had been discussed by these four laboratories together, and shortcomings of the test protocol had been identified.

27. *Phase 2 - Part 2:* After the identified shortcomings had been corrected in the test protocol, a mini ring trial (4 - 6 participants) for one test organism and two active substances was conducted. With this mini ring trial it should be checked if the corrections of the shortcomings were fruitful. In the mini ring trial the four participants of Part 1 should participate and two new laboratories, which had not been involved in the initial ring trial in phase 1 or in part 1 of phase 2. The latter was necessary in order to check to which extent a training phase is required. To facilitate the proceeding not necessarily new laboratories had to be acquired but it was also possible that a new technician (so far unfamiliar with the test protocols) carried out the tests.
28. *Phase 2 - Part 3:* Finally, after adapting the test protocols again, a confirmatory ring trial with 7 participants was planned for one completely new test organism and one active substance for bacteria. In this confirmatory ring trial, the six participants from part 2 should participate and two new laboratories, which had neither been involved in the initial ring trial in phase 1 nor in parts 1 and 2 of phase 2.
29. The results of the mini ring trial and the confirmatory ring trial could then be used to extrapolate the findings to other active substances and organisms.

## **Phase 2 - Part 1**

### Participating laboratories

30. Of the 37 laboratories that had stated their participation in the study, two laboratories from the U.S. and two laboratories from Europe, which had already participated in phase 1, were selected. Additional details on the participating laboratories and their principal investigators are summarized in the Annex *Participating laboratories*, p. 156.

### Test organism and active substance

31. As test organism *Staphylococcus aureus* and as test substance benzalkonium chloride were chosen.

### Experimental design

32. Each laboratory had to perform one run. This test run included at least four control carriers. Four concentration levels with three measurement replicates per concentration level were tested. The concentration levels differed between the European and the U.S. laboratories. The following Table 3 summarizes the concentration levels.

**Table 3: List of used concentrations in the U.S. laboratories and in the European laboratories**

	Used concentrations
U.S. laboratories	400 ppm, 1500 ppm, 2500 ppm, 5500 ppm
European laboratories	500 ppm, 1000 ppm, 2000 ppm, 3500 ppm

**Phase 2 - Part 2**Participants

33. Of the 37 laboratories that had stated their participation in the study, four laboratories performed tests in part 2 of phase 2. In one of these laboratories, two technicians carried out tests, in another one even three technicians performed tests. Thereby, in total, seven independent data sets were available at least for one combination of organism and active substance. Additional details on the participating laboratories and their principal investigators are summarized in the Annex *Participating laboratories*, p. 156.

Test organisms and active substances

34. As test organism *Staphylococcus aureus* and as test substances benzalkonium chloride and glutaraldehyde were chosen.

Experimental design

35. Each laboratory or technician had to perform one run. This test run included at least four control carriers. Four concentration levels with at least 3 measurement replicates per concentration level had to be tested. The concentration levels were specified by taking into account the results from phase 1 as well as from part 1 of phase 2. The following Table 4 summarizes these concentration levels. However, it should be noted that not all laboratories used the suggested concentrations.

**Table 4: List of used concentrations of benzalkonium chloride and of glutaraldehyde for tests with *Staphylococcus aureus***

	Used concentrations
benzalkonium chloride	400 ppm, 1500 ppm, 2500 ppm, 5500 ppm
glutaraldehyde	250 ppm, 500 ppm, 900 ppm, 1300 ppm

**Phase 2 - Part 3**Participants

36. Of the 37 laboratories that had stated their participation in the study, six laboratories performed tests in part 3 of phase 2. For the bacteria, in two of these laboratories, two technicians carried out tests. Thereby, in total, seven independent data sets were available for both bacteria test organisms. For the virus, in two laboratories two technicians carried out the tests. Thereby, in total, four independent data sets were available. Additional details on the participating laboratories and their principal investigators are summarized in the Annex *Participating laboratories*, p. 156.

Test organisms and active substances

37. For the bactericidal method, two test organisms were available: *Enterococcus hirae* and *Pseudomonas aeruginosa*. As active substances benzalkonium chloride and glutaraldehyde were used, where benzalkonium chloride has only been tested against *Enterococcus hirae*,

and glutaraldehyde has been tested against *Pseudomonas aeruginosa*. For the virus method, *Adenovirus type 5* was tested against glutaraldehyde.

#### Experimental design

38. Each laboratory or technician had to perform at least one run. This test run included at least three control carriers. Minimum four concentration levels with at least three measurement replicates per concentration level had to be tested. The concentration levels were specified by taking into account the results from phase 1 as well as from parts 1 and 2 of phase 2. The levels are summarized in the following Table 5. However, it should be noted that not all laboratories used the suggested concentrations.

**Table 5: List of used concentrations of benzalkonium chloride and of glutaraldehyde for tests with *Enterococcus hirae*, *Pseudomonas aeruginosa* and *Adenovirus type 5***

	Used concentrations
<i>Enterococcus hirae</i> versus benzalkonium chloride	250 ppm, 400 ppm, 1500 ppm, 2500 ppm, 5500 ppm
<i>Pseudomonas aeruginosa</i> versus glutaraldehyde	250 ppm, 350 ppm, 400 ppm, 600 ppm, 800 ppm
<i>Adenovirus type 5</i> versus glutaraldehyde	31 ppm, 62.5 ppm, 125 ppm, 250 ppm, 500 ppm, 1000 ppm

## Preparation of data

### Data selection

39. In two experts meetings – after the preliminary statistical analysis – six rules have been formulated to exclude data patterns which are obviously inconsistent and implausible from the microbiological point of view (see Annexes *Minutes Meeting Dresden 17th/18th November 2008*, p. 148 and *Minutes Meeting Dresden 9th/10th March 2009*, p. 152) and which would influence the statistical analysis. These rules are only applicable to single carriers and not to a set of carriers.
40. It has to be noted that the rules have to be applied in the given order, e.g. start with rule 0 and continue with rule I, rule II and so on. Please note that these rules are applied to the test carriers only and not to the water controls.
41. In order to apply the rules, the raw data had to be imported into a new database (comprising not only the aggregated data required for the ring trial analyses but also the underlying raw data, counts and the given status). This became necessary as the workbooks with the mean CFU/carrier and the log<sub>10</sub> reductions could not be used further due to changes in the algorithms.
42. Before starting data selection according to the rules, all given raw data had to be checked especially regarding the status (ok, TMTC, zero), because some laboratories did not declare the correct status to the respective plate count.
43. All corrections or adaptations of the statuses carried out by quo data are summarized in the Annex *Status correction and adaptation carried out by quo data GmbH in phase 1*, p. 164. In some cases, no status was specified at all and had to be entered by quo data.

### Rule 0

44. a) *If there is a reasonable plate count, but the laboratory entered N/A as status, the status should always be set to OK.*
45. b) *If the plate count is empty, but the laboratory entered status 0, set plate count to 0 as well. If the plate count is 0, but the laboratory entered status N/A, leave status N/A and therefore plate count will be disregarded.*
46. c) *Every plate count above 299 will be disregarded. Accept when a laboratory entered TMTC status and therefore disregard given plate count even when it is below 300.*
47. d) *Zero plate counts between OK plate counts (“Middle zeros”) will be regarded as long as no other rule applies (e.g. 5(-1 dilution)/0(-2 dilution)/1(-3 dilution)). Therefore, middle zeros will be included in calculating mean value.*
48. e) *Zero plate counts for lower dilutions (where at least one higher dilution has countable plate counts) are either*

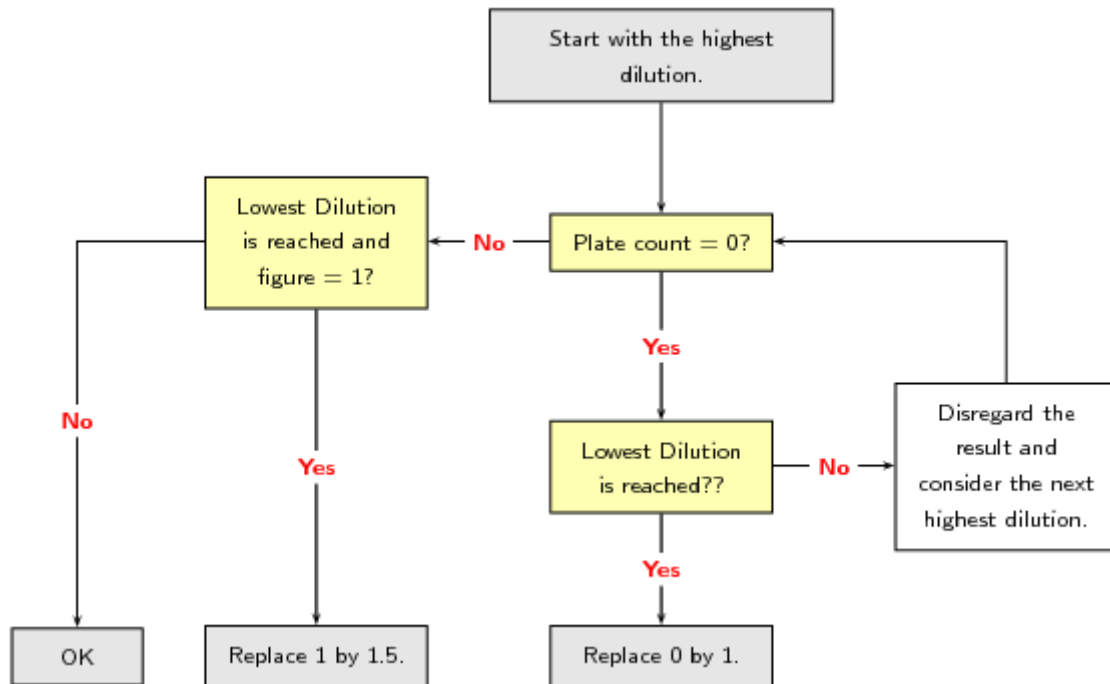
- covered by rule IV if non-zero plate counts are larger than or equal to 4 or
- for non-zero plate counts smaller than 4, the outlier tests will take care of worst case and other cases do not have a big effect.

49. f) (extension of rule IV): Any count(s) in lower dilution step(s) is/are disregarded if there is/are TMTTC in higher dilution step(s), but include colony count(s) in the higher dilution step(s).

**Rule I**

50. In the first step, for carriers with a plate count of 1 for the lowest dilution and plate counts equaling zero for all higher dilutions, the plate count for the lowest dilution is set to 1.5. For carriers with zero results only, the plate count of the lowest dilution is set to 1.

**Figure 1: Rule I of data selection**



51. Example: Before applying the rule:

Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count
1.00E+00	OK	12	1.00E+02	0	0	1.00E+03	0	0	1.00E+04	0	0
1.00E+00	OK	1	1.00E+02	0	0	1.00E+03	0	0	1.00E+04	0	0
1.00E+00	0	0	1.00E+02	0	0	1.00E+03	0	0	1.00E+04	0	0

The second line is an example for the following case: If there is only one dilution with counts and the count is equal to 1, replace it by 1.5.

The third line is an example for the following case: If there is no growth at all, replace 0 by 1 for the lowest dilution.

After applying the rule:

Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count
1.00E+00	OK	12	1.00E+02	N/A	0	1.00E+03	N/A	0	1.00E+04	N/A	0
1.00E+00	OK	1.5	1.00E+02	N/A	0	1.00E+03	N/A	0	1.00E+04	N/A	0
1.00E+00	OK	1	1.00E+02	N/A	0	1.00E+03	N/A	0	1.00E+04	N/A	0

**Rule II**

52. After applying rule I: If there are more than 3 dilutions with countable numbers, use only the three highest dilutions.

53. Example: Before applying the rules:

Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count
1.00E+00	OK	210	1.00E+02	OK	82	1.00E+03	OK	25	1.00E+04	OK	0
1.00E+00	OK	238	1.00E+02	OK	103	1.00E+03	OK	31	1.00E+04	OK	7
1.00E+00	OK	222	1.00E+02	OK	99	1.00E+03	OK	29	1.00E+04	OK	6

After applying the rules:

Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count
1.00E+00	OK	210	1.00E+02	OK	82	1.00E+03	OK	25	1.00E+04	N/A	0
1.00E+00	N/A	238	1.00E+02	OK	103	1.00E+03	OK	31	1.00E+04	OK	7
1.00E+00	N/A	222	1.00E+02	OK	99	1.00E+03	OK	29	1.00E+04	OK	6

**Rule III**

54. Carry out the following procedure for each pair of subsequent dilutions. Start with the two lowest dilutions: Check whether the figure for the lower dilution is 50 or larger. Then calculate the dilution corrected counts and divide the figure for the lower dilution by 5. If this ratio is higher than the figure for the higher dilution then reject this carrier.

55. Example: Before applying the rules:

Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count
1.00E+00	OK	310	1.00E+01	OK	6	1.00E+02	OK	1	1.00E+03	0	0

Dilution corrected plate counts (use only the 3 highest dilutions with countable numbers according to rule II): The dilution corrected counts for the two lowest dilutions are 310 and 60. Since 310 is larger than 50, it is divided by the dilution corrected count of the higher dilution:  $310/60=5.2 \rightarrow$  this ratio is larger than 5 and therefore the whole carrier needs to be rejected.

Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count
1.00E+00	OK	310	1.00E+01	OK	60	1.00E+02	OK	100	1.00E+03	N/A	0

Dilution corrected plate counts after applying the rules:

Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count
1.00E+00	N/A	310	1.00E+01	N/A	60	1.00E+02	N/A	100	1.00E+03	N/A	0

**Rule IV**

56. Start with the two highest dilutions where the figure for the higher dilution is 4 or larger. If there is no such pair, rule IV cannot be applied. If there is such a pair, calculate the dilution corrected counts.

If the corrected count for the higher dilution exceeds the corrected count for the lower dilution by 10 or more than 10 times, ignore all lower dilutions.

If not, then additionally consider the next highest dilution – if available, i.e. consider the three highest dilutions: Calculate the dilution corrected counts. If the mean of the corrected counts of the two highest dilutions exceed the corrected count for the lowest considered dilution by 10 or more than 10 times, ignore this lower dilution.

57. Example: Before applying the rules:

Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count
1.00E+00	OK	140	1.00E+01	OK	55	1.00E+02	OK	4	1.00E+03	OK	4

Dilution corrected plate counts (use only the 3 highest dilutions with countable numbers according to rule II):

Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count
1.00E+00	N/A	140	1.00E+01	OK	550	1.00E+02	OK	400	1.00E+03	OK	4000

Dilution corrected plate counts after applying the rules:

Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count
1.00E+00	N/A	140	1.00E+01	N/A	55	1.00E+02	N/A	4	1.00E+03	OK	4

**Rule V**

58. No test carrier should have log counts of the CFU's or PFU's higher than the mean of the logarithmized water control + 30% (= log water control + 0.3). In this case disregard this carrier's results!

### **Recalculation of results for statistical analysis**

59. 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**

60. After the application of the rules, the final dataset is at first checked for outliers with regard to the single log<sub>10</sub> CFU values of each carrier. After these outliers are eliminated, the responses considered for the statistical model are determined. As statistical model, so-called inactivation curves are used. At first, run-specific inactivation curves are estimated. To these run-specific inactivation curves, three laboratory-comprehensive outlier tests are applied in order to identify runs that differ significantly from the runs of the remaining laboratories. After elimination of the outlier laboratories or outlier runs, the selection of run-specific inactivation curves is complete. On the basis of this selection, the overall (laboratory-comprehensive) inactivation curve is determined. In the last step, the intra- and inter-laboratory reproducibility may be estimated and based on these, the 95%-confidence interval for the overall inactivation curve as well as the 95%-prediction interval for the run-specific inactivation curves can be determined. Of course, the whole proceeding is carried out separately for each combination of organism and substance. In the following sub-sections, each of the mentioned steps is explained in detail.

### **Response considered**

61. The responses of the statistical analysis are the mean log<sub>10</sub> reductions at a specific concentration level, separately for each run of a laboratory and – of course – separately for each combination of organism and substance. After recalculating all mean CFU values per carrier – for the water controls and the test carriers – they are logarithmized. Thereby the so-called log<sub>10</sub> CFU values per carrier are obtained. For the water controls the arithmetic mean of these log<sub>10</sub> 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 log<sub>10</sub> of the water controls or AWC. Then for the test carriers – separately for each replication at one concentration level – the log<sub>10</sub> reduction is calculated by subtracting the log<sub>10</sub> of the CFU of the test carrier from the average log<sub>10</sub> of the water controls. Finally, the mean log<sub>10</sub> reduction for one concentration level is determined over all carriers of this concentration level.

62. Two different approaches – the standard approach and the normalized approach – have been considered during the analyses. In the standard approach, the log<sub>10</sub> reduction as described above has been considered as the response, whereas in the normalized approach, these responses have additionally been normalized. The main difference between both approaches is that for the standard approach the logarithm of the ratio of test carrier and water control is calculated, while for the normalized approach, at first the logarithms are calculated and then the ratio.

63. Standard approach: the log<sub>10</sub> reductions of each test carrier are calculated as follows:

$$\begin{aligned} \log_{10} \text{ reduction/carrier} &= \text{AWC} - \log\{\text{CFU / test carrier}\} \\ &= -\log\left\{\frac{\text{CFU / test carrier}}{\text{geometric mean of CFU of water controls / carrier}}\right\} \end{aligned}$$

64. Normalized approach: the log<sub>10</sub> reductions will be corrected by the average log<sub>10</sub> of the water controls of the respective run, i.e. the log<sub>10</sub> reductions will be given as percentages of the average log<sub>10</sub> of the water controls, where the latter one is set at 100%. So, the log<sub>10</sub> reductions of each test carrier are calculated as follows:

$$\begin{aligned} \text{Normalized log}_{10} \text{ reduction/carrier} &= 1 - \frac{\log\{\text{CFU / test carrier}\}}{\text{AWC}} \\ &= 1 - \frac{\log\{\text{CFU / test carrier}\}}{\log\{\text{geometric mean of CFU of water controls / carrier}\}} \end{aligned}$$

The normalized log<sub>10</sub> reduction is not directly related to the percentage of inactivated microorganisms; it represents the inactivation rate on the log scale and depends on the water control.

Example: if the water control has an average log<sub>10</sub> of 5, 80% normalized log<sub>10</sub> reduction means a log<sub>10</sub> reduction of 4, i.e. an inactivation rate of  $1 - 10^{-4} = 99.99\%$

65. Apparently, the standard log<sub>10</sub> reduction can be easily obtained by multiplying the normalized log<sub>10</sub> reduction with the respective average log<sub>10</sub> of the water controls of the respective run. The obvious advantage of the standard approach is the closer relationship to the average log<sub>10</sub> of the water controls: As the maximum log<sub>10</sub> reduction depends on the value of the average log<sub>10</sub> of the water controls, it is easier to see if the average log<sub>10</sub> of the water control could be reached by a laboratory during the test. Furthermore differences between the water controls of each laboratory and each run can be balanced.

However, within the study, laboratories exhibited a high variation regarding the average log<sub>10</sub> of the water controls (see also section *Analysis of water controls*, p. 33). This variation is considerably higher than normally accepted and also higher than originally prescribed in the protocol for the ring trial. As for more than 50% of the data the average log<sub>10</sub> of the water control is out of the tolerance limits, it was decided to not follow the original protocol for the ring trial but to accept an exceedance of the tolerance limits. A direct consequence of this decision is another source of deviations between different participants: the log<sub>10</sub> reduction according to the standard approach is highly depending on the water control. The normalized approach compensates the influence of this error source better than the standard approach and allows identifying differences between participants which are not due to different starting titres. Thus, it had been decided that the normalized approach is the more advantageous

option within in the context of this ring trial. However for routine use, the standard approach is recommended. In this report, the proceeding for both approaches will be explained. Only the results of the normalized approach are shown, while the results according to the standard approach are given in the Annex *Results of phase 1 according to the standard approach*, p. 202.

Regulatory authorities will require the use of the standard approach for calculating the log reduction, since prospective products will need to meet the minimum water control values in order for the data to be accepted.

### **Analysis of water controls**

66. A statistical analysis of the water controls has been carried out according to ISO 5725-2 based on the values of average log<sub>10</sub> of the water controls per run of each laboratory, separately for each combination of organism and substance.

### **The inactivation curve**

67. The relationship between the mean (normalized) log<sub>10</sub> 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) log<sub>10</sub> survival rate in dependence on the concentration of the active substance for all microorganisms tested:

$$\log_{10} \text{ reduction} = R = D - \frac{D}{1 + \left(\frac{\text{conc}}{C}\right)^B},$$

where

**D** is a theoretical value. It describes the mean (normalized) log<sub>10</sub> reduction (the negative (normalized) log<sub>10</sub> 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) log<sub>10</sub> 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.

68. For the normalization of the presentation of the inactivation curves, the average log<sub>10</sub> 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 log<sub>10</sub> of the water controls.

69. The statistical analysis of inactivation curves offers the possibility to detect and assess systematic and random sources of errors separately.
70. Additionally, a so-called logit approach was tested. Applying the logit approach did not appear to be appropriate from a microbiological point of view. However, for the sake of completeness, the logit approach is explained in the Annex *Logistic approach*, p. 166.

### **Statistical outlier identification with regard to the single log<sub>10</sub> CFU values of each carrier**

71. Before estimating the run-specific inactivation curves, two outlier tests are carried out for the single log<sub>10</sub> CFU values of each test carrier. Therefore, at first, the standard deviation of the log<sub>10</sub> CFU values per carrier for each combination of run and concentration level is determined, but only if there are at least 3 carriers with log<sub>10</sub> CFU values available. For concentration levels with less than 3 values no further outlier analysis is carried out. As an example, assume that there are 3 runs with 6 concentration levels each and also assuming that at each concentration level 3 replicates are available, 18 standard deviations are obtained in total.
72. Now the Cochran test at a significance level of 1% is applied to the obtained standard deviations. If the test yields in a statistically significant result, i.e. if there is one standard deviation that is significantly higher than the other standard deviations, the Grubbs test is applied to the three single log<sub>10</sub> CFU values of the suspicious combination of run and concentration level. Therefore a significance level of 5% is assumed. If the test yields in a statistically significant result, i.e. if there is one log<sub>10</sub> CFU that differs significantly from the two other log<sub>10</sub> CFU values, the value of respective carrier will not be taken into account for estimating the inactivation curves. If outliers could be identified, the whole procedure is repeated a second time.

### **Determination of run-specific inactivation curves**

73. The models for the run-specific inactivation curves for one laboratory (and one combination of organism and active substance) are determined by applying the least squares method.
74. Log reductions are censored data, i.e. if there are no viable counts, the actual log reduction is only known to be greater or equal than the log of the water control. In order to define the final estimates for the parameters, the following correction rule is applied: At first the parameters are estimated only for the data without the complete inactivation concentrations. If the

reported log<sub>10</sub> reductions belonging to the complete inactivation concentrations lie below the curve, it can be assumed that the minimum concentration that causes a complete inactivation is smaller than the complete inactivation concentrations reported. If, however, the reported log<sub>10</sub> reductions belonging to the complete inactivation concentrations lie above the predicted inactivation curve, it can be assumed that the minimum concentration that causes a complete inactivation is larger than the complete inactivation concentration reported. Therefore, at first all complete inactivation log<sub>10</sub> reductions and the respective concentration levels are excluded. With the remaining values the run-specific inactivation curves are estimated. Successively, beginning with the smallest concentration level, the complete inactivation log<sub>10</sub> reductions are tested if it lies below the estimated inactivation curve or above. If it lies below the inactivation curve, the value stays excluded from the inactivation curve estimation. If it lies above the inactivation curve, it will be included in the final estimation of the run-specific inactivation curve. Now the whole procedure is repeated for the next highest concentration with a complete inactivation log<sub>10</sub> reduction and so on until all complete inactivation log<sub>10</sub> reductions are tested.

75. If, however, a log<sub>10</sub> reduction equaling the value of the water control is followed by a smaller log<sub>10</sub> reduction on a higher concentration level, the log<sub>10</sub> reduction equaling the water control value is included in the first and in the final estimation of the run-specific inactivation curves.
76. For fitting the curves, the following “most-likely-approach” is applied for each test substance and organism separately: at first for each run of one laboratory the parameters are estimated; if there are runs where the inactivation curves fits the empirical data only badly, i.e. if the slope of a run curve is too steep, the geometric mean of the parameters B of the runs with a satisfying fit from the same laboratory is used as estimation. In special cases, also the parameter C has to be adapted in the same way like the parameter B.
77. If the mean log<sub>10</sub> reduction of the available runs at the smallest concentration level is larger than 2, no extrapolation into the direction of zero is carried out and the run-specific inactivation curves start at the smallest concentration level measured.
78. The minimum requirement for including data is that there are at least two log<sub>10</sub> reductions of a run that do not equal the average log<sub>10</sub> of the water controls. In these cases, the least square algorithm for estimating the parameters cannot find reasonable results and therefore no estimation of the run-specific inactivation curves can be carried out. The measurement of the respective runs are excluded from the statistical analysis and in the following referred to as *not regarded*.
79. For the run-specific inactivation curves, the 95%-prediction intervals are determined by a non-parametric bootstrap method.

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

80. The following outlier tests are carried out separately for the standard approach and the normalized approach!

81. Three laboratory-comprehensive outlier tests are applied to the run-specific inactivation curves: a Cochran test with regard to the residual standard deviation and a Grubbs test and a Cochran test with regard to concentrations at specific log<sub>10</sub> reductions. By these tests, suspicious run-specific inactivation curves of one laboratory (and one combination of organism and active substance) may be identified. The order of the three tests is fixed. Identified outlier values are excluded from the dataset. The three outlier tests are explained in more detail in the following paragraphs.

### **82. Outlier test with regard to the residual standard deviations**

For each laboratory the residual standard deviation over all run-specific inactivation curves is considered if there are enough values available. Now, the Cochran test at the significance level 1% is applied to these standard deviations. If the Cochran test yields in a significant result, all data of the respective laboratory is regarded as outlier data and excluded from the data set for the following outlier tests.

### **83. Outlier test with regard to the concentration**

For each laboratory the values of the logarithmized concentrations of the run-specific inactivation curves are determined at a log<sub>10</sub> reduction of 3 in the standard approach and at a normalized log<sub>10</sub> reduction of 50% in the normalized approach, respectively.

Then

- mean value is determined and
- standard deviation (if there are at least two run-specific inactivation curves)

of these logarithmized concentrations are determined. Thereby, as many mean values as there are laboratories and as many mean standard deviations as there are laboratories with at least two run-specific inactivation curves are available. Now, the Cochran test at the significance level 1% is applied to the standard deviations. If the Cochran test yields in a significant result, the respective laboratory exhibits run-specific inactivation curves that differ more than the inactivation curves of the other laboratories for the specified log<sub>10</sub> reduction. If for the laboratory with the significant result at least 3 run-specific inactivation curves are available, a Grubbs test can be carried out in order to see if one of the inactivation curves is responsible for the high variation. The Grubbs test is applied at the significance level 5%. If the test yields in a significant result, i.e. if one of the inactivation curves differs significantly from the other run-specific inactivation curves of that laboratory, only the respective run will be marked as an outlier. If, however, the Grubbs test cannot be applied or if it does not yield in a significant result, all runs of the respective laboratory have to be considered as outlier runs. Now, the two-sided Grubbs test is applied to the mean values at the significance level 5%. The outliers from the Cochran tests are already eliminated. If the test yields in a significant result, i.e. if a laboratory mean value differs significantly from the total mean, all

data of the respective laboratory is excluded from the data set for the following outlier test.

### **Determination of overall (laboratory-comprehensive) inactivation curves**

84. The overall or laboratory-comprehensive inactivation curves are derived via a bootstrap calculation. It is assumed that the residuals of all laboratories and all runs of one combination of organism and substance 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.
85. If the parameter D has been estimated for the run-specific inactivation curve, the parameter D for the bootstrap inactivation curves will be estimated as well.
86. In order to derive the overall inactivation curve, the concentration range of one combination of organism and substance is divided into many small intervals and within each of these intervals, the mean value of the 750 inactivation curves is calculated. These mean values describe the overall inactivation curve.
87. It has to be noted that the overall inactivation curves for both approaches – standard and normalized – are calculated in parallel, i.e. the 750 single inactivation curves according to the normalized approach will be directly derived from the 750 single inactivation curves according to the standard approach by dividing the respective log10 of the water control.

### **Intra- and inter-laboratory reproducibility**

88. The general model for the mean (normalized) log10 reduction for one laboratory and one combination of organism and substance at a specific concentration level c is given by

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

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  $\sigma_r^2$ .

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  $\sigma_r^2$  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.

89. Note that only the run-specific inactivation curves are modeled as described in section 24, 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  $c$ .

90. 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.

91. 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)

92. 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.

### **Laboratory-specific sensitivity**

93. The shape of inactivation curves of different laboratories 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 delogarithmized shift is referred to as the sensitivity  $\lambda$  of the respective laboratory. If  $\lambda$  equals about 1, the sensitivity of the respective laboratory is about the same as the sensitivity over all laboratories. If  $\lambda$  is larger than 1, the sensitivity of the respective laboratory is larger than the overall sensitivity, whereas a run with a  $\lambda$  smaller than 1 exhibits a lower sensitivity than the overall sensitivity. The sensitivity  $\lambda$  is changing from laboratory to laboratory, from microorganism to microorganism, from substance to substance. A correlation analysis of these  $\lambda$ 's may reveal possible sources of error.

## Phase 1

### **Raw data**

#### **Reporting of data**

94. Several laboratories used a different quantification method than suggested or a different workbook for entering the data or even both of it. This applies mainly to *Adenovirus type 5* for which many laboratories were not able to use the workbook supplied and some of them adapted the available workbooks. If possible, these laboratories have been asked to enter their data into the provided workbook. However, not all of them followed the plea, therefore for some laboratories, data from doc-files or pdf-files needed to be transferred to the database by quo data GmbH.

#### **Overview of available data**

95. Of the thirty-five laboratories that stated their participation, 27 laboratories submitted data, at least for one of the organism/substance combination. No data were submitted by 8 laboratories: LC0003, LC0005, LC0015, LC0018, LC0024, LC0027, LC0030 and LC0034.

96. Table 6 shows the number of runs included in the statistical analysis for each combination of laboratory, organism and substance. Laboratories LC0004 and LC0019 additionally submitted data for *Pseudomonas aeruginosa* and benzalkonium chloride, which was not scheduled in the ring trial at all.

97. For *Adenovirus type 5*, two different quantification methods have been applied: TCID<sub>50</sub> method and well count method. The latter has been applied by three laboratories only, while the TCID<sub>50</sub> method has been applied by 10 laboratories.

98. For the following three laboratories, the TCID<sub>50</sub> values had to be recalculated according to Spearman and Kärber (see (3) and (4)): LC0001, LC0002 and LC0022.

**Table 6: Number of runs for which data was submitted for each laboratory/organism/substance**

Lab Code	<i>Adenovirus type 5</i> peracetic acid	<i>Adenovirus type 5</i> glutaraldehyde	<i>Aspergillus niger</i> peracetic acid	<i>Aspergillus niger</i> glutaraldehyde	<i>Bacillus subtilis</i> peracetic acid	<i>Mycobacterium terrae</i> peracetic acid	<i>Mycobacterium terrae</i> glutaraldehyde	<i>Mycobacterium terrae</i> phenol	<i>Pseudomonas aeruginosa</i> peracetic acid	<i>Pseudomonas aeruginosa</i> glutaraldehyde	<i>Staphylococcus aureus</i> peracetic acid	<i>Staphylococcus aureus</i> glutaraldehyde	<i>Staphylococcus aureus</i> benzalkonium chloride
LC0000											1	3	2
LC0001	1	1				1	1	1					
LC0002	3	3			3								
LC0004									3	5	4	3	4
LC0006	3	3											
LC0007							3	3					
LC0008					3	3	3	3			4	4	1
LC0009	3	3											
LC0010											5	5	3
LC0011			3	3	3	3	3	3	3	3	3	3	3
LC0012						2	2	2	3	3	4	3	3
LC0013									3	3	3	3	3
LC0014	2*	3*											
LC0016	3	3											
LC0017	3	3											
LC0019			1	1					1	1	1	1	1
LC0020											4	4	3
LC0021			3	3		3	3	3	3	3	3	3	3
LC0022	3	3	2	2	2						3	3	3
LC0023			3	3									
LC0025					3	3							
LC0026	3*	3*							2	3	4	4	3
LC0028									3	3	3	3	3
LC0029	3	3											
LC0031	3	3											
LC0032	3*	3*											
LC0033	3	3											
Number of labs	13	13	5	5	5	6	6	6	8	8	13	13	13
Number of runs	36	37	12	12	14	15	15	15	21	24	42	42	35

\*) well count method

**Overview of concentrations used**

99. For each organism and test substance six test concentrations were suggested by the lead laboratories for the first run (see also Table 2). However, it was suggested that the laboratories adapt the concentrations of runs 2 and 3 in order to obtain reasonable results, e.g. not only complete inactivation. Some of the participants already changed the suggested concentrations in the first run. The following Table 7 summarizes the laboratories that adapted the concentrations. In general, most of the laboratories that adapted their concentrations either had to decrease or to increase the suggested concentration levels. Several did both in order to find the most appropriate concentration range, e.g. in the second run they tried higher concentrations and in the third run they worked with lower concentrations again. Some laboratories restricted the suggested concentration range.

**Table 7: Laboratories that adapted the concentrations**

Organism	Test substance	Lab Code
<i>Adenovirus type 5</i>	glutaraldehyde	LC0001; LC0006; LC0016; LC0017; LC0026; LC0029
	peracetic acid	LC0016; LC0017; LC0022; LC0026; LC0029
<i>Aspergillus niger</i>	glutaraldehyde	LC0011; LC0021; LC0023
	peracetic acid	LC0021
<i>Bacillus subtilis</i>	peracetic acid	LC0002; LC0008; LC0011; LC0025
<i>Mycobacterium terrae</i>	glutaraldehyde	LC0008; LC0011; LC0021
	peracetic acid	LC0008; LC0011; LC0021
	phenol	LC0008; LC0011; LC0021
<i>Pseudomonas aeruginosa</i>	glutaraldehyde	LC0004; LC0011; LC0012; LC0019; LC0021; LC0026; LC0028
	peracetic acid	LC0004; LC0012; LC0019; LC0021; LC0026
<i>Staphylococcus aureus</i>	glutaraldehyde	LC0000; LC0004; LC0008; LC0010; LC0020; LC0021; LC0022; LC0026; LC0028
	peracetic acid	LC0004; LC0008; LC0010; LC0011; LC0012; LC0020; LC0021; LC0022; LC0026; LC0028
	benzalkonium chloride	LC0000; LC0004; LC0008; LC0010; LC0012; LC0022; LC0026; LC0028

**Starting titer**

100. It was suggested that the viability titer of test organism on dried carriers ranges from 4.5 to 6 log<sub>10</sub> for *Adenovirus type 5* or from 5.5 to 7.0 log<sub>10</sub> for the remaining organisms. The following Table 8 presents the number of runs and laboratories not falling outside the suggested range.

**Table 8: Total number of runs and laboratories available and number of runs and laboratories not falling outside the tolerance for the log<sub>10</sub> of water control**

Organism	Substance	Total number of available runs	Number of runs with water control within range	Total number of available labs	Number of labs with at least one run with water control within range
<i>Adenovirus type 5</i>	peracetic acid	36	14 (39%)	13	7
<i>Adenovirus type 5</i>	glutaraldehyde	37	19 (51%)	13	8
<i>Aspergillus niger</i>	peracetic acid	12	0 (0%)	5	0
<i>Aspergillus niger</i>	glutaraldehyde	12	1 (8%)	5	1
<i>Bacillus subtilis</i>	peracetic acid	14	8 (57%)	5	3
<i>Mycobacterium terrae</i>	peracetic acid	15	8 (53%)	6	4
<i>Mycobacterium terrae</i>	glutaraldehyde	15	6 (40%)	6	3
<i>Mycobacterium terrae</i>	phenol	15	8 (53%)	6	4
<i>Pseudomonas aeruginosa</i>	peracetic acid	21	11 (52%)	8	4
<i>Pseudomonas aeruginosa</i>	glutaraldehyde	24	9 (38%)	8	3
<i>Staphylococcus aureus</i>	peracetic acid	42	32 (76%)	13	10
<i>Staphylococcus aureus</i>	glutaraldehyde	41	30 (73%)	13	11
<i>Staphylococcus aureus</i>	benzalkonium chloride	35	27 (77%)	13	11

**Implausible results and data correction**

101. In a first step, the data has been checked for implausible values. Thereby, the values of laboratory LC0001 and of laboratory LC0026 for *Adenovirus type 5* attracted attention. Both participants were informed, and laboratory LC0001 corrected the values, while LC0026 stated that the submitted data were correct.

102. For laboratory LC0026 – *Staphylococcus aureus* (benzalkonium chloride (run 2) and glutaraldehyde (run 2)) a copy & paste error has occurred during the data transfer from the workbook sent by the laboratory to the database. This error has been corrected.

103. In a second step - between 8<sup>th</sup> and 10<sup>th</sup> September 2008 - all participating laboratories were provided with the database used. Thereby every participant had the chance to check if their data has been submitted correctly and also if they have been transferred correctly from the workbooks to the database.

104. Within this time window, several laboratories sent additional data, e.g. results of

additional runs not sent before. These laboratories with the respective additions are summarized in the following Table 9.

**Table 9: Additional data sent after preliminary statistical analysis**

Lab Code	Organism	Comment
LC0019	<i>Aspergillus niger</i>	detailed information supplied
	<i>Pseudomonas aeruginosa</i>	detailed information supplied
	<i>Staphylococcus aureus</i>	detailed information supplied
LC0028	<i>Staphylococcus aureus</i>	runs added

105. Furthermore, several laboratories corrected their results sent before. They are summarized in the following Table 10.

**Table 10: Corrected data sent after preliminary statistical analysis**

Lab Code	Organism	Comment
LC0001	<i>Adenovirus type 5</i>	amended workbook
LC0001	<i>Mycobacterium terrae</i>	2 values corrected (phenol run 1)
LC0002	<i>Adenovirus type 5</i>	new data supplied (workbook instead of handwritten and scanned documents)
LC0006	<i>Adenovirus type 5</i>	slight correction of values for glutaraldehyde run 1 (check if correct 20 <sup>th</sup> August)
LC0008	<i>Bacillus subtilis</i>	corrected values for 2 concentration levels (run 1 and run 2)
LC0009	<i>Adenovirus type 5</i>	correction of concentration values
LC0021	<i>Mycobacterium terrae</i>	values corrected
LC0028	<i>Pseudomonas aeruginosa</i>	corrected values for 1 concentration level (peracetic acid run 2)
LC0032	<i>Adenovirus type 5</i>	2 values corrected (peracetic acid run 1)

## **Data evaluation**

### **Analysis of water controls**

106. No data selection has been carried out before, i.e. all available data is included in the analysis. However, according to ISO 5725-2, significant outliers have been identified.

107. Please note that the average log<sub>10</sub> of the water controls have been recalculated due to the revised determination of the mean CFU/carrier, i.e. not the values submitted by the laboratories have been used.

108. For *Mycobacterium terrae* and the substances phenol and peracetic acid, the variability of the average log<sub>10</sub> reductions of the water controls of laboratory LC0008 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 log<sub>10</sub> of the water controls.

109. The following Table 11 summarizes the statistical parameters obtained in the analysis. It should be noted that the parameters for *Adenovirus type 5* (well count) are based on the values of 3 laboratories only and therefore no great importance should be attached to these values.
110. For *Adenovirus type 5* (TCID<sub>50</sub>) the overall mean values of the log<sub>10</sub> of the water controls lies within the demanded range of 4.5 - 6. For *Adenovirus type 5* (well count), the overall mean is clearly lower than the overall mean for the TCID<sub>50</sub> methods, for peracetic acid it even is below 4.5. For the other organisms, the overall mean lies within the demanded range of 5.5 - 7 log<sub>10</sub> only for *Bacillus subtilis*, *Mycobacterium terrae* and *Staphylococcus aureus*. For *Pseudomonas aeruginosa* the overall mean is slightly smaller than 5.5, whereas for *Aspergillus niger*, for both substances the overall mean values is clearly smaller than 5.5.
111. For each organism, the parameters (mean and standard deviations) for the different substances are very similar.
112. The highest reproducibility standard deviation can be observed for *Bacillus subtilis* and peracetic acid, the lowest value has been obtained for *Aspergillus niger* and glutaraldehyde.
113. The highest repeatability standard deviation can be observed for *Mycobacterium terrae* and glutaraldehyde, the lowest value has been obtained for *Mycobacterium terrae* and phenol.
114. In the Annex *Analysis of water controls in phase 1*, p. 167, for each combination of organism and substance the variability of the average log<sub>10</sub> of the water controls over the runs is shown as well as the kernel density estimation of the average log<sub>10</sub> of the water controls.
115. Highly varying water control results of the laboratories lead to the decision that the normalized approach is the more advantageous option. For example, in the initial ring trial in phase 1 for *Bacillus subtilis* and peracetic acid the average log<sub>10</sub> of the water controls ranged between 4 and 7.3. The normalized approach set all water controls to 100% with which a more uniform presentation of the results is possible.

**Table 11: Parameters of the statistical analysis of the average log<sub>10</sub> of the water controls**

Organism	Substance	number of participating laboratories	number of laboratories after outlier elimination	overall mean	reproducibility s.d.	repeatability s.d.
<i>Adenovirus type 5</i> (TCID <sub>50</sub> )	glutaraldehyde	10	10	5.98	0.76	0.29
	peracetic acid	10	10	5.83	0.94	0.24
<i>Adenovirus type 5</i> (well count)	glutaraldehyde	3	3	4.53	(0.55)	(0.13)
	peracetic acid	3	3	4.35	(0.53)	(0.53)
<i>Aspergillus niger</i>	glutaraldehyde	5	5	4.78	0.52	0.18
	peracetic acid	5	5	4.77	0.56	0.15
<i>Bacillus subtilis</i>	peracetic acid	5	5	6.19	1.16	0.17
<i>Mycobacterium terrae</i>	phenol	6	5	6.41	0.86	0.11
	glutaraldehyde	6	6	6.50	0.82	0.39
	peracetic acid	6	5	6.38	0.81	0.14
<i>Pseudomonas aeruginosa</i>	glutaraldehyde	8	8	5.30	0.78	0.31
	peracetic acid	8	8	5.44	0.71	0.28
<i>Staphylococcus aureus</i>	benzalkonium chloride	13	13	6.34	0.64	0.29
	glutaraldehyde	13	13	6.45	0.62	0.23
	peracetic acid	13	13	6.48	0.63	0.20

**Statistical outlier identification with regard to the single log<sub>10</sub> CFU values of each carrier**

116. The following table summarizes all identified outliers of the single log<sub>10</sub> CFU values of each carrier. These outlier carriers have been eliminated and do not contribute to any further statistical analysis.
117. For *Adenovirus type 5* (well count) and *Pseudomonas aeruginosa* no outliers could be identified for either substance. For *Aspergillus niger* no outliers could be identified for the measurements with glutaraldehyde.

**Table 12: Outliers with regard to the single log<sub>10</sub> CFU values of each carrier**

Organism	Substance	Lab Code	Run	Carrier	Concentration
<i>Adenovirus type 5</i> (TCID <sub>50</sub> )	peracetic acid	LC0033	3	11	75 ppm
	glutaraldehyde	LC0006	3	6	250 ppm
<i>Aspergillus niger</i>	peracetic acid	LC0011	1	4	400 ppm
		LC0022	1	16	25 ppm
<i>Bacillus subtilis</i>	peracetic acid	LC0011	1	5	1200 ppm
<i>Mycobacterium terrae</i>	phenol	LC0012	2	1	20000 ppm
	peracetic acid	LC0011	1	4	700 ppm
		LC0011	2	6	700 ppm
		LC0011	2	10	500 ppm
	glutaraldehyde	LC0008	2	5	20000 ppm
		LC0011	1	3	25000 ppm
		LC0021	1	4	20000 ppm
<i>Staphylococcus aureus</i>	peracetic acid	LC0011	1	13	20 ppm
		LC0028	1	6	110 ppm
	benzalkonium chloride	LC0004	2	15	1000 ppm
		LC0008	1	15	2500 ppm
		LC0012	1	12	2500 ppm

**Minimum requirements**

118. The following table summarizes all runs not regarded, because the minimum requirement has not been fulfilled. The last column gives the reason for the exclusion of the data. None of the data will contribute to any further statistical analysis.
119. For *Adenovirus type 5* (TCID<sub>50</sub> and well count), *Bacillus subtilis* and *Mycobacterium terrae* no runs that do not fulfill the minimum requirements have been identified.

**Table 13: Runs not regarded because the minimum requirement has not been fulfilled  
(AWC = average log<sub>10</sub> of the water controls)**

Organism	Substance	Lab Code	Run	Reason
<i>Aspergillus niger</i>	peracetic acid	LC0019	1	all values excluded due to rules 0 - V
	glutaraldehyde	LC0019	1	only two values available and both equal AWC
<i>Pseudomonas aeruginosa</i>	glutaraldehyde	LC0004	3	only one value not equaling AWC
		LC0019	1	all values equal AWC
	peracetic acid	LC0004	1	all values equal AWC
		LC0019	1	all values equal AWC
		LC0026	1 + 2	implausible values
<i>Staphylococcus aureus</i>	peracetic acid	LC0000	1	only two values available and both equal AWC
		LC0004	1	all values equal AWC
		LC0004	2 + 3	only one value not equaling AWC
		LC0008	1	only one value not equaling AWC
		LC0010	1	only one value available
		LC0012	1	only one value not equaling AWC
		LC0019	1	all values equal AWC
		LC0020	1	implausible values
		LC0022	1	all values equal AWC
		LC0028	1	all values equal AWC
		LC0028	3	only one value not equaling AWC
		glutaraldehyde	LC0010	1
	LC0019		1	only one value available
	LC0022		1	only one value not equaling AWC
	benzalkonium chloride	LC0004	2	only one value not equaling AWC
		LC0010	1	implausible values
		LC0019	1	all values equal AWC
		LC0022	1	only one value not equaling AWC

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

120. The following table summarizes the results of the three outlier tests with regard to the run-specific inactivation curves according to the normalized approach. These outlier laboratories (or runs) do not contribute to any further statistical analysis.
121. Since for *Adenovirus type 5* (well count) data of only three laboratories is available, no outlier tests are carried out.
122. The Grubbs test with regard to the mean concentration at a normalized log reduction of 50% never yielded in any significant result.
123. For *Aspergillus niger* and peracetic acid no outlier laboratories (or runs) could be identified at all.

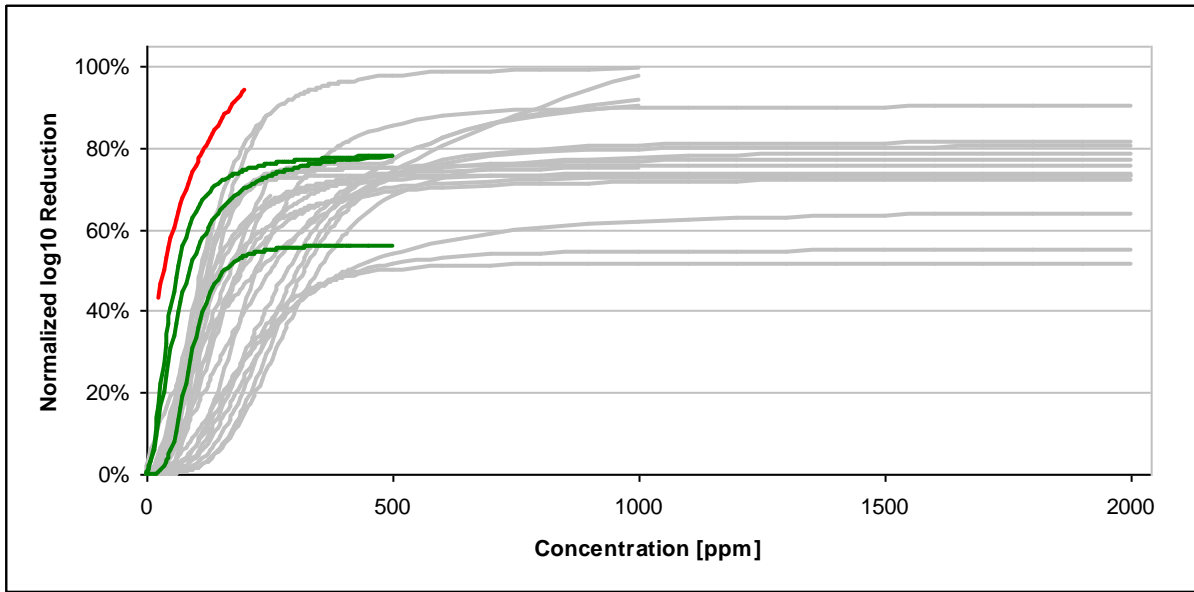
124. It has to be noted that the values of laboratory LC0007 for *Mycobacterium terrae* and phenol had to be excluded from the dataset as well. The respective values exhibit a very slow increase (see also the respective figure in the Annex *Normalized run-specific inactivation curves of phase 1*, p. 176) and including these values would cause a bias of the results. No outlier test identified all runs as outlier runs, because the standard deviation is very small and the mean value at a normalized log<sub>10</sub> reduction of 50% does not differ significantly from the mean values of the other laboratories. If, however, a different normalized log<sub>10</sub> reduction had been assumed as test level (e.g. 80%), the runs would clearly have been identified as outlier runs.

**Table 14: Eliminated outlier laboratories (or runs) due to deviations of the run-specific inactivation curves**

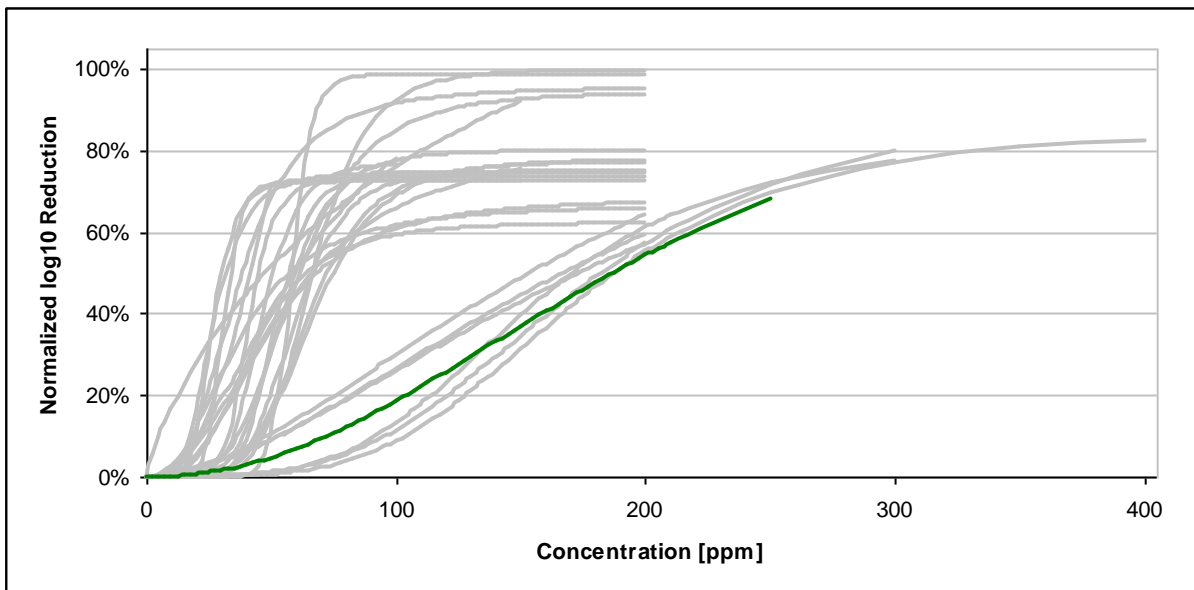
Organism	Substance	RSD (Cochran)	Mean concentration at normalized log <sub>10</sub> reduction of 50% (Grubbs)	Standard deviation of concentrations at normalized log <sub>10</sub> reduction of 50% (Cochran)
<i>Adenovirus type 5</i> (TCID <sub>50</sub> )	peracetic acid	-	-	LC0022 - Run 3
	glutaraldehyde	LC0001	-	LC0006
<i>Aspergillus niger</i>	glutaraldehyde	LC0022	-	-
	peracetic acid	-	-	-
<i>Bacillus subtilis</i>	peracetic acid	-	-	-
<i>Mycobacterium terrae</i>	peracetic acid	-	-	-
	phenol	-	-	LC0007 - Run 1 (see also paragraph 124)
	glutaraldehyde	LC0011	-	-
<i>Pseudomonas aeruginosa</i>	peracetic acid	-	-	LC0004
	glutaraldehyde	LC0026	-	-
<i>Staphylococcus aureus</i>	peracetic acid	-	-	-
	glutaraldehyde	LC0020	-	LC0010
	benzalkonium chloride	LC0012	-	-

125. The following figures show the run-specific inactivation curves for the normalized approach for each combination of organism and substance. Outlier inactivation curves that will be eliminated from further statistical analyses are marked by different colors. The respective laboratory is specified in the caption. The remaining inactivation curves, i.e. non-outliers on which all further statistical analyses will be based in, are shown in a light-grey.

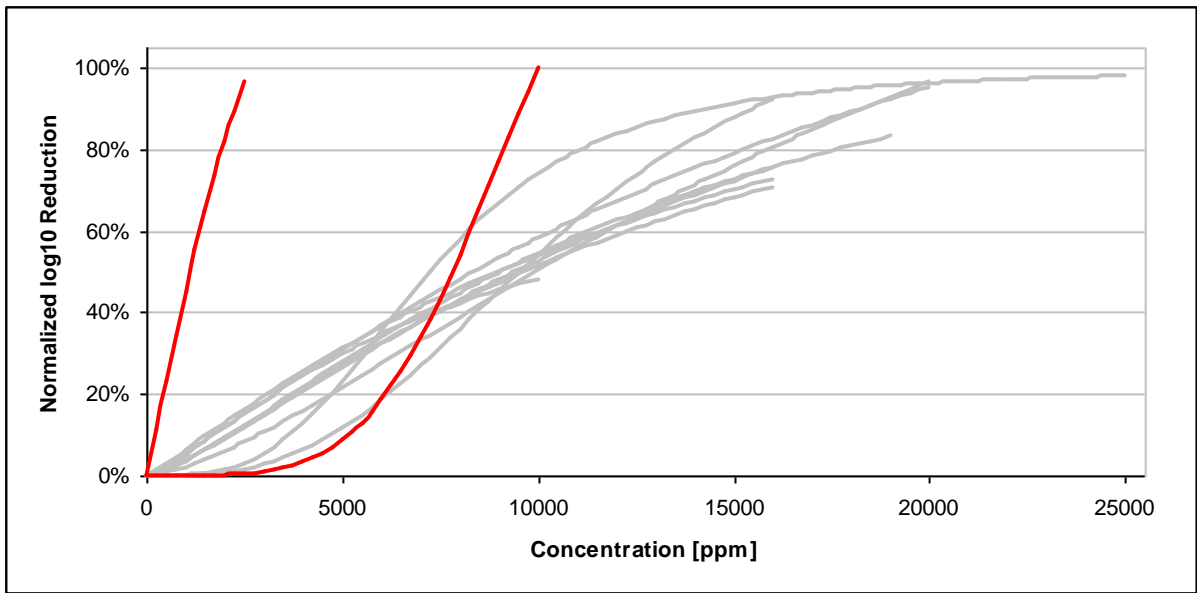
**Figure 2: Run-specific inactivation curves for *Adenovirus type 5* (TCID<sub>50</sub>) and glutaraldehyde (red: LC0001, green: LC0006)**



**Figure 3: Run-specific inactivation curves for *Adenovirus type 5* (TCID<sub>50</sub>) and peracetic acid (green: LC0022 – run 3)**



**Figure 4: Run-specific inactivation curves for *Aspergillus niger* and glutaraldehyde (red: LC0022)**



**Figure 5: Run-specific inactivation curves for *Aspergillus niger* and peracetic acid (no outliers)**

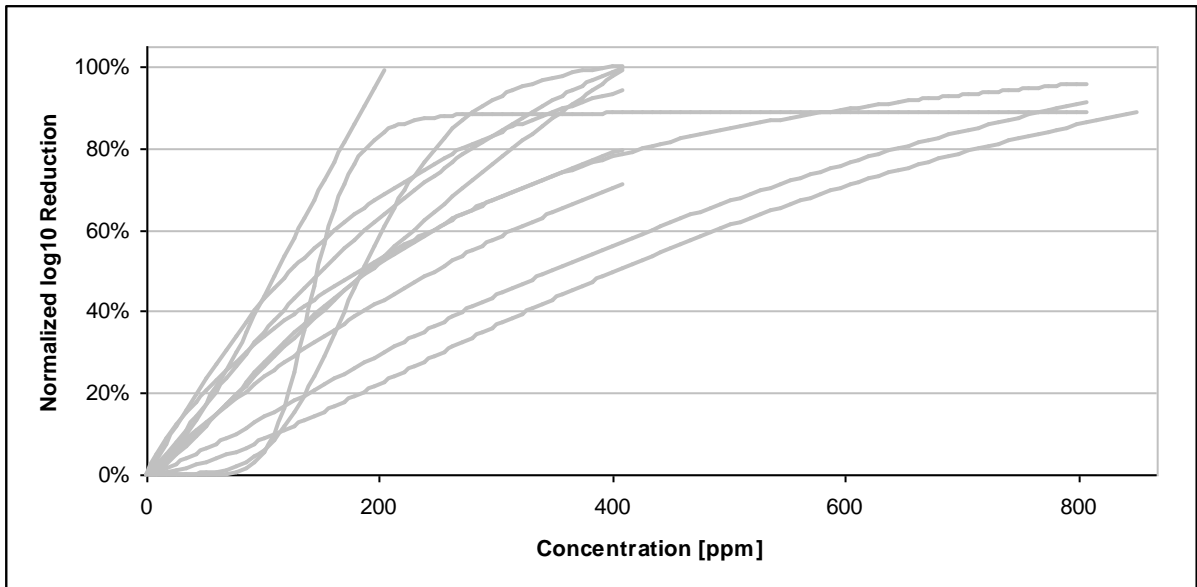


Figure 6: Run-specific inactivation curves for *Bacillus subtilis* and peracetic acid (no outliers)

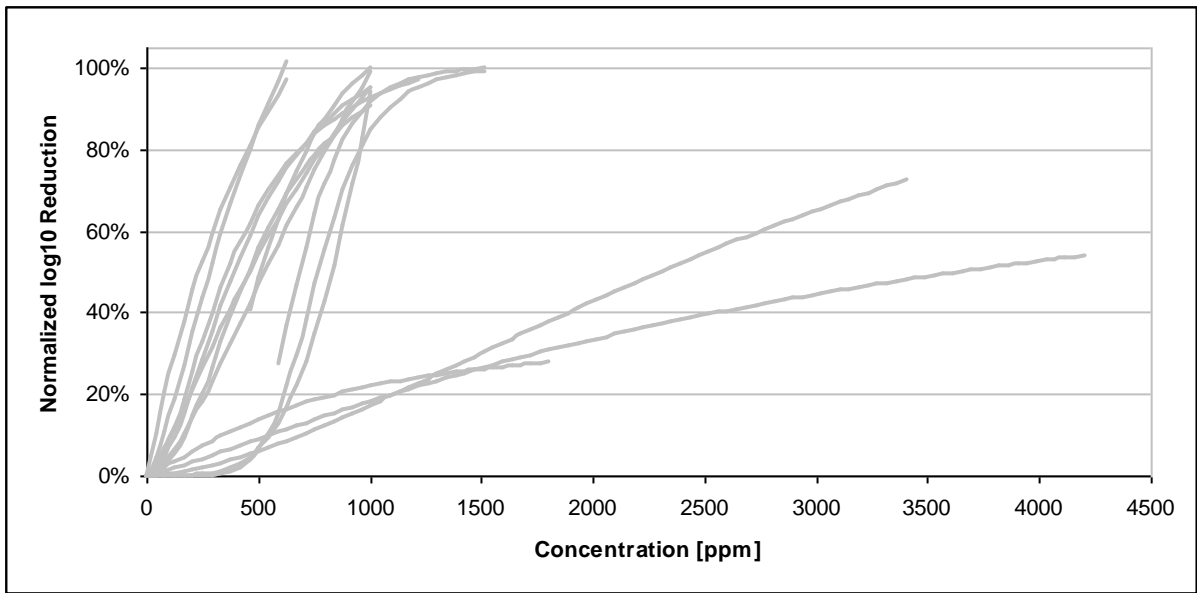


Figure 7: Run-specific inactivation curves for *Mycobacterium terrae* and glutaraldehyde (red: LC0011)

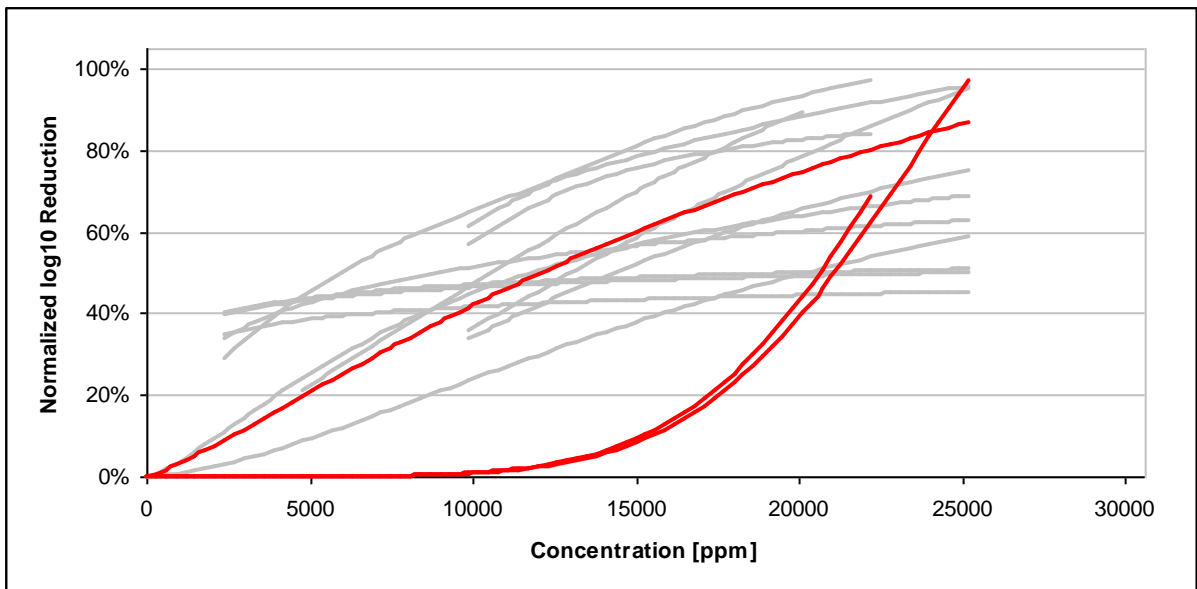


Figure 8: Run-specific inactivation curves for *Mycobacterium terrae* and peracetic acid (no outliers)

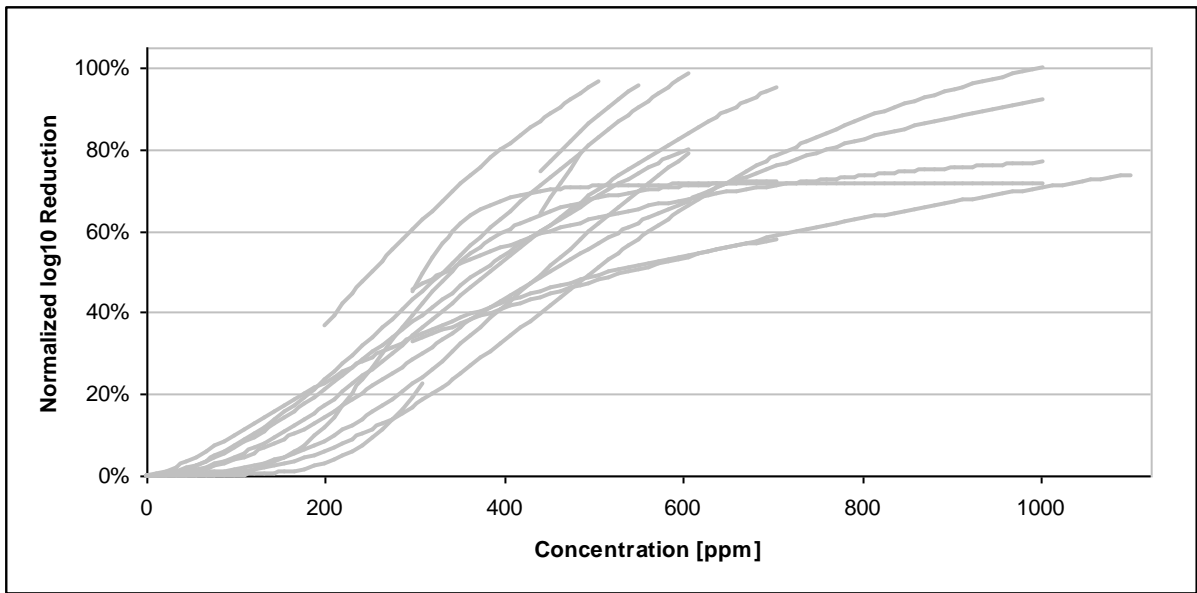


Figure 9: Run-specific inactivation curves for *Mycobacterium terrae* and phenol (blue: LC0007)

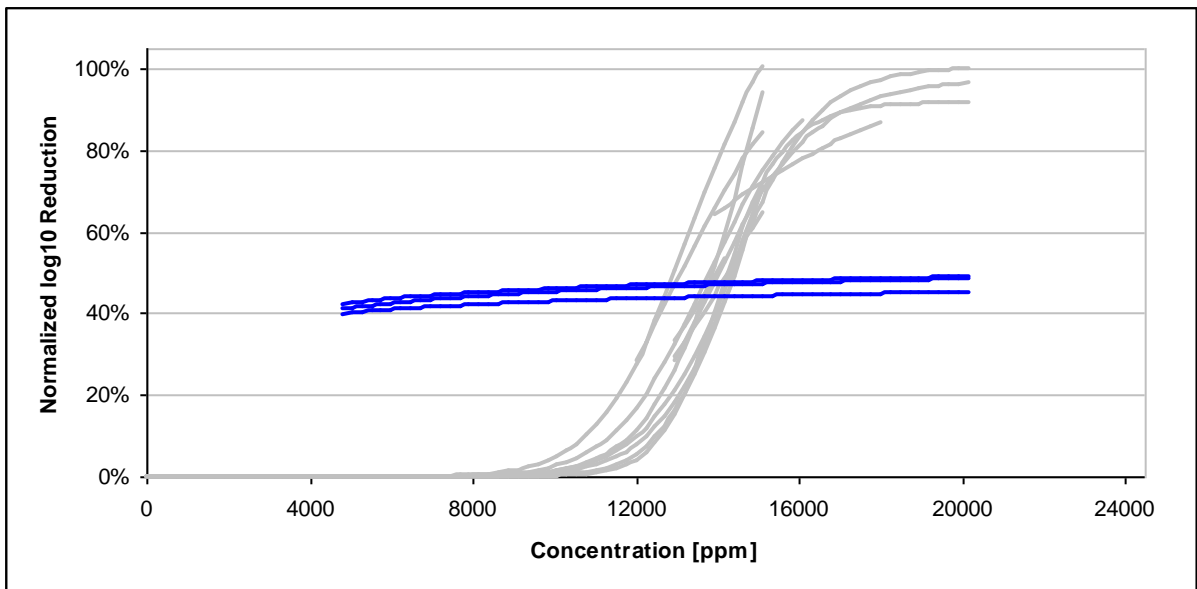


Figure 10: Run-specific inactivation curves for *Pseudomonas aeruginosa* and glutaraldehyde (red: LC0026)

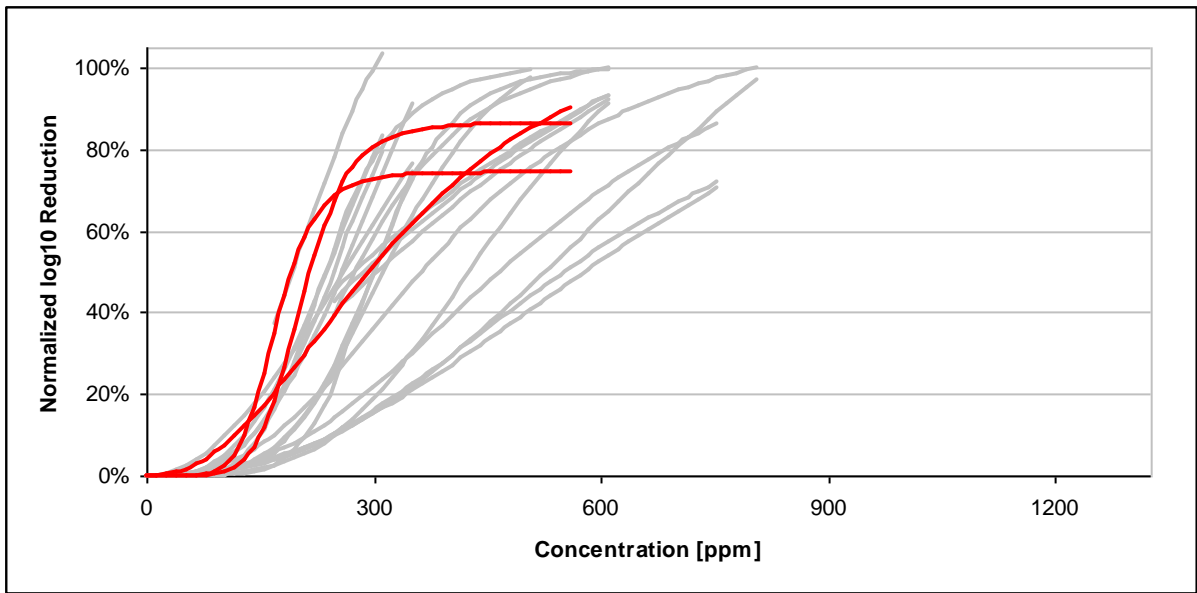


Figure 11: Run-specific inactivation curves for *Pseudomonas aeruginosa* and peracetic acid (green: LC0004)

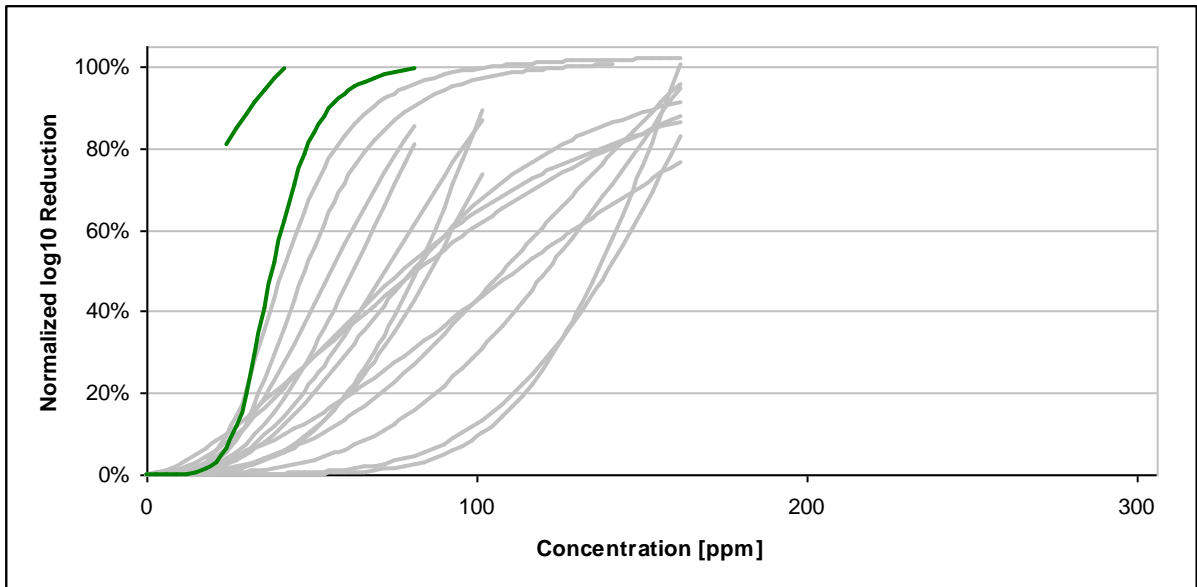


Figure 12: Run-specific inactivation curves *Staphylococcus aureus* and benzalkonium chloride (red: LC0012)

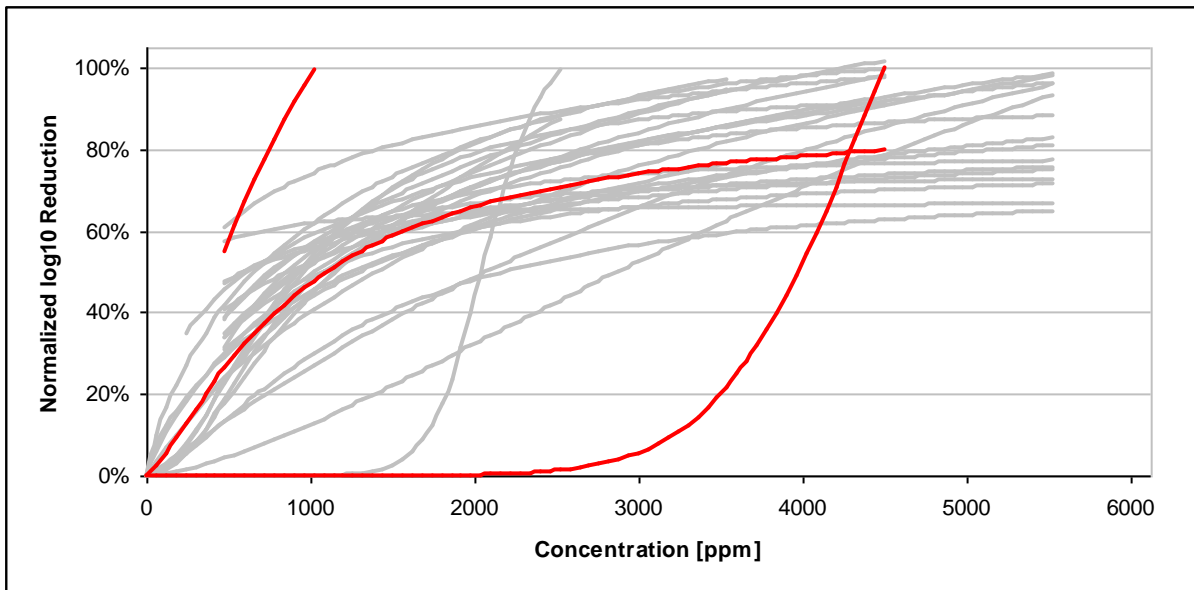
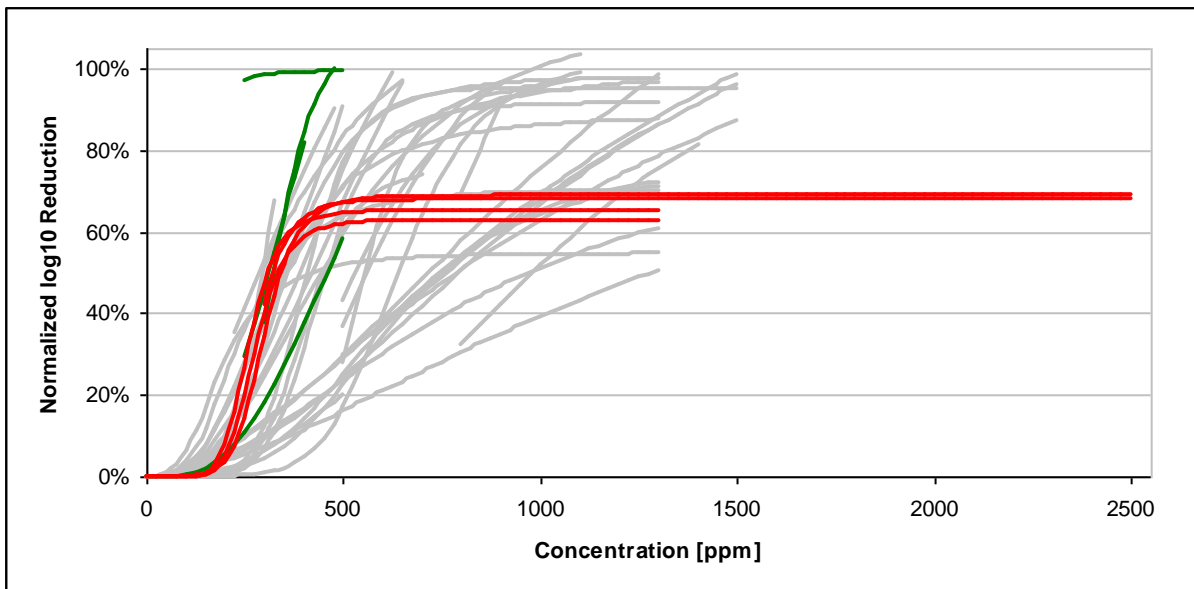
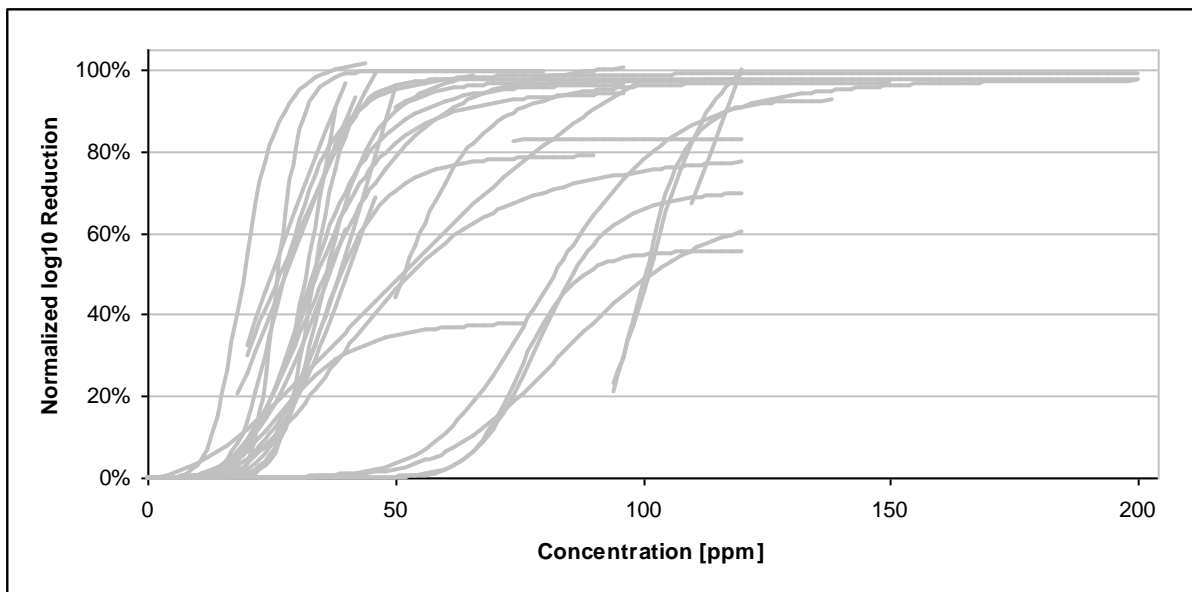


Figure 13: Run-specific inactivation curves *Staphylococcus aureus* and glutaraldehyde (green: LC0010, red: LC0020)



**Figure 14: Run-specific inactivation curves *Staphylococcus aureus* and peracetic acid (no outliers)**

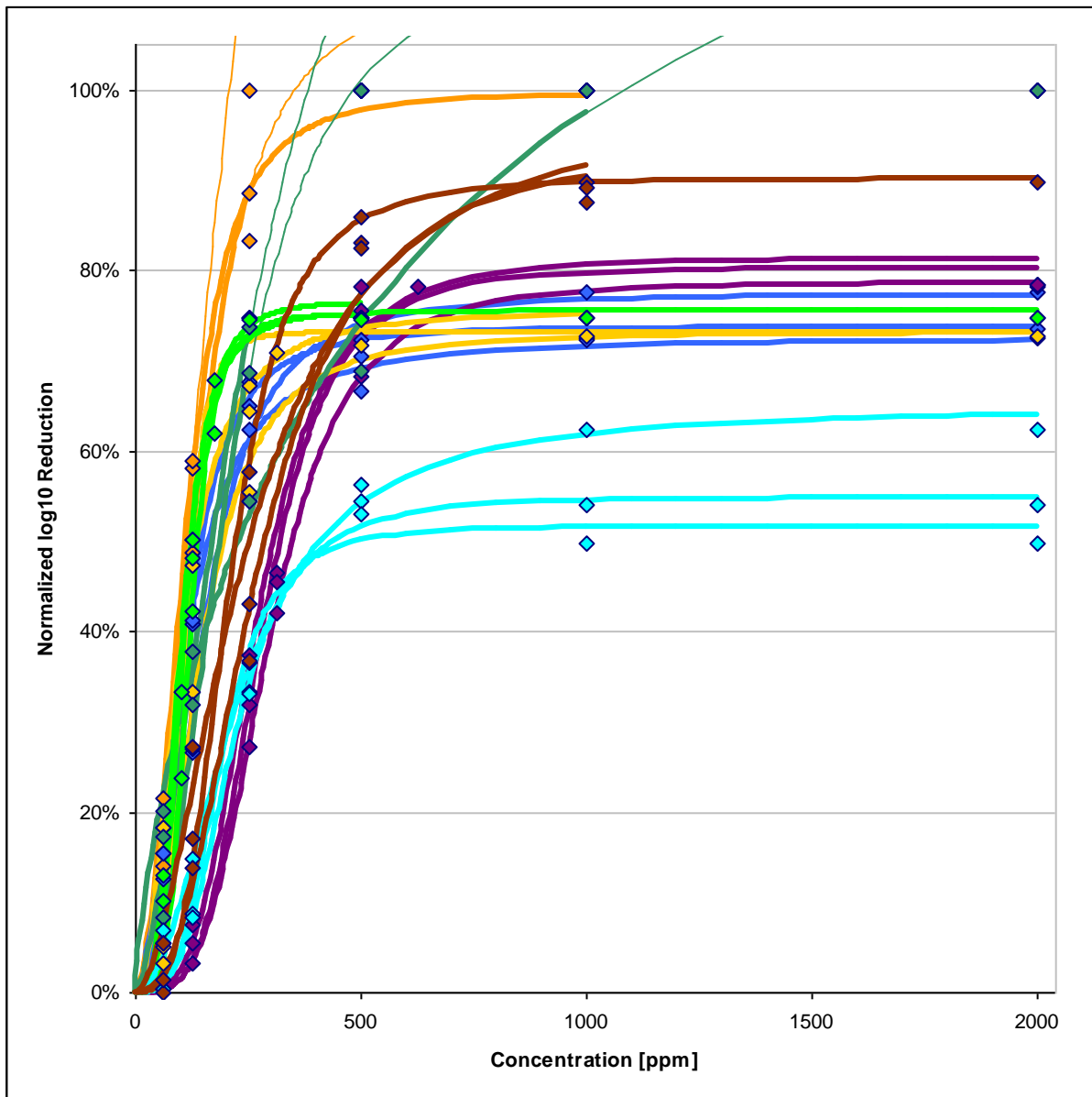


#### **Final run-specific inactivation curves**

126. In the following, for each combination of organism and substance, a figure with all final run-specific inactivation curves is shown. In this figure, the mean log<sub>10</sub> reductions at each concentration level are displayed as well. Not regarded runs as well as outlier runs are not shown in the figures.
127. Extrapolated run-specific inactivation curves are shown as thin continuations of the non-extrapolated curve. This applies to cases where the mean log<sub>10</sub> 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.
128. In the Annex *Normalized run-specific inactivation curves of phase 1*, p. 176, the run-specific inactivation curves for each laboratory are shown in separate figures.

**Figure 15: Single normalized log10 reductions and run-specific normalized inactivation curves for *Adenovirus type 5* (TCID<sub>50</sub>) and glutaraldehyde**

LC0002	LC0009	LC0016	LC0017	LC0022	LC0029	LC0031	LC0033



**Figure 16: Single normalized log<sub>10</sub> reductions and run-specific normalized inactivation curves for *Adenovirus type 5* (TCID<sub>50</sub>) and peracetic acid**

LC0001	LC0002	LC0006	LC0009	LC0016	LC0017	LC0022	LC0029	LC0031	LC0033

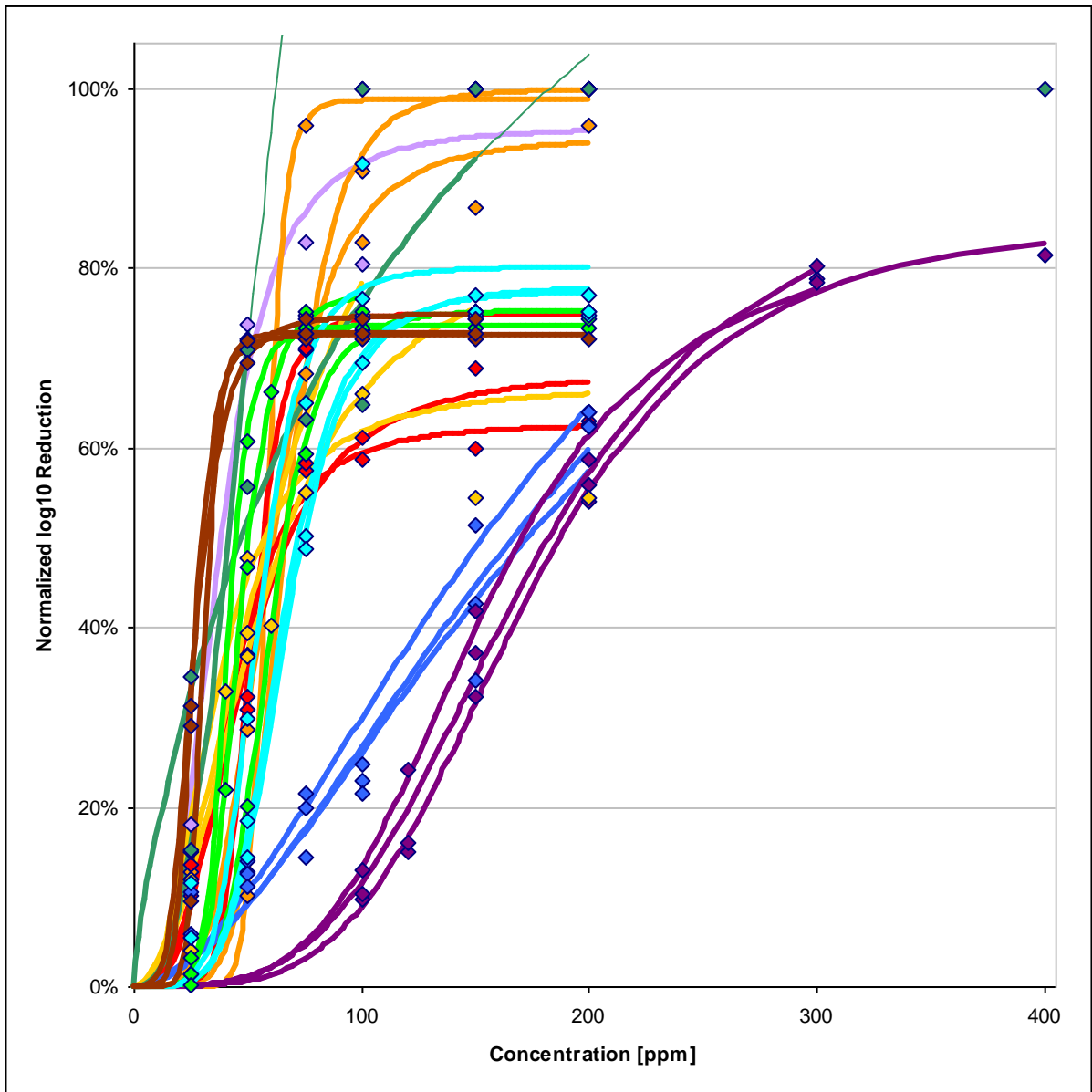
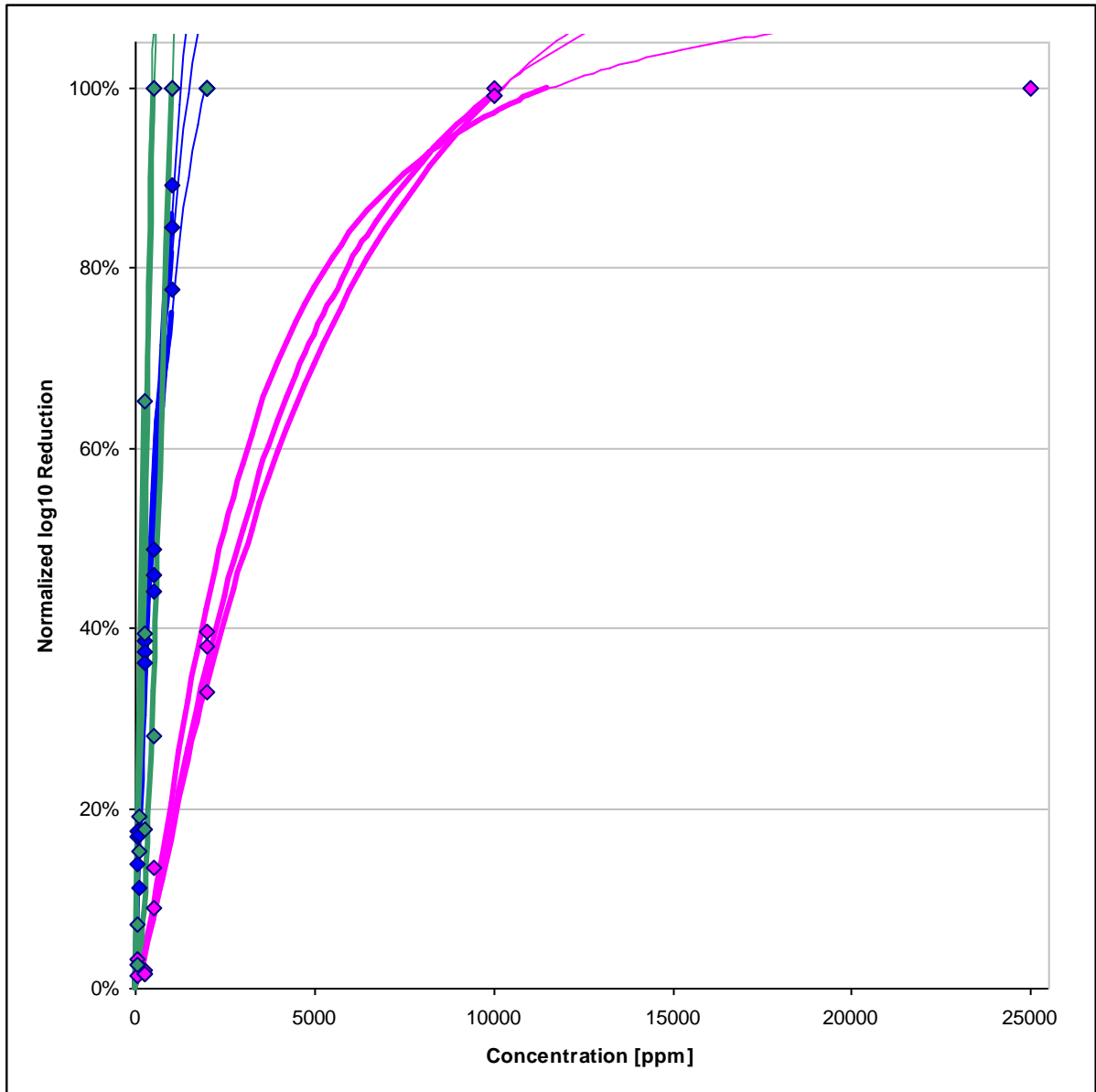


Figure 17: Single normalized log<sub>10</sub> reductions and run-specific normalized inactivation curves for *Adenovirus type 5* (well count) and glutaraldehyde

LC0014	LC0026	LC0032
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**Figure 18: Single normalized log10 reductions and run-specific normalized inactivation curves for *Adenovirus type 5* (well count) and peracetic acid**

LC0014	LC0026	LC0032
LC0014	LC0026	LC0032

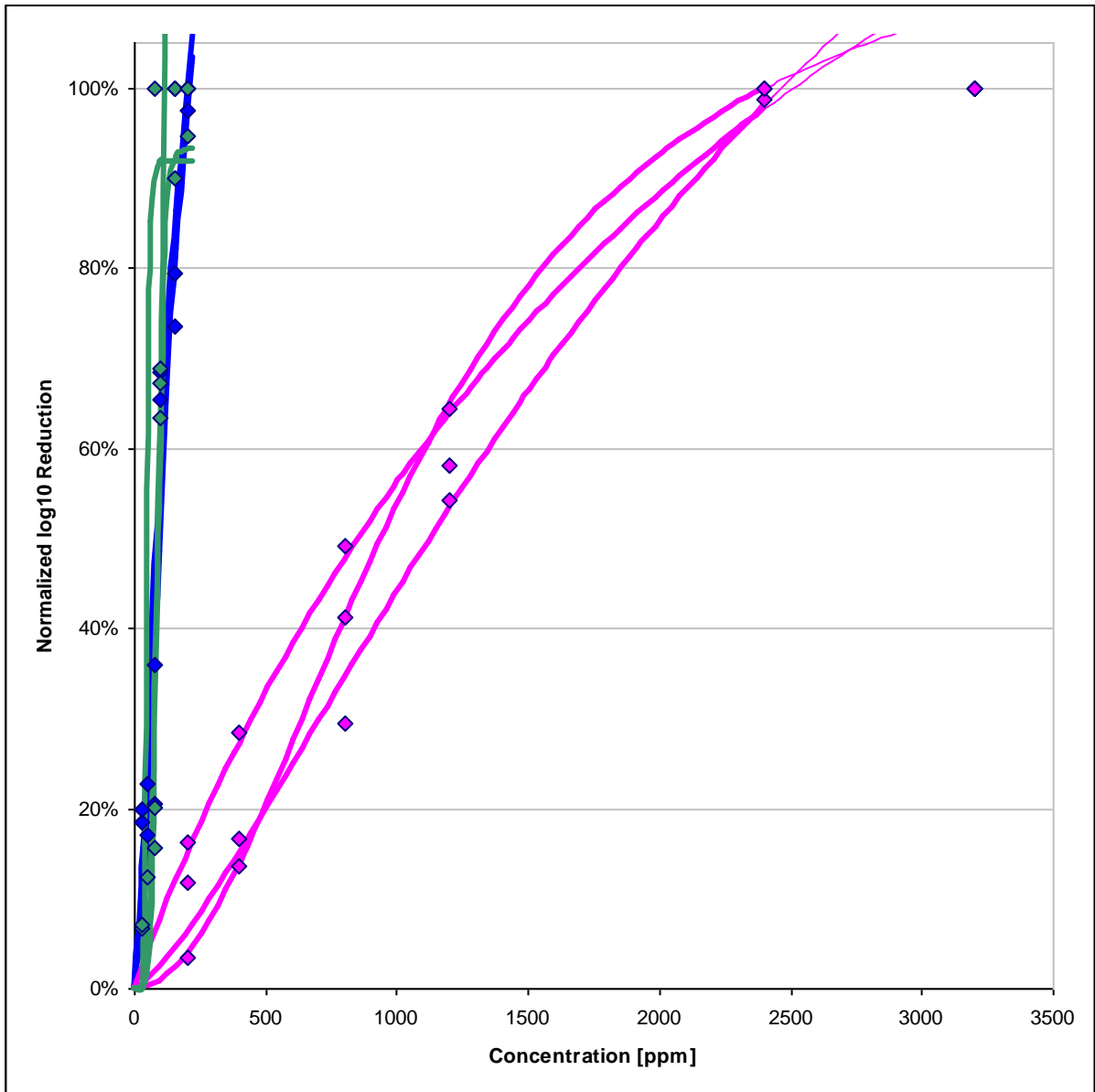
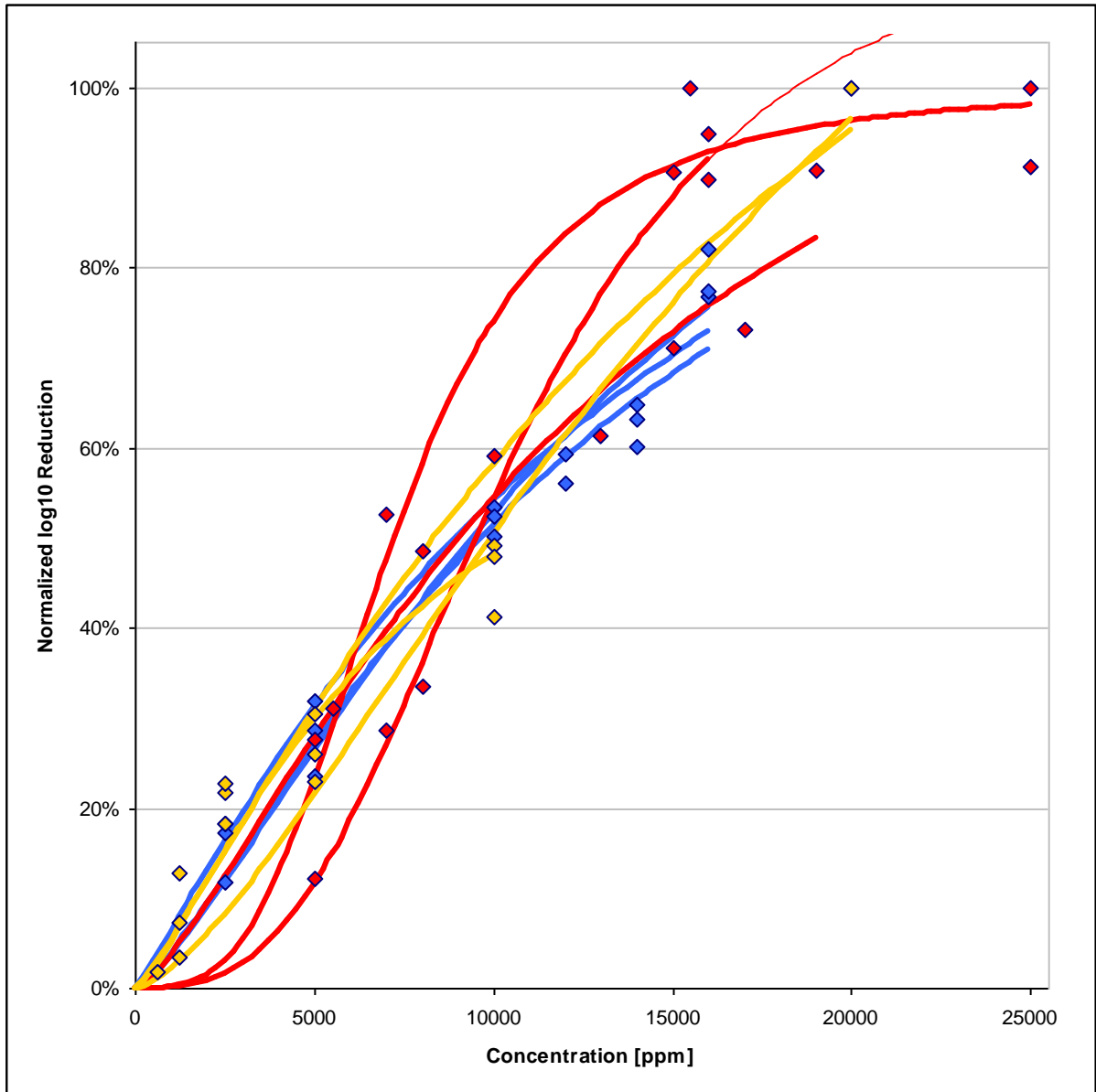


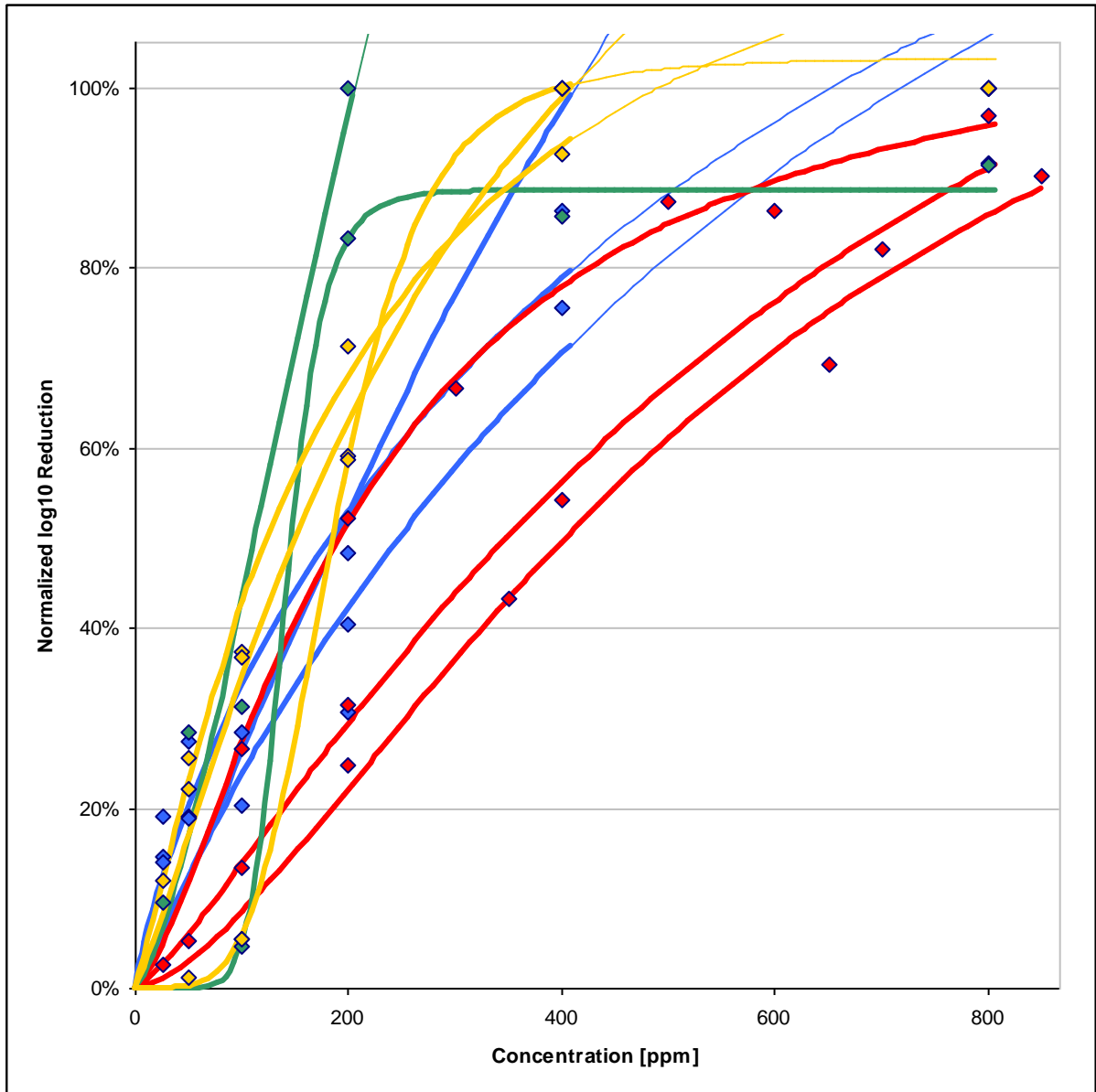
Figure 19: Single normalized log<sub>10</sub> reductions and run-specific normalized inactivation curves for *Aspergillus niger* and glutaraldehyde

LC0011	LC0021	LC0023



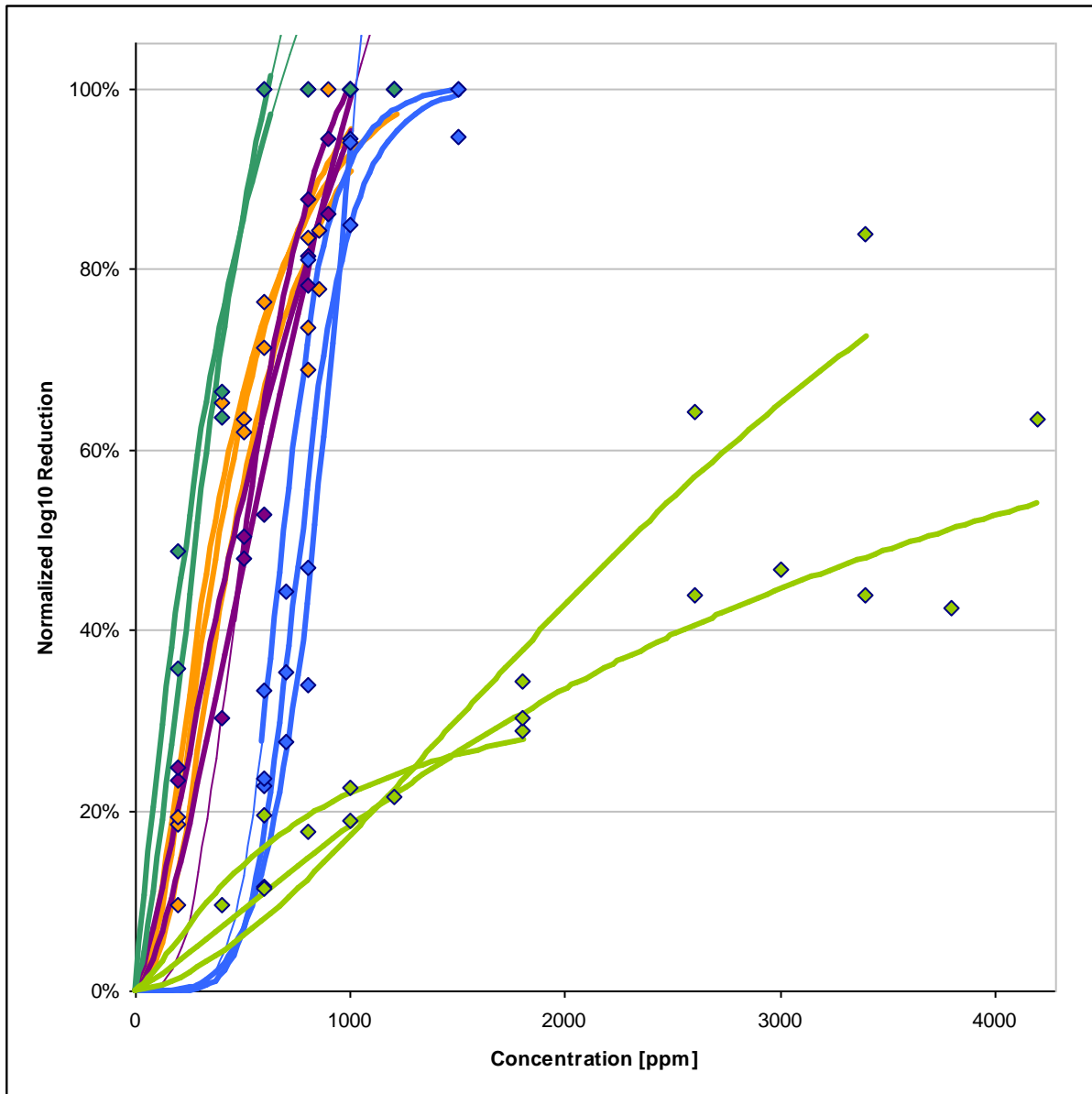
**Figure 20: Single normalized log<sub>10</sub> reductions and run-specific normalized inactivation curves for *Aspergillus niger* and peracetic acid**

LC0011	LC0021	LC0022	LC0023
LC0011	LC0021	LC0022	LC0023



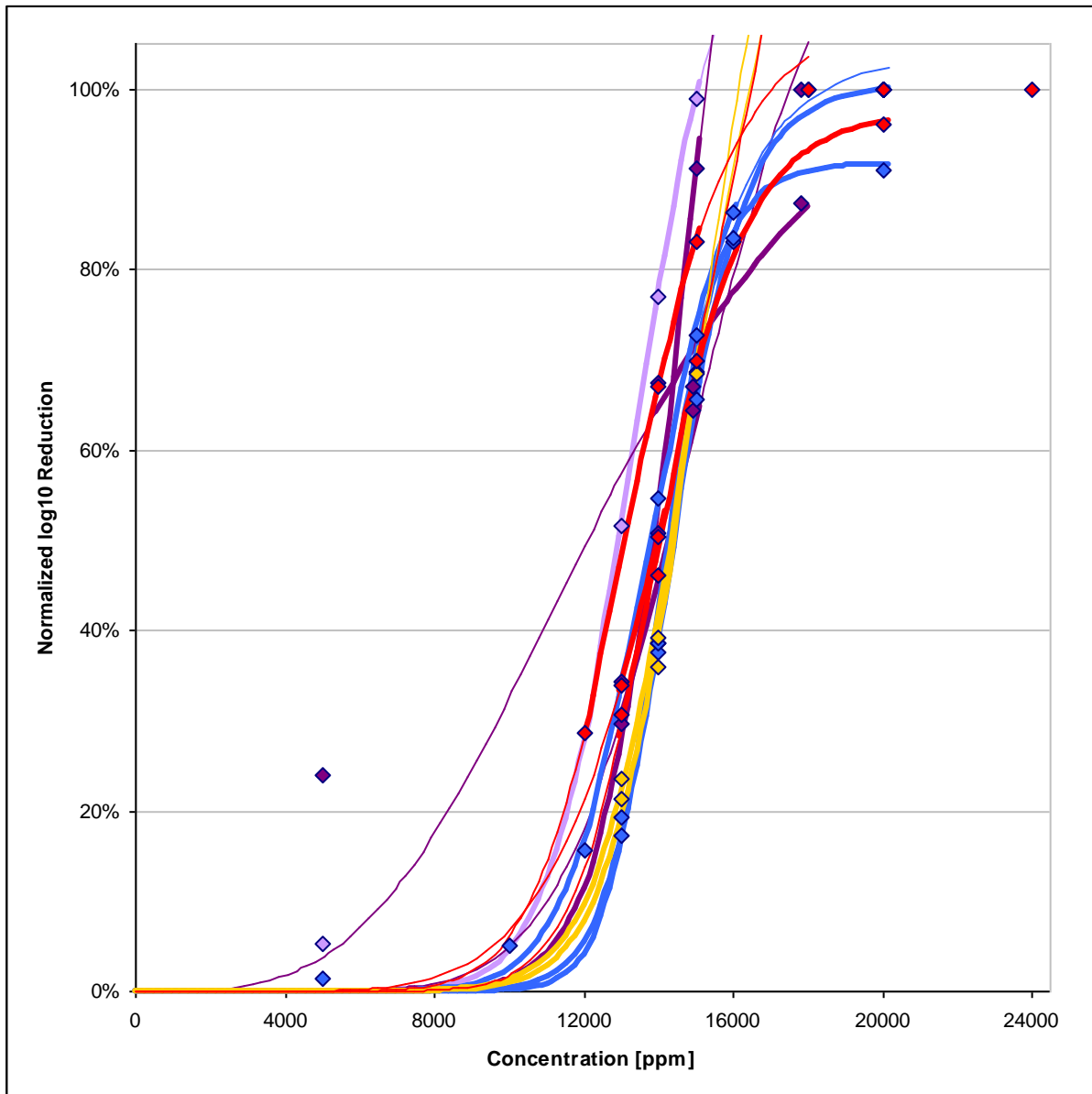
**Figure 21: Single normalized log10 reductions and run-specific normalized inactivation curves for *Bacillus subtilis* and peracetic acid**

LC0002	LC0008	LC0011	LC0022	LC0025



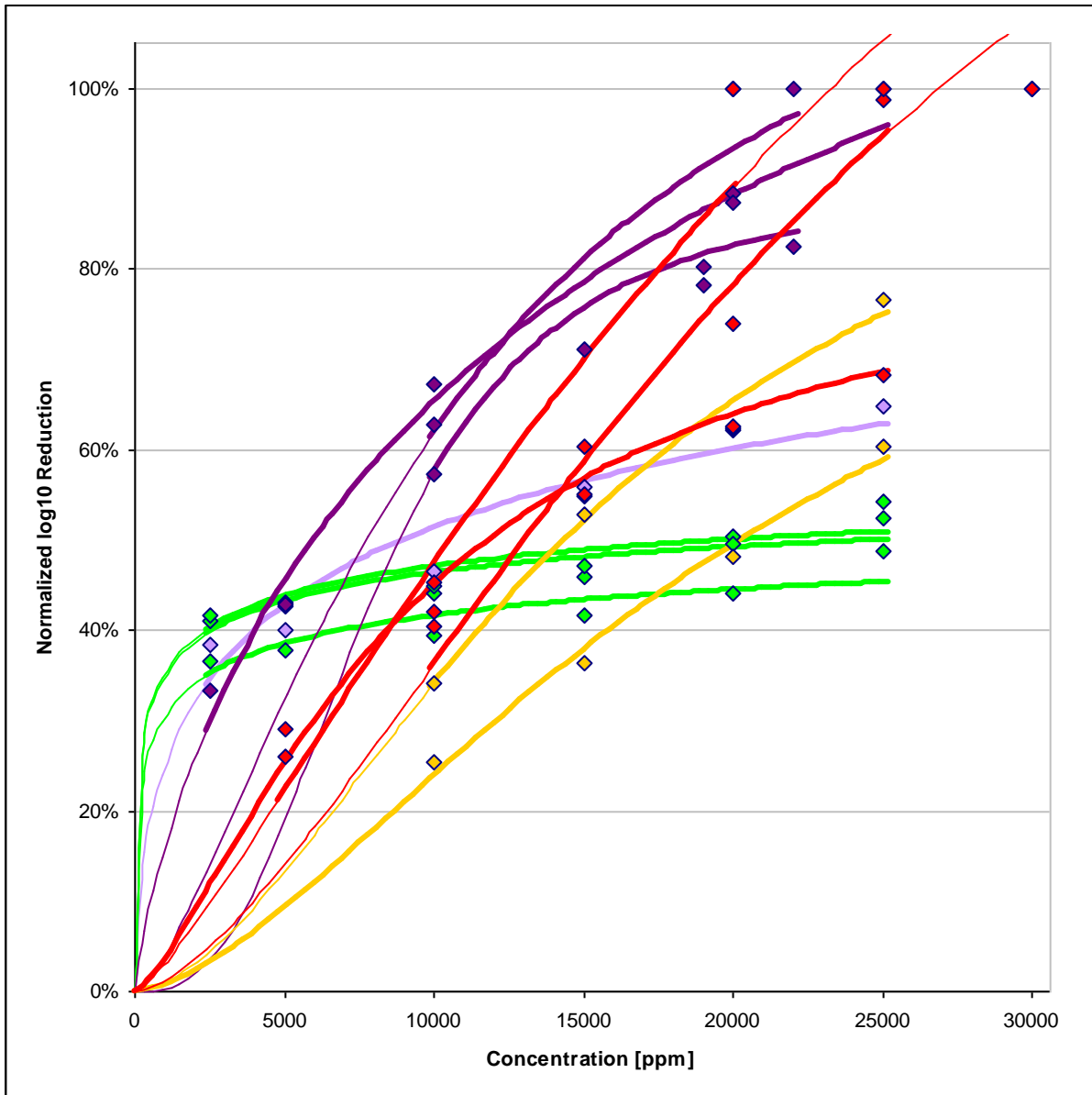
**Figure 22: Single normalized log10 reductions and run-specific normalized inactivation curves for *Mycobacterium terrae* and phenol**

LC0001	LC0008	LC0011	LC0012	LC0021



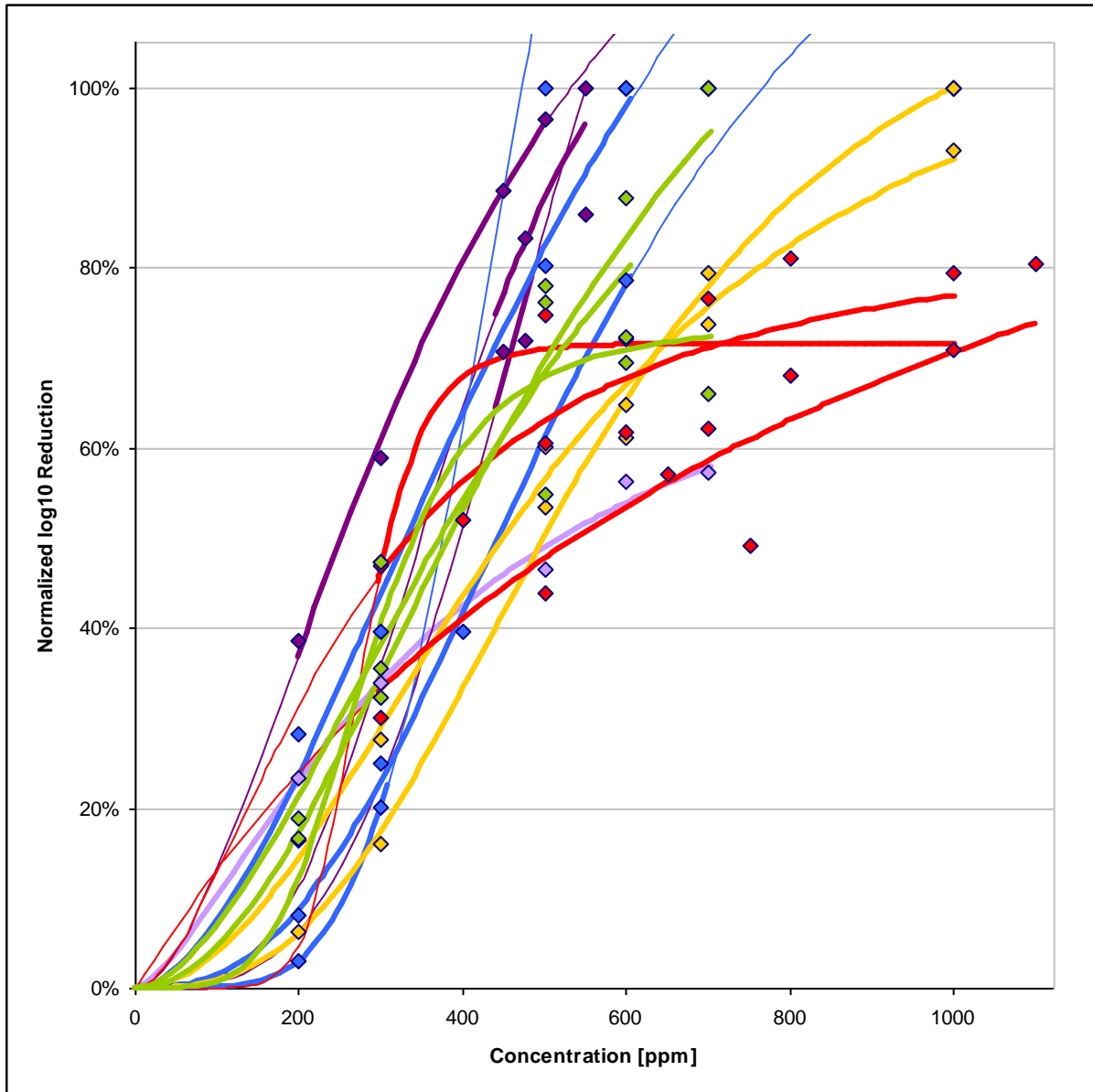
**Figure 23: Single normalized log10 reductions and run-specific normalized inactivation curves for *Mycobacterium terrae* and glutaraldehyde**

LC0001	LC0007	LC0008	LC0012	LC0021



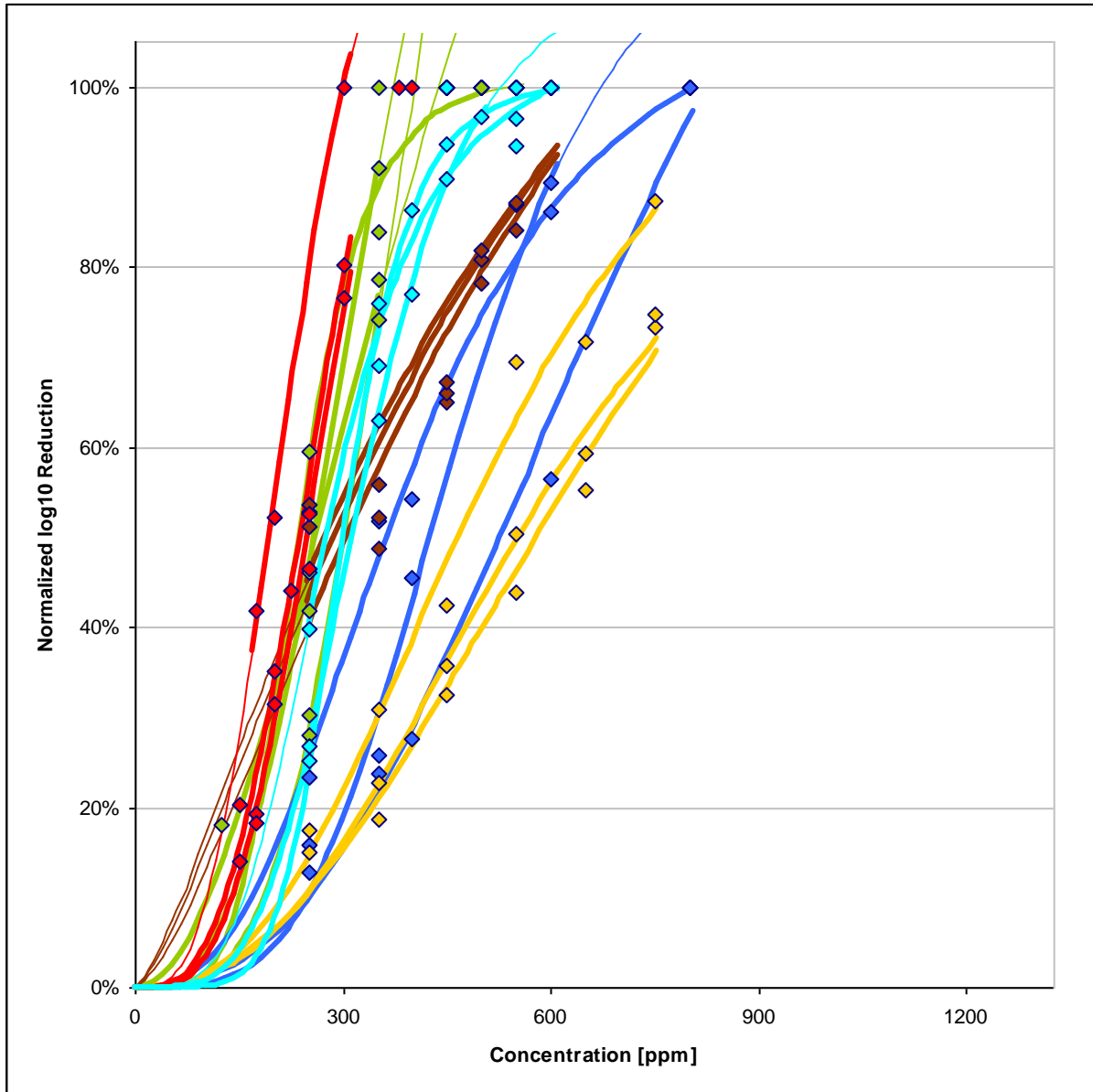
**Figure 24: Single normalized log<sub>10</sub> reductions and run-specific normalized inactivation curves for *Mycobacterium terrae* and peracetic acid**

LC0001	LC0008	LC0011	LC0012	LC0021	LC0025



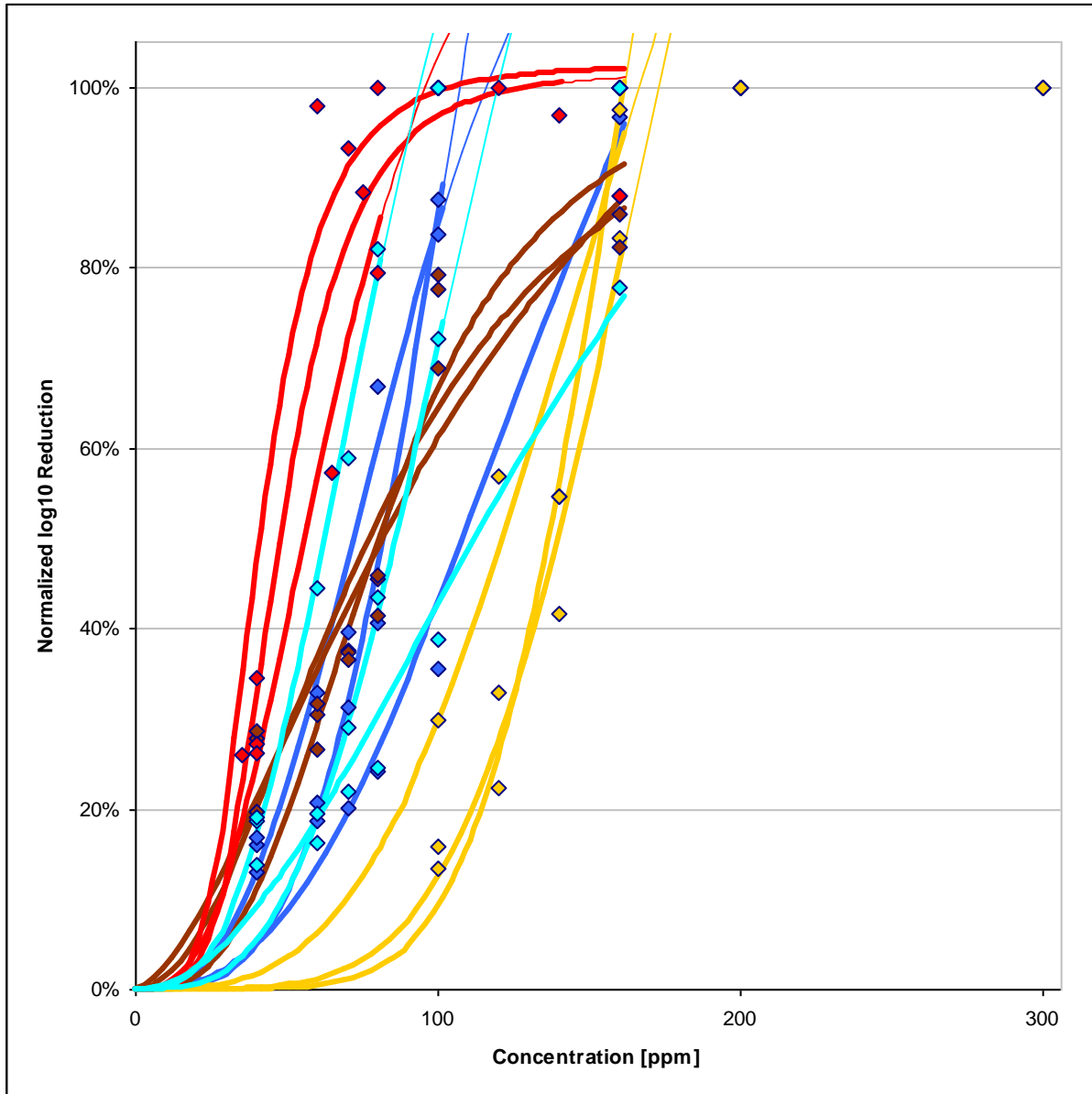
**Figure 25: Single normalized log<sub>10</sub> reductions and run-specific normalized inactivation curves for *Pseudomonas aeruginosa* and glutaraldehyde**

LC0004	LC0011	LC0012	LC0013	LC0021	LC0028



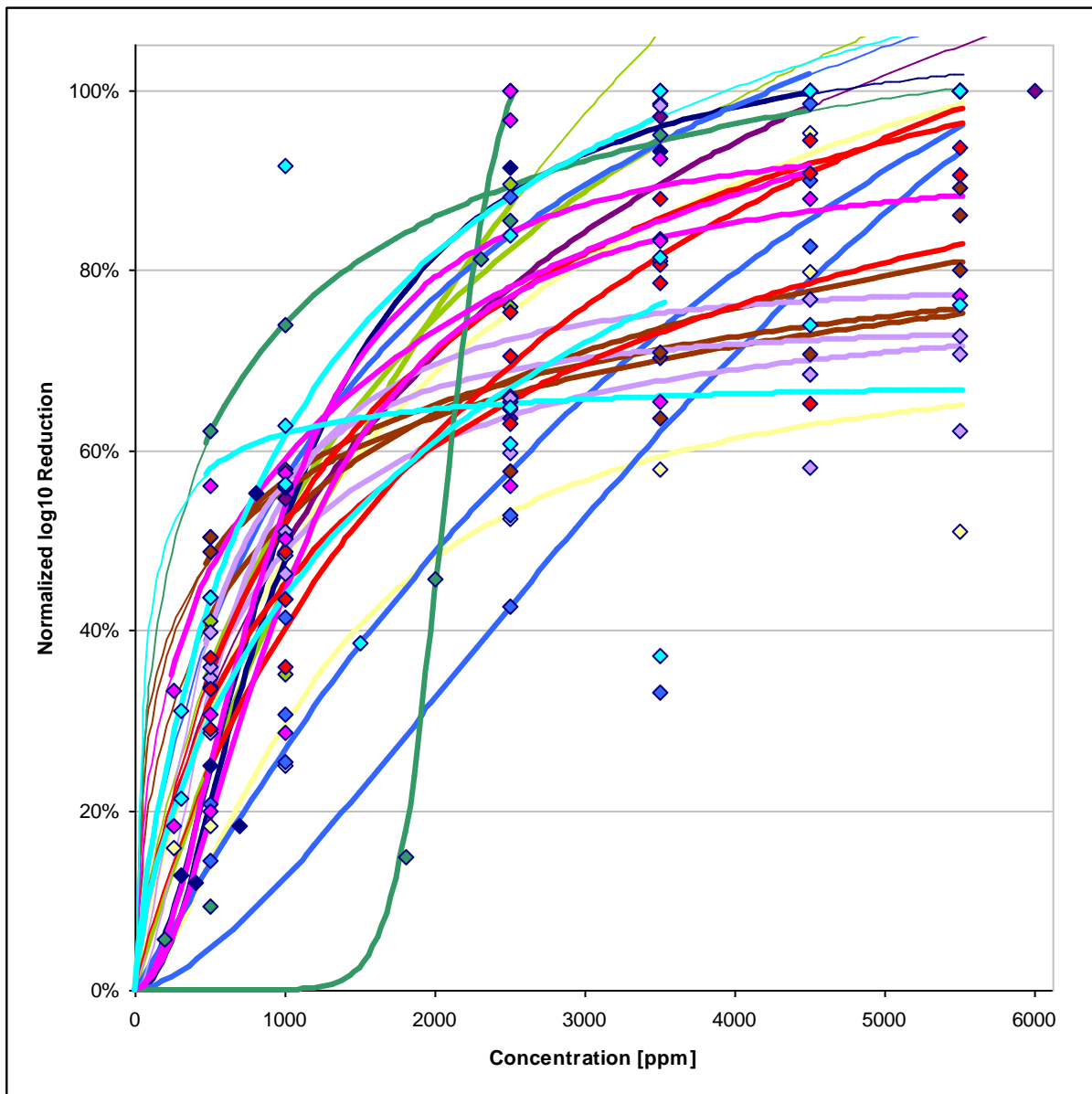
**Figure 26: Single normalized log<sub>10</sub> reductions and run-specific normalized inactivation curves for *Pseudomonas aeruginosa* and peracetic acid**

LC0011	LC0012	LC0013	LC0021	LC0028
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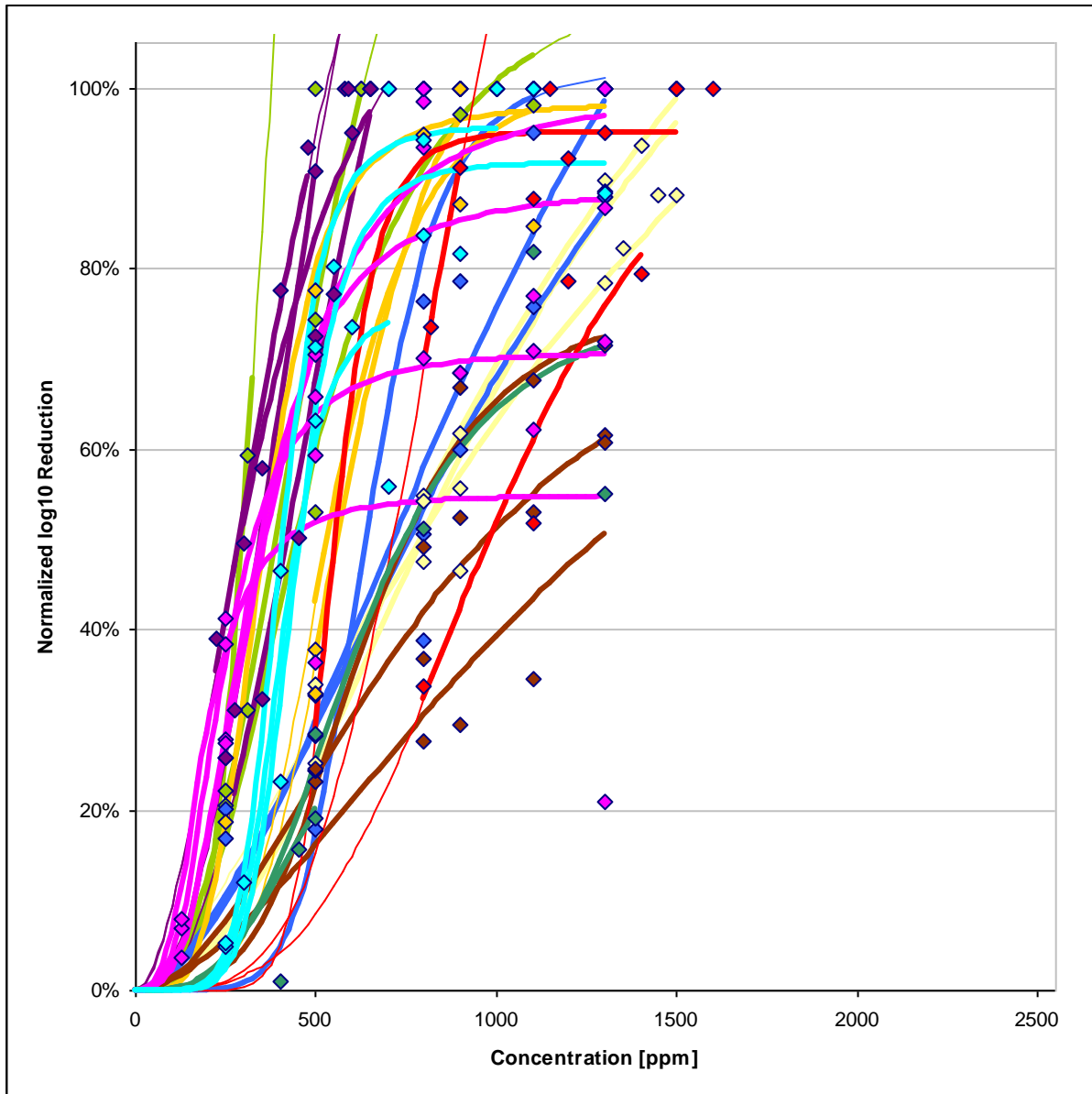
**Figure 27: Single normalized log<sub>10</sub> reductions and run-specific normalized inactivation curves for *Staphylococcus aureus* and benzalkonium chloride**

LC0000	LC0004	LC0008	LC0010	LC0011	LC0013	LC0020	LC0021	LC0022	LC0026	LC0028



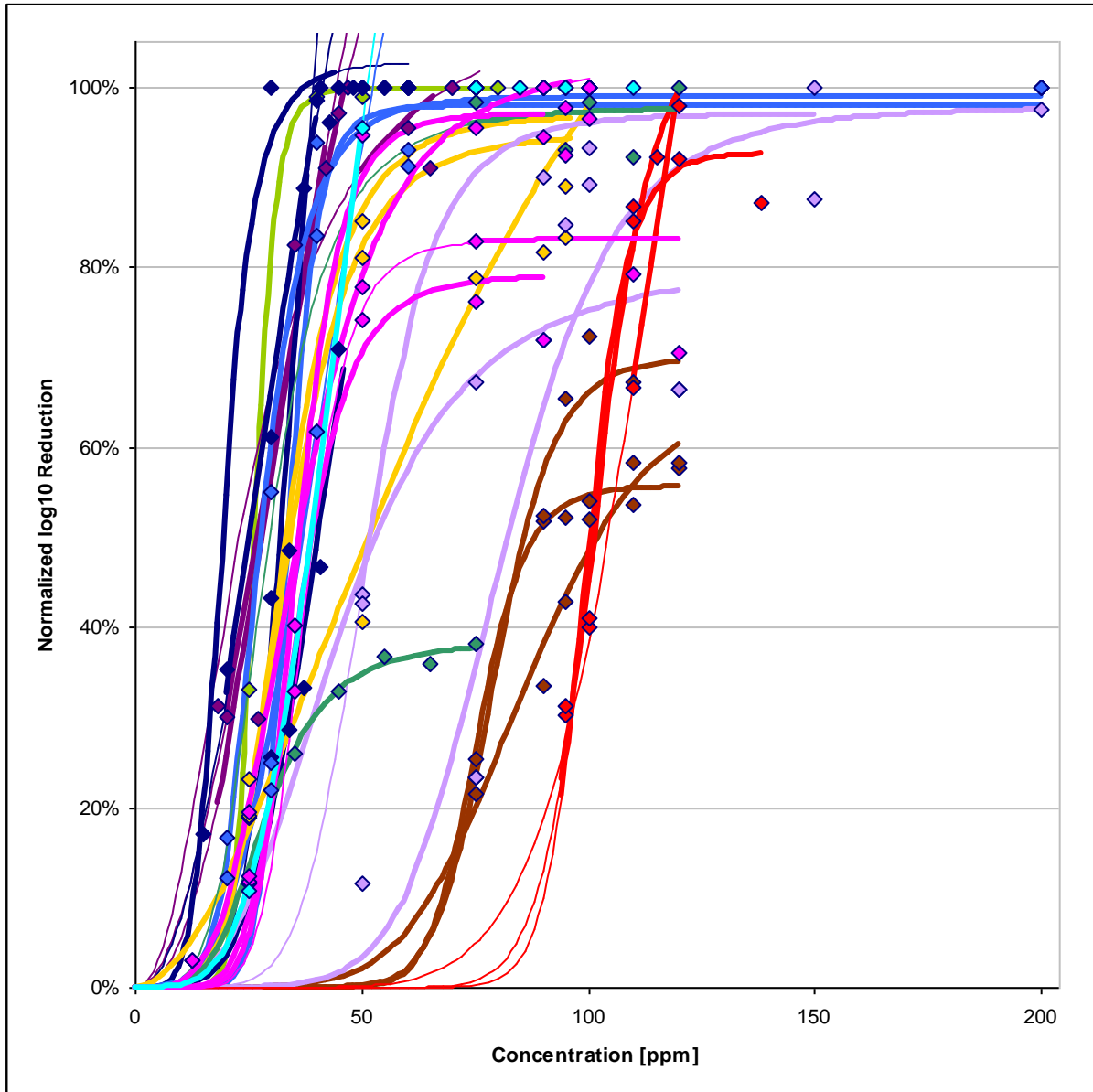
**Figure 28: Single normalized log<sub>10</sub> reductions and run-specific normalized inactivation curves for *Staphylococcus aureus* and glutaraldehyde**

LC0000	LC0004	LC0008	LC0011	LC0012	LC0013	LC0021	LC0022	LC0026	LC0028



**Figure 29: Single normalized log<sub>10</sub> reductions and run-specific normalized inactivation curves for *Staphylococcus aureus* and peracetic acid**

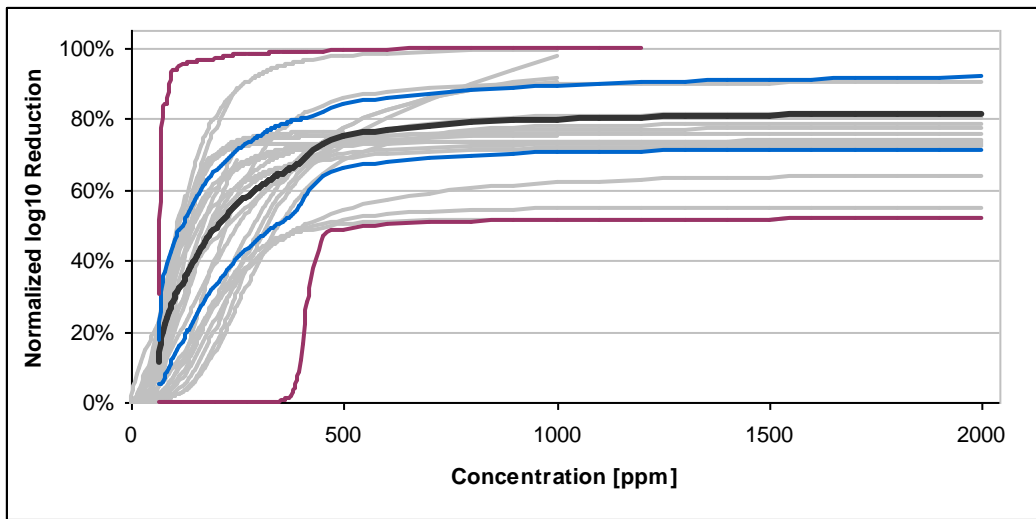
LC0004	LC0008	LC0010	LC0011	LC0012	LC0013	LC0020	LC0021	LC0022	LC0026	LC0028



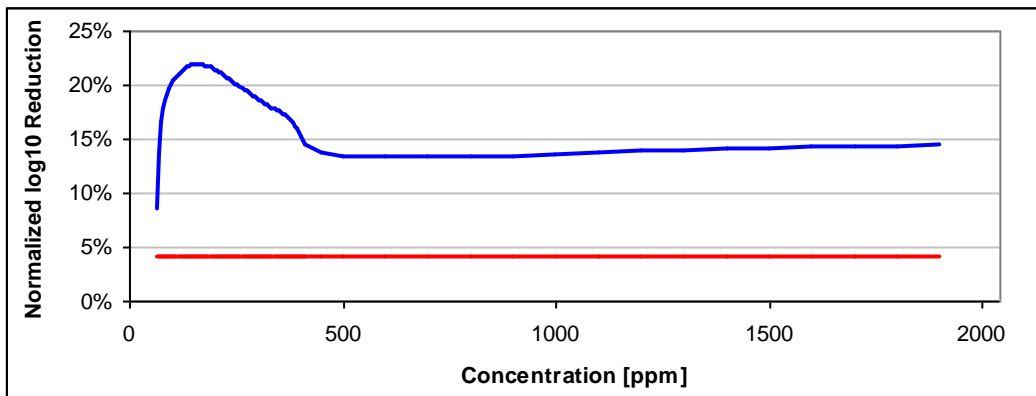
### **Overall (laboratory-comprehensive) inactivation curves and intra- and inter-laboratory reproducibility**

129. In the following figures, for each combination of organism and substance 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.
130. Each overall inactivation curve is followed by a figure 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 the smaller than the inter-laboratory reproducibility  $s_R$ .
131. Below each figure of the standard deviations a table is given in which 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  $s_t$  and intra-laboratory reproducibility within one run  $s_r$  are summarized for the six concentration levels as originally suggested to the laboratories. No values can be given if the respective concentration lies not within the concentration range considered for the bootstrap.
132. 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.
133. Since for *Adenovirus type 5* (well count) data of only three laboratories is available, neither the overall inactivation curve nor the intra- and inter-laboratory reproducibilities have been determined.
134. For all microorganisms and all substances the inter-laboratory reproducibility standard deviation is considerably larger than under intra-laboratory conditions. At a true normalized log<sub>10</sub> reduction of 80%, the inter-laboratory reproducibility is in the range between 8% and 31%, whereas the intra-laboratory reproducibility over time is in the range between 3% and 21%. The average over all test combinations equals 20% and 11% for inter- and intra-laboratory reproducibility, respectively.

**Figure 30: Overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Adenovirus type 5* (TCID<sub>50</sub>) and glutaraldehyde**



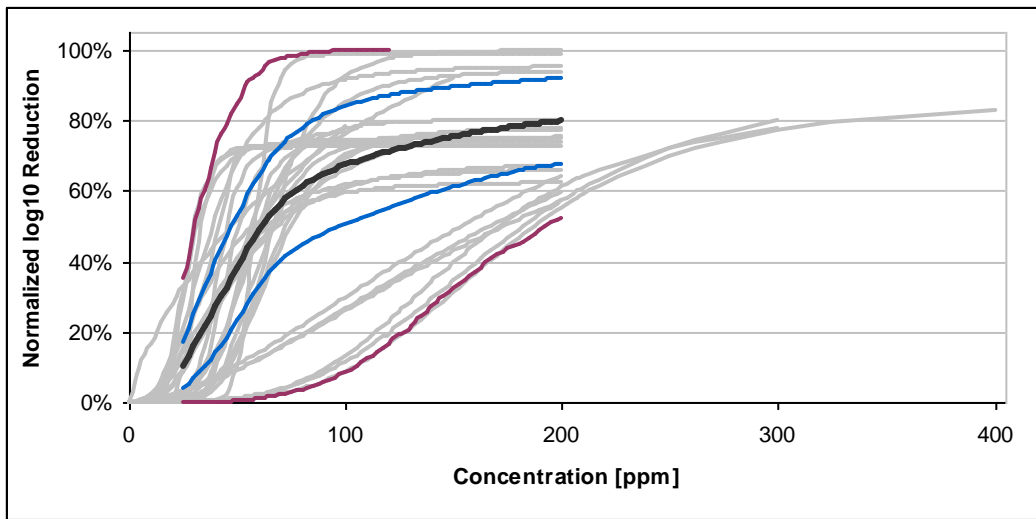
**Figure 31: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Adenovirus type 5* (TCID<sub>50</sub>) and glutaraldehyde**



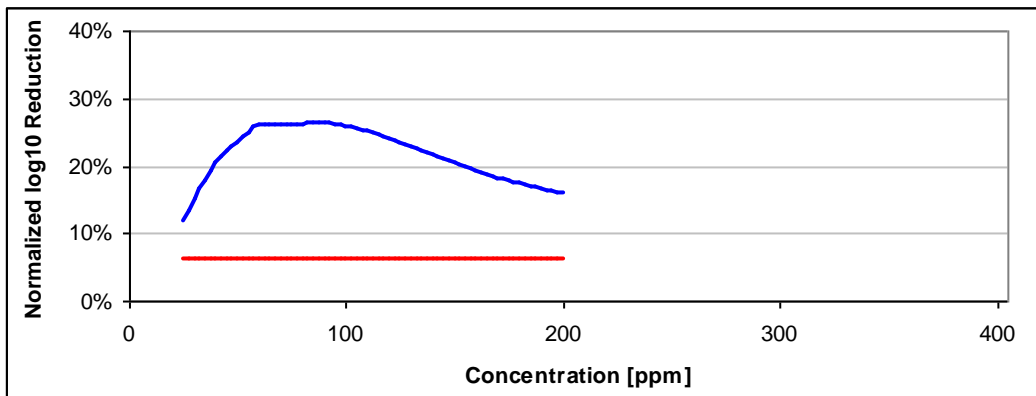
**Table 15: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Adenovirus type 5* (TCID<sub>50</sub>) and glutaraldehyde**

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 $s_t$
62.5	11%	7%	4%	4%
125	35%	21%	4%	4%
250	56%	20%	4%	4%
500	75%	13%	4%	6%
1000	80%	14%	4%	4%
2000	81%	14%	4%	4%

**Figure 32: Overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Adenovirus type 5* (TCID<sub>50</sub>) and peracetic acid**



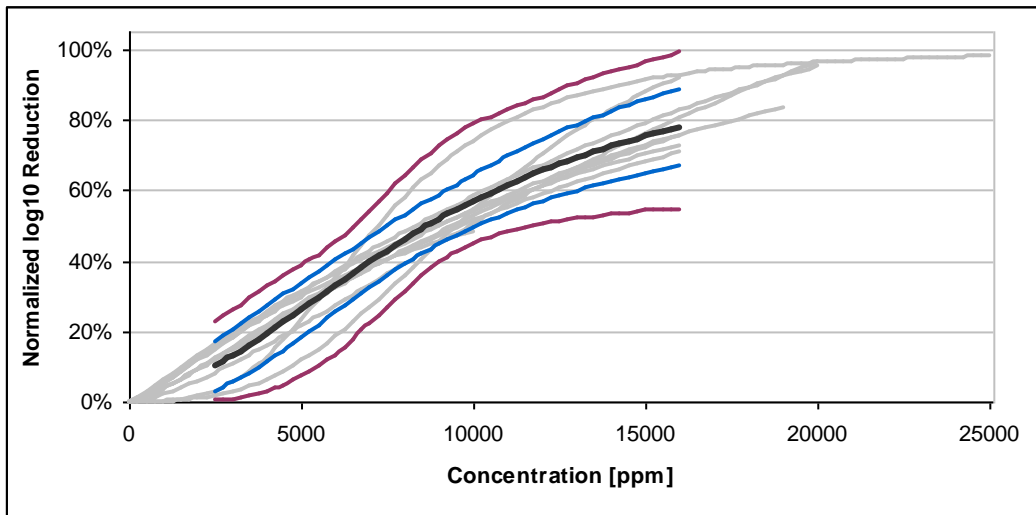
**Figure 33: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Adenovirus type 5* (TCID<sub>50</sub>) and peracetic acid**



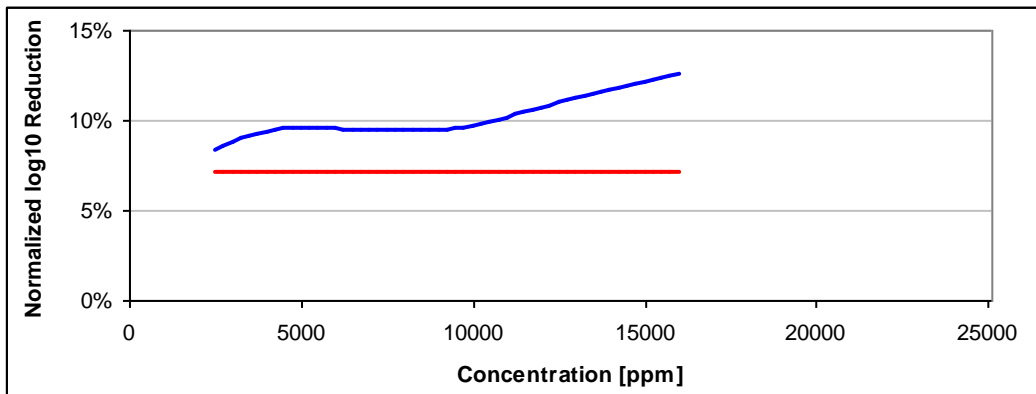
**Table 16: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Adenovirus type 5* (TCID<sub>50</sub>) and peracetic acid**

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 $s_t$
25	10%	12%	6%	7%
50	38%	24%	6%	7%
75	59%	26%	6%	7%
100	67%	26%	6%	6%
150	75%	20%	6%	6%
200	80%	16%	6%	6%

**Figure 34: 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**



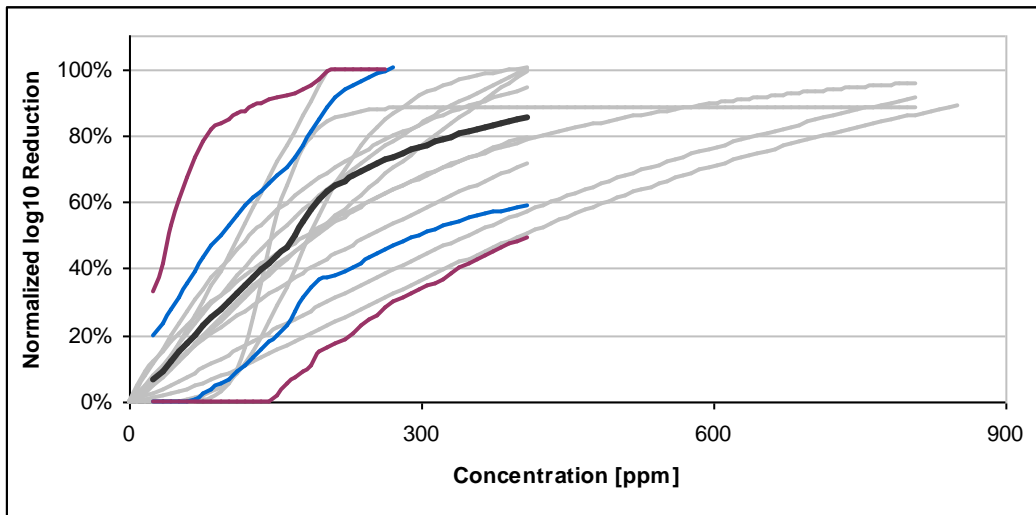
**Figure 35: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Aspergillus niger* and glutaraldehyde**



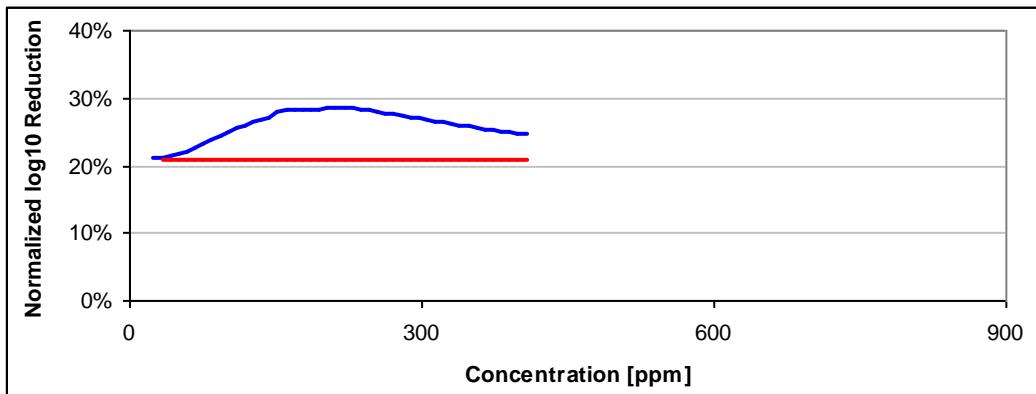
**Table 17: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Aspergillus niger* and glutaraldehyde**

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 $s_t$
312				
625				
1250				
2500	10%	8%	7%	8%
5000	26%	10%	7%	9%
10000	57%	10%	7%	9%

**Figure 36: 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 peracetic acid**



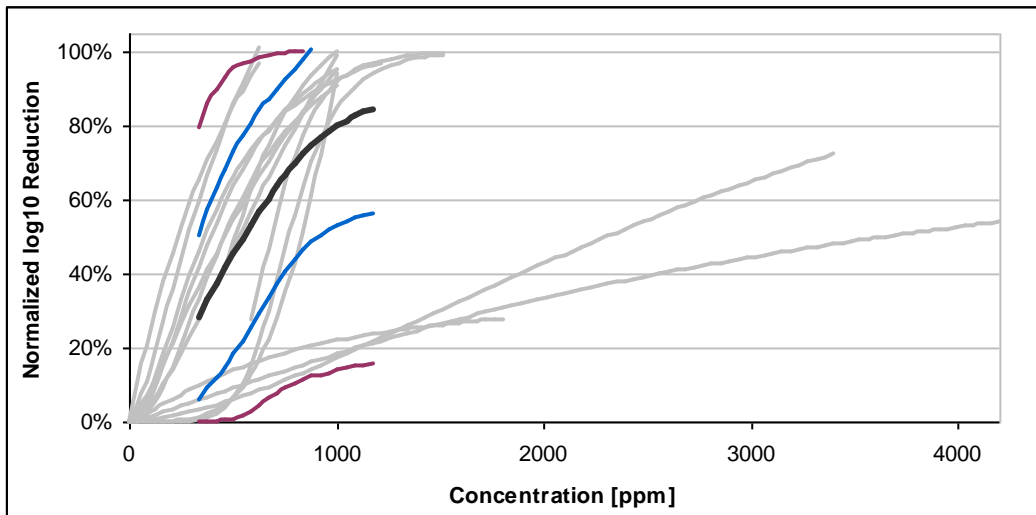
**Figure 37: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Aspergillus niger* and peracetic acid**



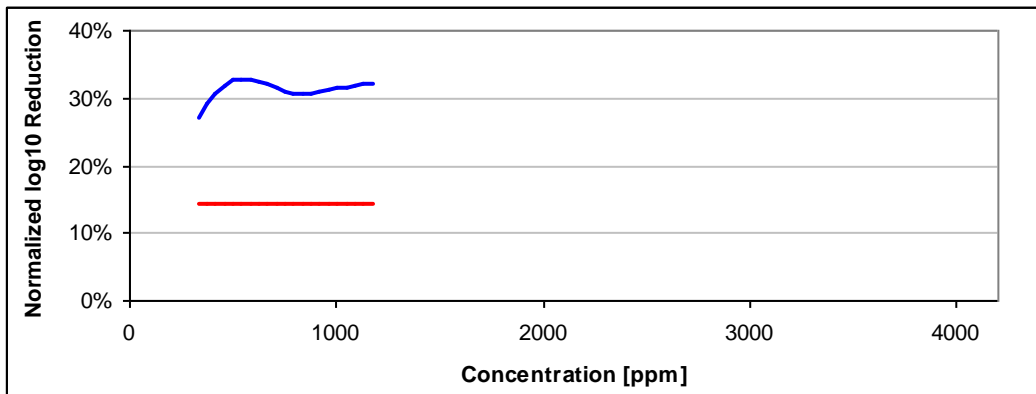
**Table 18: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Aspergillus niger* and peracetic acid**

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 $s_t$
25	7%	21%	21%	21%
50	15%	22%	21%	21%
100	30%	25%	21%	21%
200	63%	28%	21%	21%
400	85%	25%	21%	21%
800				

**Figure 38: Overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Bacillus subtilis* and peracetic acid**



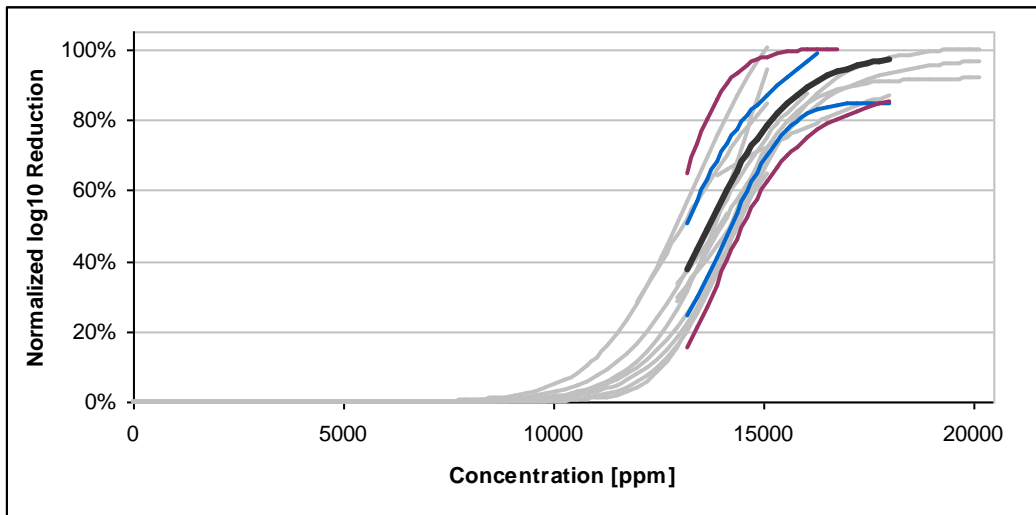
**Figure 39: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Bacillus subtilis* and peracetic acid**



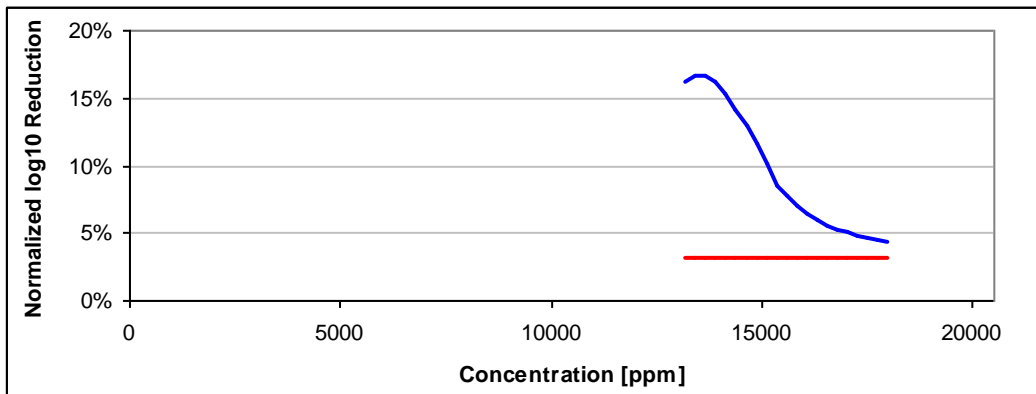
**Table 19: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Bacillus subtilis* and peracetic acid**

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 $s_t$
200				
400	33%	29%	14%	15%
600	53%	33%	14%	14%
800	70%	31%	14%	14%
1000	80%	31%	14%	14%
1200	85%	32%	14%	14%

**Figure 40: Overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Mycobacterium terrae* and phenol**



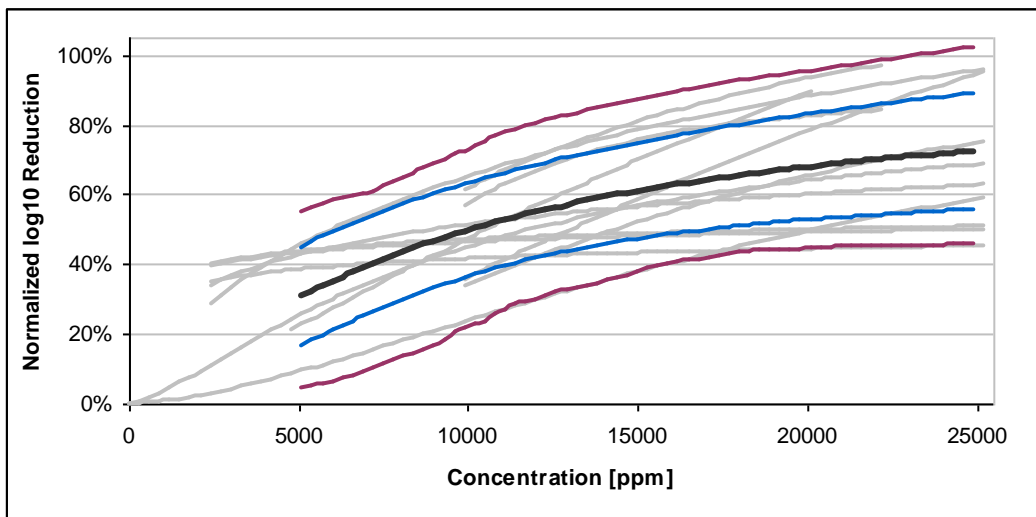
**Figure 41: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Mycobacterium terrae* and phenol**



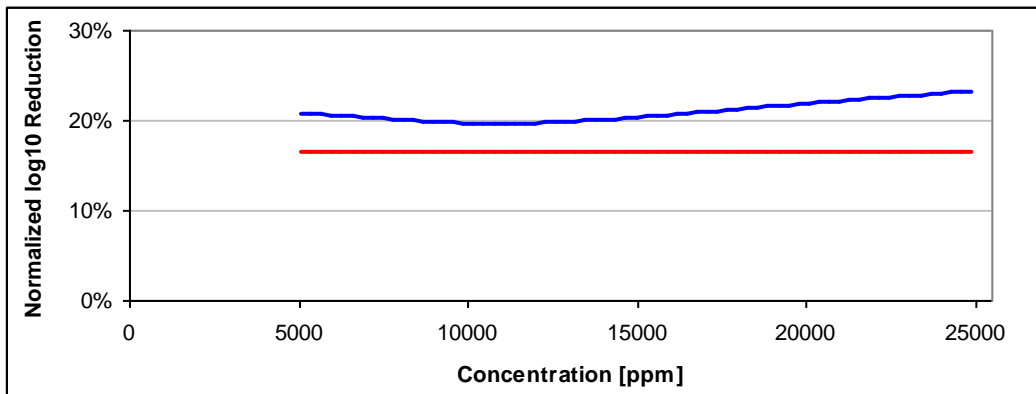
**Table 20: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Mycobacterium terrae* and phenol**

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 $s_t$
5000				
10000				
13000	37%	16%	3%	8%
14000	55%	16%	3%	7%
15000	78%	10%	3%	5%
20000				

**Figure 42: Overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Mycobacterium terrae* and glutaraldehyde**



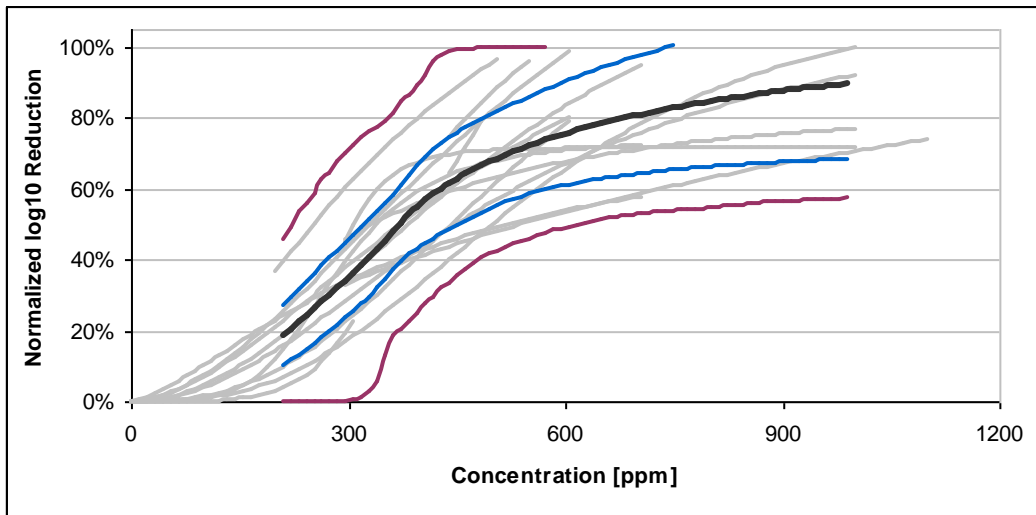
**Figure 43: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Mycobacterium terrae* and glutaraldehyde**



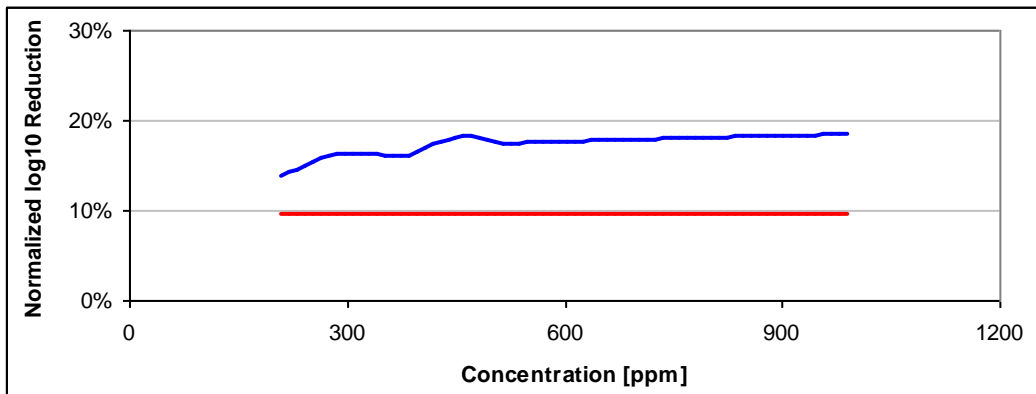
**Table 21: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Mycobacterium terrae* and glutaraldehyde**

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 $s_t$
2500				
5000	31%	21%	16%	17%
10000	50%	20%	16%	16%
15000	61%	20%	16%	17%
20000	68%	22%	16%	17%
25000	72%	23%	16%	18%

**Figure 44: Overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Mycobacterium terrae* and peracetic acid**



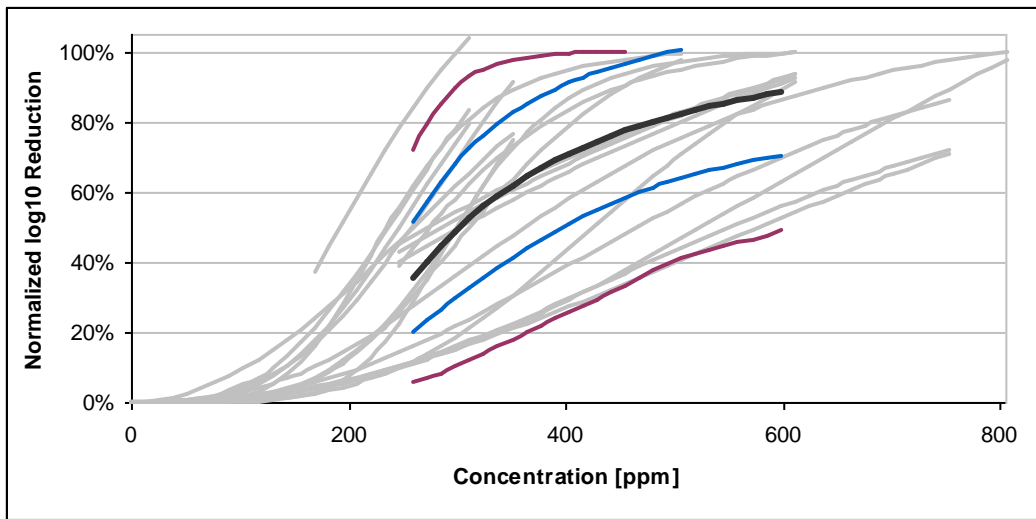
**Figure 45: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Mycobacterium terrae* and peracetic acid**



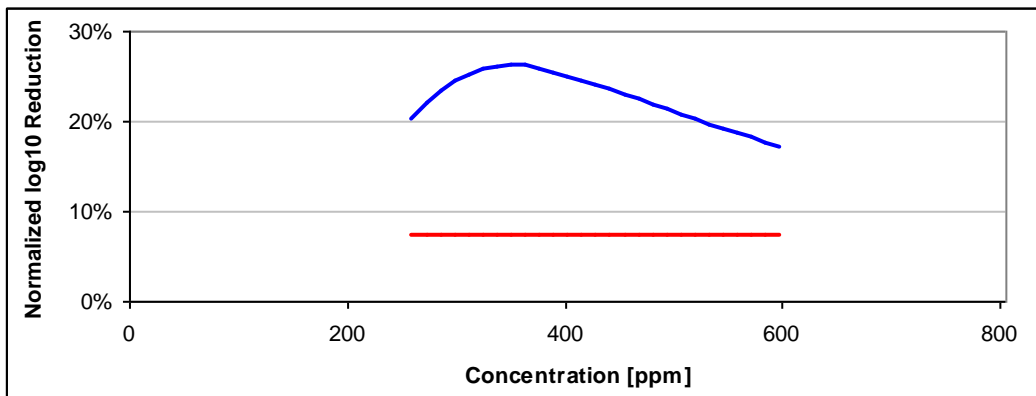
**Table 22: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Mycobacterium terrae* and peracetic acid**

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 $s_t$
200	19%	14%	10%	13%
300	34%	16%	10%	14%
500	68%	18%	10%	11%
600	76%	18%	10%	10%
700	81%	18%	10%	10%
1000	90%	18%	10%	10%

**Figure 46: Overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Pseudomonas aeruginosa* and glutaraldehyde**



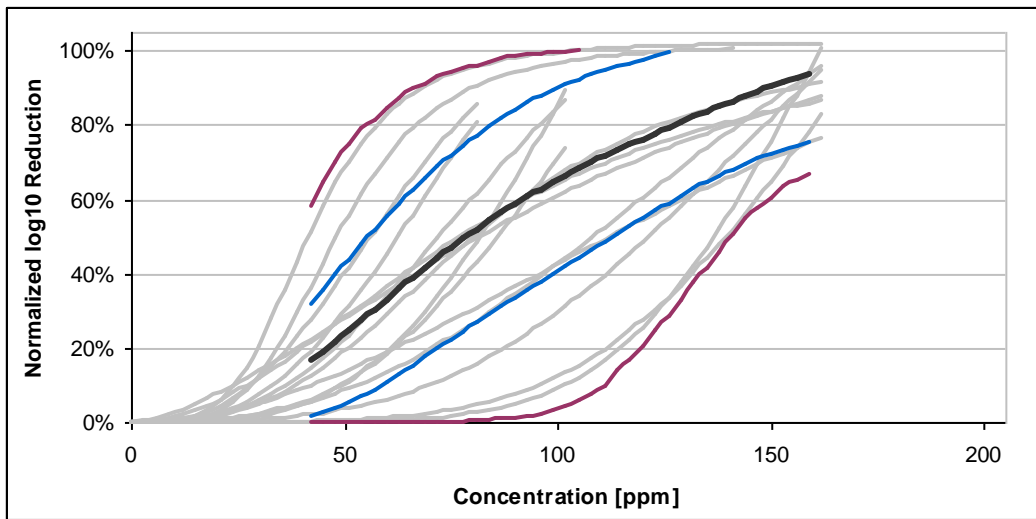
**Figure 47: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Pseudomonas aeruginosa* and glutaraldehyde**



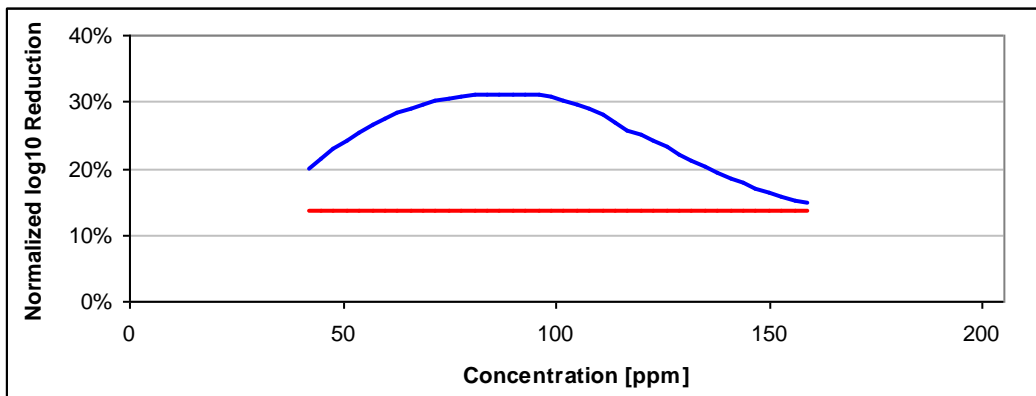
**Table 23: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Pseudomonas aeruginosa* and glutaraldehyde**

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 $s_t$
250	36%	20%	7%	10%
350	62%	26%	7%	9%
450	77%	23%	7%	7%
500	81%	21%	7%	7%
550	85%	19%	7%	7%
600	88%	17%	7%	7%

**Figure 48: Overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Pseudomonas aeruginosa* and peracetic acid**



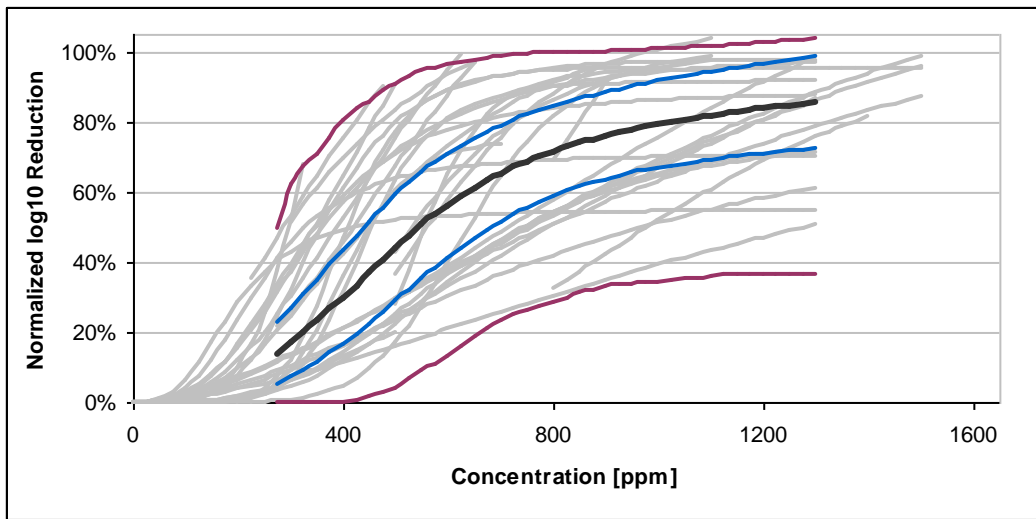
**Figure 49: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Pseudomonas aeruginosa* and peracetic acid**



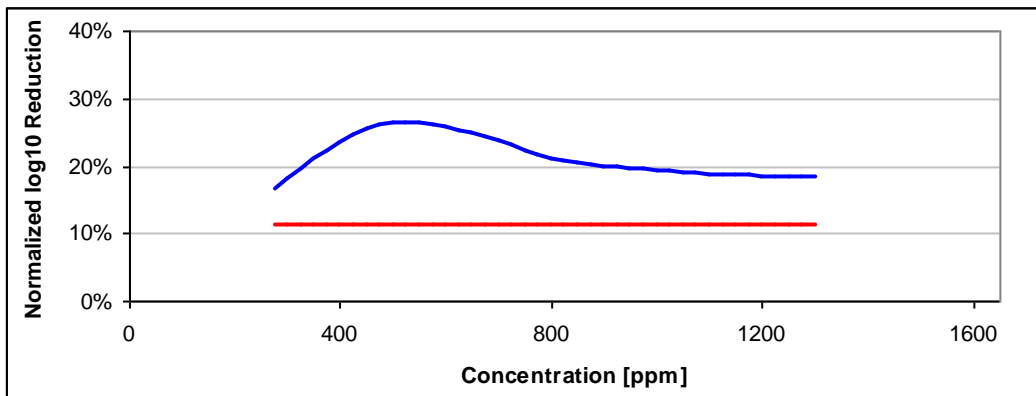
**Table 24: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Pseudomonas aeruginosa* and peracetic acid**

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 $s_t$
40	16%	20%	13%	15%
60	33%	27%	13%	16%
70	42%	29%	13%	16%
80	52%	31%	13%	17%
100	65%	31%	13%	17%
160	94%	15%	13%	13%

**Figure 50: Overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Staphylococcus aureus* and glutaraldehyde**



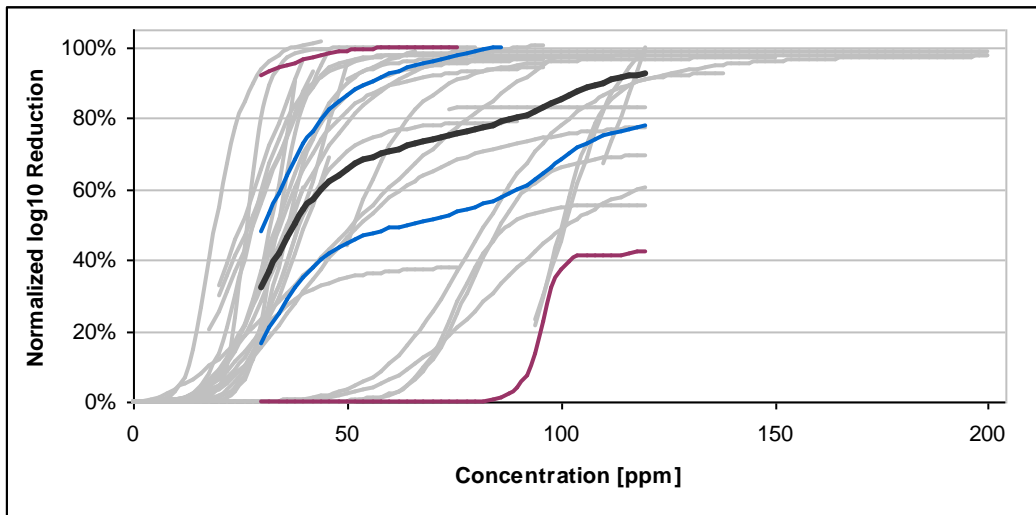
**Figure 51: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Staphylococcus aureus* and glutaraldehyde**



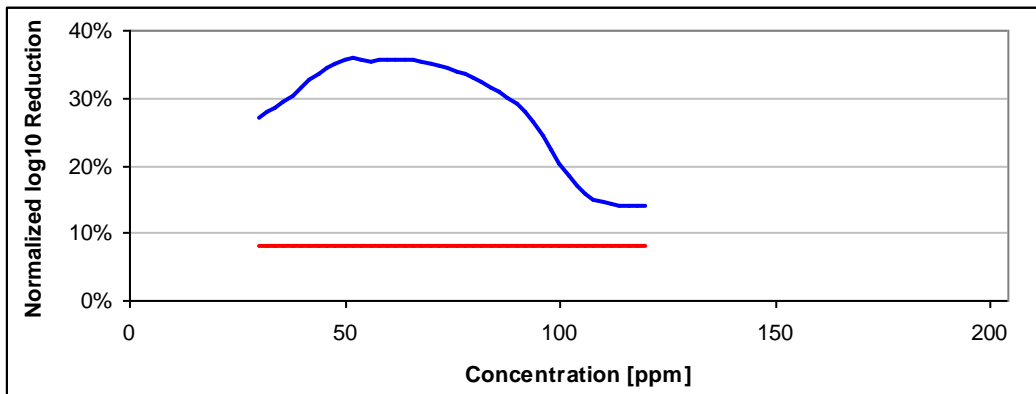
**Table 25: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Staphylococcus aureus* and glutaraldehyde**

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 $s_t$
250	14%	17%	11%	13%
500	44%	26%	11%	15%
800	72%	21%	11%	11%
900	76%	20%	11%	11%
1100	82%	19%	11%	11%
1300	85%	18%	11%	11%

**Figure 52: Overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Staphylococcus aureus* and peracetic acid**



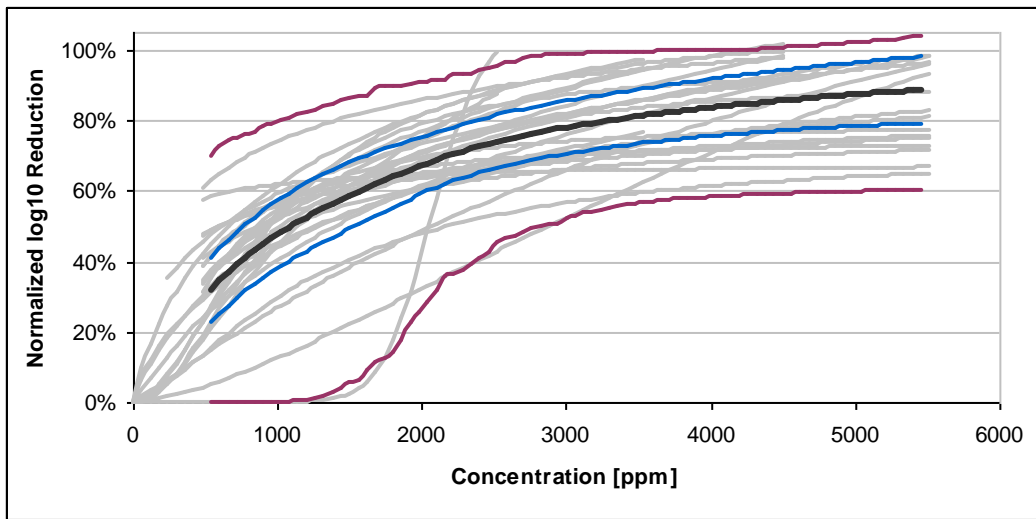
**Figure 53: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Staphylococcus aureus* and peracetic acid**



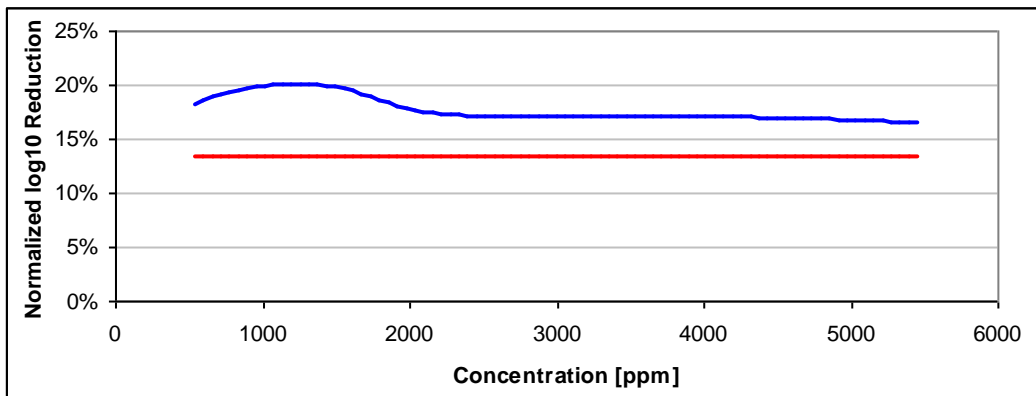
**Table 26: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Staphylococcus aureus* and peracetic acid**

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 $s_t$
75	75%	34%	8%	8%
90	80%	29%	8%	8%
95	83%	24%	8%	8%
100	85%	20%	8%	8%
110	90%	14%	8%	8%
120	93%	14%	8%	8%

**Figure 54: Overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Staphylococcus aureus* and benzalkonium chloride**



**Figure 55: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Staphylococcus aureus* and benzalkonium chloride**



**Table 27: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Staphylococcus aureus* and benzalkonium chloride**

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 $s_l$
500	32%	18%	13%	15%
1000	48%	20%	13%	17%
2500	74%	17%	13%	15%
3500	81%	17%	13%	15%
4500	86%	17%	13%	15%
5500	89%	16%	13%	14%

## Phase 2

### Part 1

#### Raw data

135. All four laboratories submitted data for one run with four concentrations used in each case. The four concentrations used in U.S. laboratories differed from the concentrations used in European laboratories (see Table 28). As can be seen, the range of concentration for the U.S. laboratories (400 ppm - 5500 ppm) is wider than for the European laboratories (500 ppm - 3500 ppm).

**Table 28: List of used concentrations in the U.S. laboratories and in the European laboratories**

	Used concentrations
U.S. laboratories	400 ppm, 1500 ppm, 2500 ppm, 5500 ppm
European laboratories	500 ppm, 1000 ppm, 2000 ppm, 3500 ppm

#### Data evaluation

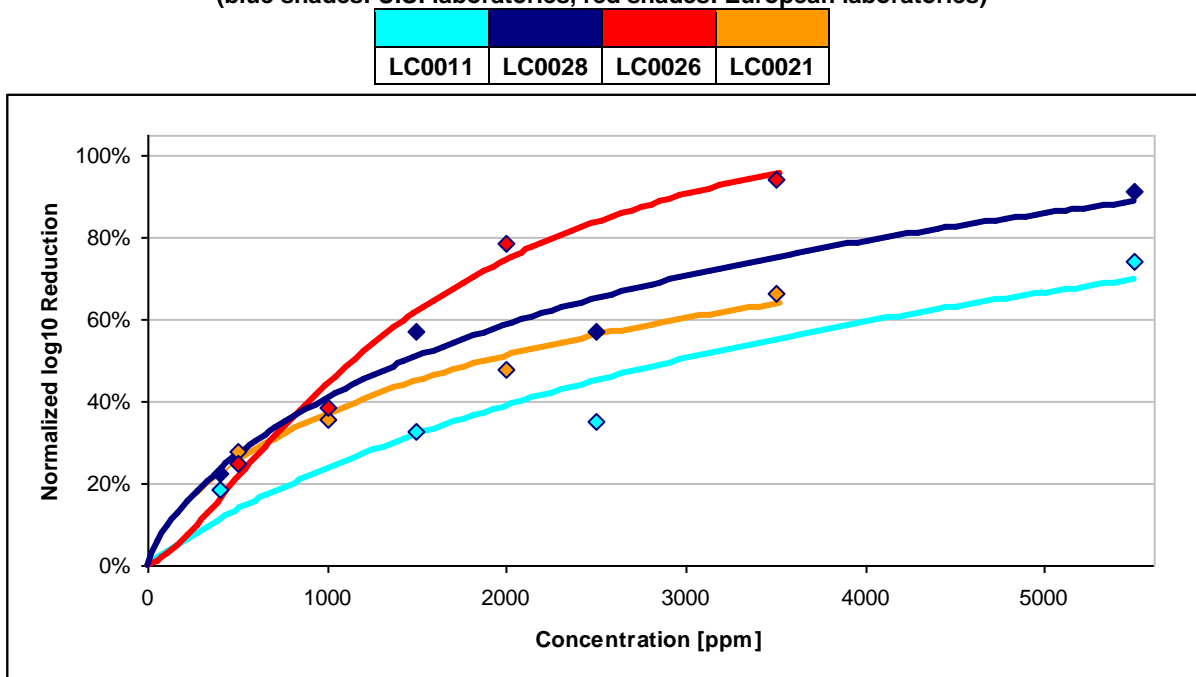
##### Outlier tests

136. No single log<sub>10</sub> CFU value could be identified as an outlier. Outlier test with regard to the residual standard deviations did not show any significant results. The Cochran test (with the embedded Grubbs test) with regard to the run-specific inactivation curves could not be applied, because there was only one run for each laboratory. The Grubbs test with regard to the run-specific inactivation curves did not show any significant results.

##### Inactivation curves

In the following Figure 56, all four run-specific inactivation curves are shown. In this figure, the mean log<sub>10</sub> reductions at each concentration level are displayed as well. The inactivation curves in blue shades belong to U.S. laboratories, whereas the inactivation curves in red shades belong to European laboratories

**Figure 56: Single normalized log<sub>10</sub> reductions and normalized inactivation curves for *Staphylococcus aureus* with benzalkonium chloride (blue shades: U.S. laboratories, red shades: European laboratories)**



137. For the U.S. laboratories LC0011 (turquoise) and LC0028 (dark blue) it can be observed that the log<sub>10</sub> reductions for the smallest concentration (400 ppm) are almost identical. For the three higher concentration levels the difference between the obtained log<sub>10</sub> reductions is almost constant for all three concentrations. Therefore, the inactivation curves show the same shape besides the difference regarding the level. The average log<sub>10</sub> reductions of the water control are also nearly the same for the two laboratories: 6.53 for laboratory LC0011 and 6.44 for laboratory LC0028.

138. For the European laboratories LC0026 (red) and LC0021 (orange), the behavior of the log<sub>10</sub> reductions for the two laboratories is similar to the behavior of the log<sub>10</sub> reductions of the other two laboratories. For the two lowest concentration levels the log<sub>10</sub> reductions do almost not differ for the two laboratories. For the two highest concentration levels, however, the difference between the log<sub>10</sub> reductions of the two laboratories is almost constant. The average log<sub>10</sub> reduction of the water control for laboratory LC0026 (red) equals 6.64 and for laboratory LC0021 (orange) it equals 7.27 – a statistically significant difference of approximately 0.6 between both laboratories (t-test, significance level 5 %).

139. It should be kept in mind that both runs of the U.S. laboratories were carried out in one U.S. laboratory and also both runs of the European laboratories were carried out in one European laboratory.

Possible causes for deviations between laboratories' inactivation curves

140. For both the European and the U.S. laboratories, one laboratory with downward deviations of the run-specific inactivation curves could be identified; the following list summarizes possible reasons for these deviations

141. Possible reasons for higher values by laboratory LC0026:

- used pour plates for the further dilutions
- had contaminated and dried all discs at the same time (thereby, the discs may have been “lying” around after the 1 h drying in the desiccator!)
- worked with measuring flasks
- had misunderstood the indication “3.5 Stage 5” and had mis-taken the 30 min - which shall not be transgressed until the end of the procedure - for the “neutralization time”
- used the  $10^3$  dilution and filtered the 10 mL of the test tube in two steps (for ex. 5 mL and 5 mL (two membranes) and 1 mL and 9 mL (also two membranes) thus filtering 200 mL PBS
- autoclaved the solution Since Mucin did not go through pore size  $0.22\ \mu\text{m}$

142. Possible reasons for lower values by laboratory LC0021:

- used spread plates only
- had not retrieved the TSB excess completely after centrifugation so that there was still residue of the TSB in the suspension
- has not reached  $10^9$  several times during preparation of the suspensions of microorganisms
- started with autoclaving, but due to “rusting” (corrosion) of some discs changed to heat sterilizing
- weighed the amounts product solutions
- used the  $10^2$  dilution and the 10mL in one step and rinsed with 100 mL PBS (according to OECD LOGO)
- filtered with pore size  $0.45\ \mu\text{m}$  (Mucin is “clear” afterwards)

143. Possible reasons for lower values by laboratory LC0011:

- contaminated on the rounded edge side of the carrier; started with the tip midpoint between the middle and the edge and drew a circle while dispensing
- wt/vol – weighed 20 g product in beaker + 180 mL HW, rinsing with some of the HW
- pipetted HW into volumetric flasks and did not have a lot of foaming
- used disposable filtration units with pore size 0.22 µm
- pre-wet filters prior to addition of neutralized sample
- dispensed 10mL neutralizer directly into the vials

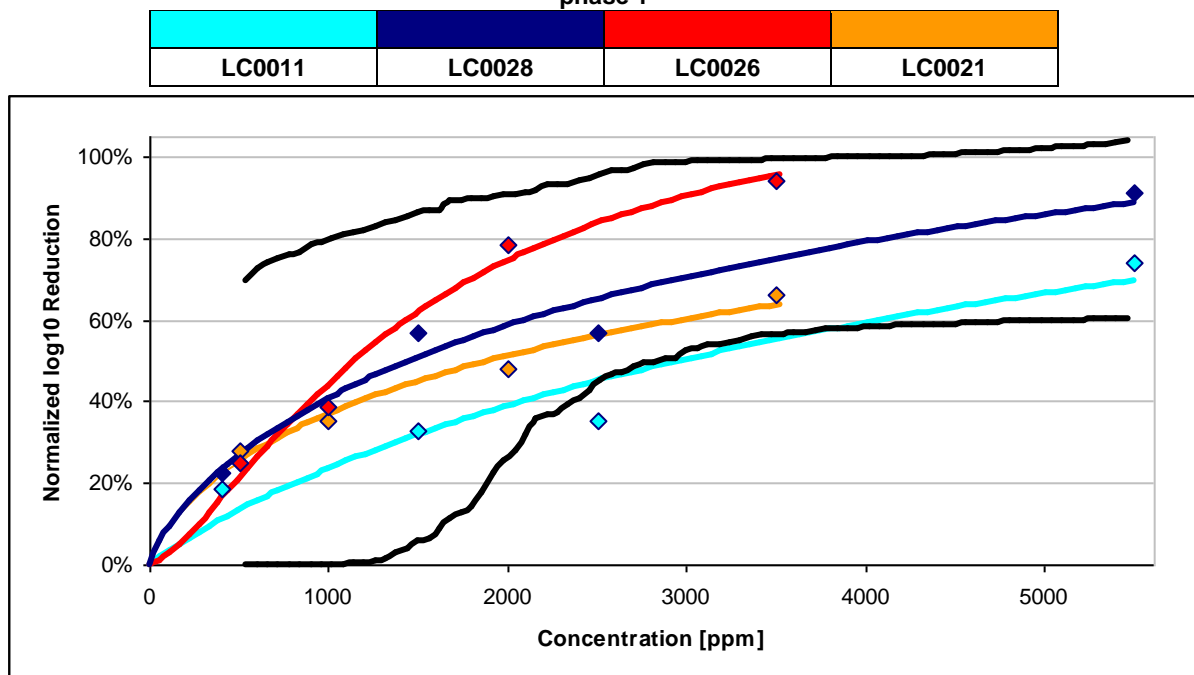
144. Possible reasons for higher values by laboratory LC0028:

- contaminated on both the rounded and flat edge side; inoculated in the middle of the carrier and spread with pipette tip
- did wt/wt - weighed 20 g product + 180 g HW
- poured HW into volumetric flasks and did have some foaming issues
- used re-usable filtration units with pore size 0.45 µm
- did not pre-wet filters; some of the plates from the lab that did not pre-wet filter had pooling of growth making counting of survivors difficult
- pre-dispensed 10mL into tubes and poured the neutralizer from the tubes to the vials

Comparison with results from initial ring trial in phase 1

145. The following Figure 57 shows the normalized inactivation curves of the part 1 of phase 2 compared to the 95%-prediction interval derived in the ring trial.

**Figure 57: Inactivation curves for part 1 of phase 2 for *Staphylococcus aureus* with benzalkonium chloride with the 95%-prediction interval for run-specific curves (black) from the initial ring trial in phase 1**

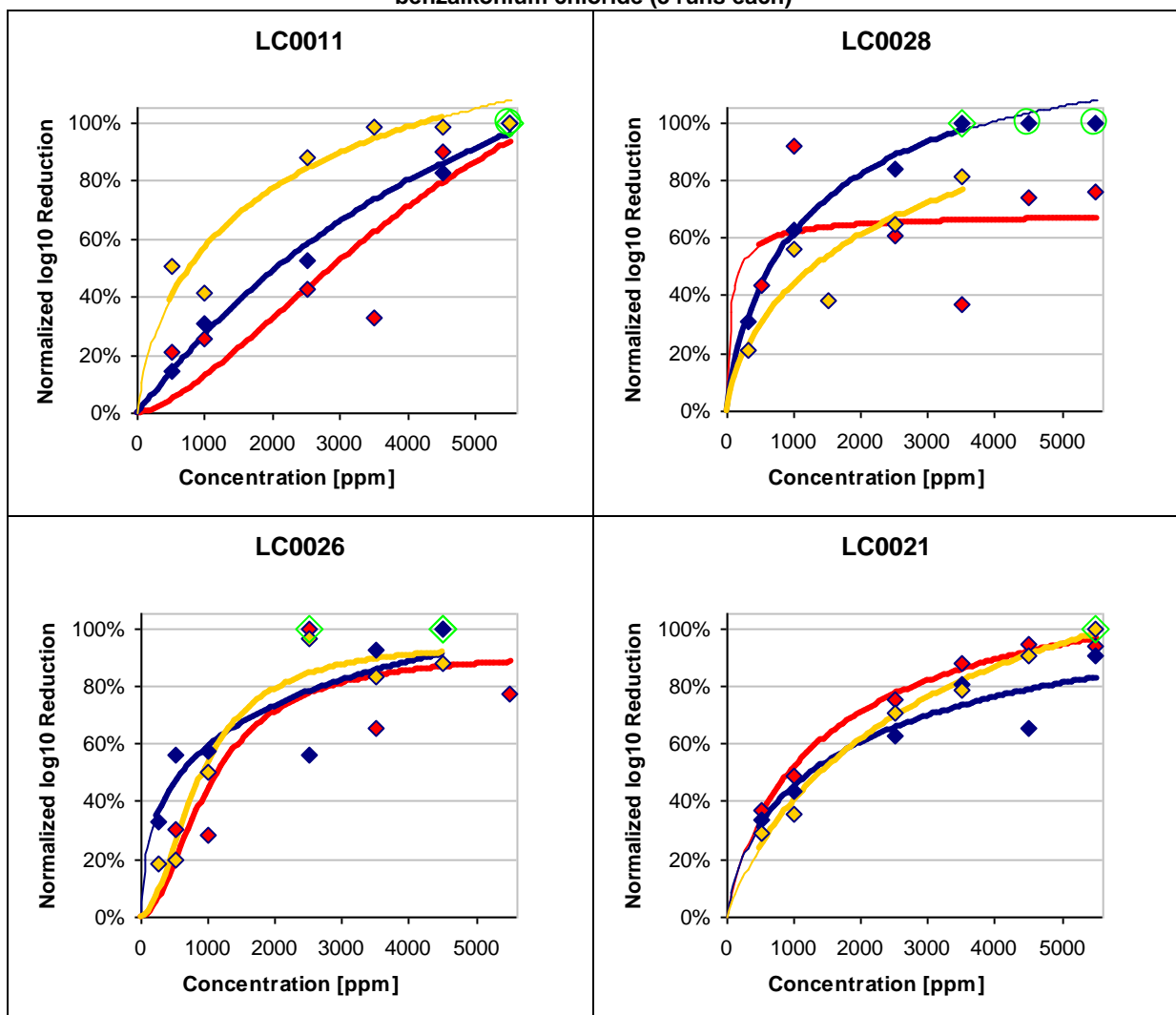


146. As can be seen, the inactivation curves of the two European laboratories and the inactivation curve of the U.S. laboratory LC0028 lie clearly within the prediction interval. The inactivation curve of laboratory LC0011 (turquoise) is touching the prediction interval.

147. In the initial ring trial of phase 1, all four laboratories had reached the 80 % level of the normalized  $\log_{10}$  reduction for each run – except for the U.S. laboratory LC0028 that reached the 80 % level only for one out of three runs (see Figure 58).

148. In part 1 of phase 2, only two out of the four laboratories accomplished to reach the 80 % level: LC0026 and LC0028.

**Figure 58: Inactivation curves for initial ring trial of phase 1 for *Staphylococcus aureus* with benzalkonium chloride (3 runs each)**



149. In the following, the average  $\log_{10}$  reductions of the water control and the residual standard deviations of the inactivation curves of part 1 in phase 2 are compared to the results of the initial ring trial in phase 1.

150. As can be seen in Table 29, in part 1 of phase 2 all laboratories could nearly reproduce their average  $\log_{10}$  reduction of the water control of the ring trial. However, for the European laboratory LC0021 as well as for the U.S. laboratory LC0028 a statistically significant difference between the average  $\log_{10}$  reductions of the water control could be observed (t-test, significance level 5 %).

**Table 29: Comparison of the average  $\log_{10}$  reductions of the water control (AWC) between the initial ring trial in phase 1 and part 1 of phase 2 (*Staphylococcus aureus* with benzalkonium chloride)**

Laboratory	AWC in the initial ring trial in phase 1	AWC in part 1 of phase 2
LC0026	6.61	6.64
LC0021	7.62	7.27
LC0011	6.47	6.53
LC0028	5.89	6.44

151. Regarding the residual standard deviation of the inactivation curves, for all four laboratories the standard deviations obtained in part 1 of phase 2 are lower than the ones of the initial ring trial in phase 1 (see Table 30). However, none of these differences is statistically significant, which is mainly caused by the low number of degrees of freedom for part 1 of phase 2 (estimating three parameters for the data of only one run with four concentration levels).

**Table 30: Comparison of the residual standard deviation between the initial ring trial in phase 1 and part 1 of phase 2 (*Staphylococcus aureus* with benzalkonium chloride)**

Laboratory	RSD in the initial ring trial in phase 1	RSD in part 1 of phase 2
LC0026	0.170	0.080
LC0021	0.066	0.051
LC0011	0.145	0.094
LC0028	0.190	0.105

## **Part 2**

### **Raw data**

152. For *Staphylococcus aureus* and glutaraldehyde, in total, seven independent data sets from four different laboratories were available. For *Staphylococcus aureus* and benzalkonium chloride only four independent data sets from three different laboratories were available. However, the number of runs, the number water controls, the number of replicates per concentration level as well as the concentration levels themselves differed. The following Table 31 and Table 32 offer an overview of the experimental design applied in each laboratory.

**Table 31: Individual experimental designs for *Staphylococcus aureus* and glutaraldehyde**

Laboratory	Number of runs	Number of water controls	Number of replicates per concentration level	Concentration levels [in ppm]
LC0011a	1	4	3	250, 500, 900, 1300
LC0011b	1	4	3	250, 500, 900, 1300
LC0021a	1	3	3	800, 1200
LC0021b	1	3	3	800, 1200
LC0021c	1	4	5	250, 500, 900, 1300
LC0028	1	3	5	250, 500, 900, 1300
LC0035	1	3	5	800, 1200

**Table 32: Individual experimental designs for *Staphylococcus aureus* and benzalkonium chloride**

Laboratory	Number of runs	Number of water controls	Number of replicates per concentration level	Concentration levels [in ppm]
LC0011a	1	4	3	400, 1500, 2500, 5500
LC0011b	1	4	3	400, 1500, 2500, 5500
LC0021	1	4	5	400, 1500, 2500, 5500
LC0028	1	3	5	400, 1500, 2500, 5500

## **Data evaluation**

### **Outlier tests**

153. According to the outlier test with regard to the single log<sub>10</sub> CFU values of each carrier for glutaraldehyde no significant outlier values could be identified. For benzalkonium chloride, however, the log<sub>10</sub> CFU value at a concentration of 2500 ppm in run 1 of laboratory LC0028 proved to be an outlier value. It has been excluded from further statistical analyses.

154. The outlier test with regard to the residual standard deviations did not show any significant results. The Cochran test (with the embedded Grubbs test) with regard to the run-specific inactivation curves could not be applied because therefore every laboratory needed at least two runs which was not given in part 2. The Grubbs test with regard to the run-specific inactivation curves did not show any significant results.

### **Inactivation curves**

155. The following Figure 59 and Figure 60 show the mean log<sub>10</sub> reductions at each concentration level and the inactivation curves if they could be estimated from the data. Furthermore, the 95%-prediction intervals derived in the initial ring trial in phase 1 are shown.

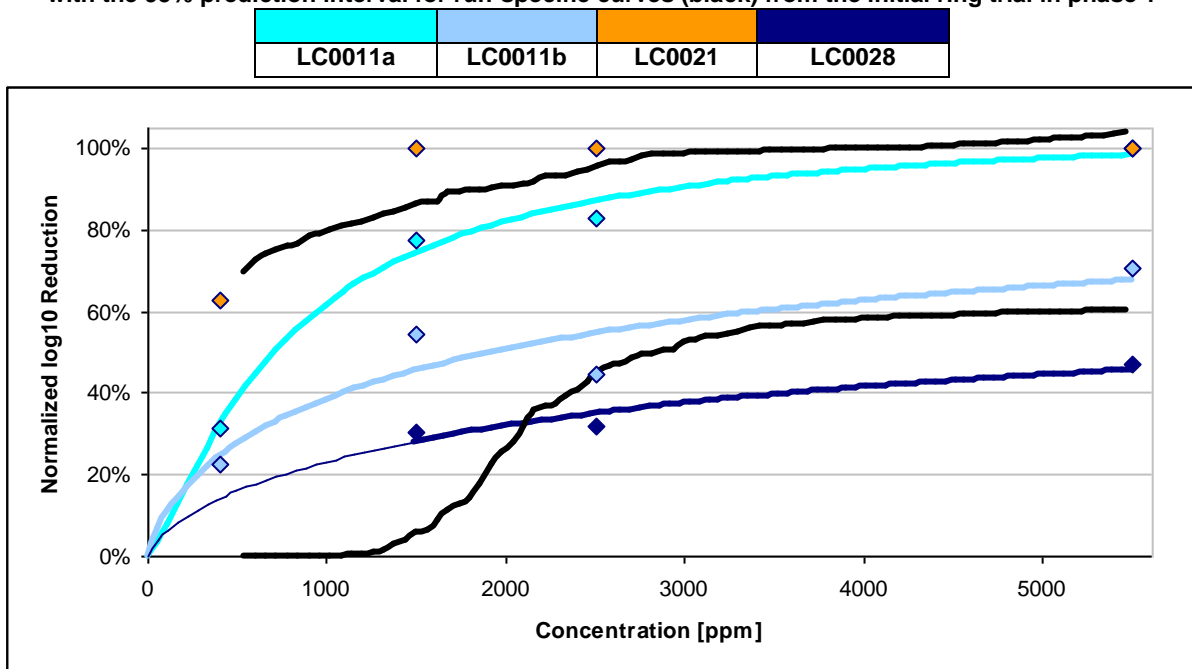
156. For *Staphylococcus aureus* with benzalkonium chloride for laboratory LC0021 no

inactivation curve could be estimated, because three of the four concentration values resulted in a log<sub>10</sub> reduction of 100% (complete kill).

157. The inactivation curves of laboratories LC0011b and LC0028 never reach the 80% level.

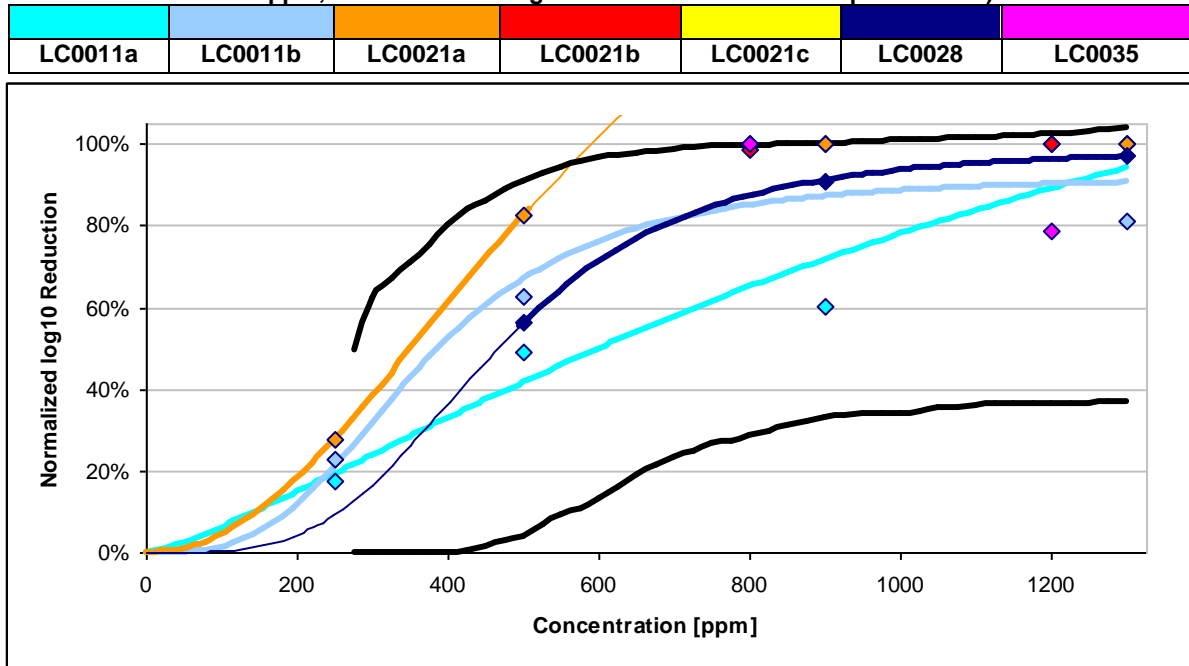
158. Two of the log<sub>10</sub> reductions of laboratory LC0021 lie within the prediction interval and two values outside. The two inactivation curves of laboratory LC0011 (a+b) lie clearly within the prediction interval from the ring trial. Above a concentration of about 2000 ppm, the inactivation curve of laboratory LC0028 lies outside the prediction interval.

**Figure 59: Inactivation curves for *Staphylococcus aureus* – benzalkonium chloride in part 2 of phase 2 with the 95%-prediction interval for run-specific curves (black) from the initial ring trial in phase 1**



159. For *Staphylococcus aureus* with glutaraldehyde for laboratories LC0021b, LC0021c and LC0035 no inactivation curve could be estimated, because either all log<sub>10</sub> reductions equaled 100% or the values were decreasing with an increasing concentration. Thus, only for four out of seven laboratories/technicians inactivation curves could be determined. All of them reach the 80% level. Furthermore, within the concentration range considered, all four inactivation curves lie within the prediction interval of the ring trial. Please note that in the figure the log<sub>10</sub> reduction values of LC0021c are covered by log<sub>10</sub> reductions of other laboratories. For both analysed concentration levels (800 ppm and 1200 ppm), the normalized log<sub>10</sub> reduction of LC0021c equaled 100%. All inactivation curves reach the 80% level.

**Figure 60: Inactivation curves for *Staphylococcus aureus* – glutaraldehyde in part 2 of phase 2 with the 95%-prediction interval for run-specific curves (black) from the initial ring trial in phase 1 (log10 reduction values of LC0021c are covered in the figure, for both concentration levels 800 ppm and 1200 ppm, the normalized log10 reduction of LC0021c equaled 100%)**



**Part 3**

**Raw data**

160. For *Pseudomonas aeruginosa* with glutaraldehyde as well as for *Enterococcus hirae* with benzalkonium chloride, in total, seven independent data sets from five different laboratories were available. However, the number of runs, the number water controls, the number of replicates per concentration level as well as the concentration levels themselves differed. The following Table 31 and Table 32 offer an overview of the experimental design applied in each laboratory.

161. For *Adenovirus type 5* with glutaraldehyde, data from two laboratories was available. In both laboratories, two technicians had carried out the tests. However, the data of both laboratories could not be combined, because laboratory LC0036 applied the well count method, while laboratory LC0006 applied the TCID<sub>50</sub> method. Details on the tests are summarized in Table 35.

**Table 33: Individual experimental designs for *Pseudomonas aeruginosa* with glutaraldehyde**

Laboratory	Number of runs	Number of water controls	Number of replicates per concentration level	Concentration levels [in ppm]
LC0011a	1	4	3	250, 350, 400, 600, 800
LC0011b	1	4	3	250, 350, 400, 600, 800
LC0012	3	3	3	250, 350, 400, 600, 800
LC0021a	3	4	3	runs 1+2: 250, 350, 400, 600, 800 run 3: 50, 200, 300, 350, 400, 600
LC0028	1	4	3	50, 100, 250, 350, 400, 600
LC0036	6	4	3	for 4 out of 6 runs: 50, 100, 200, 300, 350, 400 for 2 out of 6 Runs: 50, 100, 200, 250, 300, 350
LC0021b	1	4	3	50, 200, 300, 350, 400, 600

**Table 34: Individual experimental designs for *Enterococcus hirae* with benzalkonium chloride**

Laboratory	Number of runs	Number of water controls	Number of replicates per concentration level	Concentration levels [in ppm]
LC0011a	1	4	3	250, 400, 1500, 2500, 5500
LC0011b	1	4	3	250, 400, 1500, 2500, 5500
LC0012	3	3	3	250, 400, 1500, 2500, 5500
LC0021a	3	4	3	runs 1+2: 250, 400, 1500, 2500, 5500 run 3: 250, 500, 1000, 1500, 2500, 5500
LC0028	1	4	3	250, 500, 1000, 1500, 2500, 5500
LC0036	3	4	3	50, 250, 500, 1000, 1500, 2000
LC0021b	1	4	3	250, 500, 1000, 1500, 2500, 5500

**Table 35: Individual experimental designs for *Adenovirus type 5* with glutaraldehyde**

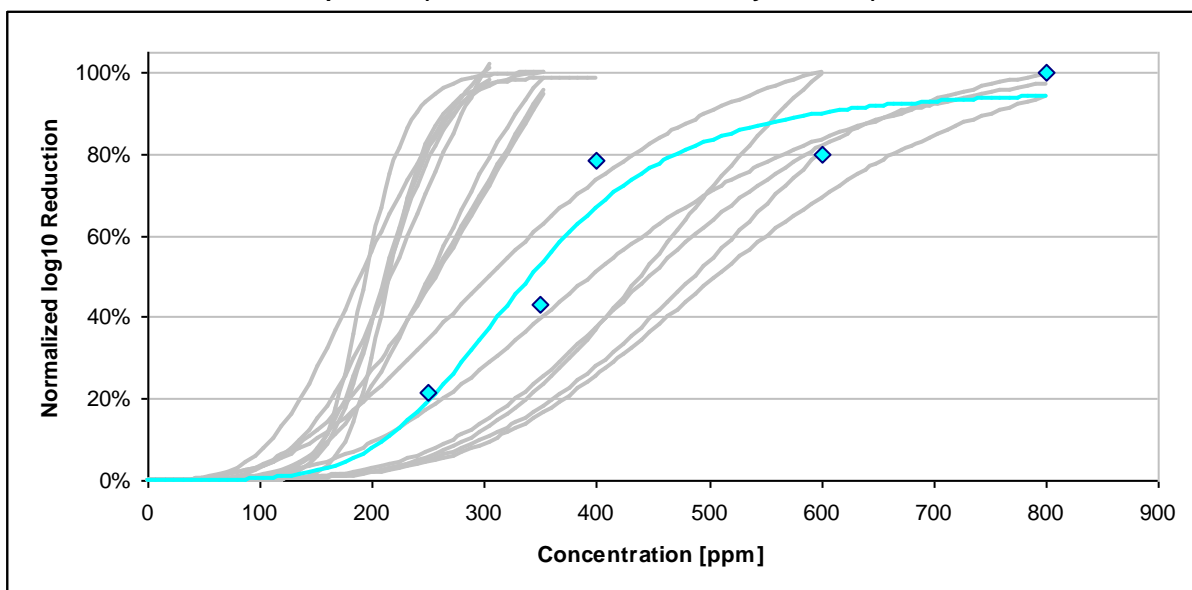
Laboratory	Number of runs	Number of water controls	Number of replicates per concentration level	Concentration levels [in ppm]
LC0036a	1	3	2	31.3, 63, 125, 250, 500, 1000
LC0036b	1	3	2	62.5, 125, 250, 500, 1000
LC0006a	2	4	3	31, 62.5, 125, 250, 500, 1000
LC0006b	2	4	3	31, 62.5, 125, 250, 500, 1000

## Data evaluation

### Outlier tests

162. The Cochran test (with the embedded Grubbs test) with regard to the run-specific inactivation curves could not be applied; therefore every laboratory needed at least two runs which was not given in phase 2 – part 3.
163. Neither for *Enterococcus hirae* nor for *Adenovirus type 5* any outliers could be identified – neither with regard to the single log<sub>10</sub> CFU values of each carrier nor with regard to the residual standard deviations nor with regard to the run-specific inactivation curves.
164. The outlier test with regard to the residual standard deviations did not show any significant results. The Cochran test (with the embedded Grubbs test) with regard to the run-specific inactivation curves could not be applied; therefore every laboratory needed at least two runs which was not given in part 2. The Grubbs test with regard to the run-specific inactivation curves did not show any significant results.
165. For *Pseudomonas aeruginosa* the single log<sub>10</sub> CFU value of laboratory LC0021a in run 1 at a concentration of 300 ppm proved to be an outlier. Furthermore, the outlier test with regard to the residual standard deviations identified the run of laboratory LC0011a as an outlier run. The single log<sub>10</sub> CFU value of laboratory LC0021a as well as the run of laboratory LC0011a (that is all data by LC0011a) have been eliminated from the data set and not been considered in any further analyses. The following Figure 61 shows the outlier run-specific inactivation curve highlighted in blue, while the remaining inactivation curves, i.e. non-outliers on which all further statistical analyses will be based on, are shown in a light-grey.

**Figure 61: Run-specific inactivation curves for *Pseudomonas aeruginosa* with glutaraldehyde in part 3 of phase 2 (blue: outlier run of laboratory LC0011a)**

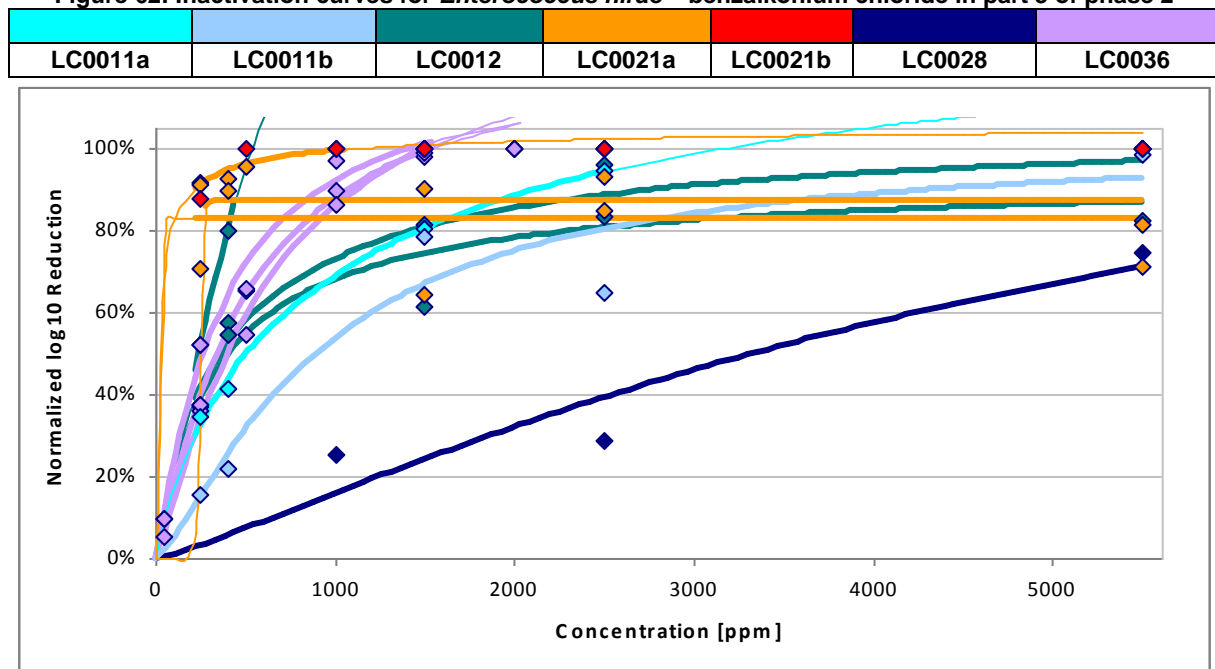


Inactivation curves

166. The following Figure 62 and Figure 63 show the mean log<sub>10</sub> reductions at each concentration level and the inactivation curves. For *Pseudomonas aeruginosa* with glutaraldehyde the 95%-prediction intervals derived in the initial ring trial in phase 1 is shown. For *Enterococcus aureus* no prediction interval can be shown, because the organism had not been analysed in the initial ring trial.

167. For *Enterococcus aureus* with benzalkonium chloride for six out of seven laboratories/technicians an inactivation curve could be estimated. Only for laboratory LC0021b (red) no inactivation curve could be estimated because for five out of six concentration levels a log<sub>10</sub> reduction of 100% (complete inactivation) was obtained. The clearly deviating inactivation curve of laboratory LC0028 had not been identified as an outlier run, because in the Grubbs test all concentrations had been logarithmized and therefore deviations had been compensated. However, the inactivation curve of this laboratory is the only one that does not reach the 80% level within the considered range of concentrations.

**Figure 62: Inactivation curves for *Enterococcus hirae* – benzalkonium chloride in part 3 of phase 2**



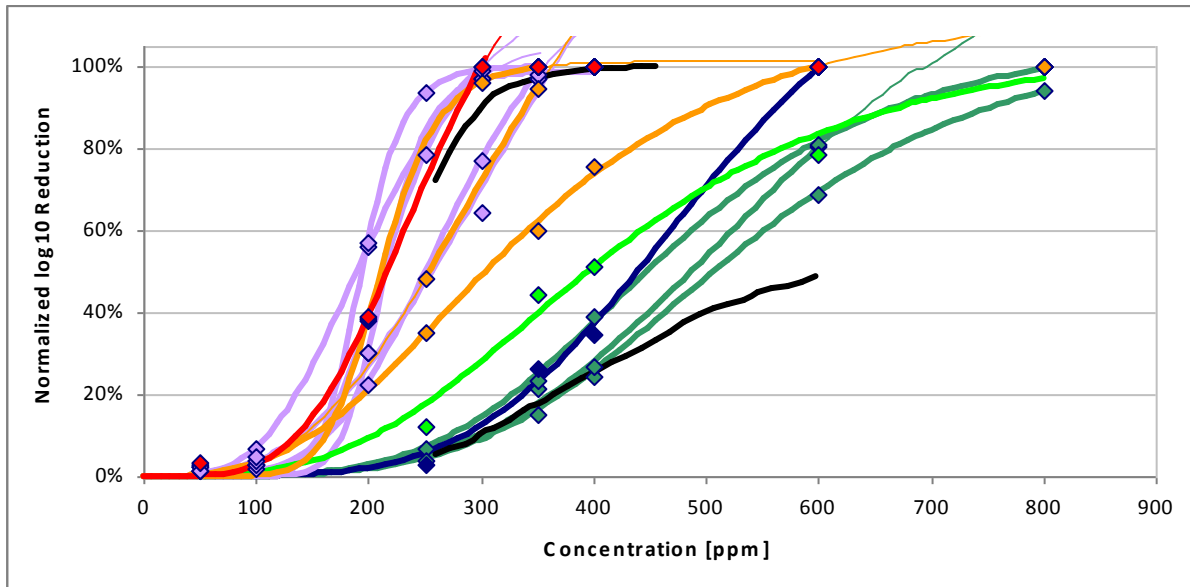
168. For *Pseudomonas aeruginosa* all run-specific inactivation curves could be estimated. Please note that one of the inactivation curves of laboratory LC0036 is partly covered by the inactivation curve of laboratory LC0021. An inexperienced technician had carried out the run of LC0011c. The run carried out by an experienced technician was identified as an outlier run and had to be excluded from the analysis. All inactivation curves reach the 80% level.

169. Regarding the prediction interval from the initial ring trial, one run of laboratory LC0012 lies slightly below the interval for concentrations between 300 ppm and 400 ppm. It is

noticeable that almost all run-specific inactivation curves of laboratory LC0036 lie outside the prediction interval. It should be noted that actually the shape of these inactivation curves looks promising. However, a lower range of concentration was used in the tests and therefore these curves do not coincide with the data of the initial ring trial very well.

**Figure 63: Inactivation curves for *Pseudomonas aeruginosa* – glutaraldehyde in part 3 of phase 2 with the 95%-prediction interval for run-specific curves (black) from the initial ring trial in phase 1**

LC0011c	LC0012	LC0021a	LC0021a	LC0028	LC0036
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170. In the following figures the mean log<sub>10</sub> reductions at each concentration level and the inactivation curves for *Adenovirus type 5* are shown.

171. Figure 64 shows the results of the well count methods of laboratory LC0036. For both runs an inactivation curve could be estimated. One of the runs was carried out by an experienced technician, the other one by an inexperienced technician. One of the inactivation curves does not reach the 80% level. A prediction interval from the initial ring trial is not available since only three laboratories had submitted data for well count method and no precision figures could be determined.

172. Figure 65 shows the results of the TCID<sub>50</sub> method of laboratory LC0006. For all four runs an inactivation curve could be estimated and all four inactivation curves reach the 80% level. Regarding the prediction interval from the initial ring trial, the inactivation curves coincide very well for concentrations larger than about 70 ppm.

Figure 64: Inactivation curves for *Adenovirus type 5* – glutaraldehyde (well count method) in part 3 of phase 2

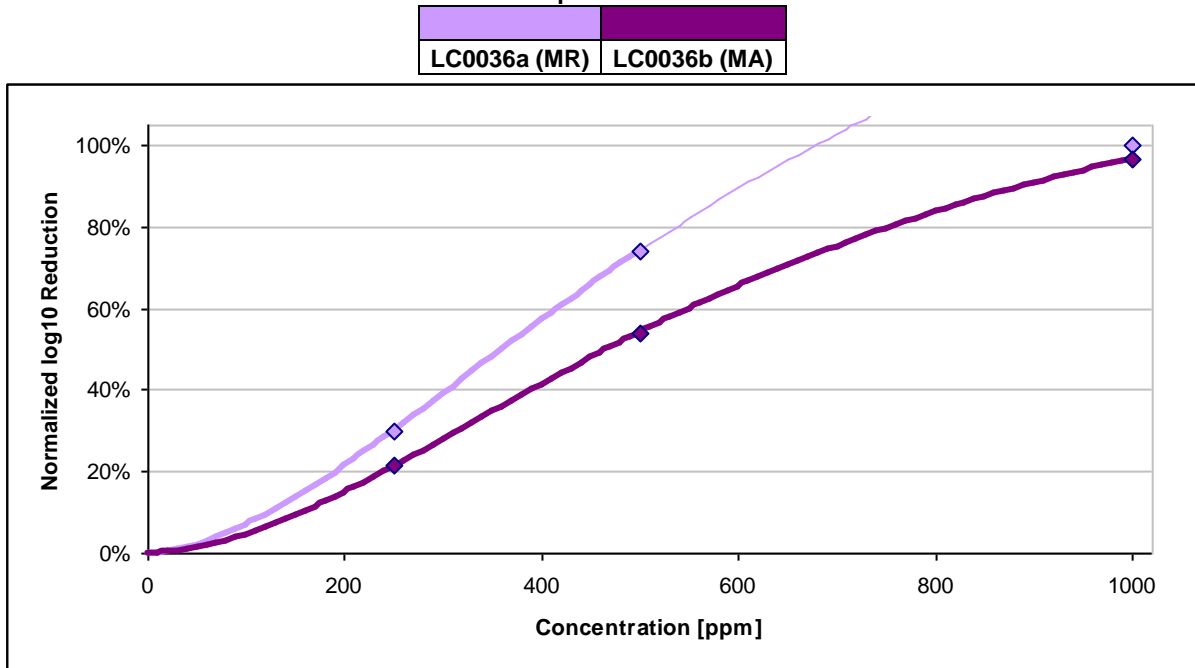
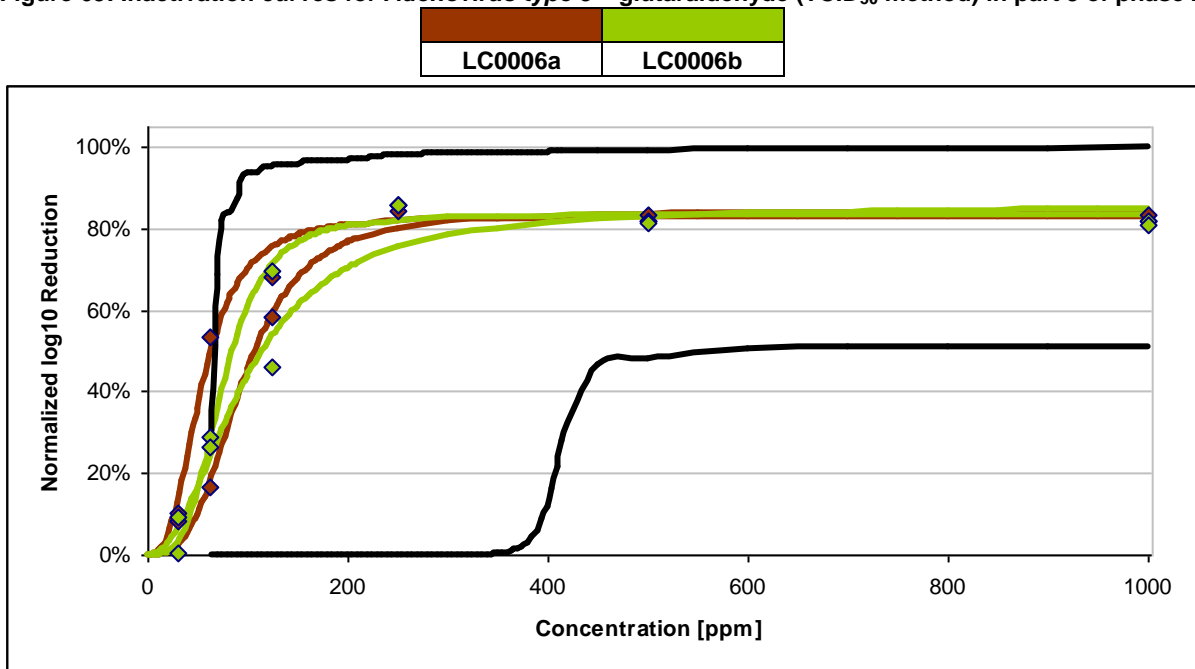


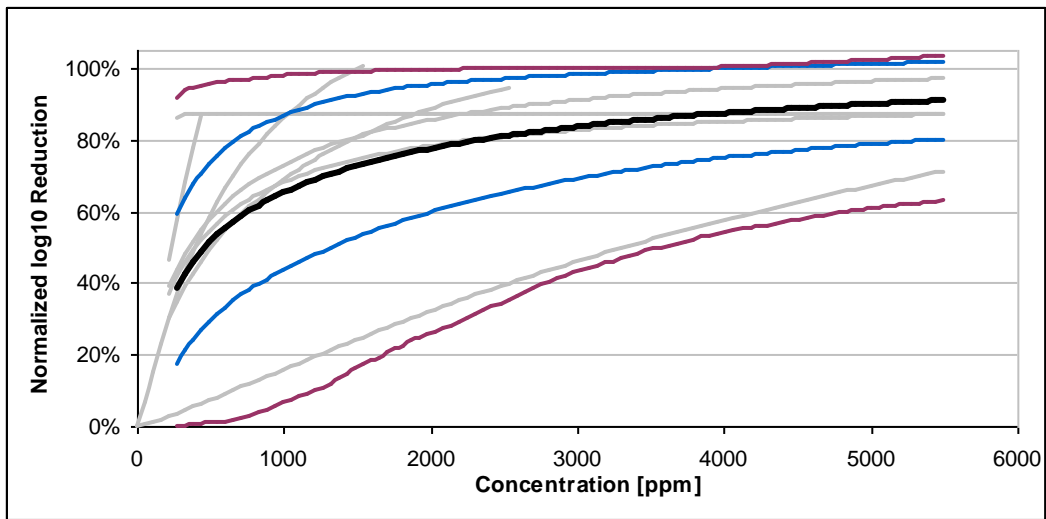
Figure 65: Inactivation curves for *Adenovirus type 5* – glutaraldehyde (TCID<sub>50</sub> method) in part 3 of phase 2



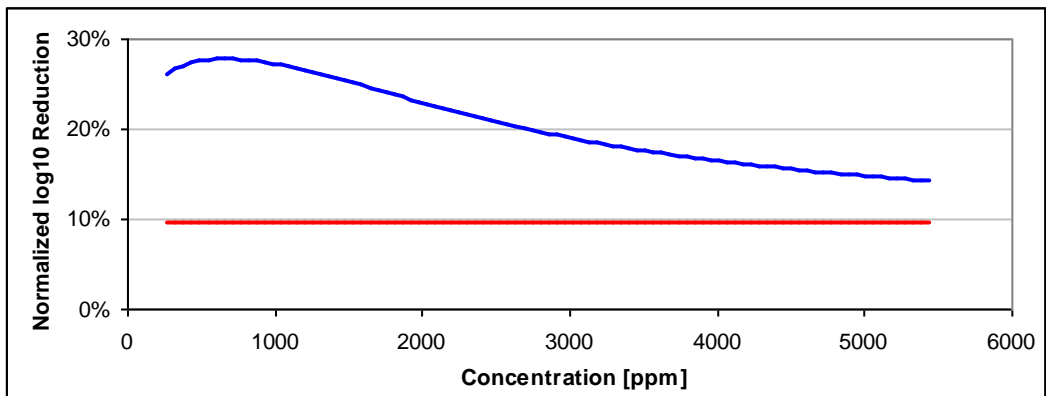
Overall (laboratory-comprehensive) inactivation curves and intra- and inter-laboratory reproducibility

173. In the following figures, for both *Enterococcus hirae* with benzalkonium chloride and *Pseudomonas aeruginosa* with glutaraldehyde 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.
174. Both overall inactivation curves are followed by a figure 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 the smaller than the inter-laboratory reproducibility  $s_R$ .
175. Below the figures of the standard deviations, a table is given in which 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  $s_t$  and intra-laboratory reproducibility within one run  $s_r$  are summarized for the six concentration levels as originally suggested to the laboratories. No values can be given if the respective concentration lies not within the concentration range considered for the bootstrap.
176. Due to the normalized response expressed in percent, the standard deviations are expressed in percent as well although they are absolute values.
177. For both microorganisms the inter-laboratory reproducibility standard deviation is considerably larger than under intra-laboratory conditions. At a true normalized log<sub>10</sub> reduction of 80%, the inter-laboratory reproducibility is about 21% for *Enterococcus hirae* with benzalkonium chloride, whereas the intra-laboratory reproducibility over time is about 10%. For *Pseudomonas aeruginosa* with glutaraldehyde an inter-laboratory reproducibility of about 26% could be obtained, which is 4% higher than the standard deviation obtained in the initial ring trial in phase 1. However, for the of intra-laboratory reproducibility over time in phase 2 – part 3 with 5% a smaller value could be obtained than in phase 1 (7%).

**Figure 66: Overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Enterococcus hirae* and benzalkonium chloride**



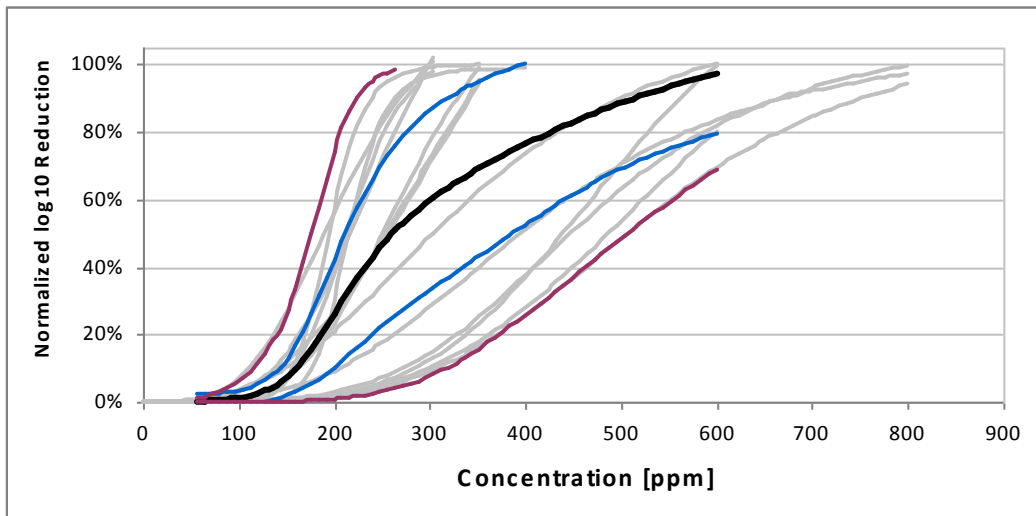
**Figure 67: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Enterococcus hirae* and benzalkonium chloride**



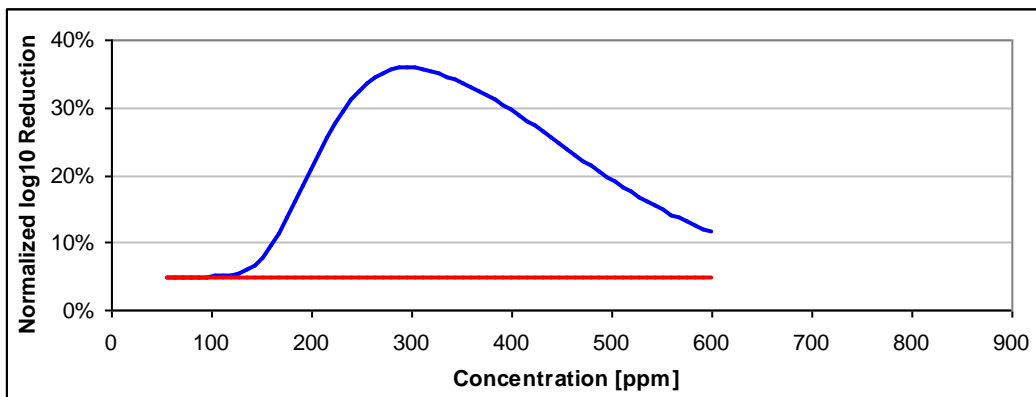
**Table 36: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Enterococcus hirae* and benzalkonium chloride**

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 $s_t$
250	38%	26%	10%	10%
400	46%	27%	10%	10%
1500	72%	25%	10%	10%
2500	81%	21%	10%	10%
5500	91%	14%	10%	10%

**Figure 68: Overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Pseudomonas aeruginosa* and glutaraldehyde**



**Figure 69: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Pseudomonas aeruginosa* and glutaraldehyde**



**Table 37: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Pseudomonas aeruginosa* and glutaraldehyde**

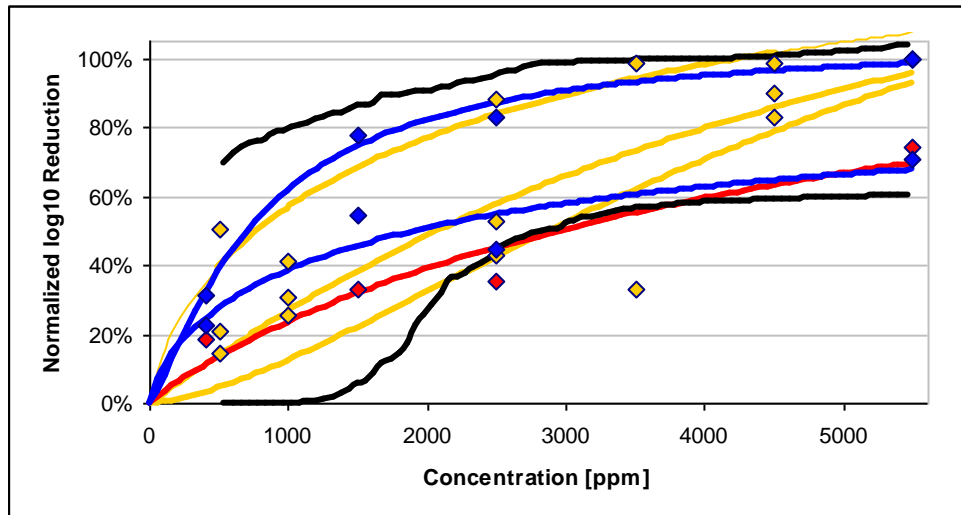
Concentration [ppm]	Overall inactivation curve (normalized log10 reduction)	Absolute inter-laboratory reproducibility $s_R$	Absolute intra-laboratory reproducibility within one run $s_r$	Absolute intra-laboratory reproducibility over time $s_t$
250	46%	32%	5%	10%
350	69%	34%	5%	5%
400	76%	30%	5%	5%
600	97%	12%	5%	5%
800	-	-	-	-

## Comparison to the results of phase 1

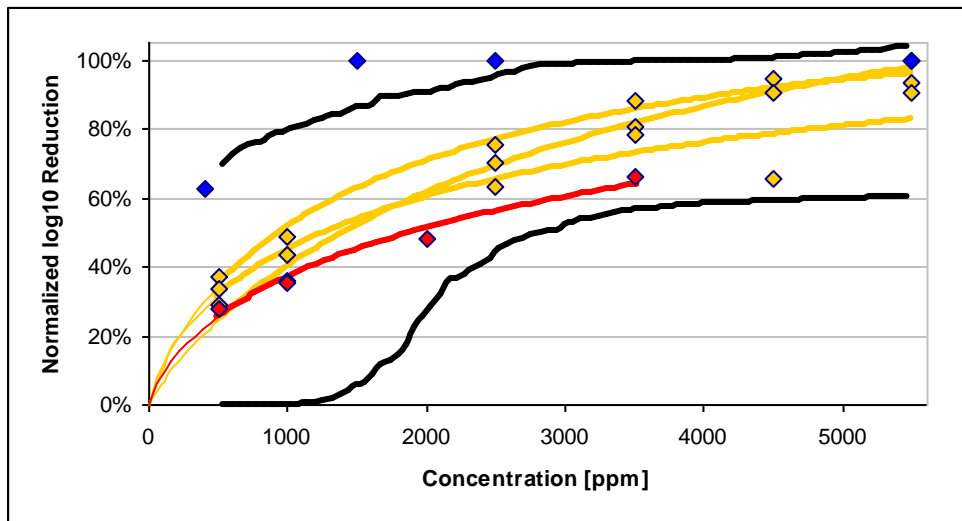
178. In the following figures, for each laboratory that has participated in at least two of the four steps (initial ring trial in phase 1, phase 2 – part 1, phase 2 – part 2 or phase 2 – part 3) all mean log<sub>10</sub> reductions and run-specific inactivation curves (if available) as well as the 95%-prediction interval from the ring trial in phase 1 (if available) are shown separately for each laboratory.

179. It is apparent that the 95%-prediction interval of phase 1 appears to be appropriate also for phase 2. For some of the laboratories inactivation curves are highly varying – within the prediction interval. Only for some exceptional cases, inactivation curves appear to be almost constant.

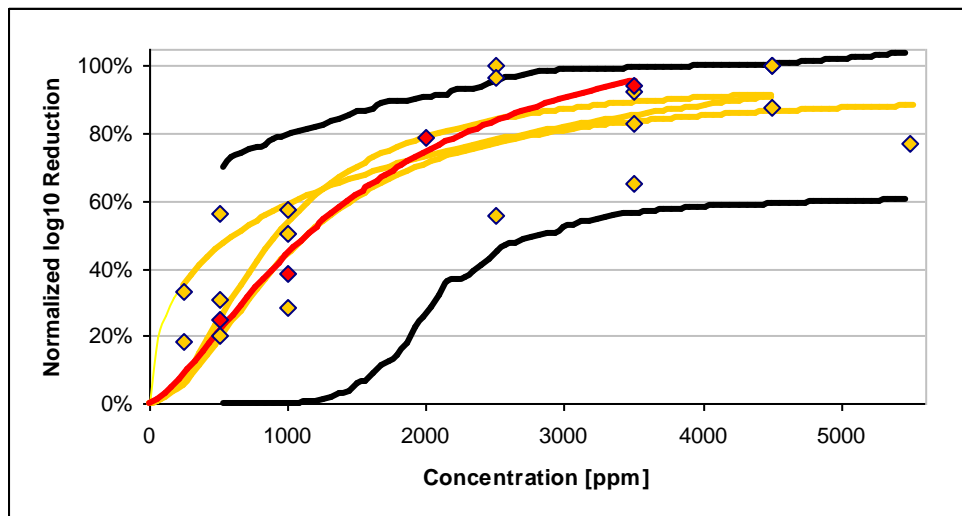
**Figure 70: Mean log<sub>10</sub> reductions with inactivation curves and 95%-prediction interval (black) of initial ring trial for *Staphylococcus aureus* vs. benzalkonium chloride – LC0011 (yellow: ring trial phase 1, red: part 1 of phase 2, blue: part 2 of phase 2)**



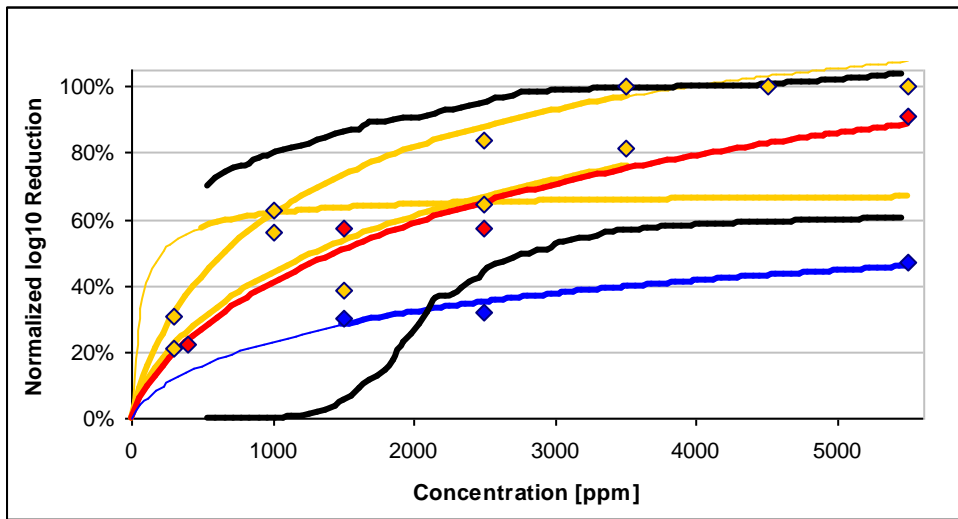
**Figure 71: Mean log<sub>10</sub> reductions with inactivation curves and 95%-prediction interval (black) of initial ring trial for *Staphylococcus aureus* vs. benzalkonium chloride – LC0021 (yellow: ring trial phase 1, red: part 1 of phase 2, blue: part 2 of phase 2)**



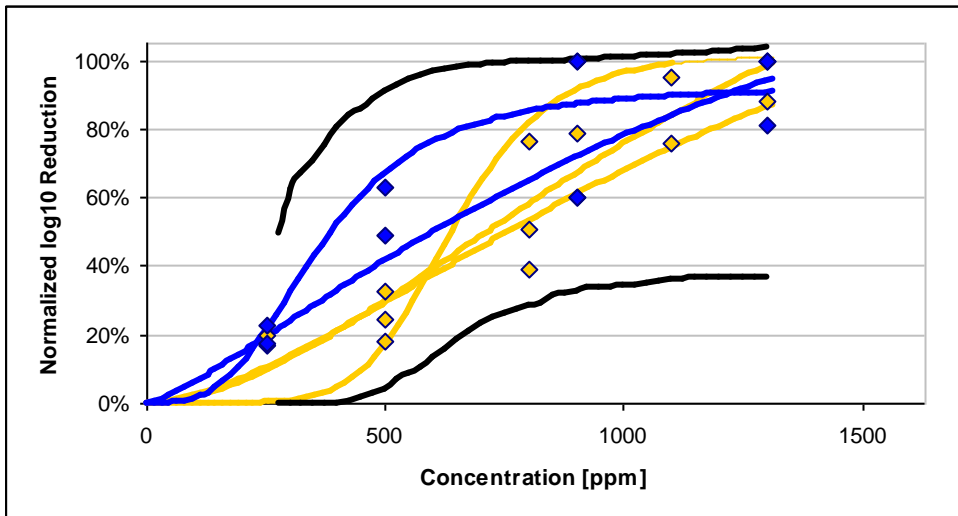
**Figure 72: Mean log<sub>10</sub> reductions with inactivation curves and 95%-prediction interval (black) of initial ring trial for *Staphylococcus aureus* vs. benzalkonium chloride – LC0026 (yellow: ring trial phase 1, red: part 1 of phase 2)**



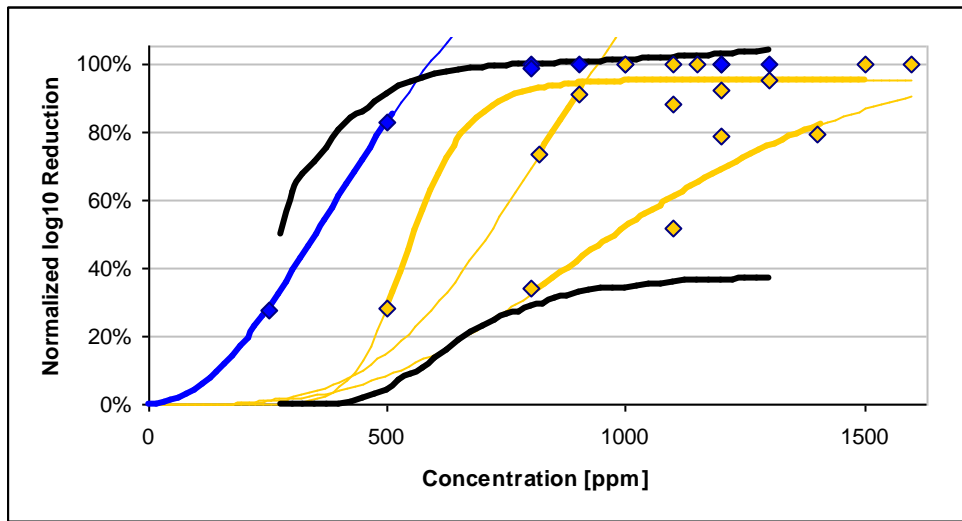
**Figure 73: Mean log<sub>10</sub> reductions with inactivation curves and 95%-prediction interval (black) of initial ring trial for *Staphylococcus aureus* vs. benzalkonium chloride – LC0028 (yellow: ring trial phase 1, red: part 1 of phase 2, blue: part 2 of phase 2)**



**Figure 74: Mean log<sub>10</sub> reductions with inactivation curves and 95%-prediction interval (black) of initial ring trial for *Staphylococcus aureus* vs. glutaraldehyde – LC0011 (yellow: ring trial phase 1, blue: part 2 of phase 2)**



**Figure 75: Mean log<sub>10</sub> reductions with inactivation curves and 95%-prediction interval (black) of initial ring trial for *Staphylococcus aureus* vs. glutaraldehyde – LC0021 (yellow: ring trial phase 1, blue: part 2 of phase 2)**



**Figure 76: Mean log<sub>10</sub> reductions with inactivation curves and 95%-prediction interval (black) of initial ring trial for *Staphylococcus aureus* vs. glutaraldehyde – LC0028 (yellow: ring trial phase 1, blue: part 2 of phase 2)**

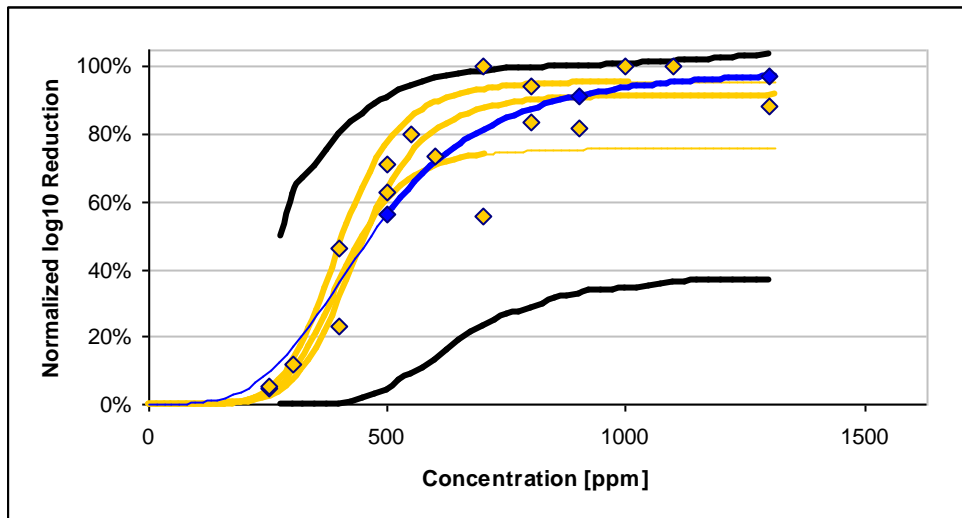


Figure 77: Mean log<sub>10</sub> reductions with inactivation curves and 95%-prediction interval (black) of initial ring trial for *Pseudomonas aeruginosa* vs. glutaraldehyde – LC0011 (yellow: ring trial phase 1, blue: part 3 of phase 2)

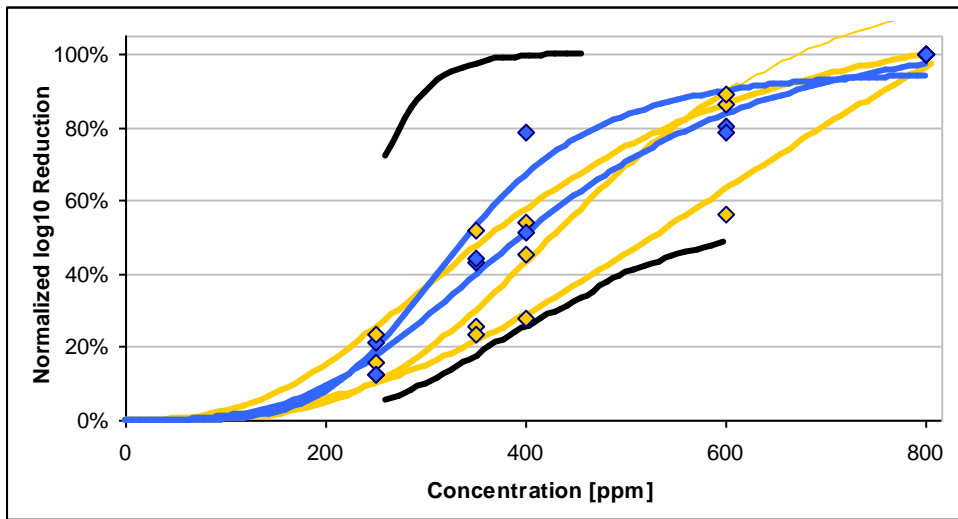
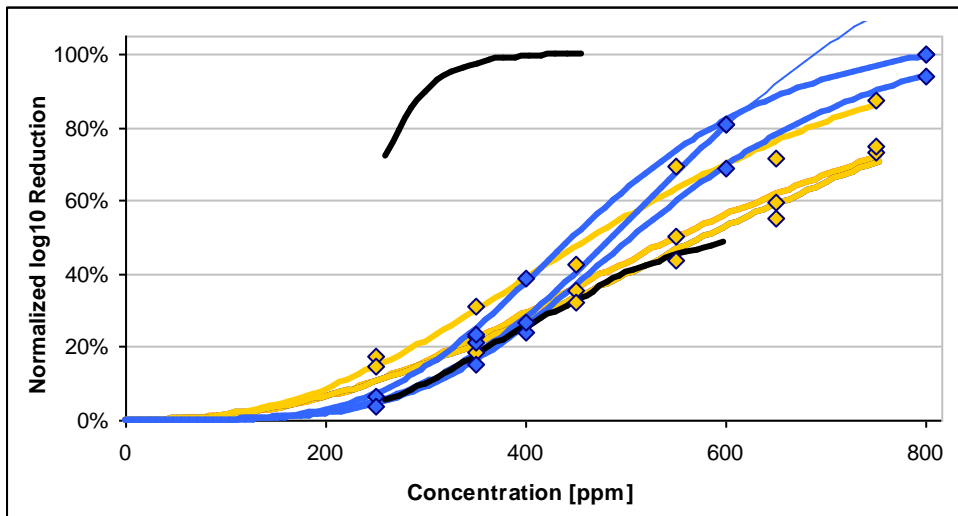
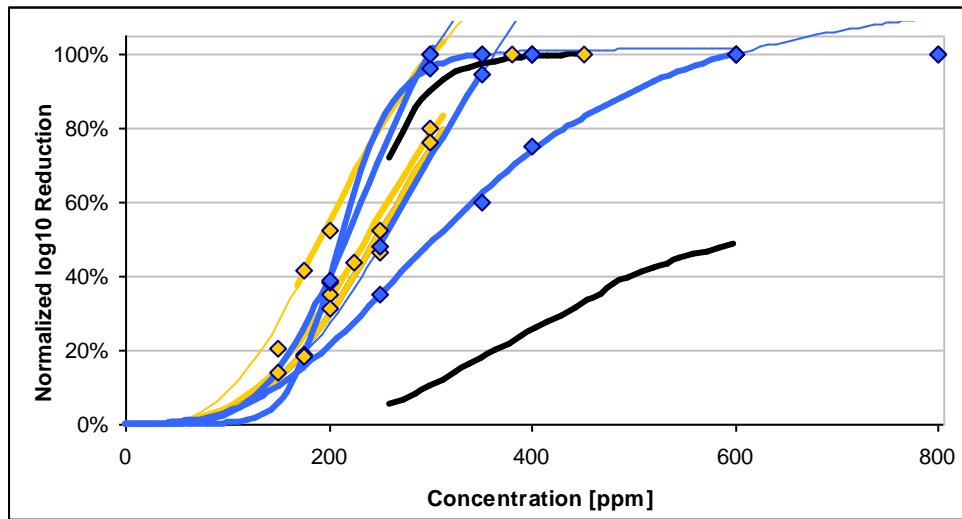


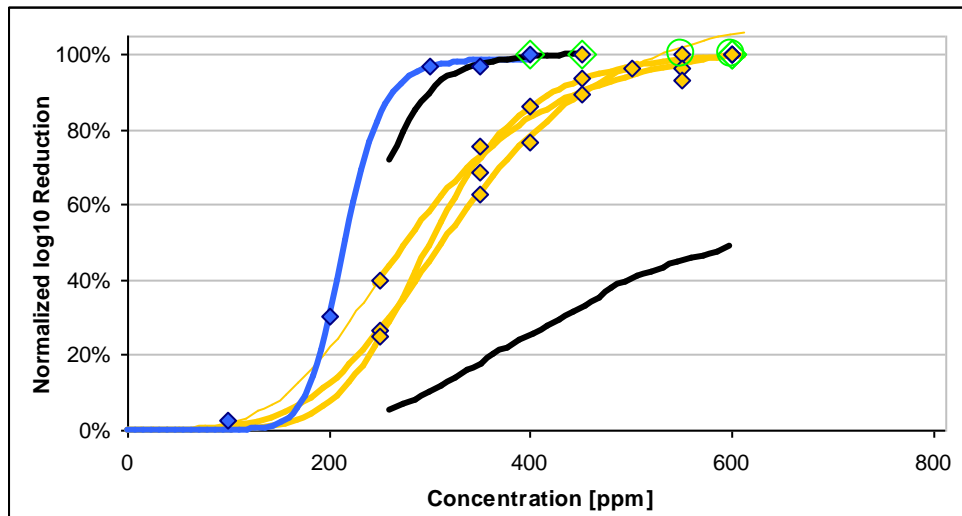
Figure 78: Mean log<sub>10</sub> reductions with inactivation curves and 95%-prediction interval (black) of initial ring trial for *Pseudomonas aeruginosa* vs. glutaraldehyde – LC0012 (yellow: ring trial phase 1, blue: part 3 of phase 2)



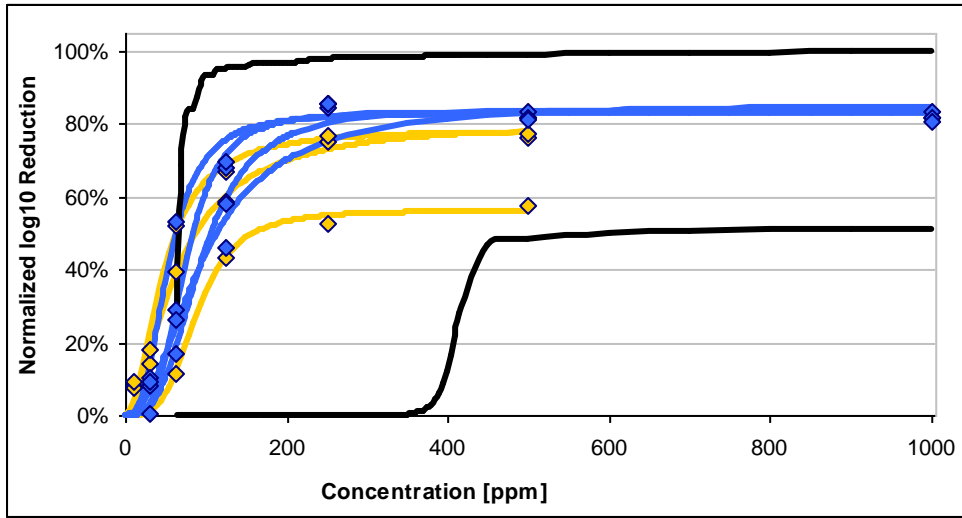
**Figure 79: Mean log<sub>10</sub> reductions with inactivation curves and 95%-prediction interval (black) of initial ring trial for *Pseudomonas aeruginosa* vs. glutaraldehyde – LC0021 (yellow: ring trial phase 1, blue: part 3 of phase 2)**



**Figure 80: Mean log<sub>10</sub> reductions with inactivation curves and 95%-prediction interval (black) of initial ring trial for *Pseudomonas aeruginosa* vs. glutaraldehyde – LC0028 (yellow: ring trial phase 1, blue: part 3 of phase 2)**



**Figure 81: Mean log<sub>10</sub> reductions with inactivation curves and 95%-prediction interval (black) of initial ring trial for *Adenovirus type 5* vs. glutaraldehyde – LC0006 (yellow: ring trial phase 1, blue: part 3 of phase 2)**



## Conclusions

180. The results of phase 2 confirm the results obtained in the initial ring trial in phase 1. The inactivation curves of phase 2 coincide very well with the prediction intervals determined in phase 1, i.e. reproducibility figures derived in phase 1 are confirmed by results of phase 2 almost perfectly well. Although, no significant improvements in the performances can be observed, also no change for the worse can be identified. It has to be noted however that almost no outliers were eliminated from the data of phase 2 – in phase 1 the rate of outliers was clearly higher. Taking into account a lower outlier rate, a somewhat higher reproducibility standard deviation may be accepted.
181. As there are no significant differences between the results of phase 1 and 2, they will be discussed together.
182. For assessing whether test methods can be considered as validated, the maximum absolute interlaboratory reproducibility standard deviation within the concentration range of the bootstrap may be consulted.
183. The following table summarizes for each combination of organism and substance the number of laboratories and the maximum absolute interlaboratory reproducibility standard deviation within the concentration range of the bootstrap. Combinations are highlighted in yellow if data of at least five laboratories were included in the analysis. Please note that these results could be determined for the initial ring trial in phase 1 and the confirmatory ring trial in phase 2 – part 3 only.
184. A possible rule for the assessment could be: If the average of the maximum absolute reproducibility standard deviations over all substances is not exceeding 30%, the method may be considered as validated for the organism tested (please remember that the response is expressed in percentages, therefore also the absolute standard deviations are expressed in percentages). If the reproducibility standard deviation exceeds 30%, the prediction interval for the measured normalized log<sub>10</sub> reduction can be very large and will always be close either to 0% or to 100% - or to both endpoints. This is apparent for *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* – for peracetic acid in the initial ring trial, but also for the measurements of *Pseudomonas aeruginosa* and glutaraldehyde in the confirmatory ring trial (part 3) in phase 2. For these combinations the measured normalized log<sub>10</sub> reduction could be used for a qualitative assessment but not for a reasonable quantitative assessment.
185. It should be noted that the statistical uncertainty of reproducibility figures is relatively high, as for 6 out of 15 combinations the minimum number of participating laboratories is below 6 – and even with more than 6 laboratories there is considerable variability in reproducibility figures if the test is repeated.
186. For *Adenovirus type 5*, *Aspergillus niger* and *Mycobacterium* the average of the maximum absolute reproducibility standard deviations over all substances is in a small range

between 19% and 24%. For *Bacillus subtilis*, *Enterococcus hirae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, the average is in the range between 27% and 33%. This difference could be considered an indication that testing bacteria is more complicated than testing other microorganisms. However, as the number of data is too small, these differences cannot be considered statistically significant.

187. Reproducibility figures indicate also that the test method may be less reproducible for peracetic acid than for other active substances. The average maximum absolute reproducibility standard deviation over all microorganisms is 31% and 23% for peracetic acid and the other active substances, respectively. However, this difference cannot be considered statistically significant.
188. Comparison of reproducibility figures with the respective results for the standard approach (see Annex *Results of phase 1 according to the standard approach*, p. 202) make it obvious that the assessment criterion is more often fulfilled for the normalized validation parameters. This indicates again that the variability of the log<sub>10</sub> reductions can be reduced if at first the logarithm of the CFU/carrier and the water control and then the ratio is taken as in the normalized approach.

**Table 38: Overview of validation parameters for initial ring trial in phase 1 and confirmatory ring trial in phase 2 – part 3**(yellow: combinations for which data from at least 5 laboratories was included in the analysis;  
red: combinations for which the interlaboratory reproducibility exceeds 30%)

Organism	Substance	Number of laboratories included in the analysis	Maximum absolute inter-laboratory reproducibility $S_R$
<i>Adenovirus type 5</i> (TCID <sub>50</sub> )	peracetic acid	10	26%
	glutaraldehyde	8	22%
<i>Aspergillus niger</i>	peracetic acid	4	29%
	glutaraldehyde	3	13%
<i>Bacillus subtilis</i>	<b>peracetic acid</b>	<b>5</b>	<b>33%</b>
<i>Enterococcus hirae</i>	benzalkonium chloride (confirmatory ring trial phase 2 – part 3)	6	28%
<i>Mycobacterium terrae</i>	phenol	5	17%
	peracetic acid	6	17%
	glutaraldehyde	5	23%
<i>Pseudomonas aeruginosa</i>	<b>peracetic acid</b>	<b>5</b>	<b>31%</b>
	glutaraldehyde (initial ring trial phase 1)	6	26%
	<b>glutaraldehyde (confirmatory ring trial phase 2 – part 3)</b>	<b>6</b>	<b>36%</b>
<i>Staphylococcus aureus</i>	<b>peracetic acid</b>	<b>12</b>	<b>36%</b>
	glutaraldehyde	10	26%
	benzalkonium chloride	11	20%

189. Of special interest is the performance of the methods for concentrations with high log<sub>10</sub> reduction factors to be expected, e.g. 80% for the normalized approach. The precision figures for this level are presented in the following table.

**Table 39: Overview of validation parameters at a concentration level at which a log reduction of 80% is expected for initial ring trial in phase 1 and confirmatory ring trial in phase 2 – part 3 (yellow: combinations for which data from at least 5 laboratories was included in the analysis; green: combinations for which the reproducibility s.d. of log<sub>10</sub> reductions does not exceed 20%)**

Organism	Substance	Mean concentration at 80% of the average water control [ppm]	Reproducibility s.d. of log <sub>10</sub> reductions	Repeatability s.d. of log <sub>10</sub> reductions	Intermediate s.d. of log <sub>10</sub> reductions
<i>Adenovirus type 5</i> (TCID <sub>50</sub> )	peracetic acid	200	16%	6%	6%
	glutaraldehyde	1100	14%	4%	4%
<i>Aspergillus niger</i>	peracetic acid	340	26%	21%	21%
	glutaraldehyde	13200 (at 70%)	11%	7%	10%
<i>Bacillus subtilis</i>	peracetic acid	1000	31%	14%	14%
<i>Enterococcus hirae</i>	benzalkonium chloride (confirmatory ring trial phase 2 – part 3)	2360	21%	10%	10%
<i>Mycobacterium terrae</i>	phenol	15200	8%	3%	3%
	peracetic acid	680	18%	10%	10%
	glutaraldehyde	22200 (at 70%)	22%	16%	17%
<i>Pseudomonas aeruginosa</i>	peracetic acid	125	22%	13%	13%
	glutaraldehyde	490	22%	7%	7%
	glutaraldehyde (confirmatory ring trial phase 2 – part 3)	430	26%	5%	5%
<i>Staphylococcus aureus</i>	peracetic acid	90	29%	8%	8%
	glutaraldehyde	1050	19%	11%	11%
	benzalkonium chloride	3350	17%	13%	15%

190. If the average of the absolute reproducibility standard deviation over all substances is not exceeding 20% (corresponds to “25% of 80%”), the method may be considered as validated for the organism tested. This is a stricter criterion than the criterion of the preceding paragraphs. Failing this criterion does not necessarily mean that the test methods are not applicable. However, variability is relatively large, so that assessments based on only one or two laboratories will be not very reliable.

191. In the following Figure 82 the correlation charts for the laboratory deviations of sensitivity ( $\lambda$ ) for organisms *Pseudomonas aeruginosa* and *Staphylococcus aureus* are shown. Only for these two organisms data from at least four laboratories that had tested either a combination of active substances for one organism or the combination of both organisms were available. For example, six laboratories had tested *Staphylococcus aureus* vs. glutaraldehyde and also *Pseudomonas aeruginosa* vs. glutaraldehyde. The highlighted sections mark the organism-specific comparisons: yellow for *Pseudomonas aeruginosa* and blue for *Staphylococcus aureus*. The white fields mark the organism-comprehensive, i.e. the

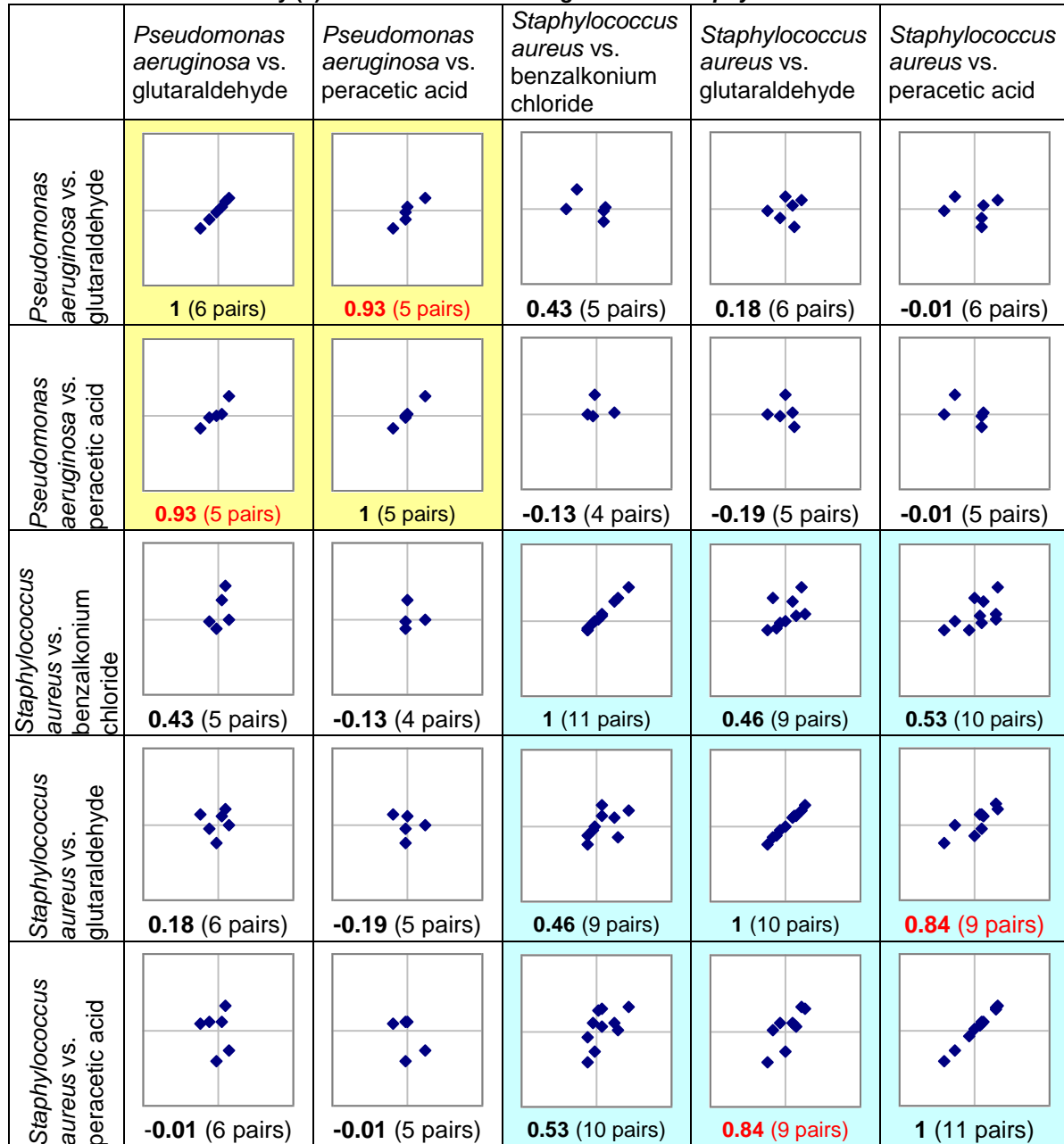
combination of both organisms, comparisons. Below each correlation chart, the correlation coefficient as well as the number of pairs, on which the analysis is based, are shown. Significant correlations are marked in red.

192. It can clearly be seen that there are only organism-specific correlations. For example, for *Pseudomonas aeruginosa*, there is a high positive correlation (0.93) between the deviations of sensitivity for glutaraldehyde and peracetic acid. It can therefore be concluded that if the laboratory deviations of sensitivity increase for glutaraldehyde, they also increase for peracetic acid and vice versa. For *Staphylococcus aureus* a similar result can be observed: the laboratory deviations of sensitivity between peracetic acid and glutaraldehyde are highly (and significantly) correlated. If the deviations of sensitivity increase for peracetic acid, they also increase for glutaraldehyde and vice versa. For the two other combinations of active substances for *Staphylococcus aureus* also positive correlations can be observed, however, the correlation coefficients are smaller and statistically not significant.
193. No organism-comprehensive correlations between the laboratory deviations of sensitivity can be observed. Except for the combination *Pseudomonas aeruginosa* vs. glutaraldehyde and *Staphylococcus aureus* vs. benzalkonium chloride, the respective absolute correlation coefficients are smaller than 0.2. Although, the correlation for the mentioned combination is higher, none of the correlation coefficients for the organism-comprehensive comparison are statistically significant.
194. The average over all correlation coefficient of sensitivities for microorganism-specific correlations is 0.69. In other words: on average 69% of the variance of the laboratory-specific sensitivity is due to microorganisms-specific effects. The average over all correlation coefficients of sensitivities for microorganism-comprehensive correlations is 0.045. In other words: on average only 4.5% of the variance of the laboratory-specific sensitivity is due to microorganisms-comprehensive effects. The remaining variance component of the laboratory-specific sensitivity, 26.5%, cannot be assigned to systematic effects.
195. Reproducibility can be substantially improved only by reducing microorganisms-specific effects. Microorganism-specific effects could be caused by different cultivation and shape of microorganisms. High correlation between different substances suggests using a reference substance in order to improve the test method: If test results of a reference substance suggest that the sensitivity of the microorganism is, say, one log lower than expected (in terms of the log<sub>10</sub> of the reduction), the same could be concluded for the test results of the product under consideration - as long as the log reduction for reference substance and test substance are similar.

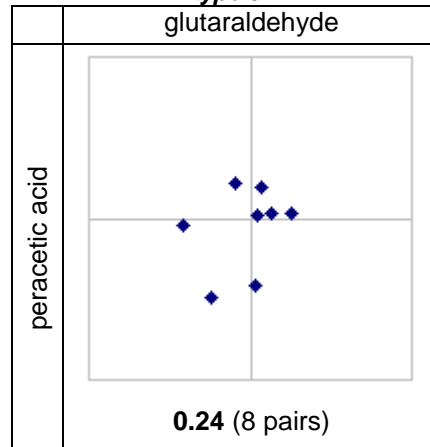
It should be noted that there are different options to make use of the test results of reference substances. Whether a correction reduces imprecision substantially, depends on the correlation between the reference substance and the test substance. More data (e.g. from proficiency tests) are required in order to decide under which conditions a correction can be carried out.

A guidance document should explain how to implement this suggestion.

**Figure 82: Organism-specific and organism-comprehensive correlation analysis of laboratory deviations of sensitivity ( $\lambda$ ) for *Pseudomonas aeruginosa* and *Staphylococcus aureus***



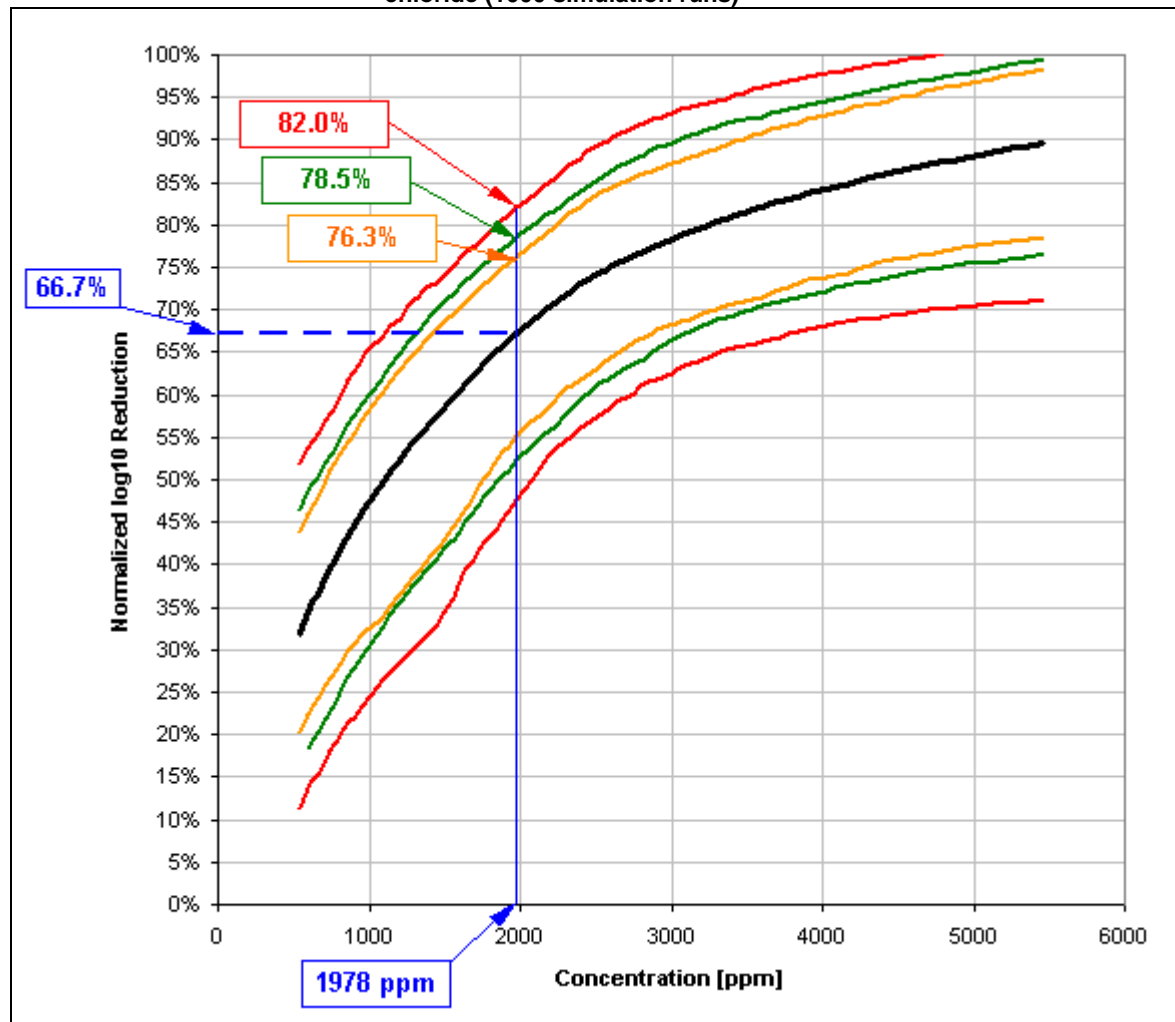
196. As mentioned before, no correlation analysis for the remaining organisms was possible due to missing data – except for *Adenovirus type 5*. The respective correlation chart is given in Figure 83. However, no significant correlation between the laboratory deviations of sensitivity of peracetic acid and glutaraldehyde can be observed.

**Figure 83: Organism-specific correlation analysis of laboratory deviations of sensitivity ( $\lambda$ ) for *Adenovirus type 5***

197. Generally it may be concluded that despite the extensive attempts to standardize the tests, only minor improvements could be obtained. An essential characteristic of the whole validation process is the fact that not the insufficiently homogenized analytical methods are responsible for high variability of results, but biological variability itself. It is a fundamental problem that a high precision of data always depends on the current condition of the test organism.
198. Variability can only be minimized when tests are repeated in different laboratories. The analysis of the results from the initial ring trial in phase 1 suggests that measurement values from three laboratories might be necessary in order to ensure reasonably verified precision figures. The results of *Staphylococcus aureus* with benzalkonium chloride are taken to illustrate the necessity of three laboratories. In the following Figure 84, the overall inactivation curve is shown with the 90% prediction intervals for one, two and three laboratories. It is obvious that the prediction intervals become narrower with an increasing number of laboratories. At a concentration of 5000 ppm an inactivation of 87% is reached. The prediction interval for one laboratory at this concentration is about 70% - 100%, i.e. with one laboratory only it is not possible to distinguish between 70% log reduction and complete kill. With three laboratories, the respective prediction interval is about 78% - 97%. In this example it has been assumed that three runs have been carried out in each laboratory. In case of a reduced number of runs per laboratory, the size of the prediction band will increase.
199. If the outcome of the assessment is only “yes” or “no”, results from only one or two laboratories might be sufficient, as long as at least three runs are taken into account: Such a qualitative statistical decision rule, also referred to as hypothesis testing, is examining whether the true, unknown log reduction (which is not equivalent with the “measured” log reduction) is below or above a given limit. Only if the measured log reduction is significantly exceeding this limit, it can be concluded that the log reduction is higher than the given limit. The decision is depending on the upper prediction limit of the measured log reduction under the assumption that the true log reduction equals the given limit. An example is given in the next paragraph.

200. Significance limits for a qualitative statistical decision rule are always depending on organism and substance. For a qualitative assessment of *Staphylococcus aureus* and benzalkonium chloride the limits can be derived from Figure 84: If the log starting titre equals six and the limit equals four, the relative limit is 66.7%. This limit corresponds to 1978 ppm, and the respective upper 90% prediction interval of the normalized inactivation curve for three laboratories equals 76.3% or 4.58 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 82.0% or 4.92 in absolute terms. This is clearly higher than the upper limit for two or three laboratories. It should be noted that for other substances and organisms this prediction interval is considerably wider than for *Staphylococcus aureus* and benzalkonium chloride.

**Figure 84: Overall inactivation curve with 90%-prediction intervals for one (red), two (green) and three (yellow) laboratories (each with three independent runs) for *Staphylococcus aureus* and benzalkonium chloride (1000 simulation runs)**



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## Symbols and abbreviations

<b>a</b>	Intercept parameter
<b>A. niger</b>	<i>Aspergillus niger</i>
<b><math>\alpha_i(c)</math></b>	Laboratory specific deviation of the inactivation curve of laboratory i from the overall inactivation curve $\mu(c)$
<b>ATCC</b>	American Type Culture Collection
<b>AWC</b>	Average log10 of the water controls
<b>b</b>	Slope parameter
<b>B</b>	Parameter of steepness of the inactivation curve
<b><math>\beta_{ij}(c)</math></b>	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
<b>c</b>	Concentration level
<b>C</b>	Mean concentration at which the inactivation curve is changing from being convex to concave
<b>CFU</b>	Colony forming unit
<b>CFU<sub>0</sub></b>	CFU of control carrier
<b>CFU<sub>c</sub></b>	CFU of test carrier
<b>conc</b>	Concentration
<b>CREM</b>	Centre for Research on Environmental Microbiology
<b>Ctrl</b>	Control
<b>D</b>	Asymptote of the inactivation curve
<b>Dr.</b>	Doctor
<b>DVV</b>	Deutsche Vereinigung zur Bekämpfung der Viruskrankheiten
<b><math>e_{ij}(c)</math></b>	Random deviation of laboratory i at run j and concentration c from the respective inactivation curve $\mu(c) + \alpha_i(c) + \beta_{ij}(c)$
<b>GLP</b>	Good laboratory practice
<b>H<sub>2</sub>O<sub>2</sub></b>	Hydrogen peroxide
<b>ISO</b>	International Organisation of Standardization
<b><math>\lambda</math></b>	Sensitivity of the inactivation curves
<b>LG</b>	Logarithm to the base 10
<b>log</b>	Logarithm to the base 10
<b>M</b>	Location parameter of the logit model
<b>max</b>	Maximum
<b>min</b>	Minimum
<b>mL</b>	Millilitre
<b><math>\mu\text{m}</math></b>	Micrometer
<b><math>\mu(c)</math></b>	Overall inactivation curve at concentration level c

<b>N/A</b>	No test result available.
<b>No.</b>	Number
<b>OECD</b>	Organisation for Economic Co-Operation and Development
<b>p</b>	Survival probability
<b>PBS</b>	Phosphate buffered saline
<b>PFU</b>	Plaque forming unit
<b>ppm</b>	Parts per million
<b>Prof.</b>	Professor
<b>R</b>	Mean (normalized) log <sub>10</sub> reduction
<b>R<sub>control</sub></b>	Mean log <sub>10</sub> reduction for the control carriers
<b>Rel.</b>	Relative
<b>R<sub>ij(c)</sub></b>	Mean (normalized) log <sub>10</sub> reduction of laboratory i at run j
<b>RSD</b>	Residual standard deviation
<b>S</b>	Slope parameter of the logit model
<b>S. aureus</b>	<i>Staphylococcus aureus</i>
<b>s.d.</b>	Standard deviation
<b>s<sub>i</sub></b>	Intra-laboratory reproducibility over time (ISO 5725: Intermediate standard deviation)
<b>s<sub>R</sub></b>	Inter-laboratory reproducibility (ISO 5725: Reproducibility standard deviation)
<b>s<sub>r</sub></b>	Intra-laboratory reproducibility within one run (ISO 5725: Repeatability standard deviation)
<b><math>\sigma_r^2</math></b>	Repeatability variance
<b><math>\sigma_\alpha^2(c)</math></b>	Variance of the laboratory specific deviation at the concentration level c
<b><math>\sigma_\beta^2(c)</math></b>	Variance of the run specific deviation at the concentration level c
<b><math>\sigma_L^2(c)</math></b>	True, but unknown variance between laboratories at the concentration level c
<b><math>\hat{\sigma}_L^2(c)</math></b>	Estimator of $\sigma_L^2(c)$
<b><math>\sigma_{run}^2(c)</math></b>	True, but unknown variance between runs at the concentration level c
<b><math>\hat{\sigma}_{run}^2(c)</math></b>	Estimator of $\sigma_{run}^2(c)$
<b>TCID<sub>50</sub></b>	50% tissue culture infectious dose
<b>TMTC</b>	Too many colonies to be counted.
<b>TSB</b>	Tryptic Soy Broth
<b>U.S.</b>	United States
<b>UK</b>	United Kingdom
<b>vol</b>	Volume

<b>vs.</b>	Versus
<b>wt</b>	Weight
<b>x</b>	Depending variable
<b>y</b>	Response variable

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## Annex

### **Pre-validation report (by quo data GmbH, 8<sup>th</sup> January 2008)**

#### ***Test validation***

The analysis of the pre-validation data aims on providing criteria for establishing an appropriate ring trial design. Therefore at first models for the relationship between concentration and the log<sub>10</sub> reduction (R), are derived. With these models, e.g., appropriate concentrations for the ring trial can be obtained. Within the statistical analysis, also sources of errors which may be relevant for the ring trial are identified. In addition, the concentration-R curve can also be used to identify implausible data.

#### ***Methods***

In one laboratory, up to 5 different test formulations have been tested on 6 different kinds of organisms. For each organism/formulation combination are 3 CFU values for the control carrier and up to 3 CFU values for the test carrier available.

The contact time in all experiments was 5 minutes.

The following table gives an overview of the organism/formulation combinations analysed in the pre-validation.

**Table 40: Combinations of organisms and formulations in pre-validation**

<b>Method</b>	<b>Test organism</b>	<b>Test formulation</b>
<b>Bactericidal</b>	<i>Staphylococcus aureus</i>	benzalkonium chloride
	<i>Staphylococcus aureus</i>	peracetic acid
	<i>Staphylococcus aureus</i>	sodium hypochlorite
	<i>Staphylococcus aureus</i>	glutaraldehyde
	<i>Staphylococcus aureus</i>	accelerated H <sub>2</sub> O <sub>2</sub>
<b>Yeasticidal</b>	<i>Candida albicans</i>	benzalkonium chloride
	<i>Candida albicans</i>	peracetic acid
	<i>Candida albicans</i>	sodium hypochlorite
	<i>Candida albicans</i>	glutaraldehyde
<b>Fungicidal</b>	<i>Aspergillus niger</i>	benzalkonium chloride
	<i>Aspergillus niger</i>	peracetic acid
	<i>Aspergillus niger</i>	sodium hypochlorite
	<i>Aspergillus niger</i>	glutaraldehyde
	<i>Aspergillus niger</i>	accelerated H <sub>2</sub> O <sub>2</sub>
<b>Mycobactericidal</b>	<i>Mycobacterium terrae</i>	benzalkonium chloride
	<i>Mycobacterium terrae</i>	peracetic acid
	<i>Mycobacterium terrae</i>	sodium hypochlorite
	<i>Mycobacterium terrae</i>	glutaraldehyde
<b>Virucidal</b>	<i>Human Adenovirus type 5</i>	benzalkonium chloride
	<i>Human Adenovirus type 5</i>	glutaraldehyde
	<i>Human Adenovirus type 5</i>	peracetic acid
	<i>Human Adenovirus type 5</i>	sodium hypochlorite
<b>Sporicidal</b>	<i>Bacillus subtilis</i>	peracetic acid

## Results

### How to read the figures?

The following figures show the kill curves, i.e. the relationship between the concentration and the log<sub>10</sub> reduction  $R$  for each organism/formulation combination. Details on the statistical methods are given in section **Data evaluation**.

From the survival probability  $p$

$$\frac{\text{CFU of test carrier}}{\text{CFU of control carrier}} = \frac{\text{CFU}_c}{\text{CFU}_0} = p = \frac{1}{1 + \exp\{-a - b \cdot \ln(\text{conc})\}}$$

the log<sub>10</sub> reduction  $R$  is determined by

$$R = \log\left(\frac{\text{CFU}_0}{\text{CFU}_c}\right) = \log\left[1 + \exp\{-a - b \cdot \ln(\text{conc})\}\right]$$

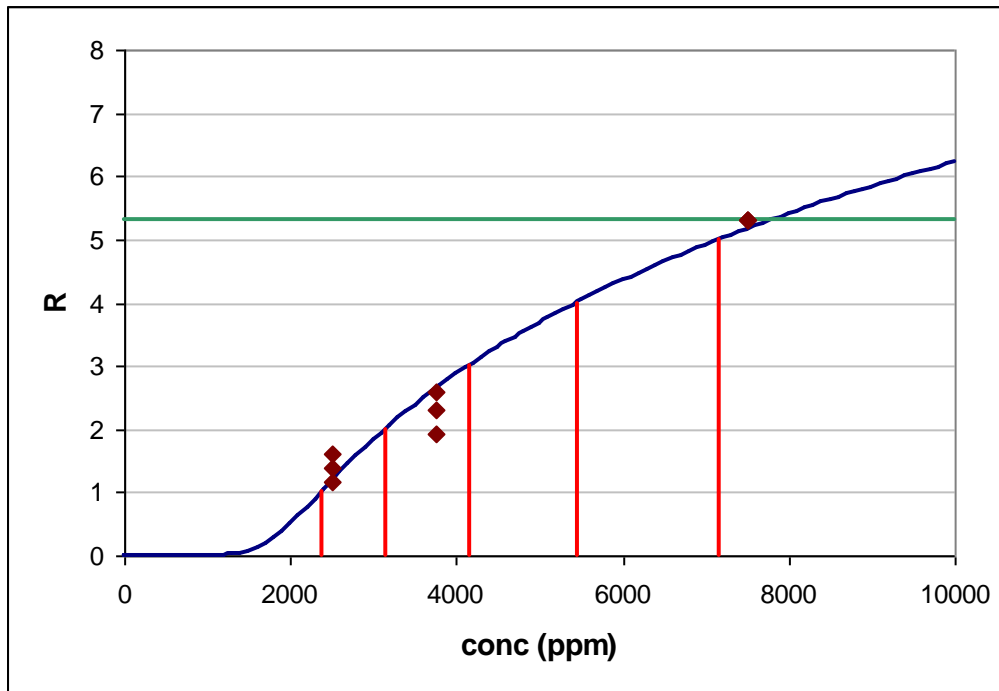
from which the respective concentration can be determined as

$$c_R = \exp\left\{-\frac{\ln(10^R - 1) + a}{b}\right\}.$$

The green lines in the figure identify the mean of the logarithmized CFU's of the control carrier (water control). The red solid lines show the log<sub>10</sub> reductions, their length equals  $R$  beginning on the left  $R=1$ , continuing with  $R=2$  and so on. The red squares are the log<sub>10</sub> reductions obtained in the actual experiment and finally the blue curve presents the estimated kill curve.

**Aspergillus niger**

**Figure 85: Estimated log10 reduction R depending on concentration for *Aspergillus niger* and accelerated H<sub>2</sub>O<sub>2</sub>**



**Figure 86: Estimated log10 reduction R depending on concentration for *Aspergillus niger* and benzalkonium chloride**

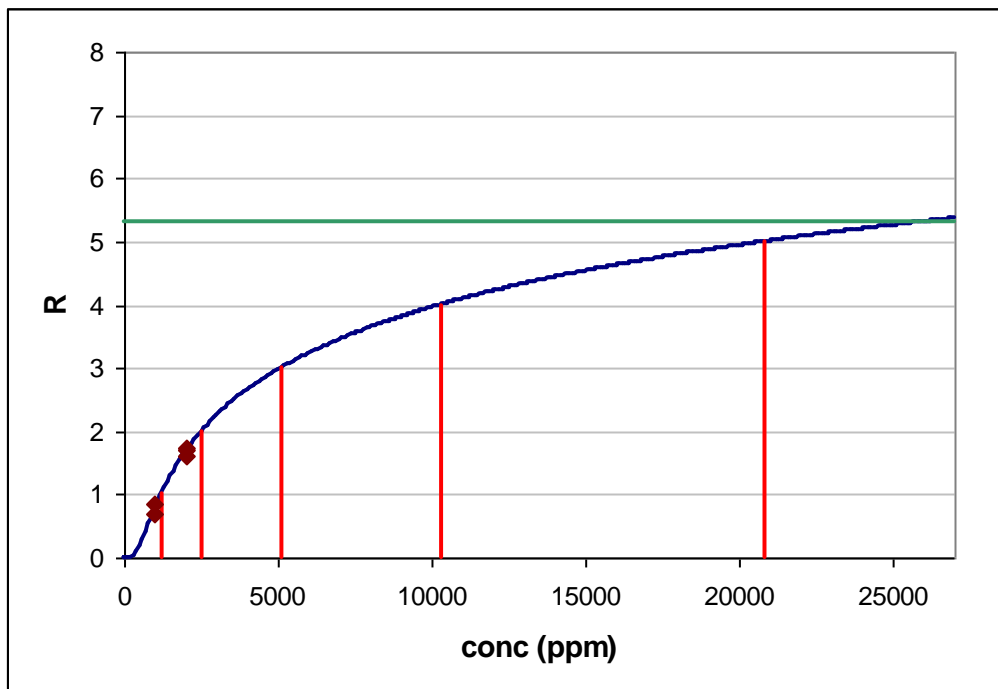


Figure 87: Estimated log<sub>10</sub> reduction R depending on concentration for *Aspergillus niger* and glutaraldehyde; experiments in July 2007

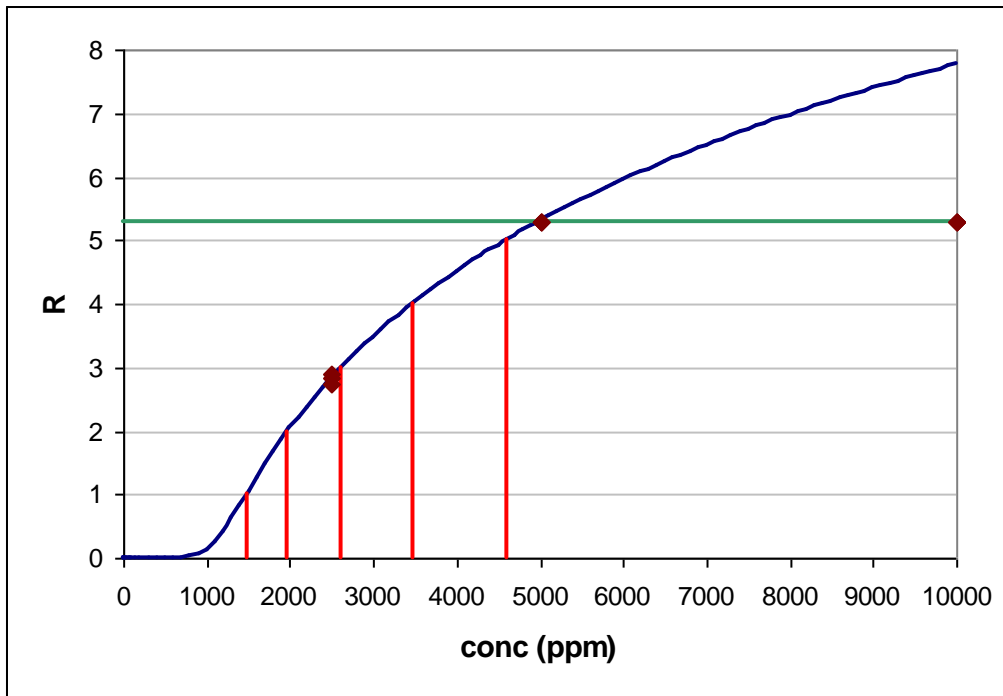


Figure 88: Estimated log<sub>10</sub> reduction R depending on concentration for *Aspergillus niger* and glutaraldehyde; repeated experiments in November 2007

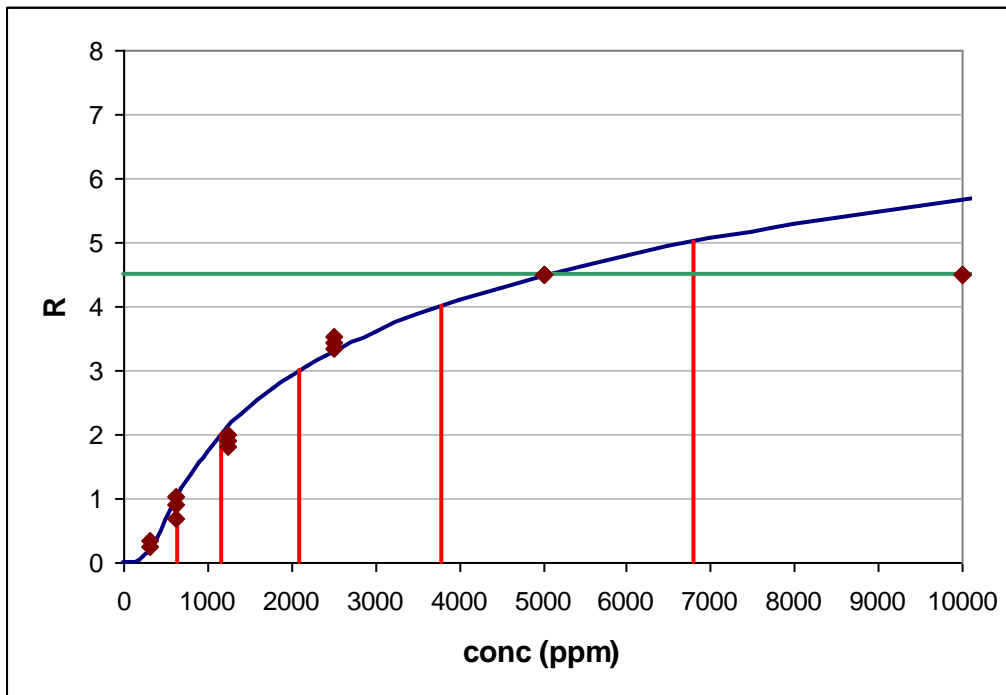


Figure 89: Comparison of estimated log<sub>10</sub> reduction R depending on concentration for *Aspergillus niger* and glutaraldehyde: experiment 1 in July 2007, experiment 2 in November 2007

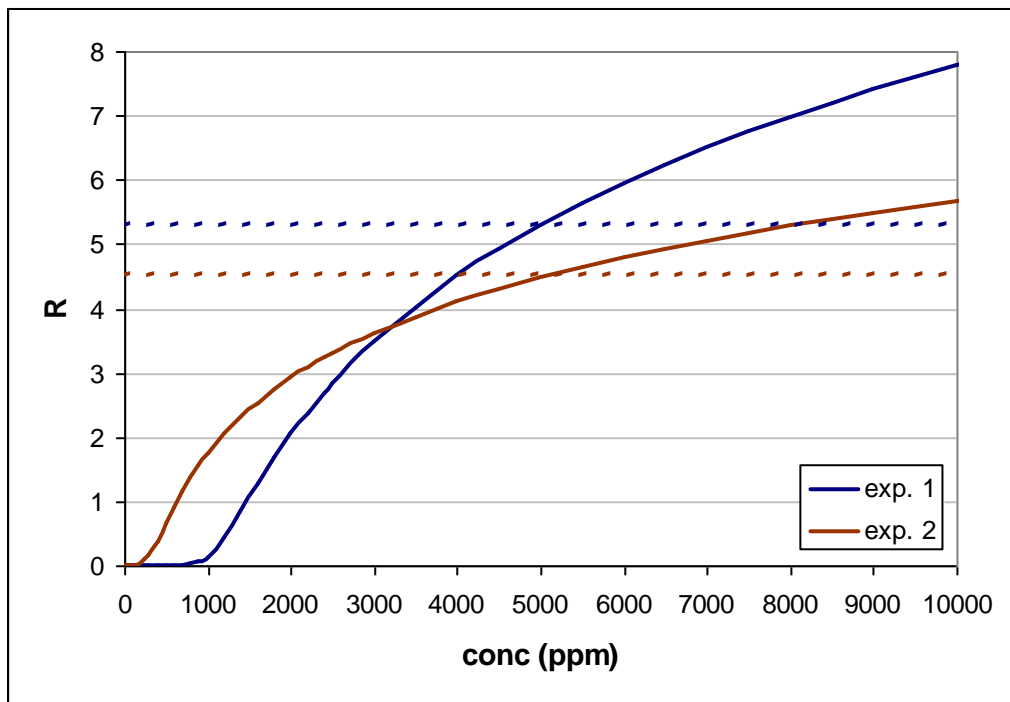
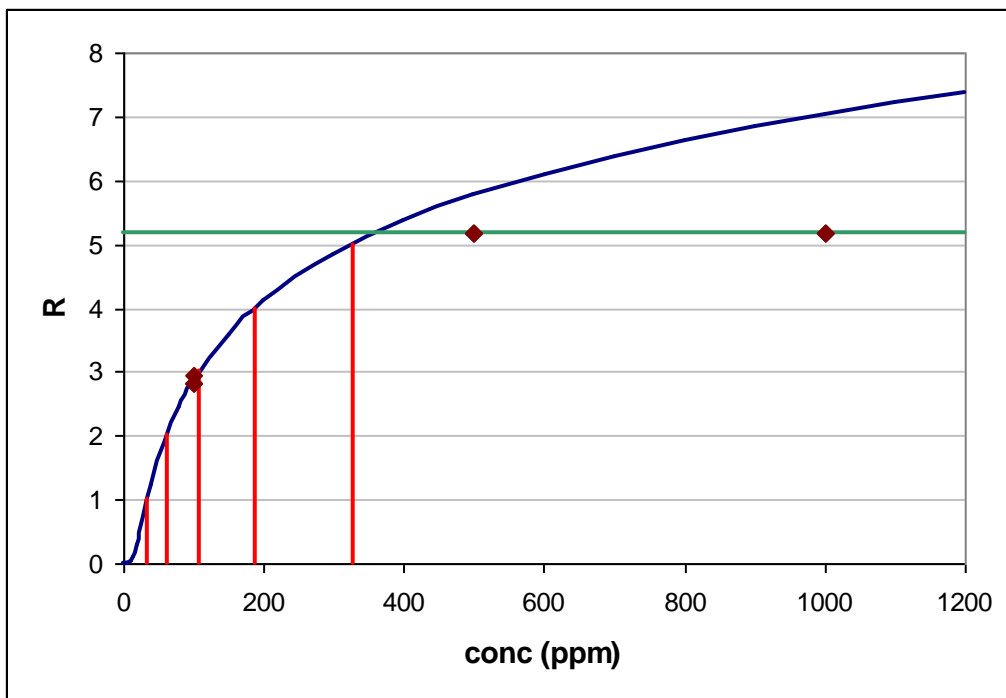
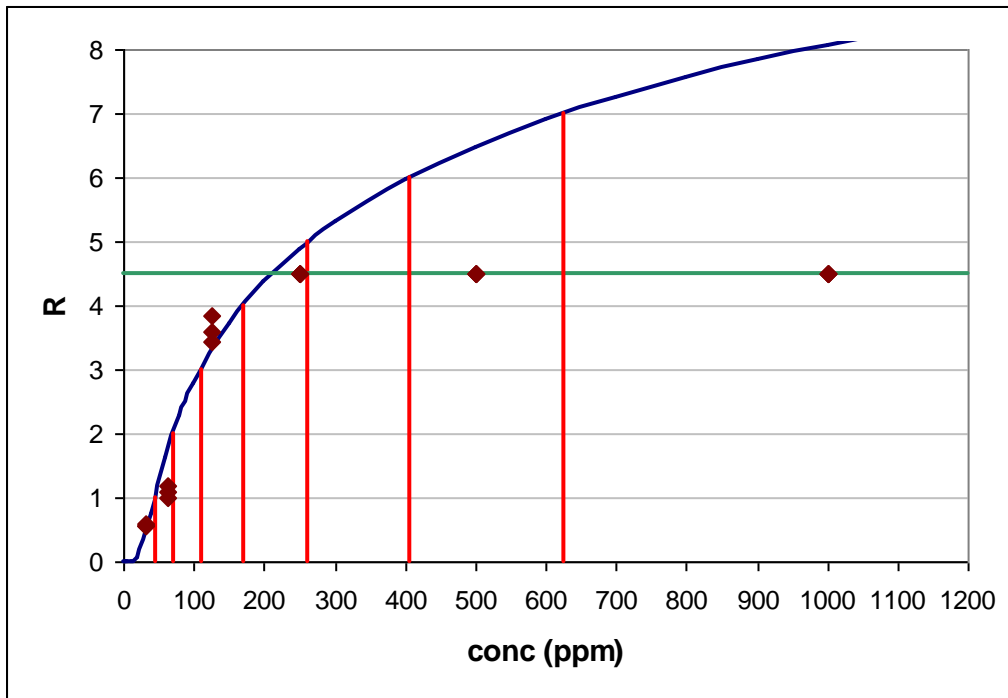


Figure 90: Estimated log<sub>10</sub> reduction R depending on concentration for *Aspergillus niger* and peracetic acid; experiments in July 2007



**Figure 91: Estimated log<sub>10</sub> reduction R depending on concentration for *Aspergillus niger* and peracetic acid; repeated experiments in November 2007**



**Figure 92: Comparison of estimated log<sub>10</sub> reduction R depending on concentration for *Aspergillus niger* and peracetic acid: experiment 1 in July 2007, experiment 2 in November 2007**

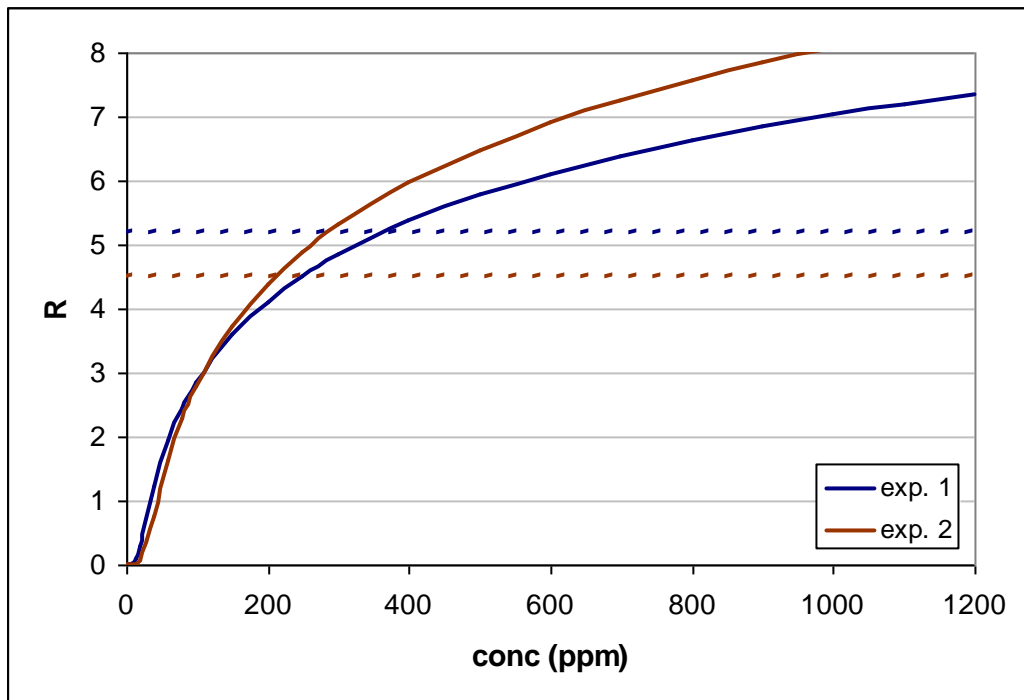
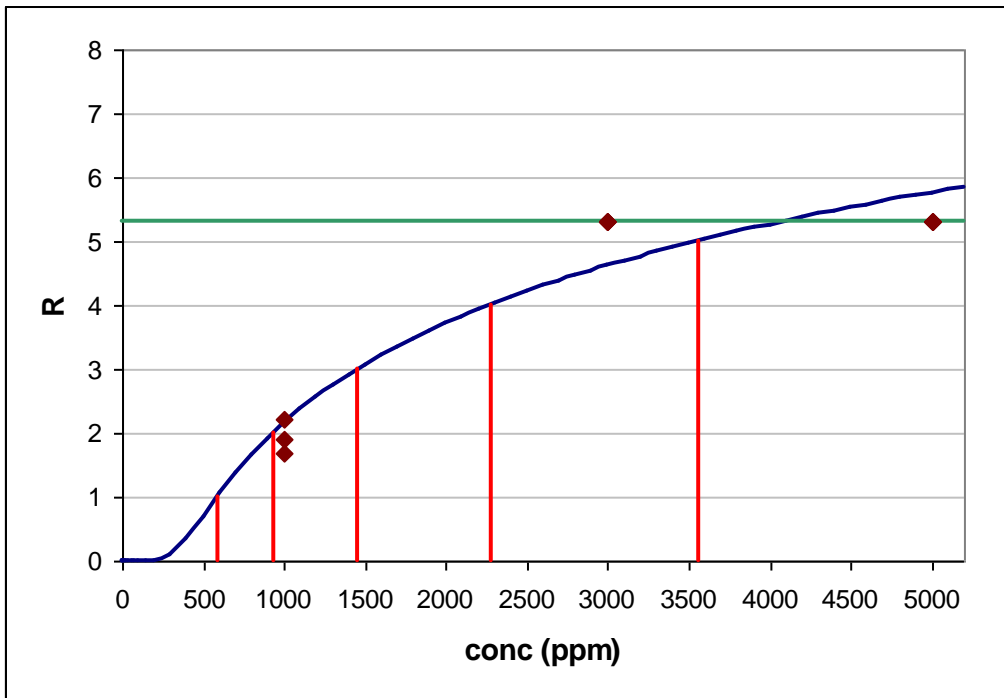


Figure 93: Estimated log<sub>10</sub> reduction R depending on concentration for *Aspergillus niger* and sodium hypochlorite



*Mycobacterium terrae*

Figure 94: Estimated log<sub>10</sub> reduction R depending on concentration for *Mycobacterium terrae* and glutaraldehyde

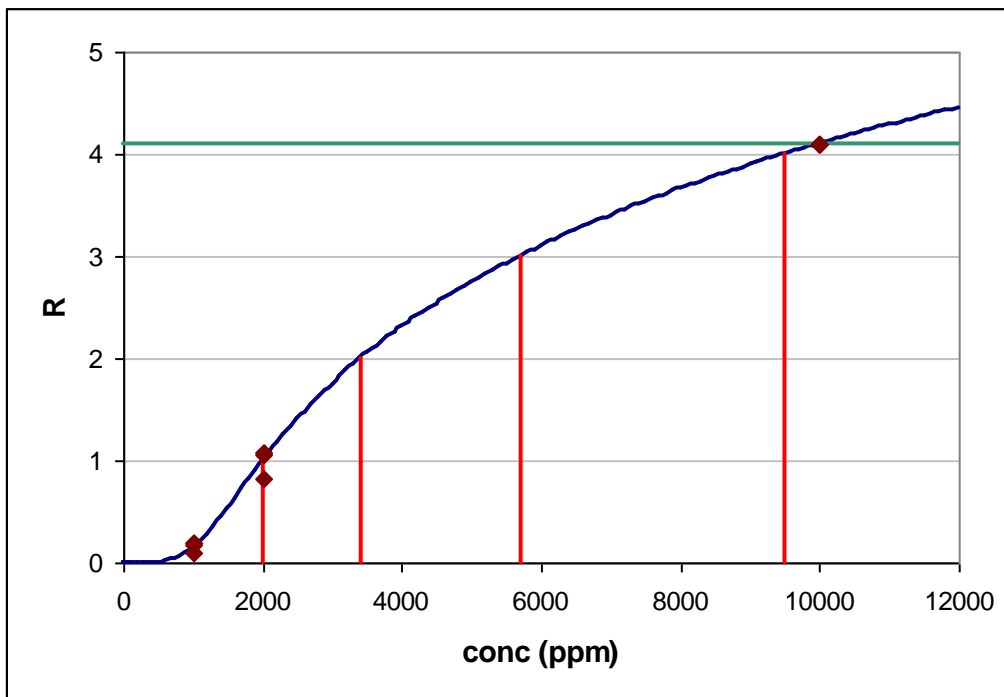


Figure 95: Estimated log<sub>10</sub> reduction R depending on concentration for *Mycobacterium terrae* and peracetic acid

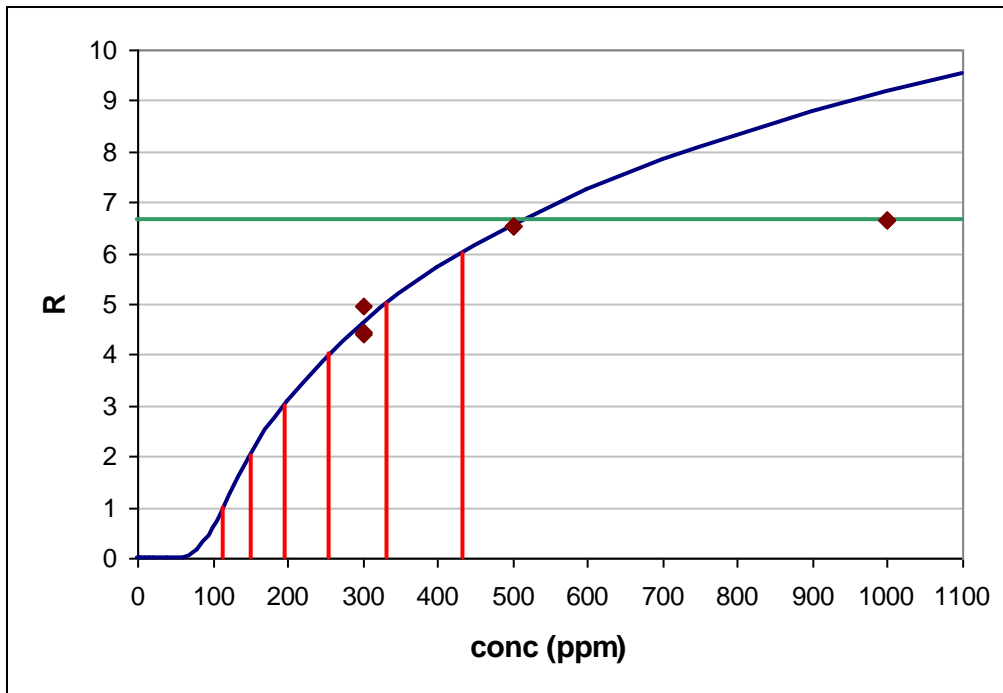


Figure 96: Estimated log<sub>10</sub> reduction R depending on concentration for *Mycobacterium terrae* and sodium hypochlorite

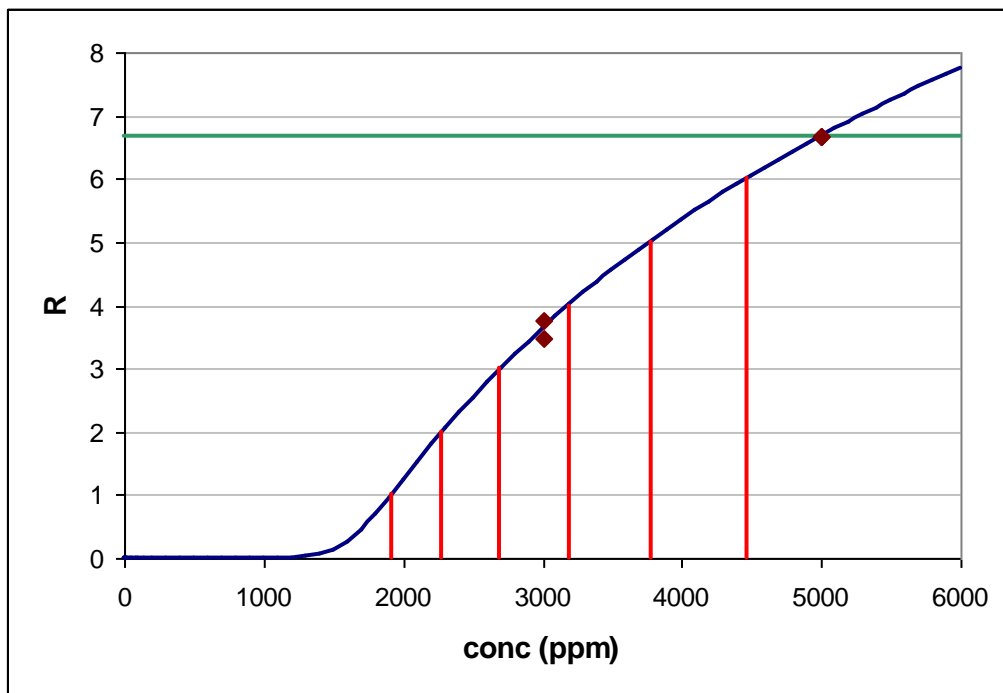
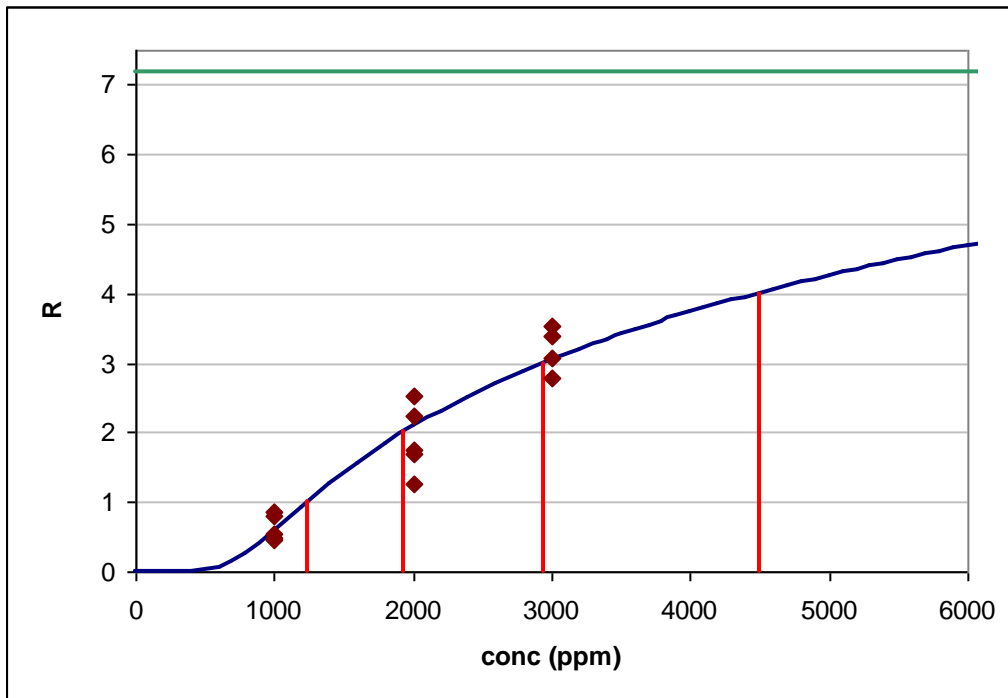


Figure 97: Former experiment (22/10/1999, 10 min contact time) - Estimated log<sub>10</sub> reduction R depending on concentration for *Mycobacterium terrae* and sodium hypochlorite



*Staphylococcus aureus*

Figure 98: Estimated log<sub>10</sub> reduction R depending on concentration for *Staphylococcus aureus* and accelerated H<sub>2</sub>O<sub>2</sub>

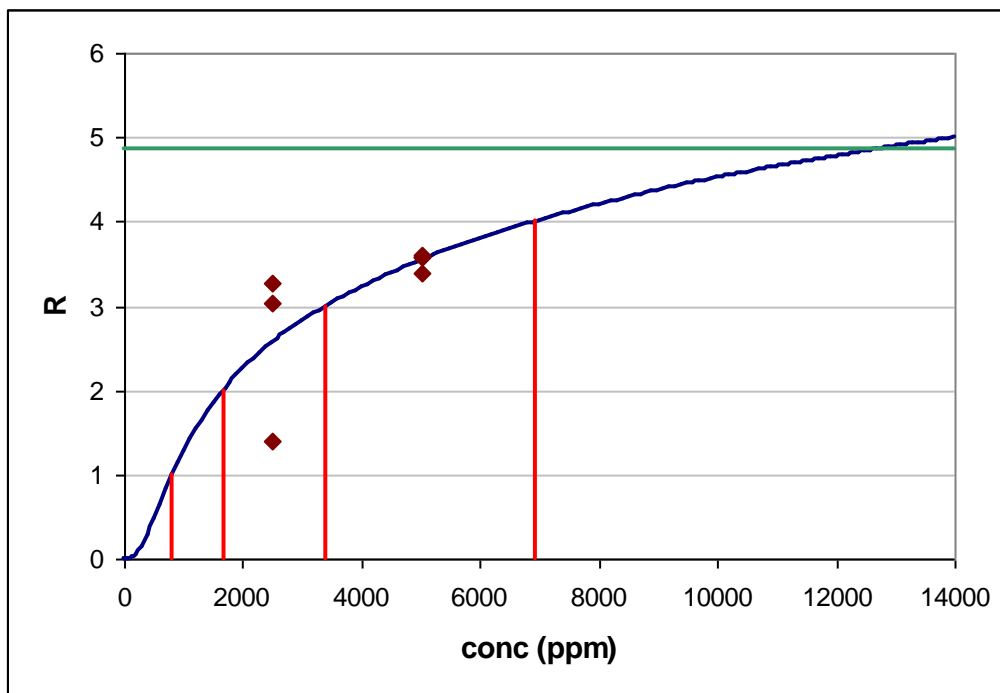


Figure 99: Estimated log<sub>10</sub> reduction R depending on concentration for *Staphylococcus aureus* and benzalkonium chloride

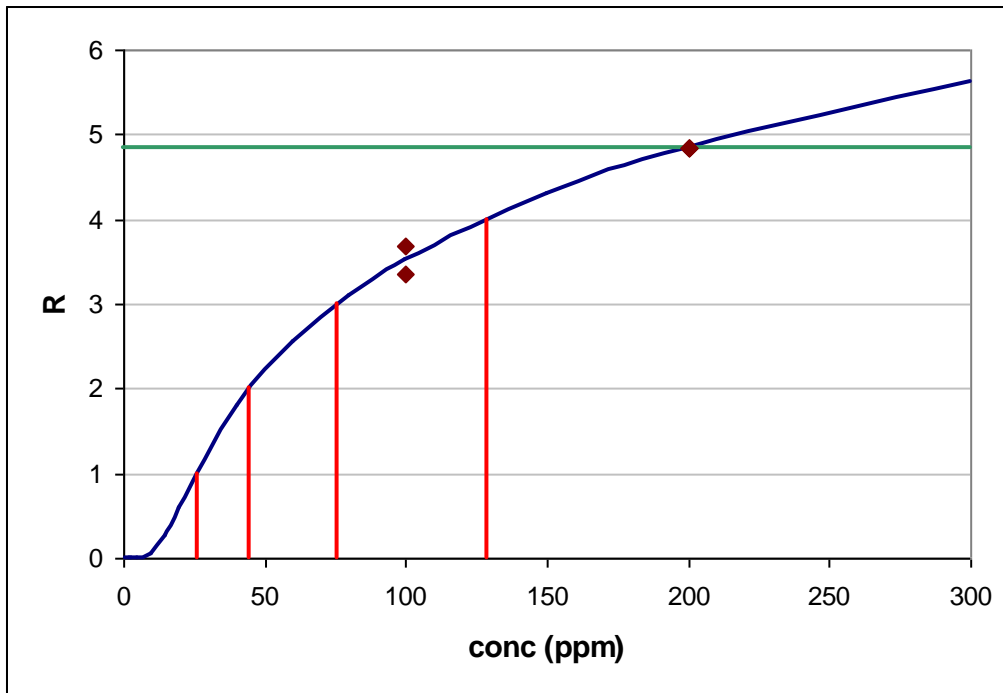


Figure 100: Estimated log<sub>10</sub> reduction R depending on concentration for *Staphylococcus aureus* and glutaraldehyde

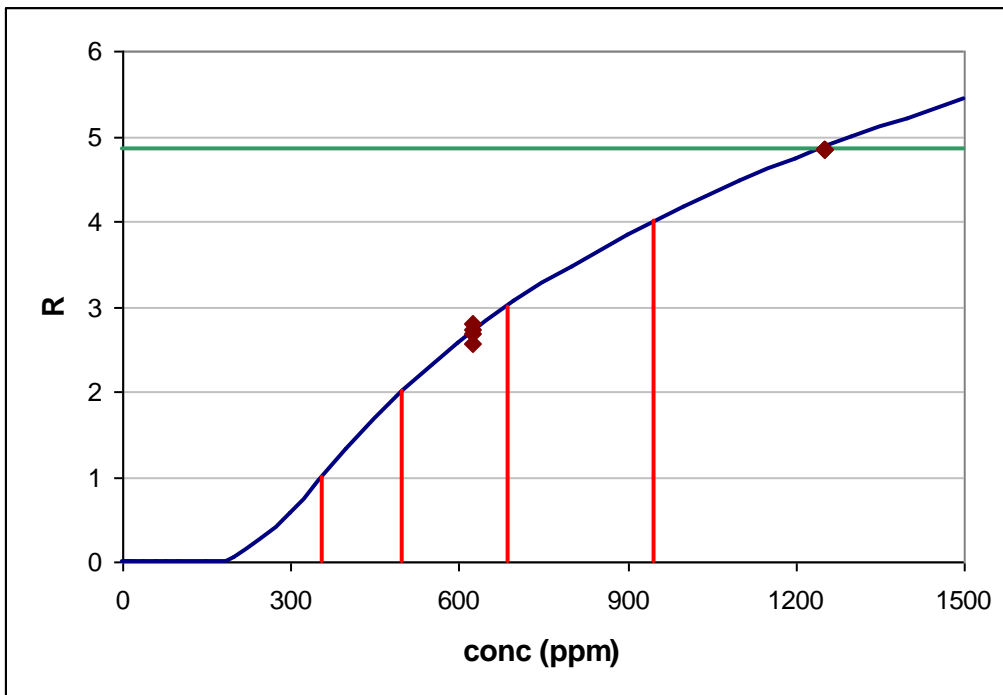


Figure 101: Estimated log<sub>10</sub> reduction R depending on concentration for *Staphylococcus aureus* and peracetic acid

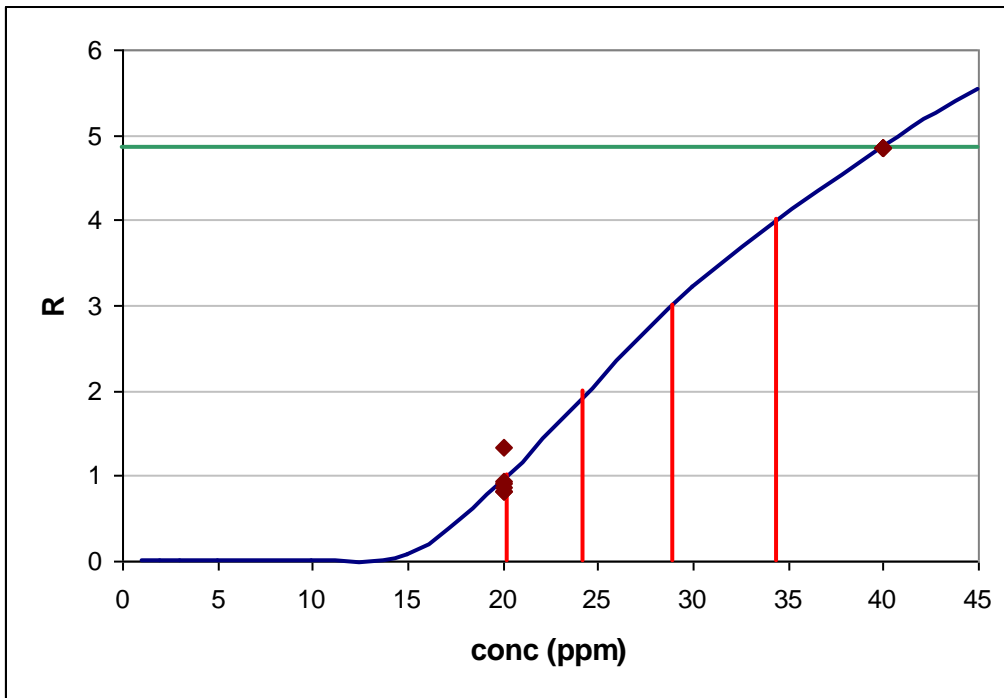
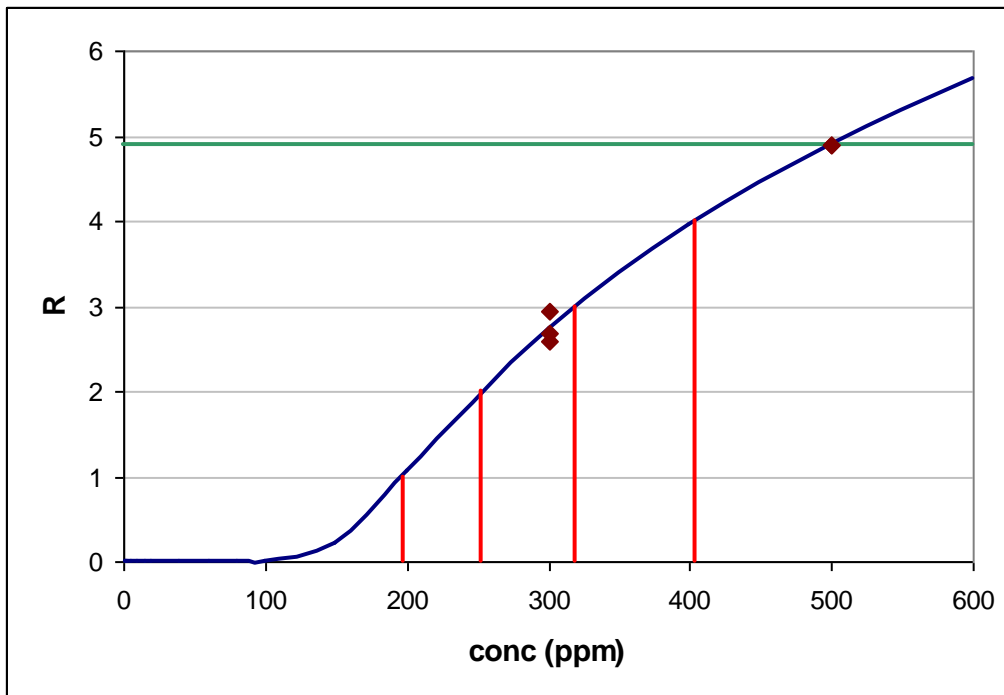
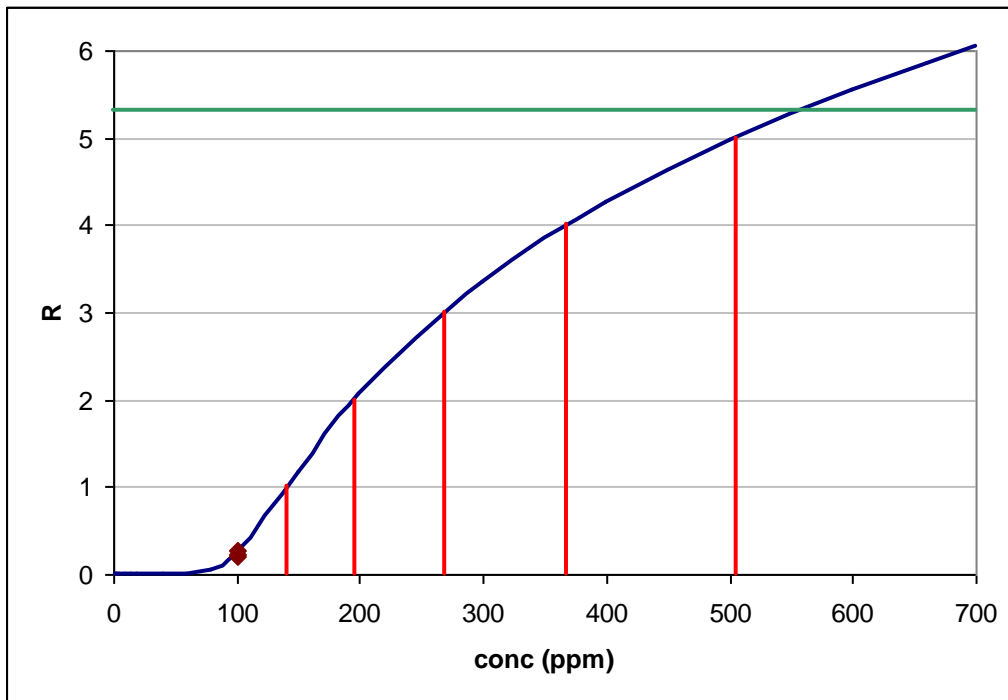


Figure 102: Estimated log<sub>10</sub> reduction R depending on concentration for *Staphylococcus aureus* and sodium hypochlorite



**Candida albicans**

**Figure 103: Estimated log10 reduction R depending on concentration for *Candida albicans* and benzalkonium chloride**



**Figure 104: Estimated log10 reduction R depending on concentration for *Candida albicans* and glutaraldehyde**

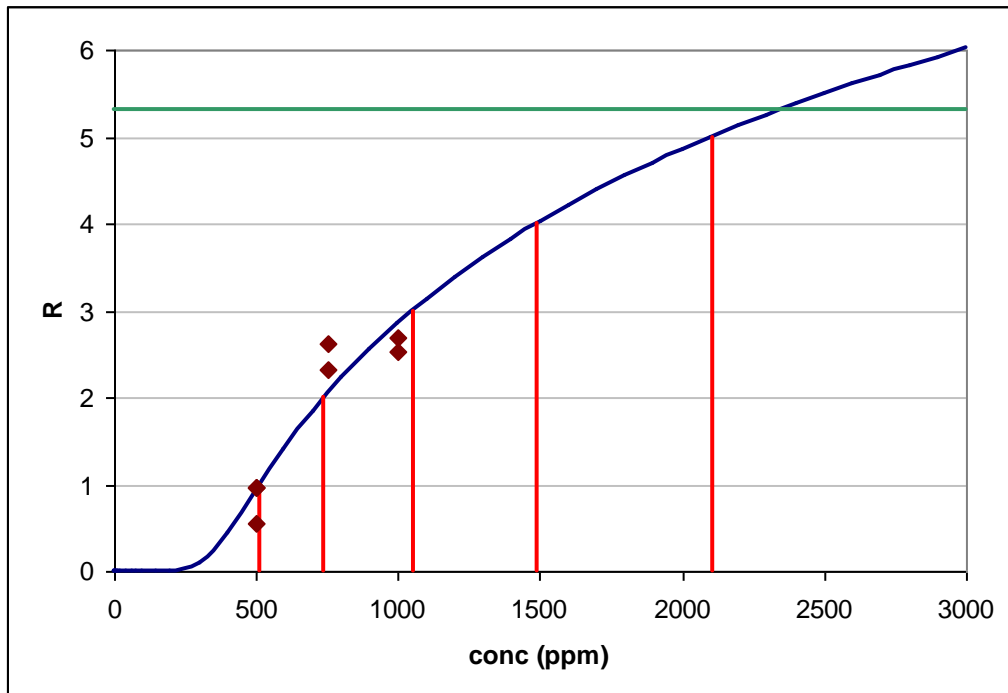


Figure 105: Estimated log<sub>10</sub> reduction R depending on concentration for *Candida albicans* and sodium hypochlorite

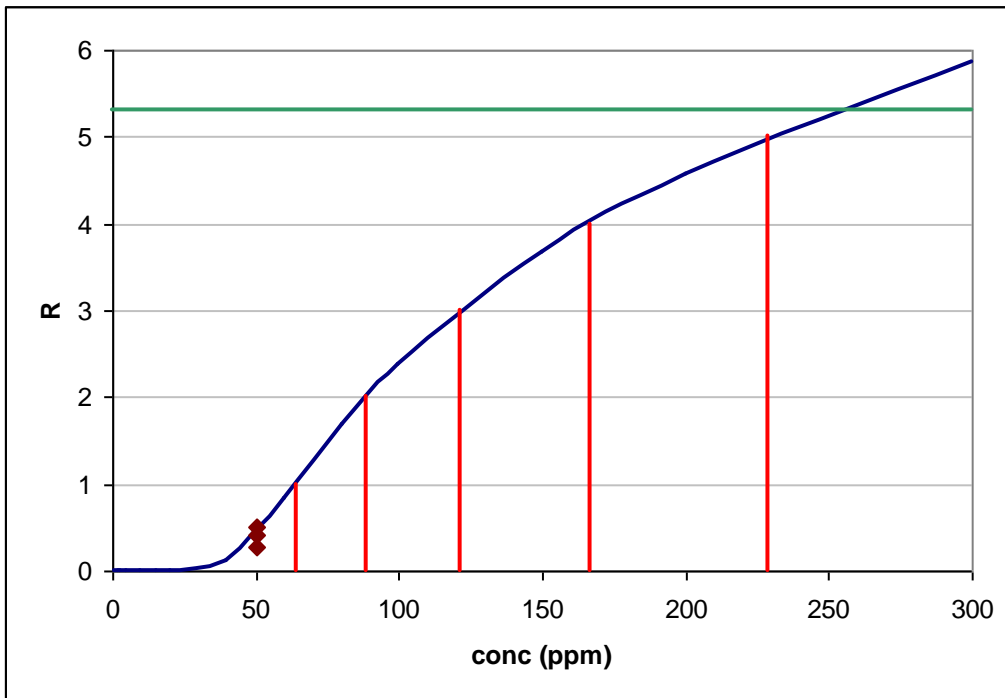
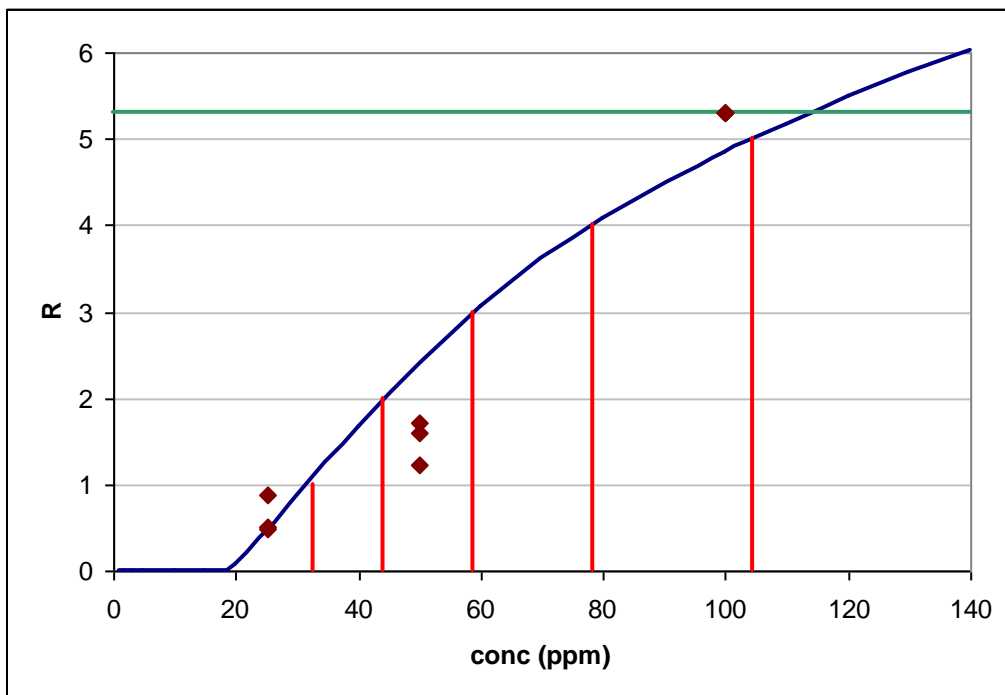
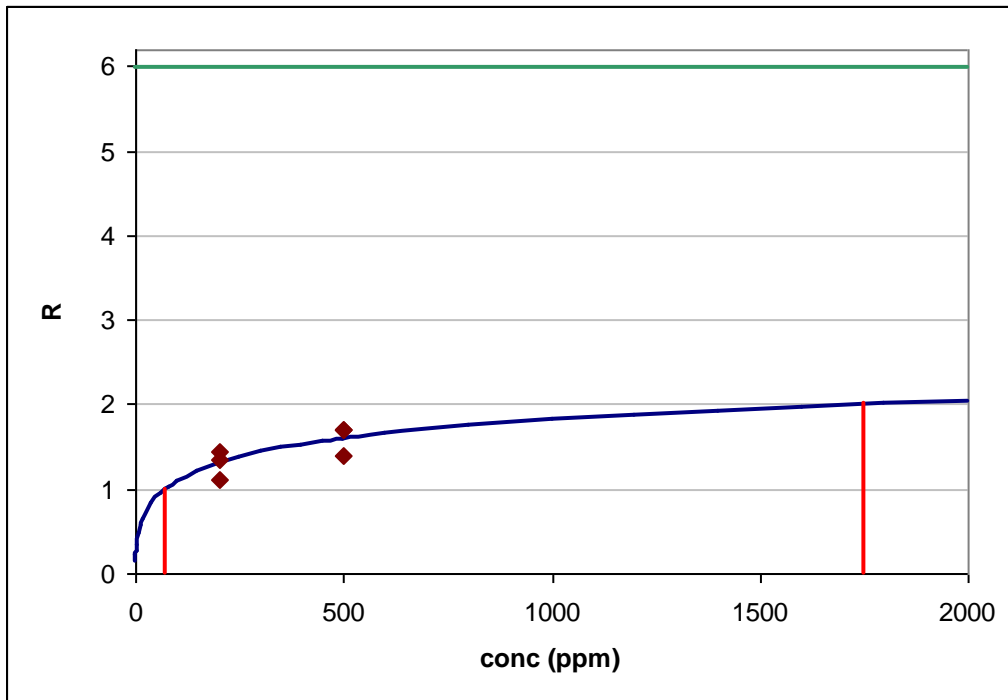


Figure 106: Estimated log<sub>10</sub> reduction R depending on concentration for *Candida albicans* and peracetic acid

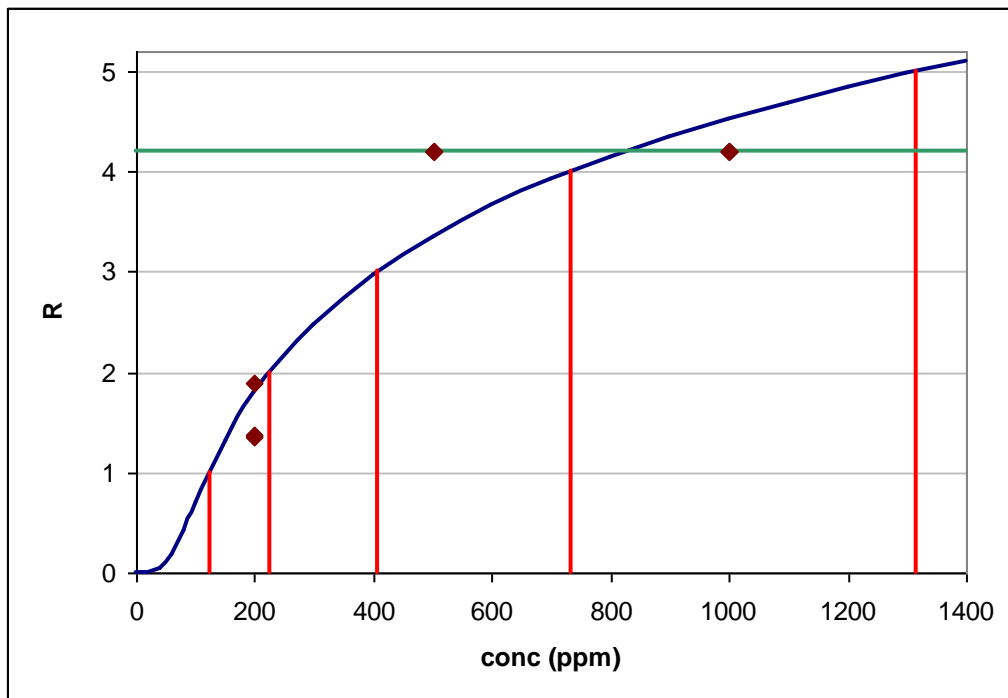


**Human Adenovirus type 5**

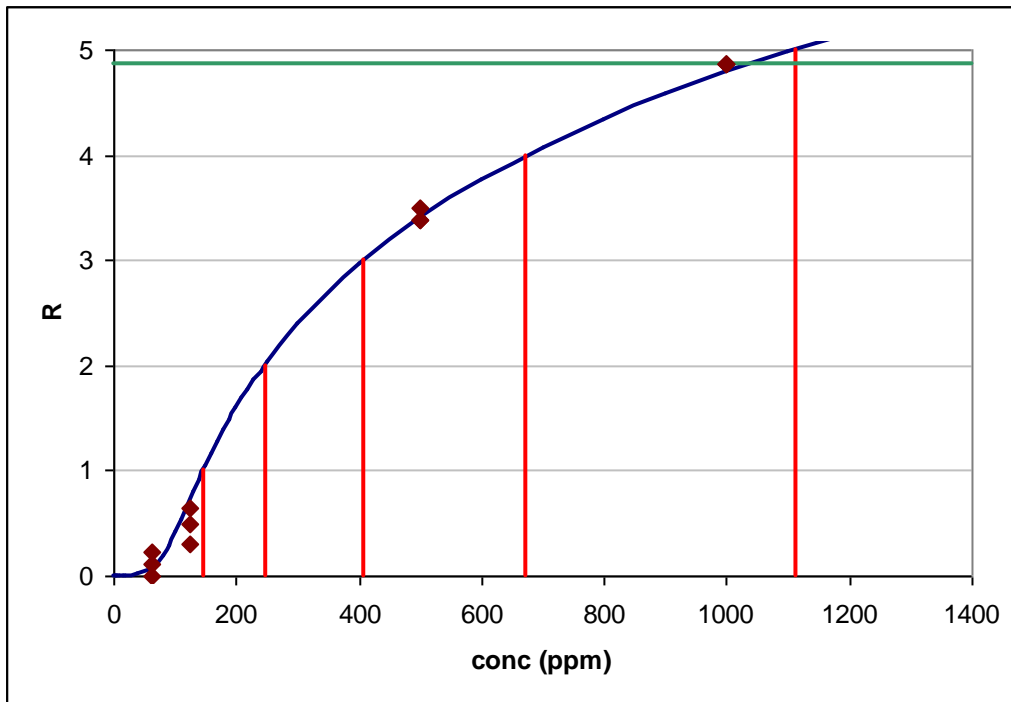
**Figure 107: Estimated log<sub>10</sub> reduction R depending on concentration for *Human Adenovirus type 5* and benzalkonium chloride**



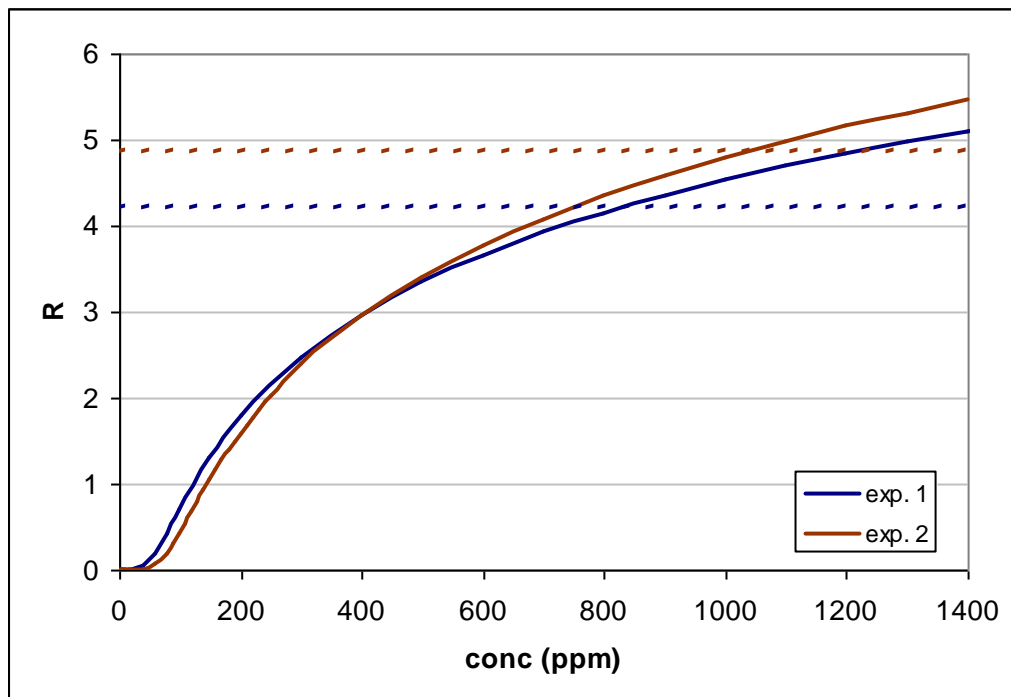
**Figure 108: Estimated log<sub>10</sub> reduction R depending on concentration for *Human Adenovirus type 5* and glutaraldehyde; experiments in July/August 2007**



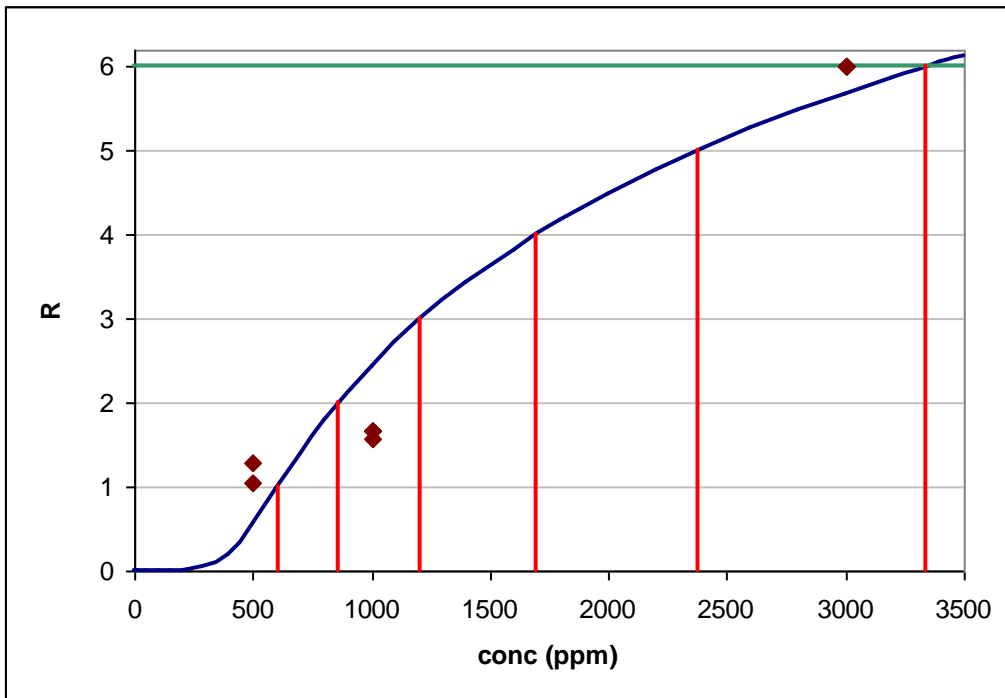
**Figure 109: Estimated log<sub>10</sub> reduction R depending on concentration for *Human Adenovirus type 5* and glutaraldehyde; repeated experiments in November 2007**



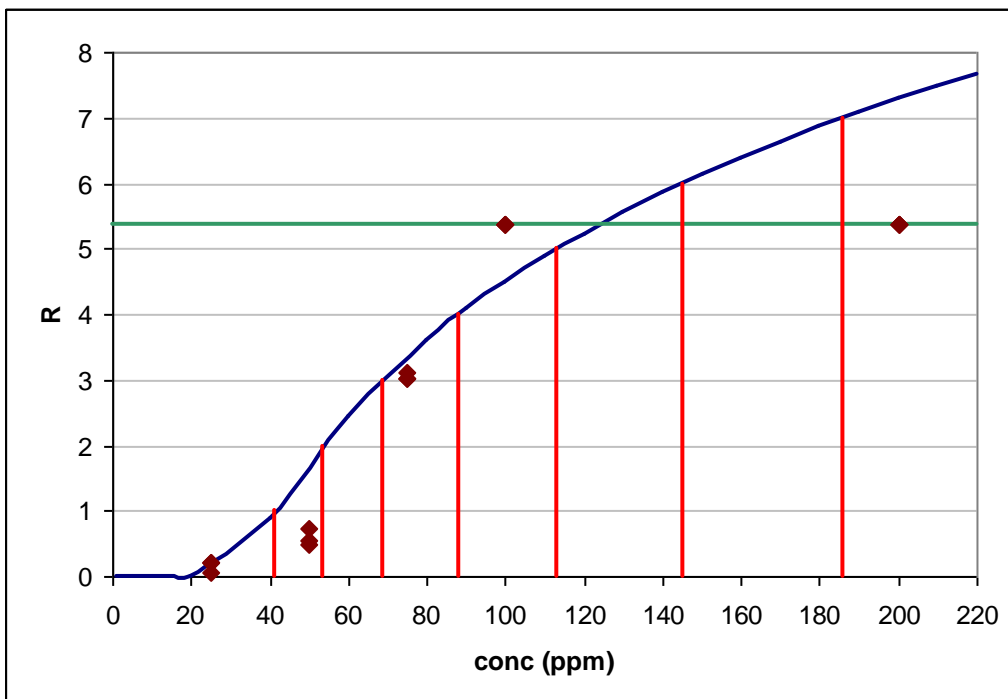
**Figure 110: Comparison of estimated log<sub>10</sub> reduction R depending on concentration for *Human Adenovirus type 5* and glutaraldehyde: experiment 1 in July/August 2007 and experiment 2 in November 2007**



**Figure 111: Estimated log<sub>10</sub> reduction R depending on concentration for *Human Adenovirus type 5* and sodium hypochlorite**

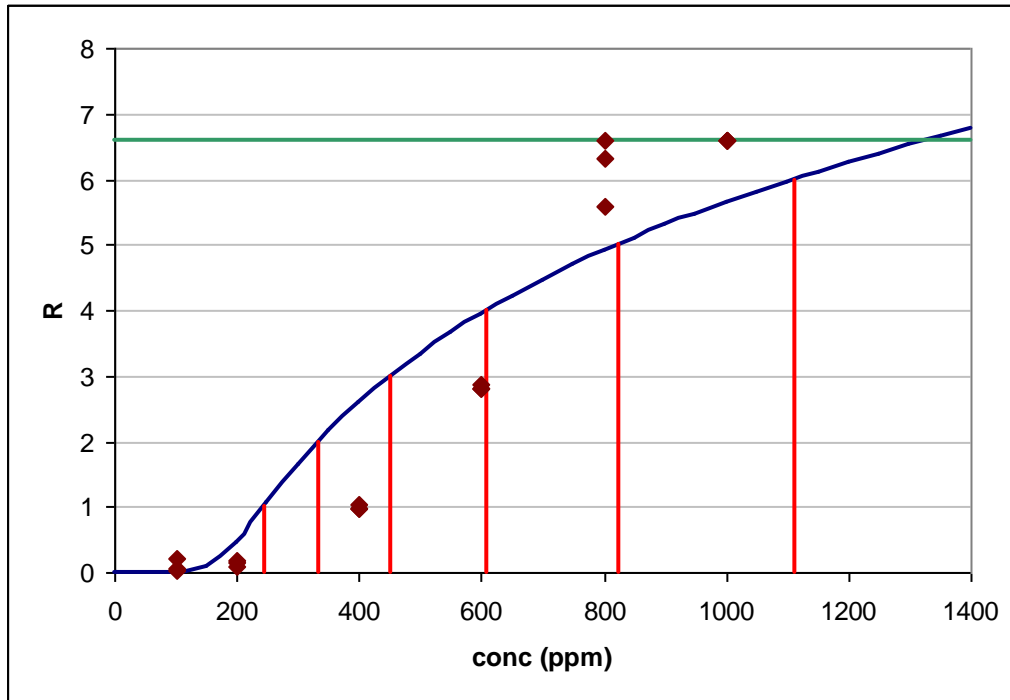


**Figure 112: Estimated log<sub>10</sub> reduction R depending on concentration for *Human Adenovirus type 5* and peracetic acid; experiments in November 2007**



**Bacillus subtilis**

**Figure 113: Estimated log<sub>10</sub> reduction R depending on concentration for *Bacillus subtilis* and peracetic acid**



## Data evaluation

### Explorative data analysis

Within the explorative data analysis, the data was observed for noticeable discrepancies. Statistical tests have been carried out in order to see if deviations are significant or not.

### Establishing the kill curve

#### Logit model

In order to establish the kill curve, i.e. a relationship between the concentration and the log<sub>10</sub> reduction, a logit model for the survival probabilities  $p$  is applied.

The survival probability is defined as

$$p = \frac{\text{CFU of test carrier}}{\text{CFU of controlcarrier}} .$$

Generally, the logit model is defined as:

$$\text{logit}(p) := \ln\left(\frac{p}{1-p}\right) = a + bx$$

with a probability  $p \in (0,1)$ , model parameters  $a$  and  $b$  and the concentration  $x$ .

Therefore the assumed model is a log-linear model  $y = a + bx$  with  $y = \ln\left(\frac{p}{1-p}\right)$ , where  $a$  is the

intercept and  $b$  is the slope.

Solving for  $p$  yields

$$p = \frac{1}{1 + \exp\{-a - bx\}} .$$

Applying the logit model to the current problem of modeling the log<sub>10</sub> reduction, the following model is obtained

$$y = \ln\left(\frac{\frac{CFU_c}{CFU_0}}{1 - \frac{CFU_c}{CFU_0}}\right) = a + b \cdot \ln(\text{conc}) .$$

From the survival probability  $p$

$$\frac{CFU_c}{CFU_0} = p = \frac{1}{1 + \exp\{-a - b \cdot \ln(\text{conc})\}} ,$$

the log<sub>10</sub> reduction  $R$  is determined by

$$R = \log\left(\frac{CFU_0}{CFU_c}\right) = \log\left[1 + \exp\{-a - b \cdot \ln(\text{conc})\}\right]$$

from which the respective concentration can be determined as

$$c_R = \exp\left\{-\frac{\ln(10^R - 1) + a}{b}\right\} .$$

It has to be noted that the applied logit model for logarithmized concentrations can be written as

$$y = \frac{1}{1 + \left(\frac{x}{M}\right)^S}.$$

It is easy to show that this model is equivalent to the above mentioned logit model, in fact the following holds:

$$\frac{CFU_c}{CFU_0} = p = \frac{1}{1 + \exp\{-a - b \cdot \ln(\text{conc})\}} = \frac{1}{1 + \left(\frac{\text{conc}}{M}\right)^S}$$

and the parameters  $S$  and  $M$  can be obtained from  $a$  and  $b$ :

$$S = -b$$

$$M = \exp\left\{-\frac{a}{b}\right\}.$$

One advantage of this model is the fact that it can also be applied for a concentration 0, then  $p$  simply equals 1. This is not possible for the logit model with logarithmized concentrations, since the logarithm for 0 is not defined.

#### Estimation of parameters

The parameters  $a$  and  $b$  of the logit model can be estimated from the empirical data by a least squares estimator. The applied estimator takes into account that the data are partially left censored, i.e. the actual concentration which causes a complete kill of the organisms is only known to be smaller than a concentration analysed in the experiment which left no visible colonies. Considering the example *A. niger* + accelerated  $H_2O_2$ , at the concentration of 3750 ppm between 500 and 2400 colonies were visible while at a concentration of 7500 ppm no colonies were visible anymore. However, we do not know if 7500 ppm really is the smallest concentration which causes a complete kill, it might be more probable that this concentration is somewhere between 3750 ppm and 7500 ppm.

## **Overall conclusions**

For the following three combinations of organism and active substance, the pre-validation experiments have been repeated:

- *Aspergillus niger* / peracetic acid
- *Aspergillus niger* / glutaraldehyde
- *Human Adenovirus type 5* / glutaraldehyde.

A 2-sample t-test showed that for all three combinations the mean of the control carriers differs significantly ( $p=5\%$ ), the mean of the test carriers differs significantly for the two combinations with glutaraldehyde. Only for *Aspergillus niger* with peracetic acid, no significant difference between the means of the test carries could be detected.

For the repeated experiments differences between the predicted log<sub>10</sub> Reduction R can be observed. However – considering only the concentration range below log<sub>10</sub> Reduction R=5 – these differences are less than 0.6 for the two combinations *Aspergillus niger* with peracetic acid and *Human Adenovirus type 5* / glutaraldehyde; for the combination *Aspergillus niger* with glutaraldehyde a difference of less than 1.2 can be observed.

## **Outline of ring trial**

### **General setting**

For the ring trial, the following general requirements for the setting are proposed:

- for each combination of organism and active substance two runs have to be performed;
- the order of these runs is randomized;
- one run consists of
  - 4 measurement replicates for the control carriers;
  - 5 concentration levels: 4 concentration levels within the range at which no complete kill is expected with 3 measurement replicates per concentration level and one concentration level with only one measurement replicate for a concentration at which a complete kill can be expected;
  - (  $4+3+3+3+3+1=17$  measurement replicates per run in total)

### **Requirements on participants**

- Single values are necessary, not only averages!
- Raw data must be supplied (template + individual result sheets)
- The participants should provide all data they have produced including those with invalid results.
- Participants have to report the sources of microorganisms and media.

**Determining the concentration levels for the ring trial**

For determining the concentration levels to be used in the ring trial, the estimated log<sub>10</sub> reduction R curves from the pre-validation are used. From these curves, concentrations can be calculated for a given log<sub>10</sub> reduction R. In order to assure that 4 of the 5 concentration levels lie within a range within which no complete kill can be expected, the following rule is applied:

1. Define mean log<sub>10</sub> reduction for the control carriers:  $R_{\text{control}}$ .
2. From  $R_{\text{control}}$  the concentration level at which a complete kill can be expected is derived by:  
 $c_1 = \text{concentration at } R_{\text{control}} + 1$ .
3. Also from  $R_{\text{control}}$  three of the five concentration levels (in the middle of the concentration range) are determined by:  
 $c_2 = \text{concentration at } R_{\text{control}} - 0.7$ ,  
 $c_3 = \text{concentration at } R_{\text{control}} - 1.2$  and  
 $c_4 = \text{concentration at } R_{\text{control}} - 1.6$ .
4. The fifth concentration level (in the lower concentration range) is set to  
 $c_5 = \text{concentration at } R = 1.5$ .

By a computer simulation, the precision of the estimated curve can be derived. This precision is expressed by the standard error which is the standard deviation of the estimated parameters.

For the simulation it is assumed that the CFU's of the control carrier are normally distributed with  $R_{\text{control}}$  as the mean and 0.075 as the standard deviation (empirical value).

The CFU's of the test carriers are assumed to follow a negative binomial distribution which takes into account the effect of over-dispersion, i.e. the effect that the observed variance of the data is larger than the predicted variance). Assuming an over-dispersion of 3, the parameters for the simulation according to the negative binomial distribution are  $p=1/9$  and  $k=(1/8)*\text{CFU}$  of test carrier.

**Proposed concentrations for the ring trial**

As an example we consider the combination *Human Adenovirus type 5* / glutaraldehyde. As the mean log<sub>10</sub> reduction for the control carriers we suggest  $R_{\text{control}} = 5$ . Therefore we need to calculate the concentrations for the log<sub>10</sub> reductions 6, 4.3, 3.8, 3.4 and 1.5.

We obtain:

R	Concentration from estimated kill curve (in ppm)	Applicable concentration level for ring trial (in ppm)
6	2100	2000
4.3	829	800
3.8	631	600
3.4	507	500
1.5	180	200

For this setting, the standard deviation of the estimated curve equals 0.25 at 800 ppm (about  $R = 4.3$ ) and 0.32 at 1200 ppm (about  $R = R_{\text{control}} = 5$ ).

**Appendix: Questions to be addressed**

- (1) The control carrier used at the concentration of 100 ppm (*A. niger* and peracetic acid) was different from the control carrier used at the two other concentrations. Why?

Statistical analyses (Grubbs tests) showed that the mean for 100 ppm is a statistically significant outlier ( $p=5\%$ ) and the Grubbs test for single logarithmized values showed that the 1<sup>st</sup> replicate at 100 ppm is a statistically significant outlier ( $p=5\%$ ). However, for the first analyses, no data was eliminated, also because if the data of concentration level 100 ppm had been eliminated, for the test carriers no CFU's greater than 1 would have been left.

**Table 41: Concentrations and CFU of control carrier for *A. niger* and peracetic acid**

conc	Replicate	CFU of control carrier
100	1	4.00E+04
100	2	1.30E+05
100	3	(0.00E+00)
300	1	1.80E+05
300	2	2.20E+05
300	3	2.00E+05
500	1	1.80E+05
500	2	2.20E+05
500	3	2.00E+05

*Independent from the above-presented findings, we ask if a control carrier can demonstrate zero growth? It may be considered to reject such a measurement series.*

- (2) We have seen in the original data (e.g. *A. niger* and peracetic acid, 100 ppm) that some 10-fold dilution series are not consistent (53/18/2 deviates statistically significant from 10-fold). Could this inconsistency indicate a systematic deviation?
- (3) Could a specific value (min/max?) for the CFU of the control carrier be demanded in the ring trial or could at least be demanded that the same controls are used for all concentrations within one organism/formulation combination?
- (4) For *S. aureus* and 4 of the test formulations 2 experiments have been carried out on 2 different days. A 2-sample t-test has been carried out to analyze if the mean value of the logarithmized CFU's of the controls of experiment 1 differs from the mean value of the logarithmized CFU's of experiment 2. A second 2-sample t-test has been carried out in order to analyze if the mean values of the logarithmized CFU's of the test carriers differ between the two experiments. The following table shows the obtained p-values. For a significance level of 5%, a p-value smaller than 0.05 identifies statistically significant differences between the mean values. These values are marked in red.

**Table 42: p-values for 2-sample t-tests**

	<b>log CFU control carrier</b>	<b>log CFU test carrier</b>
<b>accelerated H<sub>2</sub>O<sub>2</sub></b>	0.073	0.140
<b>glutaraldehyde</b>	0.044	0.469
<b>benzalkonium chloride</b>	0.159	0.094
<b>peracetic acid</b>	0.044	0.471

As there are at least two significant differences between the means of the logarithmized control CFU's, the need for an evaluation of the intermediate precision (standard deviation between different days in one lab) already for the pre-validation tests appears to be necessary. As already mentioned in the first telephone conference, there is also a need to confirm the results at least for one product per microorganism in another lab.

## **Minutes Meeting Dresden 17<sup>th</sup>/18<sup>th</sup> November 2008**

### ***Discussion of selected data***

#### ***Staphylococcus aureus***

LC0000 – glutaraldehyde – Run 1:

- Rounded plate counts → have they been counted or estimated??
- If estimated, disregard data
- How did you count?

LC0008 – peracetic acid:

- membrane filtration?
- How did you count?
- Run 3: 10 fold dilution steps, plate count for lower dilution is 50 times higher than plate count of higher dilution → disregard whole carrier

LC0011 – peracetic – Run 1:

- 100 fold dilution
- Carrier 1: disregard
- Carrier 8: take the „40“

LC0020 – peracetic acid /glutaraldehyde:

- strange results, however, none of the data is regarded
- inexperienced lab??

LC0026 – glutaraldehyde:

- use only 0 value on the very left (concentration 800 ppm) for estimating kill curve
- non-zero value for higher concentration (1300 ppm) needs to be ignored!

#### ***Bacillus subtilis***

LC0025: experiments will be repeated, new data in the next weeks → comparison of data before and after learning

#### ***Pseudomonas aeruginosa***

- LC0004 – benzalkonium chloride – Run 1: wrong concentration entered by lab (35000 instead of 3500 ppm) → has been changed, however, does not make any difference, because this combination of substance and organism was not scheduled in the ring test and has therefore not been analyzed

#### ***Adenovirus***

- if all dilutions are positive (all analyzed wells showed positive result) → no endpoint reached, data cannot be used

- some plaque tests, some TCID<sub>50</sub> methods,
- LC0026: EXCLUDE BEFORE ANY ANALYSIS (Assumption: they did not use the recommended strain of virus)

### **Mini ring trial**

Organism: *Staphylococcus aureus*

4 labs: with good consistent kill curves but different concentrations at a log reduction of 5. See outline with more details.

### **Statistical analyses**

#### **Negative logs**

For zero counts, an imaginary 1 is added in order to calculate the logarithm.

For really small counts, e.g. 1 for the 1+E01-dilution, a mean carrier between 0 and 1 and therefore a negative log is obtained, thus the log reduction becomes higher than the average water control.

Mathematically correct, but not in the microbiological sense.

Solution: replace plate counts of 1 by 1.5 (rule #4).

#### **Calculation rule for mean CFU/carrier**

In the Excel workbooks, the traditional weighted mean according to Dr. Sattar had been used. The new analysis shall be carried out on the basis of the unweighted arithmetic mean of the dilution-corrected counts. The question is: Is this approach relevant only for test carriers? Shall the water controls be aggregated according to the traditional weighted mean approach?

#### **Data to be excluded**

- *Staphylococcus aureus* - LC0020 – all substances: carry out two statistical analyses, one with the data of this lab one without, just for a comparison
- *Adenovirus type 5*: LC0026: EXCLUDE BEFORE ANY ANALYSIS (Assumption: they did not use the recommended strain of virus)

#### **Data selection**

(See outline for corrected procedure)

6 rules have been obtained that will guarantee a reasonable set of data according to microbiological point of view. They will be applied to the test carriers only, not to the water controls.

Rule #1:

Check whether the figure for the lower dilution is 50 or larger. Then calculate the dilution corrected counts and divide the figure for lower dilution by 5. If this is higher than the figure for the higher dilution then reject this carrier.

Rule #2

Check whether the figure for the higher dilution is 4 or larger. Calculate the dilution corrected counts. If

the figure for the higher dilution exceeds the figure for the lower dilution by 10 or more than 10 times, ignore the lower dilution.

Then repeat with the next highest dilution and compare the average of the two highest dilutions with the lower dilution.

#### Rule #3

Start with highest dilution:

If the count is zero, disregard the result. Then check the next highest dilution and repeat procedure. If there is no growth at all, replace 0 by 1.

#### Rule #4

If there is only one dilution with counts and if this is equal to 1, replace by 1.5

#### Rule #5

If there are more than 3 dilutions with countable numbers, use only the three highest dilutions.

#### Rule #6

No test carrier should have counts of the CFU's or PFUs higher than the geometric mean of the water control + 30%. (log water control + 0.3).

#### **Preparation of raw data**

- "Less than" is ignored and only the actual value is taken into account
- *Adenovirus type 5*: for the following laboratories, the TCID<sub>50</sub> need to be recalculated Spearman and Kärber (see Leitlinie DVV): LC0001, LC0002, LC0022

#### **Analysis**

- kill curves are calculated with the average water control as the maximum level for each run (= standard kill curve)
- additionally a normalization is carried out where the average of the water control of each run equals 100% (= normalized kill curve)
- these two kinds of kill curves show the same, but in a different way, HOWEVER, the determination of the validation parameters differs completely → it should be decided on which of the curves the validation should be based
- for standard kill curve, the validation parameters will be based
  - (1) on concentration dependent standard deviation (as hitherto) and additionally
  - (2) on the concentrations at which a log reduction of 5 is obtained (*Pseudomonas aeruginosa*: Corridor of water control:  $\geq 4.5$ )
- *Adenovirus type 5*: separate analysis of Plaque test results and TCID<sub>50</sub> results

## **To do**

### **Data**

- Import of raw data (counts) to a new database (comprising not only the aggregated data required for the ring trial analyses but also the underlying base data, counts and statuses). This is necessary as the workbooks cannot be used further due to changes in the algorithms.
- Check of the raw data, especially the statuses
- Selection of raw data according to the rules
- Recalculation of CFU/Carrier
- Recalculation of mean CFU/Carrier
- Recalculation of log reductions
- For *Adenovirus*: recalculate TCID<sub>50</sub> values

### **Theory**

- New concepts need to be derived for outlier analysis and for validation. Not yet discussed and decided.

### **Analysis**

- Recalculate run-specific kill curves: both standard kill curves and normalized kill curves
- Check outliers within and between run-specific kill-curves
- Recalculate validation parameters (inter-laboratory reproducibility, intra-laboratory reproducibility over time, intra-laboratory reproducibility within one run)

### **Additional tasks**

- Determine two laboratories for mini ring trial
- LC0025: experiments will be repeated, new data in the next weeks → comparison of data before and after learning

## **Minutes Meeting Dresden 9<sup>th</sup>/10<sup>th</sup> March 2009**

### ***Participants***

- Dr. Rödger
- Dr. Sattar
- Dr. Schwebke
- Dr. Gebel
- Dr. Uhlig
- S. Antoni (quo data)
- K. Kunath (quo data)
- U. Köser (quo data)

### ***Extension of rules***

Since the application of rules after the last meeting in Dresden (November 2008) showed that there are still some unclear cases, following additional rules (referred to as Rules 0a-f) have been designed. Furthermore it has been clarified that zero plate counts for higher dilutions are disregarded and do not contribute to the mean CFU/carrier (does not apply to “middle zeros” or zero plate counts for lower dilutions).

#### **Rule 0a:**

If there is a reasonable plate count, but the laboratory entered N/A as status, the status should always be set to OK.

#### **Rule 0b:**

If the plate count is empty, but the laboratory entered status 0, set plate count to 0 as well.

If the plate count is 0, but the laboratory entered status N/A, leave status N/A and therefore plate count will be disregarded.

#### **Rule 0c:**

Every plate count above 299 will be disregarded.

Accept when a laboratory entered TMTC status and therefore disregard given plate count even when it is below 300.

#### **Rule 0d:**

Zero plate counts between OK plate counts (“Middle zeros”) will be regarded as long as no other rule applies (e.g. 5(-1 dilution)/0(-2 dilution)/1(-3 dilution)). Therefore, middle zeros will be included in calculating mean value

#### **Rule 0e:**

Zero plate counts for lower dilutions (where at least one higher dilution has countable plate counts) are either

- covered by rule IV if non-zero plate counts are larger than or equal to 4 or

- for non-zero plate counts smaller than 4, the outlier tests will take care of worst case and other cases do not have an big effect.

e.g.

Carrier 1: 0001->outlier, carrier will be disregarded

Carrier 2: 0000

Carrier 3: 0000

Carrier 1: 0010 or Carrier 1: 0010 -> no outlier, but no problem

Carrier 2: 0000 Carrier 1: 0000

Carrier 3: 0000 Carrier 1: 0000

**Rule 0f** (extension of rule IV):

Any count(s) in lower dilution step(s) is/are disregarded if there is/are TMTC in higher dilution step(s), but include colony count(s) in the higher dilution step(s).

**Motivation for rules**

In two experts meetings after the first statistical analysis, 6 rules have been formulated to exclude data patterns which are obviously inconsistent and implausible from the microbiological point of view (see “Minutes Meeting Dresden 17./18.11.2008” + “Minutes Meeting Dresden 9./10.03.2009”) and which would influence the statistical analysis.

These rules are only applicable to single carriers and not to a set of carriers.

***Corrections in future ring trial***

- If membrane filtration is used to capture bacteria from diluted samples, give the number of colonies per plate.
- Give number of spread plates used per ml (for the dilution steps if membrane filtration is not used).
- “Count per ml” instead of “plate count”
- Give explanation for N/A
- Option “ND” for “not done”, but not for “not detected”
- Submit original data as well (besides sending it in a digital form)!

***Discussion of report and statistical results***

Remarks by the participants and by Susan Springthorpe have been discussed and some of them have been added to the report already or the respective paragraphs have been corrected.

More comments:

- Kill curve function is not expected to be exactly true, but due to measurement errors any deviations between the model curve and the true kill curve (which is unknown) are negligible.
- Regarding the benefit of the kill curve, the lab-specific kill curve balances out many of the random deviations between different measurements so that systematic differences between

different labs may be revealed.

- There are kill curves that should not be regarded in future analyses due to insufficient data (lab LC0008, *S.aureus*, benzalkonium), these kill curves are referred to as “not regarded”
- Do not show “not regarded” run kill curves in the figure with all run-specific kill curves
- Change colours of runs in the figures with the run-specific kill curves separate for each lab (blue colours are difficult to distinguish)
- All organisms in *italics*
- “kill curve” = “inactivation curve” in future
- benzalkonium chloride: curves never seem to reach 100% (complete kill), problem with active substance; possible reason: different mode of action of compared to aldehydes or peracetic acid because of detergent character
- *S. aureus*, peracetic acid, LC0021: very insensitive to active substance due to high water control
- Water control: high water control yields in normalized log<sub>10</sub> reductions clearly smaller than 100%
- *Adenovirus*: gap between water control and highest log<sub>10</sub> reduction is caused by cytotoxicity problems
- Discussion on outliers: what to do with outlier runs within one lab when run shows good fit compared to runs of all laboratories? Different sources of error (within one run of a lab, between runs of a lab, between labs); we don't know the actual biological range, this might be clarified after the exercise of phase 2 part 1;
- In tables with lower and upper limits for reproducibility intervals better show actually reproducibility s.d. than limits; maybe another picture of s.d. in dependence of concentration
- Complete kill = 5 for *S. aureus* and between 3 and 4 for *Adenovirus*, complete kill is not necessarily water control.

### **Suggestions for future analyses**

- It might be very useful to consider active substance comprehensive effects and/or micro-organism comprehensive effects as these might be explained by lab-specific deviations from the test method.
- It is therefore recommended not only to consider kill curves for one organism and one substance only but to compare systematic differences throughout tested organisms and substances (Z scores based on B, C and D).

The following suggestions could be applied to the data of *S. aureus* and benzalkonium chloride in order to find out if the ideas are reasonable and could be applied in the statistical analyses:

- Recalculate run-specific kill curves: try to establish another approach allowing for a D value higher than water control and to ignore higher concentrations showing complete kill.
- Analysis: normalized and standard; separate outlier tests (results might differ for normalized and standard curve)
- Suggestion: D from estimation, but normalize: water control=100%; show whole curve between 0 and D, but extrapolated (below log<sub>10</sub> red=2 and above 100%) show as dashed

curve

- how many times needs the test to be repeated to reach +/- 1 log<sub>10</sub> (?)
- Reproducibilities should be based on kill curves or on single values? If based on kill curves, only half the number of labs is necessary; try both and decide later which one should be taken into account
- Microbiologist assumption: s-shaped curve that should cross water control left of first complete kill (i.e. concentration when curve reaches average log<sub>10</sub> of water controls should be smaller than the first concentration where complete kill is reached)
- “Most-likely-approach” for each test substance and organism separately: “good” runs will be fitted at first and the fitting of “bad” runs will be adapted. This approach can be compared with the approach applied in the first analysis (draft report of 26<sup>th</sup> September 2008).

### ***Conference calls on both days***

- Short update on proceedings in Dresden
- Discussion of next steps
- Details see minutes of calls by Steve Smith

**Participating laboratories**

<b>Laboratory</b>	<b>Country</b>	<b>Principal investigator</b>
Abbot Analytical, New Brighton	UK	Dave Watson
AFSSA FOUGERES, Fougères	France	Pierre Maris
Arch, Manchester	UK	Paula McGeechan
ATS, Eagan	USA	Dave Rottjakob
BluScientific, Glasgow	UK	Chris Woodall
Bode Chemie GmbH	Germany	
Campden & Chorlywood Food Research Association, Chipping Campden	UK	John Hollah
Ecolab, Eagan	USA	Mary Bennett
Eurovir Hygiene-Institut	Germany	O. Thraenhart
Health And Safety Laboratory (UK HSE), Buxton	UK	Alan Beswick
Institute of Virology and Antiviral Therapy, University of Jena	Germany	Andreas Sauerbrei
Institute for Medical Virology, Frankfurt	Germany	Holger F. Rabenau
Johnson Diversey, Sturtevant	USA	Kay Bixler
Labor Dr. Merk & Kollegen, Ochsenhausen	Germany	Ingrid Rapp
Labor Prof. G. Enders & Partner	Germany	Maren Eggers
Lonza, Allendale	USA	Crystal Arlea
Lysoform, Berlin	Germany	Hans-Joachim Rödger
Microbiology and Clinical Microbiology Lab, Department of Preclinical Science Disp "LITA", University of Milan, Milano	Italy	Lorenzo Drago
Mikrolab GmbH, Bremen	Germany	Jochen Steinmann
Mircobiotest, Sterling	USA	Angela L. Hollingsworth
National Reference Laboratory on Disinfection and Sterilization of Dept. for Epidemiology and Microbiology of National Institute of Public Health, Praha	Czech Republic	Frantisek Rettich
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Robert Koch-Institut, Berlin	Germany	Ingeborg Schwebke
Therapeutic Goods Administration Laboratories (TGAL), Canberra	Australia	Kate Rusbridge
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Universität Hohenheim, Institut für Umwelt und Tierhygiene, Hohenheim	Germany	Birgit Hunsinger, Rachel Marchang
University of Bonn, Institute for Hygiene and Public Health; Disinfectant testing, Bonn	Germany	Jürgen Gebel
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US EPA OPP BEAD Laboratory, Ft. Meade	USA	Stephen Tomasino

**Receipt of data in phase 1****Table 43: Dates of receipt of data at quo data in phase 1; data received after the final deadline are highlighted in orange**

<b>Date (dd.mm.yyyy)</b>	<b>Lab Code</b>	<b>Organism</b>
30.04.2008	LC0023	<i>Aspergillus niger</i>
01.05.2008	LC0025	<i>Mycobacterium terrae</i>
02.05.2008	LC0031	<i>Adenovirus type 5</i>
03.05.2008	LC0019	<i>Aspergillus niger</i>
04.05.2008	LC0019	<i>Pseudomonas aeruginosa</i>
05.05.2008	LC0019	<i>Staphylococcus aureus</i>
06.05.2008	LC0009	<i>Adenovirus type 5</i>
09.06.2008	LC0011	<i>Aspergillus niger</i>
09.06.2008	LC0011	<i>Bacillus subtilis</i>
09.06.2008	LC0011	<i>Mycobacterium terrae</i>
09.06.2008	LC0011	<i>Pseudomonas aeruginosa</i>
09.06.2008	LC0011	<i>Staphylococcus aureus</i>
09.06.2008	LC0012	<i>Staphylococcus aureus</i>
09.06.2008	LC0029	<i>Adenovirus type 5</i>
09.06.2008	LC0025	<i>Bacillus subtilis</i>
09.06.2008	LC0007	<i>Mycobacterium terrae</i>
10.06.2008	LC0035	<i>Pseudomonas aeruginosa</i>
10.06.2008	LC0035	<i>Staphylococcus aureus</i>
10.06.2008	LC0012	<i>Mycobacterium terrae</i>
11.06.2008	LC0021	<i>Staphylococcus aureus</i>
23.06.2008	LC0009	<i>Adenovirus type 5</i>
26.06.2008	LC0014	<i>Adenovirus type 5</i>
27.06.2008	LC0026	<i>Staphylococcus aureus</i>
28.06.2008	LC0008	<i>Bacillus subtilis</i>
30.06.2008	LC0033	<i>Adenovirus type 5</i>
01.07.2008	LC0029	<i>Adenovirus type 5</i>
01.07.2008	LC0002	<i>Bacillus subtilis</i>
01.07.2008	LC0028	<i>Staphylococcus aureus</i>
02.07.2008	LC0017	<i>Adenovirus type 5</i>
03.07.2008	LC0007	<i>Mycobacterium terrae</i>
03.07.2008	LC0016	all data that are available
05.07.2008	LC0001	<i>Mycobacterium terrae</i>
11.07.2008	LC0012	<i>Pseudomonas aeruginosa</i>
15.07.2008	LC0029	<i>Adenovirus type 5</i>
30.07.2008	LC0001	<i>Adenovirus type 5</i>
31.07.2008	LC0001	<i>Adenovirus type 5</i>
01.08.2008	LC0026	<i>Pseudomonas aeruginosa</i>

<b>Date (dd.mm.yyyy)</b>	<b>Lab Code</b>	<b>Organism</b>
01.08.2008	LC0026	<i>Adenovirus type 5</i>
06.08.2008	LC0028	<i>Pseudomonas aeruginosa</i>
13.08.2008	LC0011	<i>Aspergillus niger</i>
13.08.2008	LC0011	<i>Mycobacterium terrae</i>
13.08.2008	LC0011	<i>Pseudomonas aeruginosa</i>
14.08.2008	LC0010	<i>Staphylococcus aureus</i>
18.08.2008	LC0011	invalid results
18.08.2008	LC0019	benzalkonium chloride
18.08.2008	LC0021	<i>Mycobacterium terrae</i>
18.08.2008	LC0021	<i>Staphylococcus aureus</i>
18.08.2008	LC0021	<i>Aspergillus niger</i>
19.08.2008	LC0021	<i>Pseudomonas aeruginosa</i>
19.08.2008	LC0022	<i>Adenovirus type 5</i>
19.08.2008	LC0004	<i>Staphylococcus aureus</i>
20.08.2008	LC0004	<i>Pseudomonas aeruginosa</i>
20.08.2008	LC0010	<i>Staphylococcus aureus</i>
20.08.2008	LC0006	<i>Adenovirus type 5</i>
21.08.2008	LC0020	<i>Staphylococcus aureus</i>
22.08.2008	LC0014	<i>Adenovirus type 5</i>
22.08.2008	LC0002	<i>Adenovirus type 5</i>
Latest deadline for data submission to Diane: 20.08.2008 Data transfer to quo data: 22.08/2008		
27.08.2008	LC0008	<i>Mycobacterium terrae</i>
27.08.2008	LC0008	<i>Staphylococcus aureus</i>
29.08.2008	LC0022	<i>Staphylococcus aureus</i>
29.08.2008	LC0022	<i>Bacillus subtilis</i>
29.08.2008	LC0022	<i>Aspergillus niger</i>
29.08.2008	LC0000	<i>Staphylococcus aureus</i>
02.09.2008	LC0032	<i>Adenovirus type 5</i>

**Receipt of data in phase 2****Table 44: Dates of receipt of data at quo data in phase 2 for part 2**

<b>Date (dd.mm.yyyy)</b>	<b>Lab Code</b>	<b>Organism</b>	<b>Test substance</b>
15.07.2009	LC0011	<i>Staphylococcus aureus</i>	benzalkonium chloride
15.07.2009	LC0011	<i>Staphylococcus aureus</i>	glutaraldehyde
20.07.2009	LC0021	<i>Staphylococcus aureus</i>	glutaraldehyde
20.07.2009	LC0021	<i>Staphylococcus aureus</i>	glutaraldehyde
20.07.2009	LC0035	<i>Staphylococcus aureus</i>	glutaraldehyde
27.07.2009	LC0028	<i>Staphylococcus aureus</i>	benzalkonium chloride
27.07.2009	LC0028	<i>Staphylococcus aureus</i>	glutaraldehyde
28.07.2009	LC0011	<i>Staphylococcus aureus</i>	benzalkonium chloride
28.07.2009	LC0011	<i>Staphylococcus aureus</i>	glutaraldehyde
12.08.2009	LC0021	<i>Staphylococcus aureus</i>	benzalkonium chloride
12.08.2009	LC0021	<i>Staphylococcus aureus</i>	glutaraldehyde

**Table 45: Dates of receipt of data at quo data in phase 2 for part 3**

<b>Date (dd.mm.yyyy)</b>	<b>Lab Code</b>	<b>Organism</b>	<b>Test substance</b>
02.08.2009	LC0011	<i>Enterococcus hirae</i>	benzalkonium chloride
02.08.2009	LC0011	<i>Pseudomonas aeruginosa</i>	glutaraldehyde
18.08.2009	LC0021	<i>Enterococcus hirae</i>	benzalkonium chloride
18.08.2009	LC0021	<i>Pseudomonas aeruginosa</i>	glutaraldehyde
27.08.2009	LC0012	<i>Pseudomonas aeruginosa</i>	glutaraldehyde
31.08.2009	LC0036	<i>Pseudomonas aeruginosa</i>	glutaraldehyde
31.08.2009	LC0036	<i>Enterococcus hirae</i>	benzalkonium chloride
02.09.2009	LC0028	<i>Pseudomonas aeruginosa</i>	glutaraldehyde
04.09.2009	LC0028	<i>Enterococcus hirae</i>	benzalkonium chloride
04.09.2009	LC0036	<i>Adenovirus type 5</i>	glutaraldehyde
04.09.2009	LC0021	<i>Enterococcus hirae</i>	benzalkonium chloride
04.09.2009	LC0021	<i>Pseudomonas aeruginosa</i>	glutaraldehyde
04.09.2009	LC0006	<i>Adenovirus type 5</i>	glutaraldehyde

### **Original criteria**

202. The 4 criteria for *Mycobacterium terrae*, *Aspergillus niger*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus* are:

- Criterion #1: One concentration demonstrating "almost complete inactivation". It is preferred for statistical reasons to have at least 2 different concentrations within the range of 5 to 30 viable counts;
- Criterion #2: One concentration demonstrating "almost no inactivation", (Log Reduction = 1 to 1.8);
- Criterion #3: Two concentrations demonstrating "partial inactivation" (Log Reduction >1.8 and more than 30 viable counts);
- Criterion #4: One concentration shall give no detectable survivors ("complete inactivation"), but that concentration should not be higher than 20% of the concentration in criterion #1;

203. For *Adenovirus type 5* the 4 criteria needed to be adapted and are

- Criterion #1: One concentration demonstrating "almost complete inactivation". It is preferred for statistical reasons to have at least 2 different concentrations within the range of 1 to 8 viable counts;
- Criterion #2: This concentration demonstrating "almost no inactivation", (Log Reduction = 0.6 to 1.5);
- Criterion #3: Two concentrations demonstrating "partial inactivation" (Log Reduction >1.5 and more than 8 viable counts);
- Criterion #4: One concentration shall give no detectable survivors ("complete inactivation"), but that concentration should not be higher than 35% of the concentration in criterion #1;

## **Workbook (Example)**

### **OECD Template For Assessment Of Microbicidal Activity Of Chemical Germicide**

Lab ID:	LC0035
Organism / ATCC:	Bacillus subtilis
Source of ATCC / Isolate / Date of Delivery:	
Soil Load:	
Hard Water:	
Test Substance:	
Neutralizer:	
Contact Time:	
Exposure Temperature in °C:	
Humidity in %:	
Incubation Time:	
Incubation Temperature:	
Type of media / Lot numbers (broth and solid):	
Carrier Drying Time (including Transfer Time):	
Carrier Drying Temperature:	
Dessicator type/age:	
Dates of Experiment and Counting:	
Test Run: (1, 2 or 3)	

#### **Notes:**

*Please enter concentrations [ppm] starting with concentration 1 (highest level), concentration 2 (2nd highest level), etc.*

*Please perform tests in the order indicated (carrier).*

*A minimum of 5 concentration levels has to be tested (see letter description).*

*With 6 test concentrations you might achieve a higher reliability of your results. Therefore 6 concentrations are recommended.*

*Enter plate count for each cell and select the appropriate status from the drop down menu for each of the plate counts:*

*OK Colonies on plate counted completely. Please enter the respective count as plate count.*

*TMTC Too many colonies to be counted. Please enter the minimum count (the count where you stopped) as plate count.*

*0 No colonies seen on the plate. Please enter 0 as plate count.*

*N/A No test result available, e.g. because of contamination. Please leave plate count empty.*

*You may also change the dilution factor if needed.*

*CFU/carrier and LOG RED are calculated automatically on the basis of all data with status OK or 0.*

*For the next run please check the evaluation of selected concentrations below and adjust concentrations if necessary.*

Water Ctrl	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	CFU/Carrier	Log CFU/Carrier	Average LG
Carrier - 19	1.00E+03	OK	85	1.00E+04	OK	22	1.00E+05	N/A		1.00E+06	N/A		9.73E+04	4.99	5.03
Carrier - 20	1.00E+03	OK	105	1.00E+04	OK	30	1.00E+05	N/A		1.00E+06	N/A		1.23E+05	5.09	
Carrier - 21	1.00E+03	OK	102	1.00E+04	OK	25	1.00E+05	N/A		1.00E+06	N/A		1.15E+05	5.06	
Carrier - 22	1.00E+03	OK	88	1.00E+04	OK	18	1.00E+05	N/A		1.00E+06	N/A		9.64E+04	4.98	

Concentration [ppm]	Carriers	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	Dilution factor	Status	Plate Count	CFU/Carrier	Log CFU/Carrier	LOG RED
1 1200	Carrier - 1	1.00E+00	0	0	1.00E+01	0	0	1.00E+02	0	0	1.00E+03	0	0	0.00E+00	0.00	5.03
	Carrier - 2	1.00E+00	0	0	1.00E+01	0	0	1.00E+02	0	0	1.00E+03	0	0	0.00E+00	0.00	5.03
	Carrier - 3	1.00E+00	0	0	1.00E+01	0	0	1.00E+02	0	0	1.00E+03	0	0	0.00E+00	0.00	5.03
2 1000	Carrier - 4	1.00E+00	OK	4	1.00E+01	0	0	1.00E+02	0	0	1.00E+03	0	0	3.60E+00	0.56	4.47
	Carrier - 5	1.00E+00	OK	12	1.00E+01	0	0	1.00E+02	0	0	1.00E+03	0	0	1.08E+01	1.03	4.00
	Carrier - 6	1.00E+00	OK	14	1.00E+01	OK	1	1.00E+02	0	0	1.00E+03	OK	1	1.44E+01	1.16	3.87
3 800	Carrier - 7	1.00E+00	OK	15	1.00E+01	OK	3	1.00E+02	0	0	1.00E+03	OK	0	1.62E+01	1.21	3.82
	Carrier - 8	1.00E+00	OK	20	1.00E+01	OK	2	1.00E+02	0	0	1.00E+03	OK	0	1.98E+01	1.30	3.73
	Carrier - 9	1.00E+00	N/A		1.00E+01	N/A		1.00E+02	N/A		1.00E+03	N/A		N/A		
4 600	Carrier - 10	1.00E+00	OK	100	1.00E+01	OK	20	1.00E+02	OK	2	1.00E+03	0	0	1.10E+02	2.04	2.99
	Carrier - 11	1.00E+00	OK	110	1.00E+01	OK	10	1.00E+02	OK	4	1.00E+03	OK	1	1.13E+02	2.05	2.98
	Carrier - 12	1.00E+00	OK	100	1.00E+01	OK	5	1.00E+02	OK	1	1.00E+03	0	0	9.54E+01	1.98	3.05
5 400	Carrier - 13	1.00E+00	OK	130	1.00E+01	OK	40	1.00E+02	OK	4	1.00E+03	OK	2	1.58E+02	2.20	2.83
	Carrier - 14	1.00E+00	OK	140	1.00E+01	OK	30	1.00E+02	OK	4	1.00E+03	OK	1	1.58E+02	2.20	2.83
	Carrier - 15	1.00E+00	OK	250	1.00E+01	OK	20	1.00E+02	OK	3	1.00E+03	OK	1	2.47E+02	2.39	2.64
6 200	Carrier - 16	1.00E+00	TMTC	330	1.00E+01	TMTC	330	1.00E+02	OK	80	1.00E+03	OK	34	1.04E+04	4.02	1.02
	Carrier - 17	1.00E+00	TMTC	330	1.00E+01	TMTC	330	1.00E+02	OK	120	1.00E+03	OK	14	1.22E+04	4.09	0.95

	Carrier - 18	1.00E+00	TMTC	330	1.00E+01	TMTC	330	1.00E+02	OK	120	1.00E+03	OK	15	1.23E+04	4.09	0.94
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Evaluation of selected concentrations				
Concentration [ppm]	Viable Counts	LOG RED	Criterion	
1	1200	0	5.03	Complete kill
2	1000	10	4.11	Almost complete kill
3	800	18	3.78	Almost complete kill
4	600	106	3.01	Partial kill
5	400	188	2.77	Partial kill
6	200	11606	0.97	

Evaluation	Description	#	Status	Remarks
Criterion #1	concentrations with 'almost complete kill' (5-30 viable counts):	2	OK	
Criterion #2	concentrations with 'almost no kill' (LOG RED=1-1.8):	0	Not OK	(concentration with 'almost no kill' not available)
Criterion #3	concentrations with 'partial kill' (LOG RED>1.8 and more than 30 viable counts)	2	OK	
Criterion #4	concentrations with 'complete kill'	1	OK	

**Status correction and adaptation carried out by quo data GmbH in phase 1****Table 46: Status correction and adaptation carried out by quo data GmbH**

Organism	Lab	Substance	Run	Concentration levels	Carrier	Correction
<i>Aspergillus niger</i>	11	glutaraldehyde	2	2500	16-18	10: OK-->TMTC
<i>Aspergillus niger</i>	11	glutaraldehyde	2	5000	13-15	10: OK-->TMTC
<i>Aspergillus niger</i>	11	peracetic acid	1	200	7-9	100: 0-->OK
<i>Aspergillus niger</i>	19	all				no status specifications at all, have been entered manually by quo data
<i>Bacillus subtilis</i>	2	peracetic acid	1	900 + 1200	1-6	1-1000: OK-->0
<i>Bacillus subtilis</i>	2	peracetic acid	2	1000	1-3	1-1000: OK-->0
<i>Bacillus subtilis</i>	2	peracetic acid	3	1000	1-3	1-1000: OK-->0
<i>Bacillus subtilis</i>	25	peracetic acid	2	3400	1	1: OK-->TMTC
<i>Bacillus subtilis</i>	25	peracetic acid	1	1800	2	1: OK-->TMTC
<i>Bacillus subtilis</i>	2	peracetic acid	1	800	8	10-1000: OK-->0
<i>Bacillus subtilis</i>	2	peracetic acid	1	600	10-12	10-1000: OK-->0
<i>Bacillus subtilis</i>	2	peracetic acid	2	600, 800, 850	4-12	10-1000: OK-->0
<i>Bacillus subtilis</i>	2	peracetic acid	3	600, 800, 850	4-12	10-1000: OK-->0
<i>Bacillus subtilis</i>	25	peracetic acid	2	3000	6	10-100: OK-->0
<i>Bacillus subtilis</i>	25	peracetic acid	3	4200	3	10-1000: OK-->0
<i>Bacillus subtilis</i>	11	peracetic acid	3	800	7-9	1:0-->TMTC
<i>Bacillus subtilis</i>	11	peracetic acid	3	700	11	1:0-->TMTC
<i>Bacillus subtilis</i>	11	peracetic acid	2	700	13+14	1: OK-->TMTC
<i>Bacillus subtilis</i>	11	peracetic acid	3	700	10+12	1: OK-->TMTC
<i>Bacillus subtilis</i>	8	peracetic acid	2	800	8+9	100: N/A-->0
<i>Bacillus subtilis</i>	2	peracetic acid	1	800	7	100+1000: OK-->0
<i>Bacillus subtilis</i>	2	peracetic acid	1	400	13+15	100+1000: OK-->0
<i>Bacillus subtilis</i>	2	peracetic acid	2	500	13-15	100+1000: OK-->0
<i>Bacillus subtilis</i>	2	peracetic acid	3	500	15	100+1000: OK-->0
<i>Bacillus subtilis</i>	2	peracetic acid	1	800	9	1000: OK-->0
<i>Bacillus subtilis</i>	2	peracetic acid	1	400	14	1000: OK-->0
<i>Bacillus subtilis</i>	2	peracetic acid	3	500	13+14	1000: OK-->0
<i>Bacillus subtilis</i>	2	peracetic acid	2	2600	8	1000: OK-->0
<i>Bacillus subtilis</i>	11	peracetic acid	1			dilution 1e+3 exists 2 times: highest dilution has been set to 1e+4
<i>Mycobacterium terrae</i>	7	all				lab seems to have misunderstood how to enter statuses correctly: N/A-->OK if plate count >0; N/A-->0 if plate count =0; TMTC has been deleted from the plate count fields if there is the status N/A
<i>Mycobacterium terrae</i>	8	phenol	3	17800	2+3	10: N/A-->0
<i>Mycobacterium terrae</i>	12	glutaraldehyde	2	25000	2+3	100: OK-->0
<i>Mycobacterium terrae</i>	25	peracetic acid	2	300	14	100: 0-->OK
<i>Mycobacterium terrae</i>	21	all				empty status field --> N/A
<i>Pseudomonas aeruginosa</i>	26	glutaraldehyde	3	125	16+17	1: N/A-->TMTC
<i>Pseudomonas aeruginosa</i>	13	glutaraldehyde	1	600	2	1: OK-->0
<i>Pseudomonas aeruginosa</i>	11	peracetic acid	2	160	3	1: OK-->0

Organism	Lab	Substance	Run	Concentration levels	Carrier	Correction
<i>Pseudomonas aeruginosa</i>	26	glutaraldehyde	2	250	13-15	100: OK-->0
<i>Pseudomonas aeruginosa</i>	26	glutaraldehyde	1	500	4	1000: OK-->0
<i>Pseudomonas aeruginosa</i>	26	peracetic acid	2	160	2	1000: OK-->0
<i>Pseudomonas aeruginosa</i>	12	peracetic acid	3	120	14+15	1000: OK-->0
<i>Pseudomonas aeruginosa</i>	19	all				no status specifications at all, have been entered manually by quo data
<i>Staphylococcus aureus</i>	19	all				no status specifications at all, have been entered manually by quo data
<i>Staphylococcus aureus</i>	12	benzalkonium chloride	3	3500	6	1+10: OK-->0
<i>Staphylococcus aureus</i>	20	benzalkonium chloride	1	2500	10	10: 0-->OK
<i>Staphylococcus aureus</i>	26	peracetic acid	1	75	16	10: 0-->OK
<i>Staphylococcus aureus</i>	20	glutaraldehyde	3	1300	7-9	10: OK-->0
<i>Staphylococcus aureus</i>	26	glutaraldehyde	4	1100	6	100: 0-->OK
<i>Staphylococcus aureus</i>	22	glutaraldehyde	3	800	10 +11	100: 0-->OK
<i>Staphylococcus aureus</i>	0	glutaraldehyde	3	800	13	1000: TMTC-->0
<i>Staphylococcus aureus</i>	many	all				when 0 as plate count, but status OK, the status has been set to 0
<i>Adenovirus type 5</i> (well count)	14	glutaraldehyde	3	1000	5	10: status 0 -> OK since plate count=1
<i>Adenovirus type 5</i> (well count)	14	peracetic acid	2	200	1 - 3	10: OK -> 0 (status)
<i>Adenovirus type 5</i> (well count)	14	glutaraldehyde	3	500	7	10: status 0 -> OK since plate count=1
<i>Adenovirus type 5</i> (well count)	26	peracetic acid	2 +3	400	13 + 14	10000: 0->OK
<i>Adenovirus type 5</i> (well count)	26	glutaraldehyde	3	500	10	10000: OK->0
<i>Adenovirus type 5</i> (well count)	14	peracetic acid	2	200	1-3	1000: OK->0

**Logistic approach**

204. Besides the actual approach to fit an inactivation curve, a further approach – the Logistic approach – had been investigated. The Logistic approach is applying logistic regression for the fit of survival probabilities. For this approach, it was assumed that the colonies have been considered directly, so that a Bernoulli experiment is applicable with which the required probabilities can be calculated.

205. Firstly, the number of colonies, which originally exist, is calculated by taking the mean value of the dilution-corrected positive plate counts over all dilutions and all carriers considered in the water control. This number constitutes the initial population of the Bernoulli experiment.

206. For each carrier the probability that one colony does survive is calculated in three steps:

- Step 1: For each dilution the number of colonies which could be available is determined by taking the dilution corrected initial population, i.e. in an undiluted samples all colonies identified in the water control could be available. In a dilution with dilution factor 1000, the dilution corrected initial population is a thousandth of the initial population. For example: Assume that the initial population of colonies, i.e. the average number of colonies in the water control, equals 70.000. The following table gives the respective dilution corrected initial populations for 3 dilutions:

Dilution factor	Dilution-corrected initial population size
1	70.000 (= 70.000/1)
10	7.000 (= 70.000/10)
1.000	70 (=70.000/1.000)

Hence, the population size of one carrier is just the sum of the dilution corrected initial population of each considered dilution with positive plate counts, e.g. 77.070 in the example above under the assumption that the mentioned dilutions would be considered.

- Step 2: The sum of plate counts for all considered dilutions is taken. Here, no dilution-correction is necessary.
  - Step 3: The probability that one colony does survive in the respective carrier is calculated by taking the ratio of the sum plate counts obtained in step 2 and the population size obtained in step 1.
207. For each concentration, at least one survival probability is available. Now, the Logistic approach can be applied to fit the survival probabilities depending on the logarithmized concentrations.

208. From the survival probabilities the respective log<sub>10</sub> reduction can be calculated directly by logarithmizing and afterwards multiplying by -1. It has to be noted that the log<sub>10</sub> reductions obtained in this way generally differ from the log<sub>10</sub> reductions used for the analyses presented in the report.

209. The special advantage of the Logistic approach is its applicability also in case of zero counts: it is using count data in a very effective way.
210. Unfortunately, the Logistic approach puts much less weight to the plate counts of higher dilutions than to the plate counts of lower dilutions. In order to avoid extra errors, it had been decided by the experts to give all dilutions the same weight. For this reason the Logistic approach has not been applied in the validation process.

### **Analysis of water controls in phase 1**

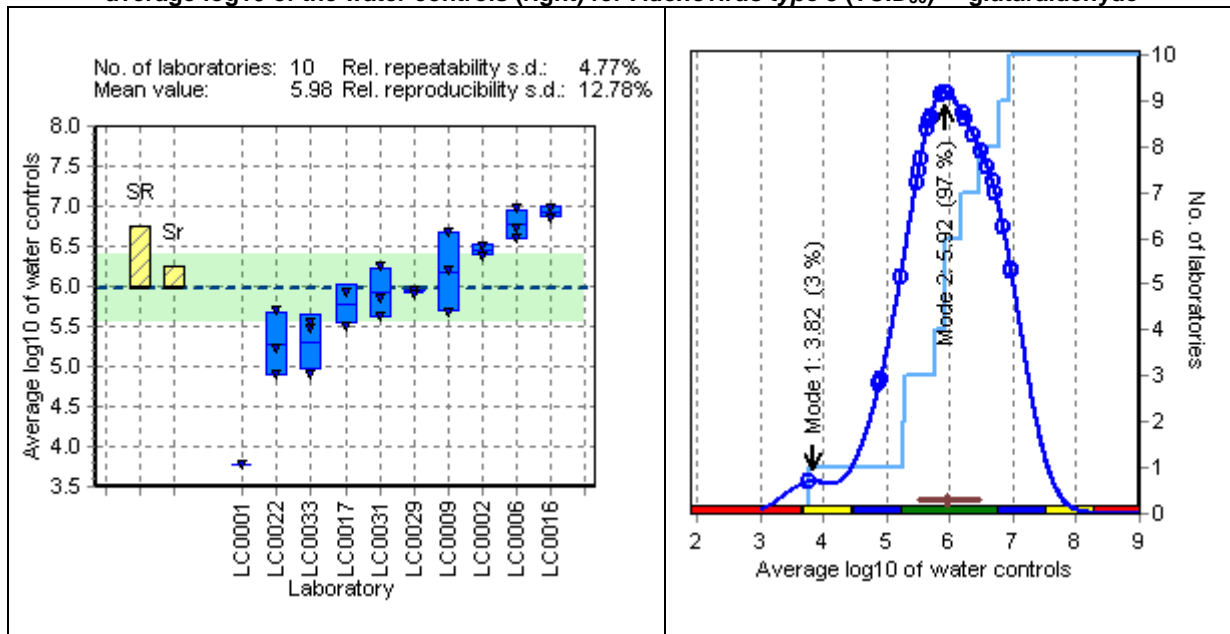
211. In the following, for each combination of organism and substance the variability of the average  $\log_{10}$  of the water controls over the runs is shown as well as the kernel density estimation of the average  $\log_{10}$  of the water controls. However, if there is data from six laboratories or less, it is not reasonable to estimate the kernel. Both kinds of figures are explained in more detail in the following.
212. *Variability of average  $\log_{10}$  of the water controls:* The variability of the average  $\log_{10}$  of the water controls of each laboratory over all available runs is shown as blue boxes. The larger the blue box, the higher is the variability of the average  $\log_{10}$  of the water controls between the runs of one laboratory. Outlier laboratories are marked by red boxes. The dark blue line in the blue boxes indicates the mean average  $\log_{10}$  of the water controls over all available runs. The small blue triangles mark the single average  $\log_{10}$  of the water controls of each run. The mean average  $\log_{10}$  of the water controls over all laboratories and all runs is marked as a dashed blue line over the whole range of laboratories, the respective 95% confidence interval is shown as a light-green band. Furthermore, the reproducibility standard deviation ( $s_R$ ) and the repeatability standard deviation ( $s_r$ ) are shown as yellow bars. In the header of the figures, the number of included laboratories, the mean of the average  $\log_{10}$  of the water controls over all laboratories and all runs as well as the relative reproducibility and repeatability standard deviations are given.
213. *Kernel density estimation of average  $\log_{10}$  of the water controls:* The kernel density estimation of the average  $\log_{10}$  of the water controls is shown as a dark blue curve, it is based on the data without the determined outliers. If outliers have been detected, the respective distribution is shown as a red curve. The sum of the blue and the red curve (i.e. the sum of the distributions of the data without outliers and of the outlier data) is shown as a black curve. It might be covered by the red or the blue curve sometimes. On the curves, the single average  $\log_{10}$  of the water controls are marked as small circles. The empirical distribution of the laboratory mean values is shown as a light-blue step function, the number of laboratories can be determined on the right axis. The horizontal brown bar indicates the overall mean value with the respective 95% confidence interval. On the bottom axis, the following ranges around the overall mean are marked by different colors:

Values that differ less than the reproducibility standard deviation from the overall mean	Green
Values that differ more than one time the reproducibility standard deviation but less than two times the reproducibility standard deviation from the overall mean	Blue
Values that differ more than two times the reproducibility standard deviation but less than three times the reproducibility standard deviation from the overall mean	Yellow
Values that differ more than three time the reproducibility standard deviation from the overall mean	Red

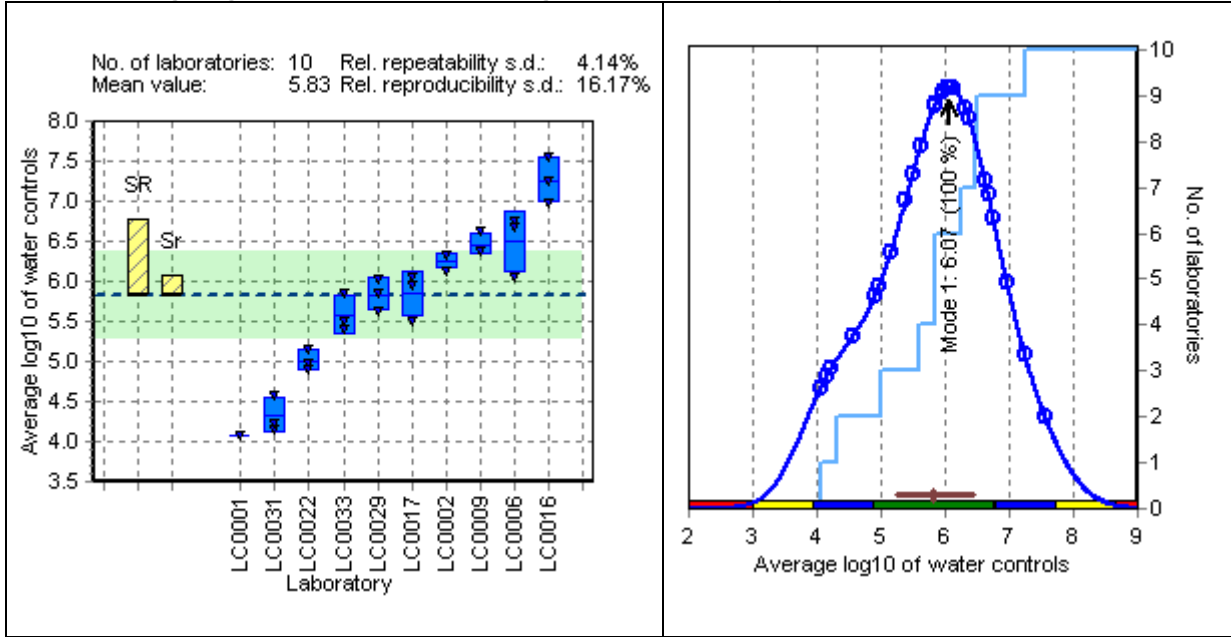
214. Furthermore, the modi (maxima) of the curves are marked by arrows and the respective value is given. Distributions with only one modus are called unimodal, while distributions with two modi are called bimodal. Bimodal distributions indicate that there might be 2 populations of laboratories, i.e. that there might be two groups of laboratories with clearly differing results.

215. For *Adenovirus type 5* (TCID<sub>50</sub>) and both analyzed substances, slightly left-skewed distributions can be observed. For glutaraldehyde a second mode can be seen, however, this is based on the values of only one laboratory (LC0001) with a clearly smaller average log<sub>10</sub> of the water controls. Still, the mean value of this laboratory did not prove to be a significant outlier but only a straggler (significant at the significance level of 5%) and has therefore not been eliminated.

**Figure 114: Variability of average log<sub>10</sub> of the water controls (left) and kernel density estimation of average log<sub>10</sub> of the water controls (right) for *Adenovirus type 5* (TCID<sub>50</sub>) – glutaraldehyde**

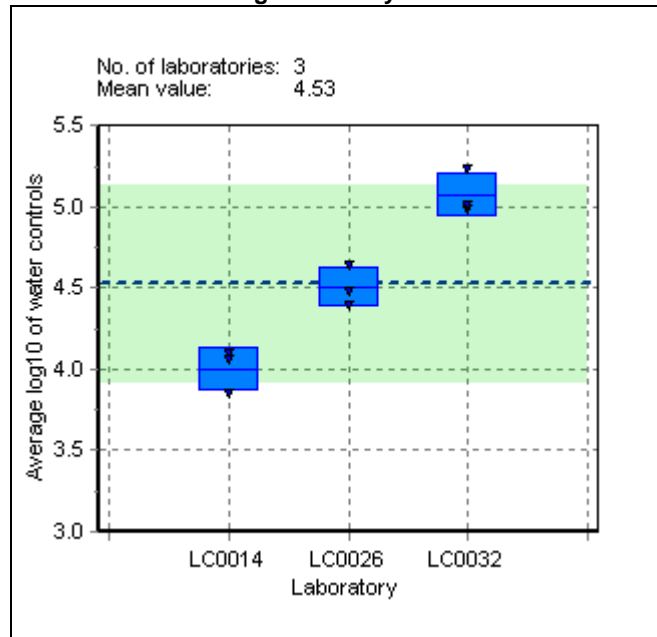


**Figure 115: Variability of average log<sub>10</sub> of the water controls (left) and kernel density estimation of average log<sub>10</sub> of the water controls (right) for *Adenovirus type 5* (TCID<sub>50</sub>) – peracetic acid**

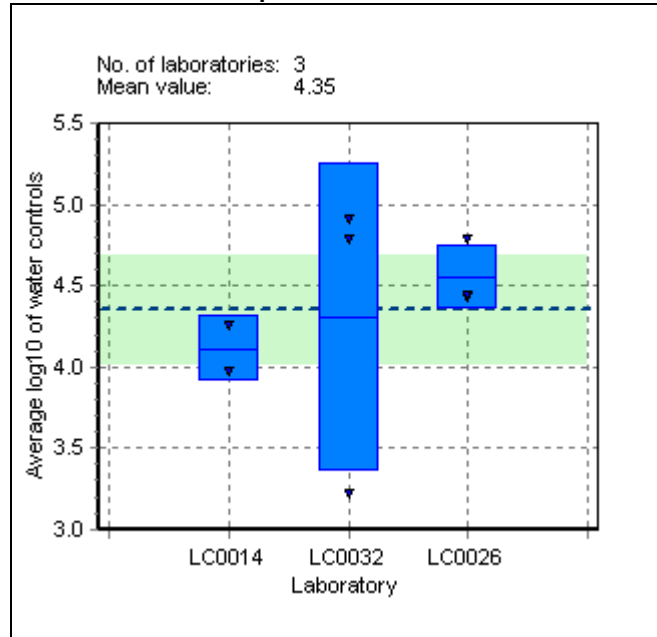


216. For *Adenovirus type 5* (well count) only the variability of the average log<sub>10</sub> of the water controls of the three laboratories are shown. Due to the small number of laboratories it is not reasonable to estimate the kernel density or determine the standard deviations. For peracetic acid, laboratory LC0032 exhibits a clearly higher variability of the average log<sub>10</sub> of the water controls than the other two laboratories. However, the Cochran test yielded in a significant result only on the significance level of 5% and therefore the laboratory cannot be considered as an outlier but as a straggler.

**Figure 116: Variability of average log<sub>10</sub> of the water controls for *Adenovirus type 5* (well count) – glutaraldehyde**

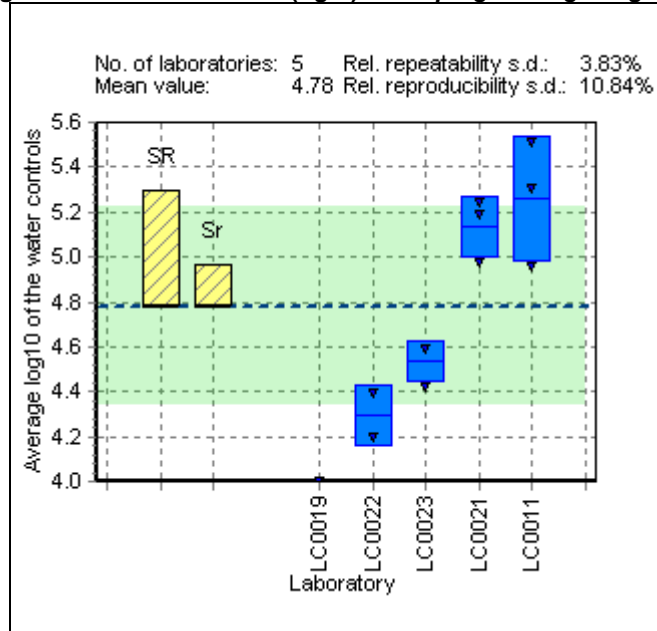


**Figure 117: Variability of average log<sub>10</sub> of the water controls for *Adenovirus type 5* (well count) – peracetic acid**

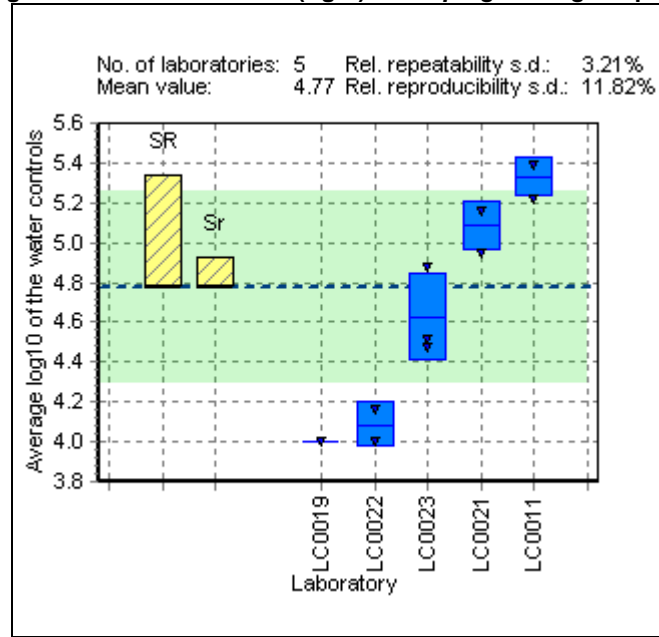


217. For *Aspergillus niger* it is not reasonable to estimate the kernel density due to the small number of laboratories.

**Figure 118: Variability of average log<sub>10</sub> of the water controls (left) and kernel density estimation of average log<sub>10</sub> of the water controls (right) for *Aspergillus niger* – glutaraldehyde**

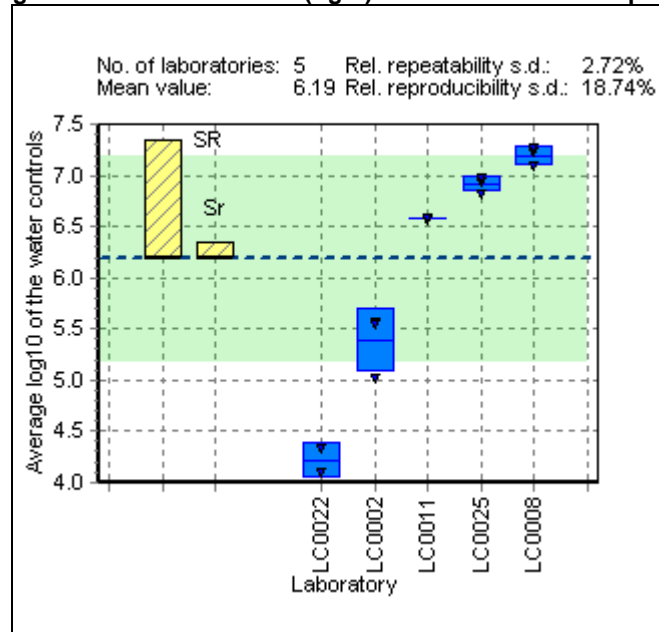


**Figure 119: Variability of average log<sub>10</sub> of the water controls (left) and kernel density estimation of average log<sub>10</sub> of the water controls (right) for *Aspergillus niger* – peracetic acid**



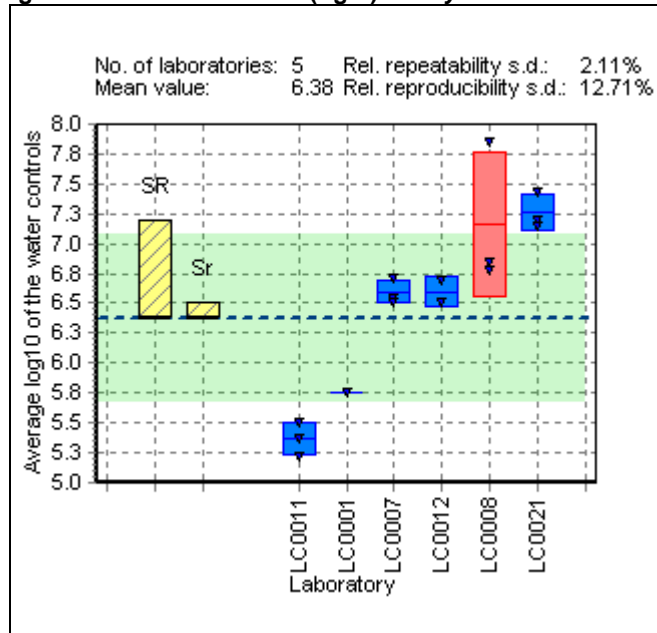
218. For *Bacillus subtilis* it is not reasonable to estimate the kernel density due to the small number of laboratories.

**Figure 120: Variability of average log<sub>10</sub> of the water controls (left) and kernel density estimation of average log<sub>10</sub> of the water controls (right) for *Bacillus subtilis* – peracetic acid**

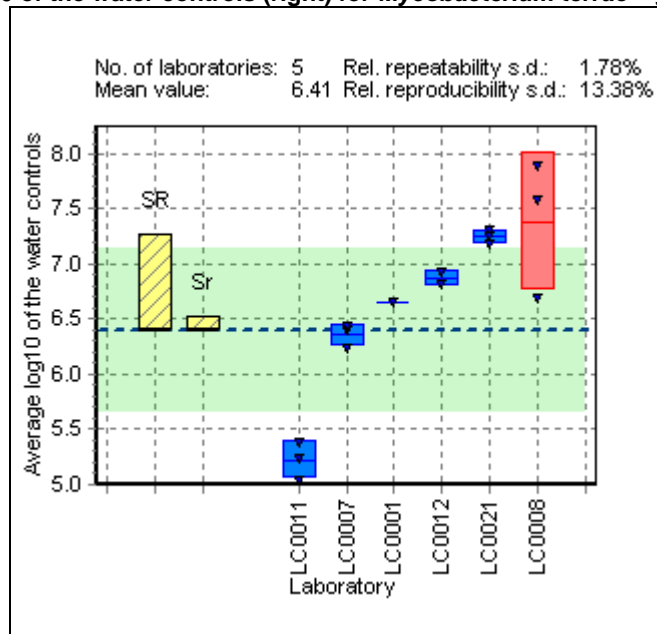


219. For *Mycobacterium terrae* it is not reasonable to estimate the kernel density due to the small number of laboratories.

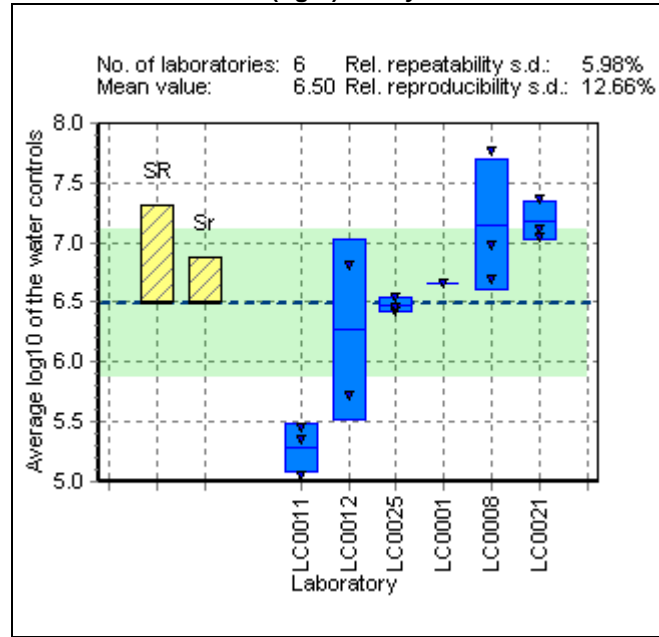
**Figure 121: Variability of average log<sub>10</sub> of the water controls (left) and kernel density estimation of average log<sub>10</sub> of the water controls (right) for *Mycobacterium terrae* – phenol**



**Figure 122: Variability of average log<sub>10</sub> of the water controls (left) and kernel density estimation of average log<sub>10</sub> of the water controls (right) for *Mycobacterium terrae* – glutaraldehyde**

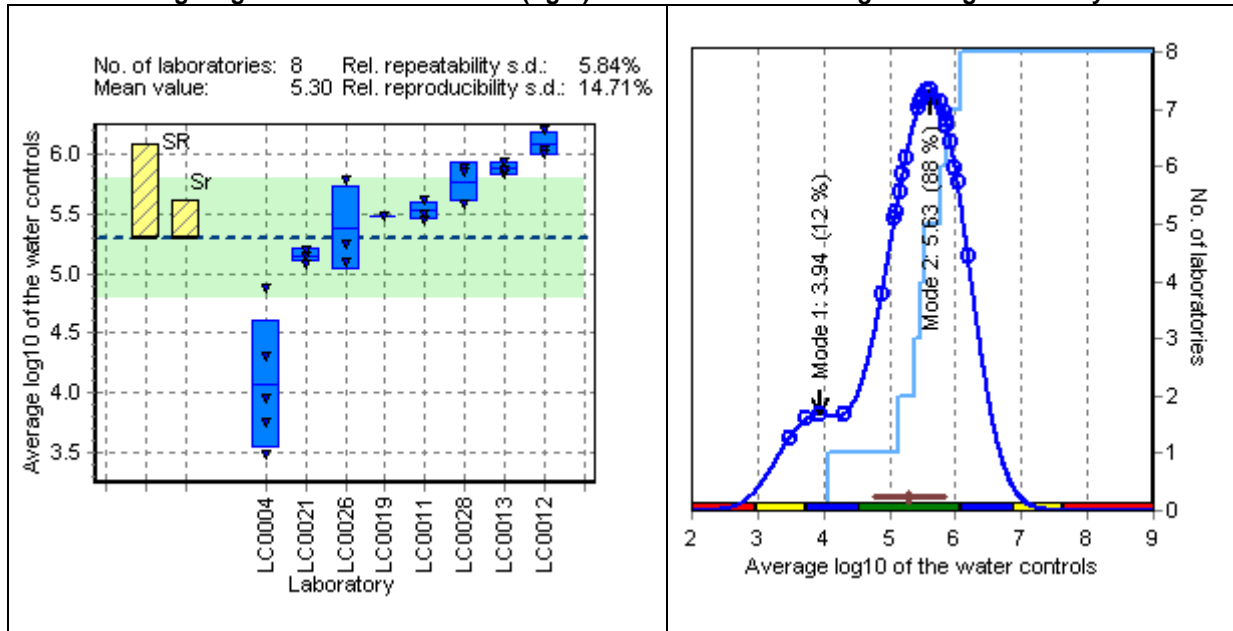


**Figure 123: Variability of average log<sub>10</sub> of the water controls (left) and kernel density estimation of average log<sub>10</sub> of the water controls (right) for *Mycobacterium terrae* – peracetic acid**

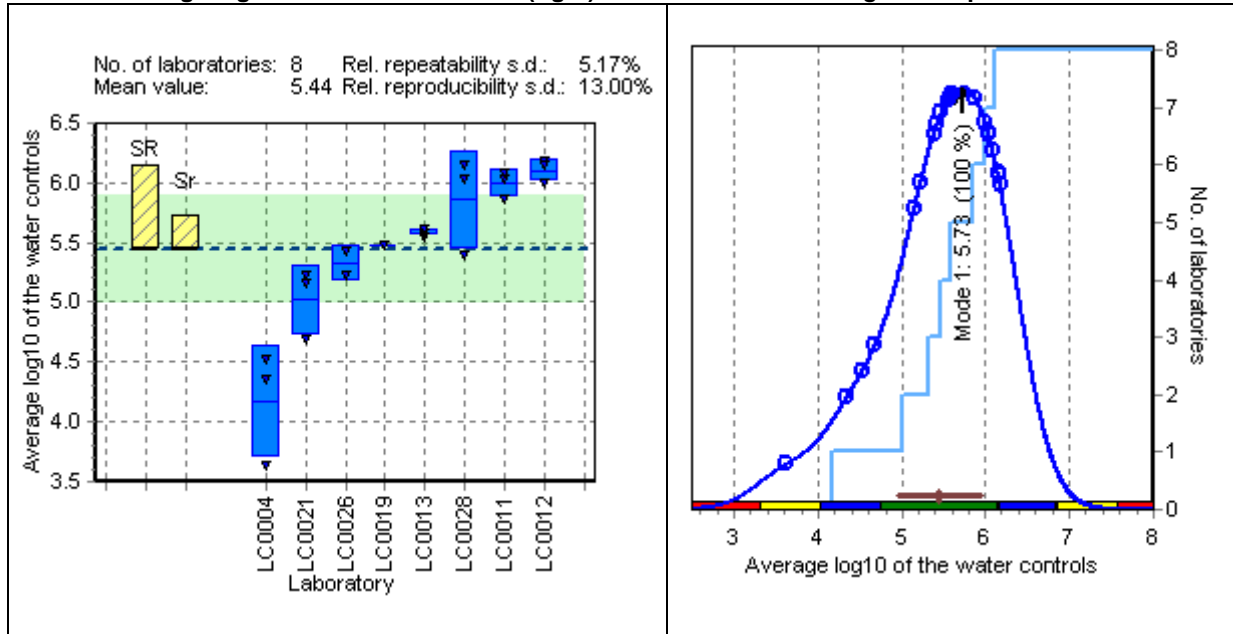


220. For *Pseudomonas aeruginosa* and peracetic acid a slightly left-skewed unimodal distribution can be observed. For glutaraldehyde, a second mode can be observed, which however, is based on 12 % of the measurement values only and cannot be considered as a second group of laboratories with clearly differing results.

**Figure 124: Variability of average log<sub>10</sub> of the water controls (left) and kernel density estimation of average log<sub>10</sub> of the water controls (right) for *Pseudomonas aeruginosa* – glutaraldehyde**

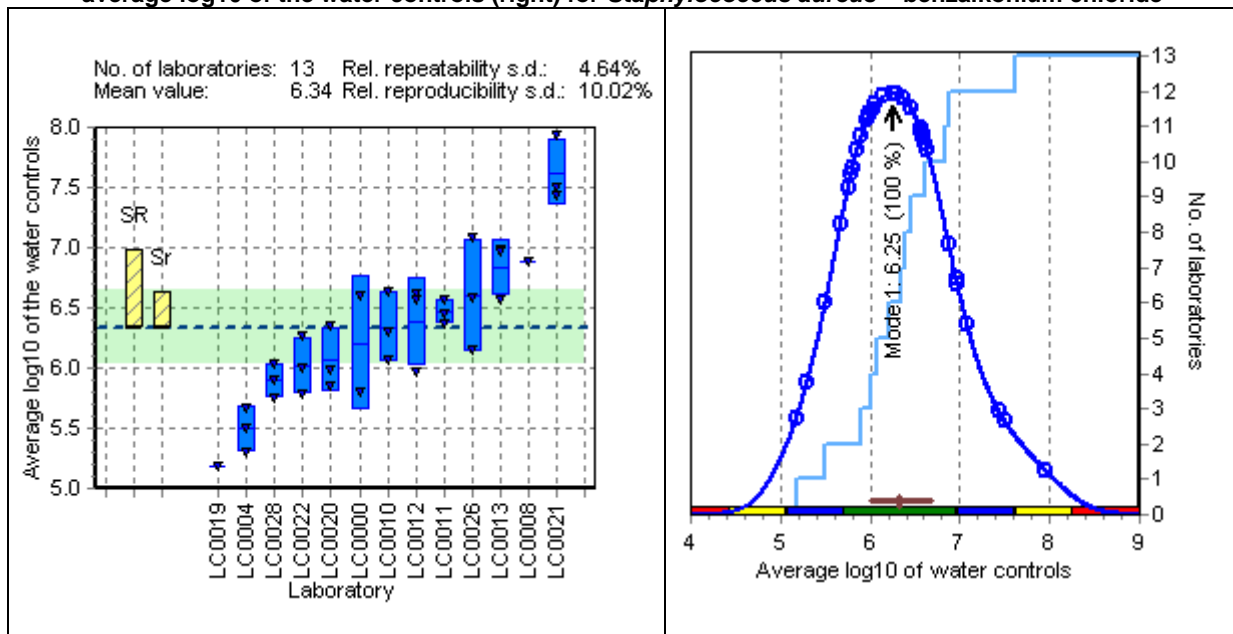


**Figure 125: Variability of average log<sub>10</sub> of the water controls (left) and kernel density estimation of average log<sub>10</sub> of the water controls (right) for *Pseudomonas aeruginosa* – peracetic acid**

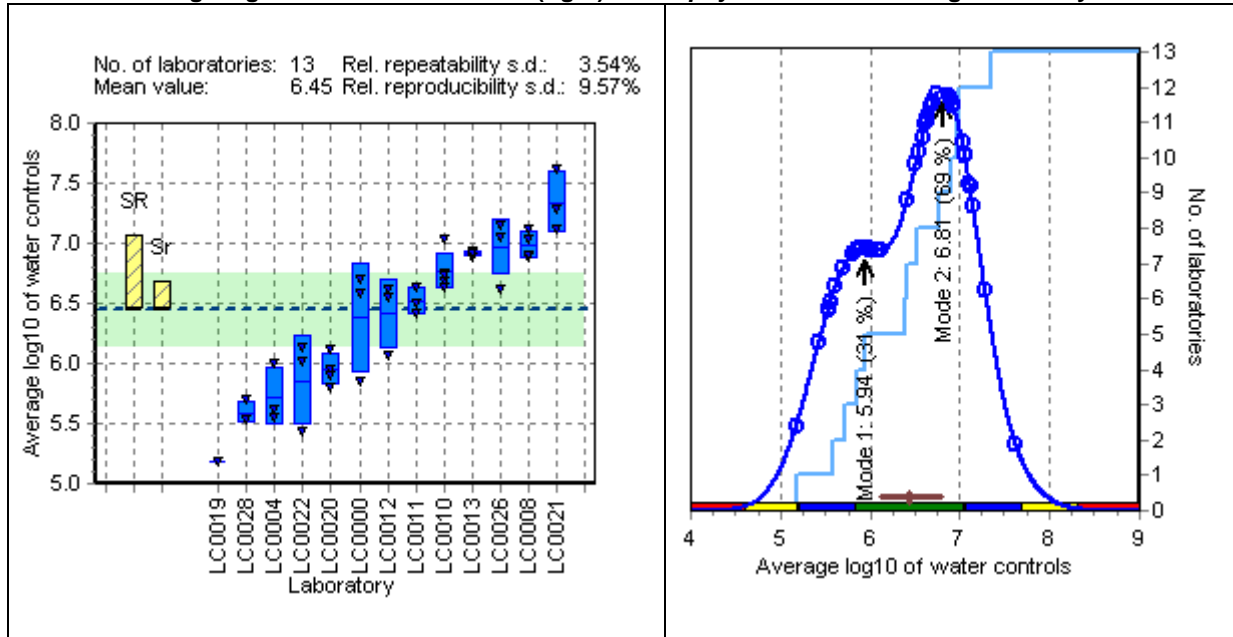


221. For *Staphylococcus aureus* and benzalkonium chloride as well as peracetic acid, unimodal distributions can be observed which for benzalkonium chloride coincides very well the normal distribution. For peracetic acid, however, a slight tendency to a second modus can be seen. The distribution of the average log<sub>10</sub> of the water controls for glutaraldehyde is bimodal and differs from the normal distribution. For the latter substance there might be two groups of laboratories with clearly differing results.

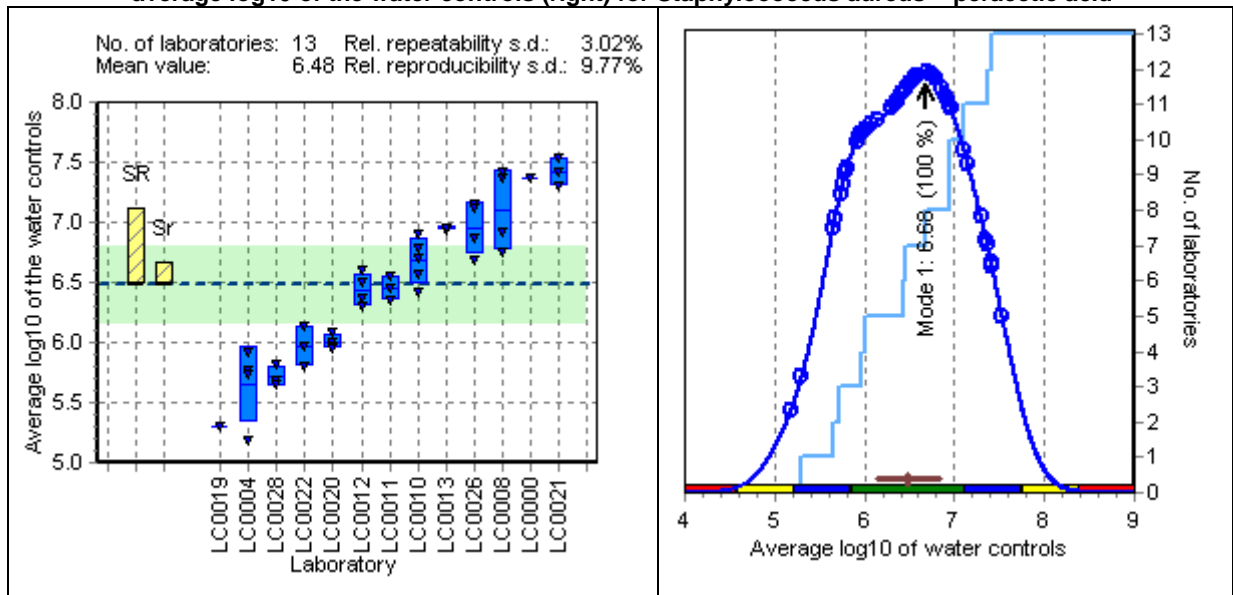
**Figure 126: Variability of average log<sub>10</sub> of the water controls (left) and kernel density estimation of average log<sub>10</sub> of the water controls (right) for *Staphylococcus aureus* – benzalkonium chloride**



**Figure 127: Variability of average log<sub>10</sub> of the water controls (left) and kernel density estimation of average log<sub>10</sub> of the water controls (right) for *Staphylococcus aureus* – glutaraldehyde**



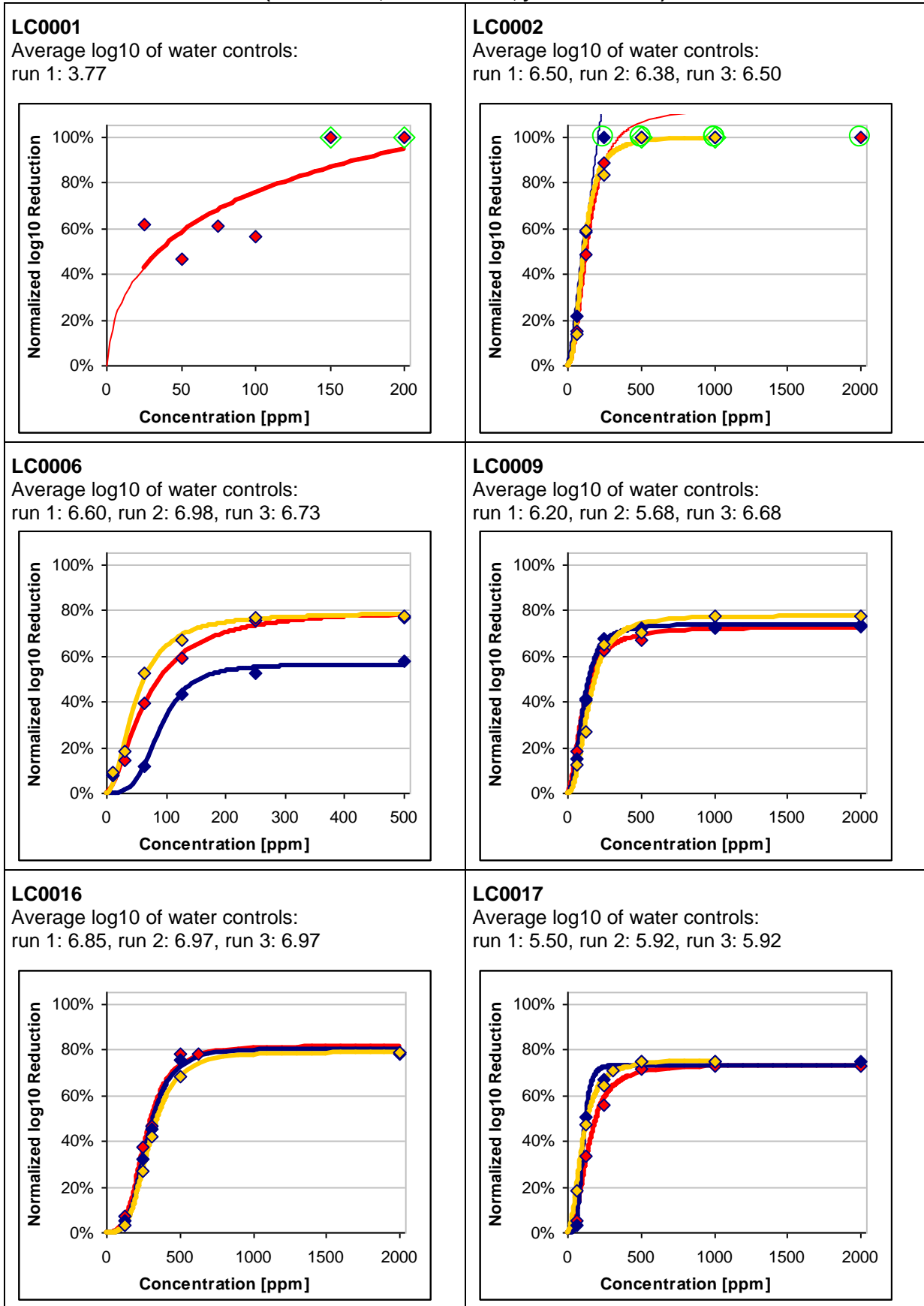
**Figure 128: Variability of average log<sub>10</sub> of the water controls (left) and kernel density estimation of average log<sub>10</sub> of the water controls (right) for *Staphylococcus aureus* – peracetic acid**



### **Normalized run-specific inactivation curves of phase 1**

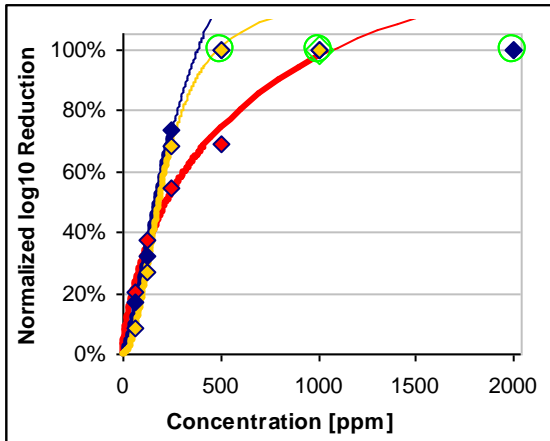
222. In the following, for each combination of organism and substance the run-specific inactivation curves and the mean log<sub>10</sub> reductions at each concentration level are shown separately for each laboratory. All available data is being displayed, i.e. also not-regarded runs and outlier runs. Extrapolated run-specific inactivation curves are shown as thin continuations of the non-extrapolated curve. This applies to cases where the mean log<sub>10</sub> 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 log<sub>10</sub> 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 129: Normalized run-specific kill curves for *Adenovirus type 5* (TCID<sub>50</sub>) – glutaraldehyde (red = 1st run, blue = 2nd run, yellow = 3rd run)**



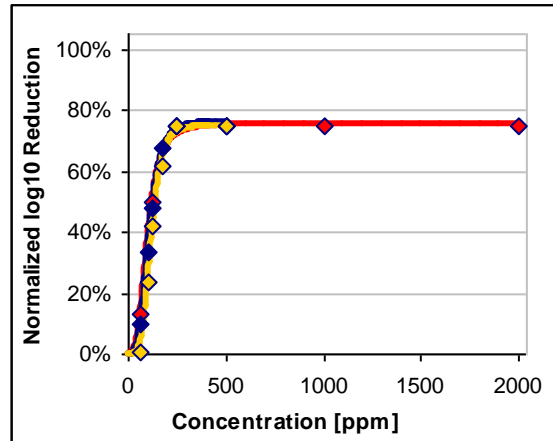
**LC0022**

Average log<sub>10</sub> of water controls:  
run 1: 5.22, run 2: 5.69, run 3: 4.91



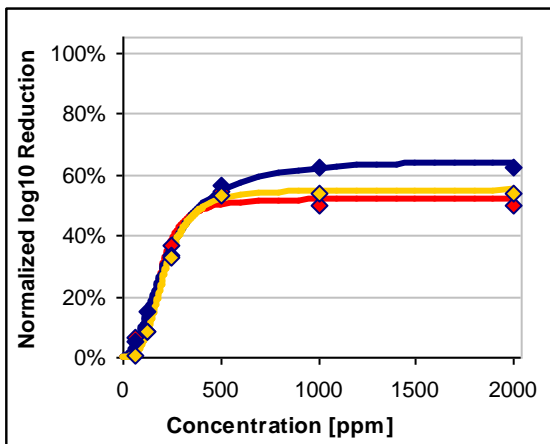
**LC0029**

Average log<sub>10</sub> of water controls:  
run 1: 5.94, run 2: 5.94, run 3: 5.91



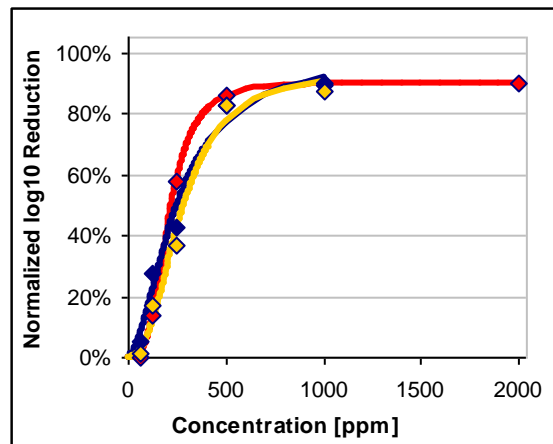
**LC0031**

Average log<sub>10</sub> of water controls:  
run 1: 6.25, run 2: 5.64, run 3: 5.86

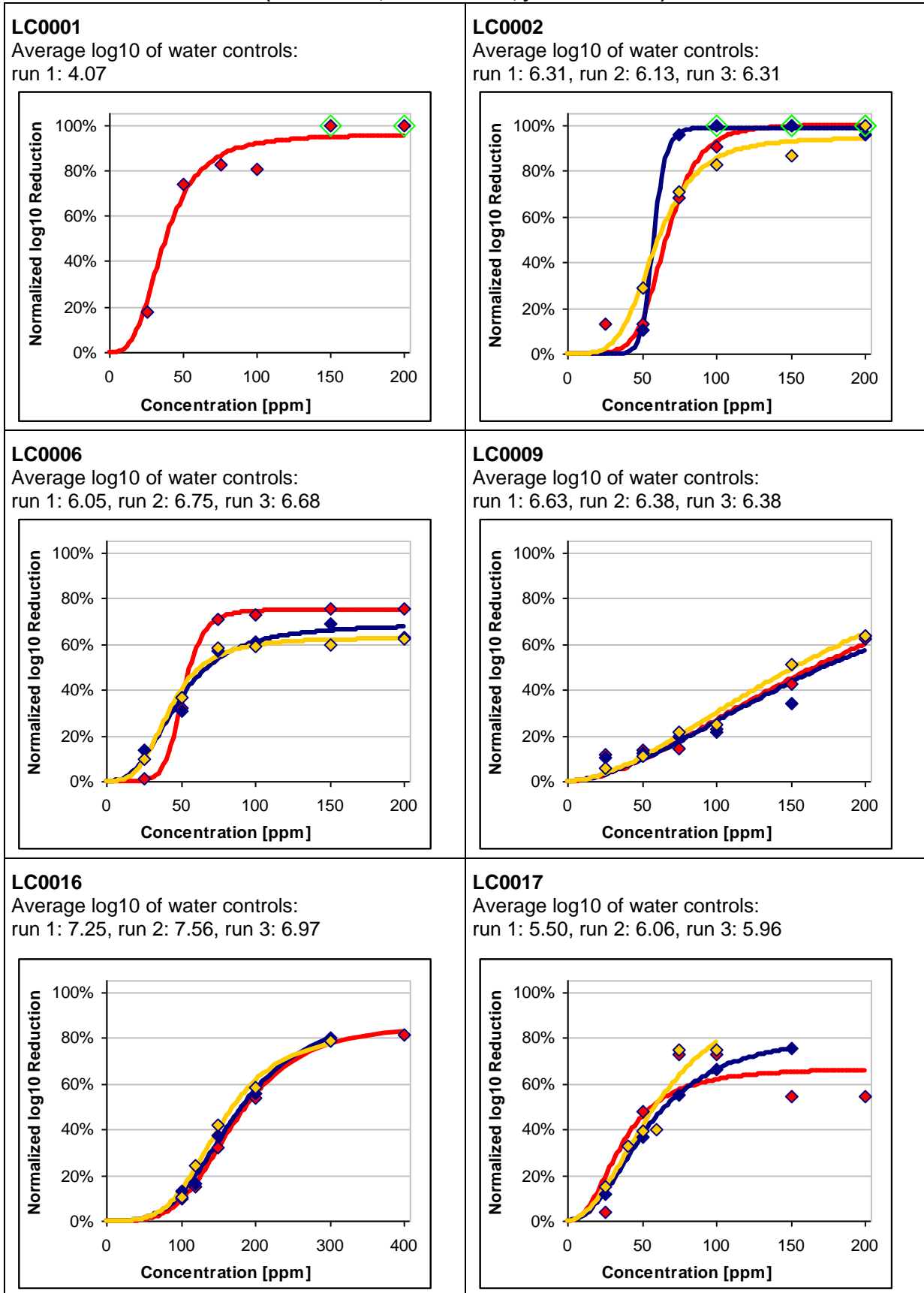


**LC0033**

Average log<sub>10</sub> of water controls:  
run 1: 4.89, run 2: 5.47, run 3: 5.54

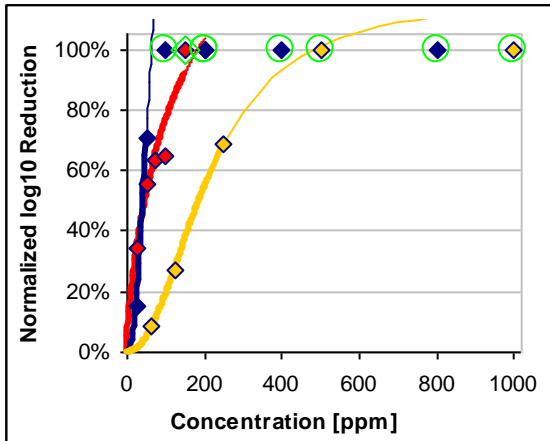


**Figure 130: Normalized run-specific kill curves for *Adenovirus type 5* (TCID<sub>50</sub>) – peracetic acid (red = 1st run, blue = 2nd run, yellow = 3rd run)**



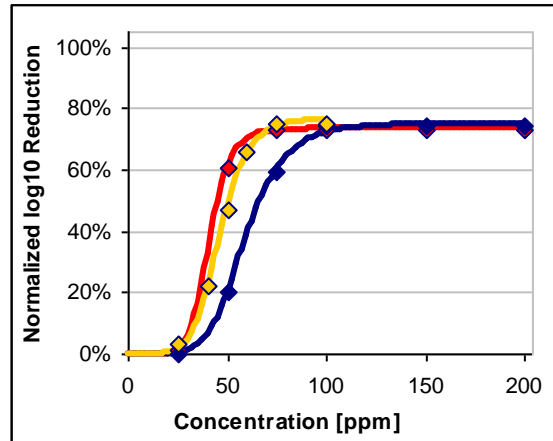
**LC0022**

Average log<sub>10</sub> of water controls:  
run 1: 4.97, run 2: 5.16, run 3: 4.91



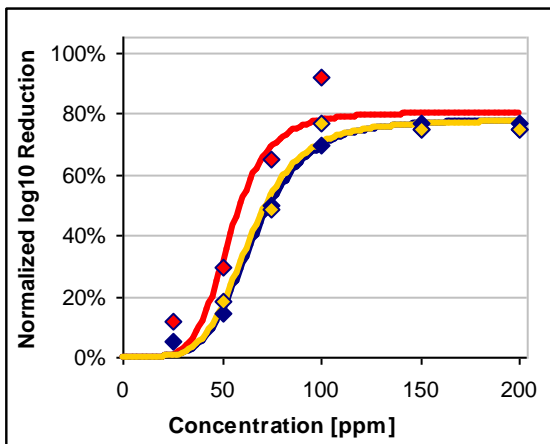
**LC0029**

Average log<sub>10</sub> of water controls:  
run 1: 5.63, run 2: 5.85, run 3: 6.03



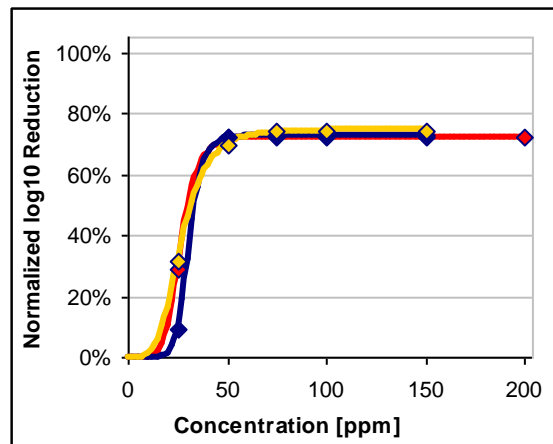
**LC0031**

Average log<sub>10</sub> of water controls:  
run 1: 4.16, run 2: 4.57, run 3: 4.22

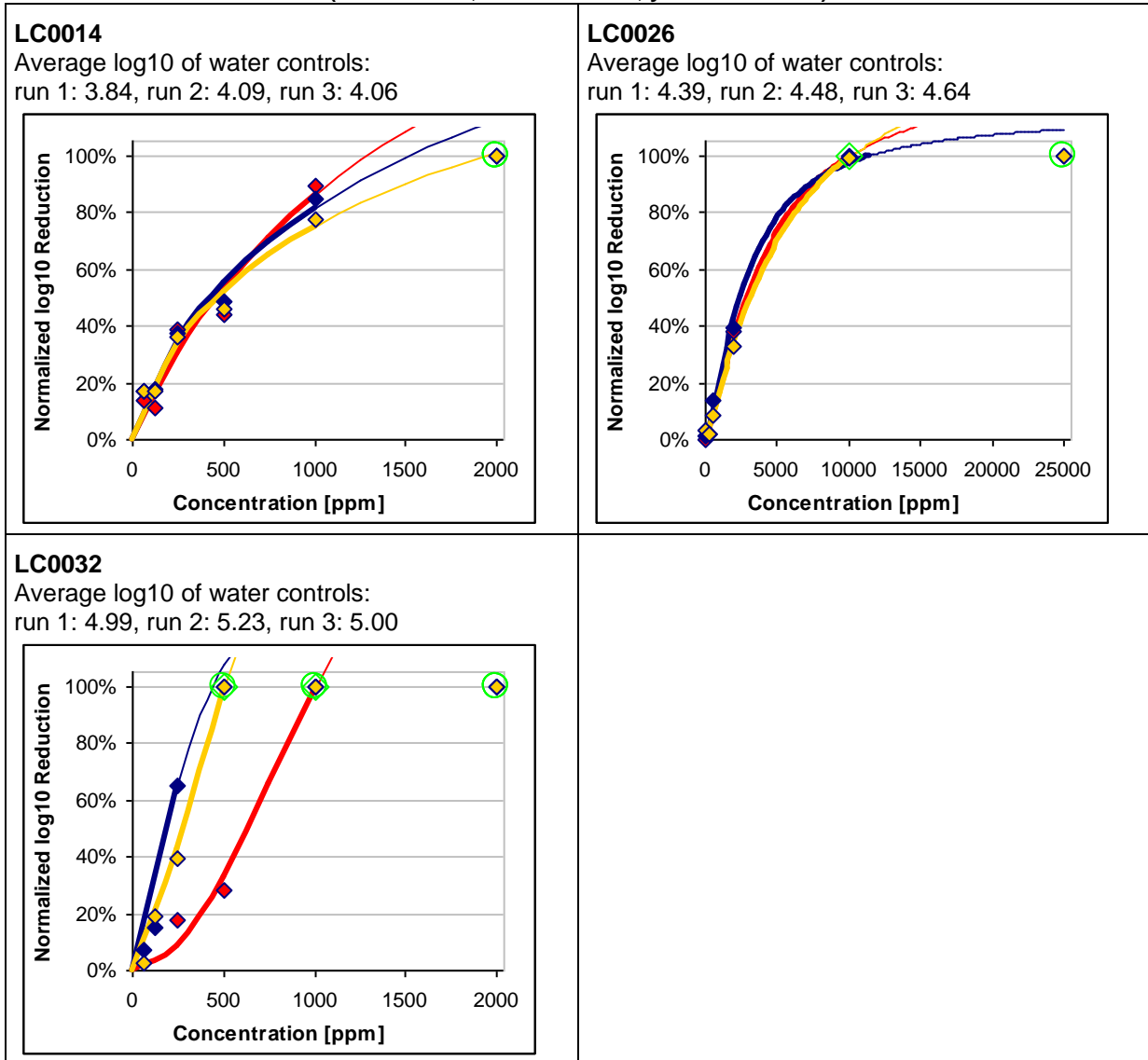


**LC0033**

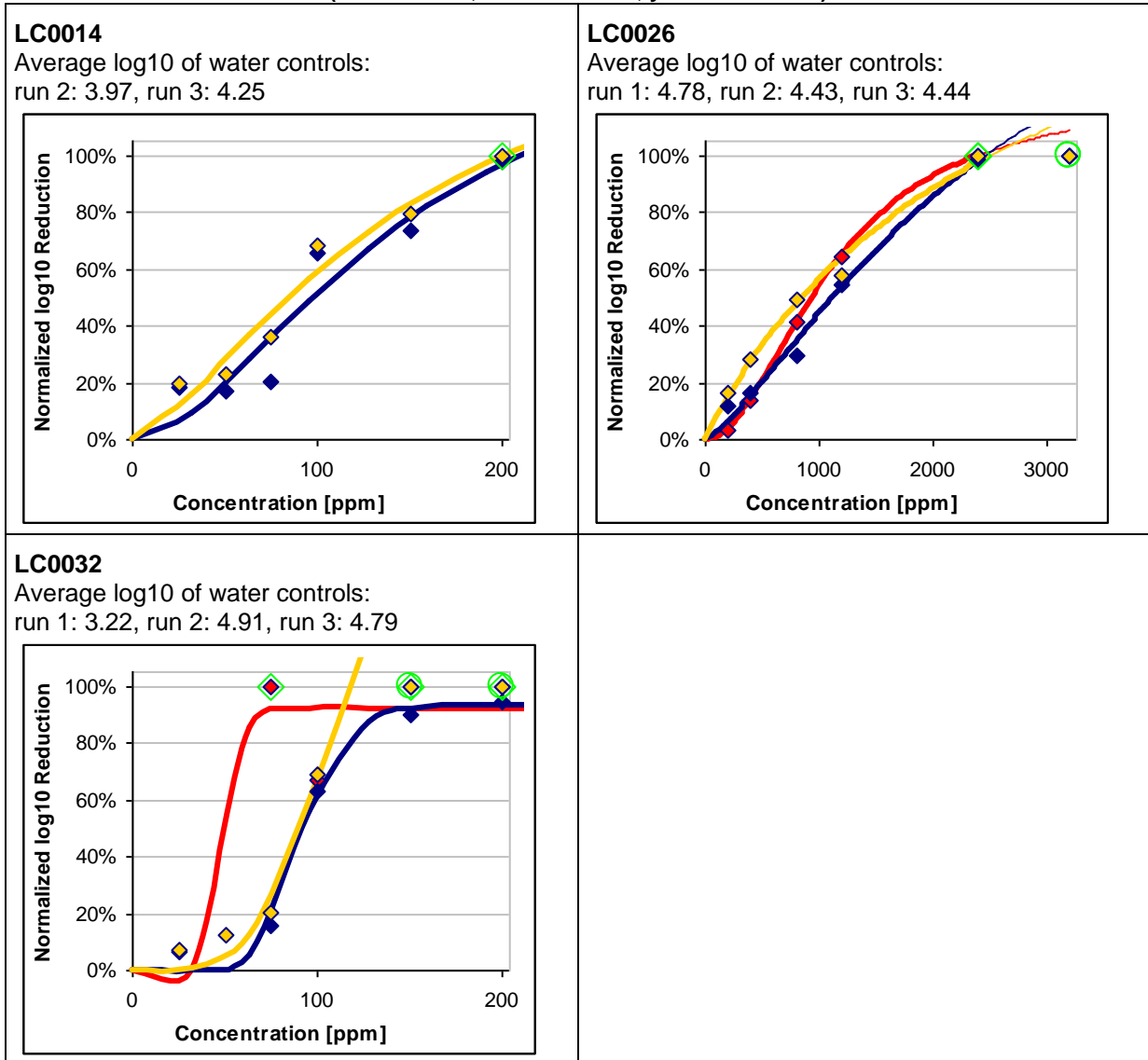
Average log<sub>10</sub> of water controls:  
run 1: 5.39, run 2: 5.50, run 3: 5.86



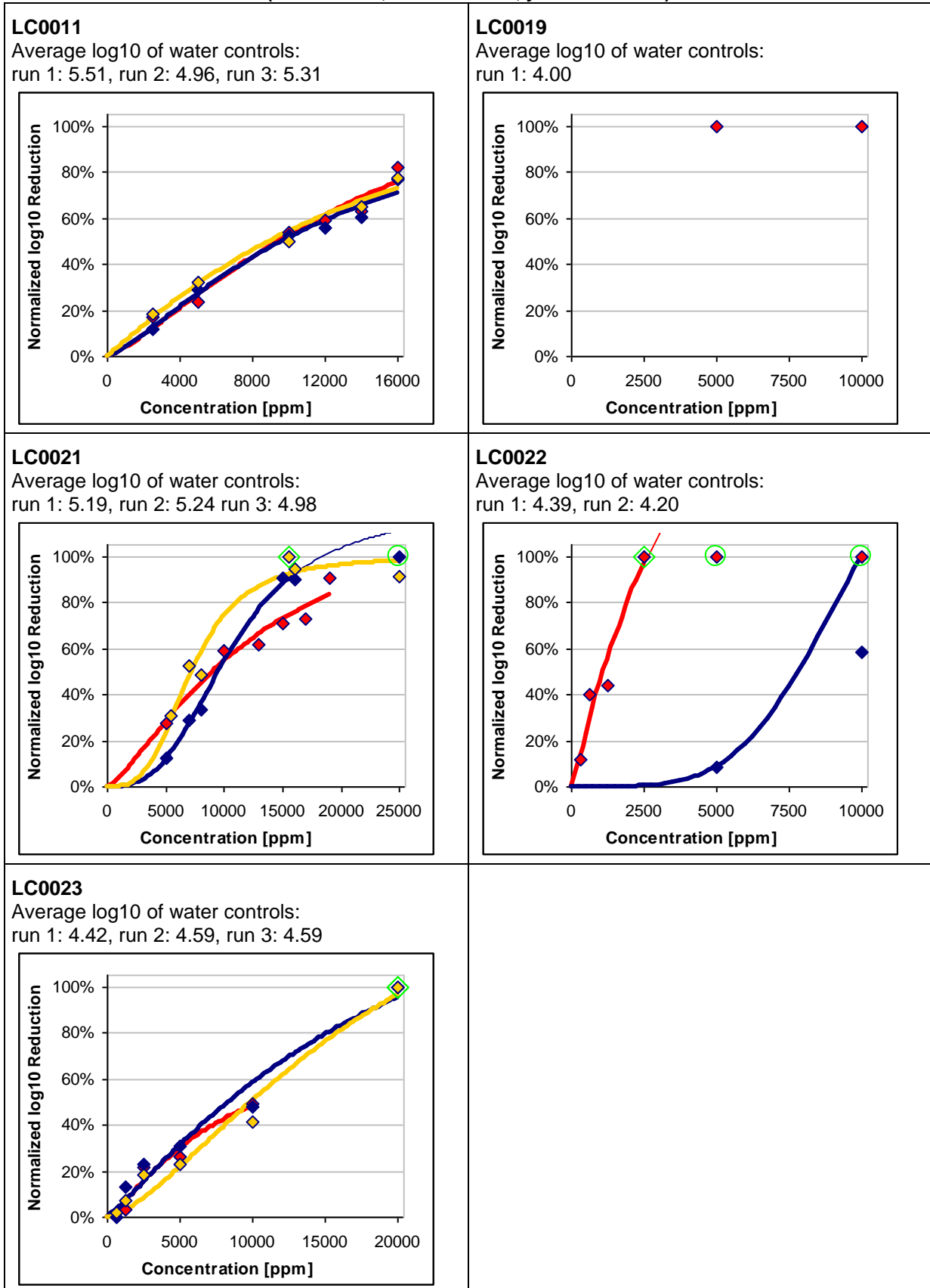
**Figure 131: Normalized run-specific kill curves for *Adenovirus type 5* (well count) – glutaraldehyde**  
 (red = 1st run, blue = 2nd run, yellow = 3rd run)



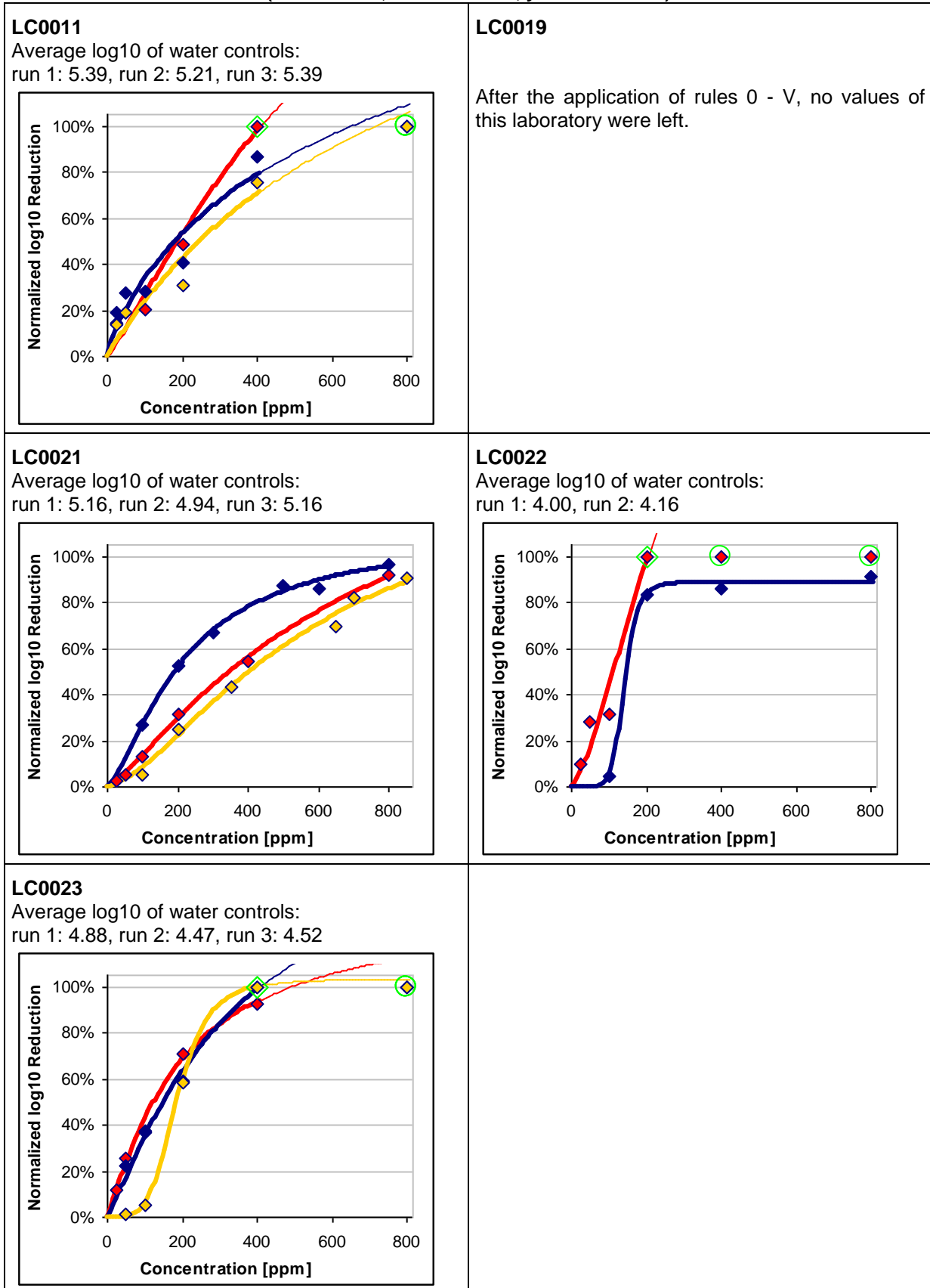
**Figure 132: Normalized run-specific kill curves for *Adenovirus type 5* (well count) – peracetic acid (red = 1st run, blue = 2nd run, yellow = 3rd run)**



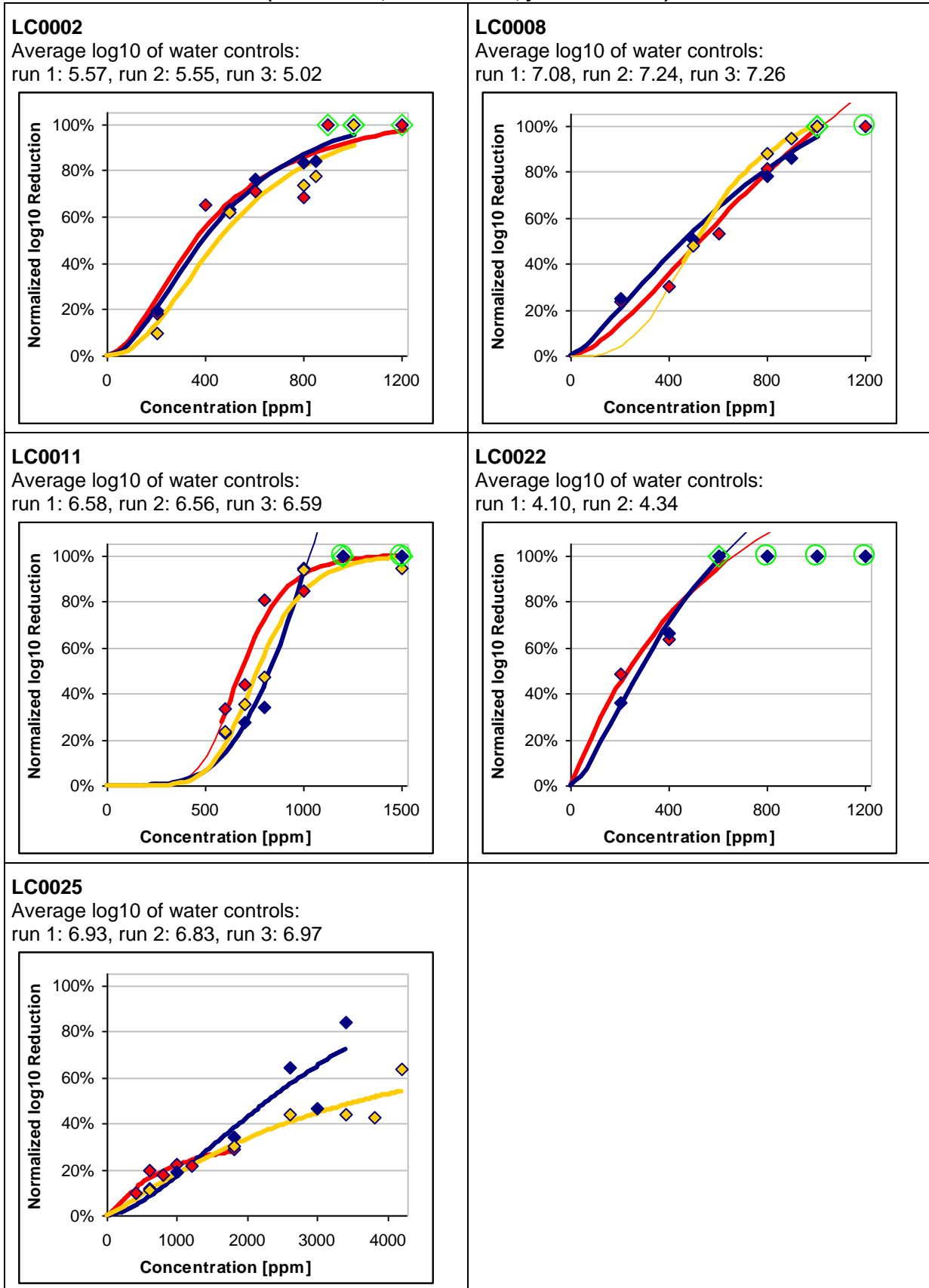
**Figure 133: Normalized run-specific kill curves for *Aspergillus niger* – glutaraldehyde (red = 1st run, blue = 2nd run, yellow = 3rd run)**



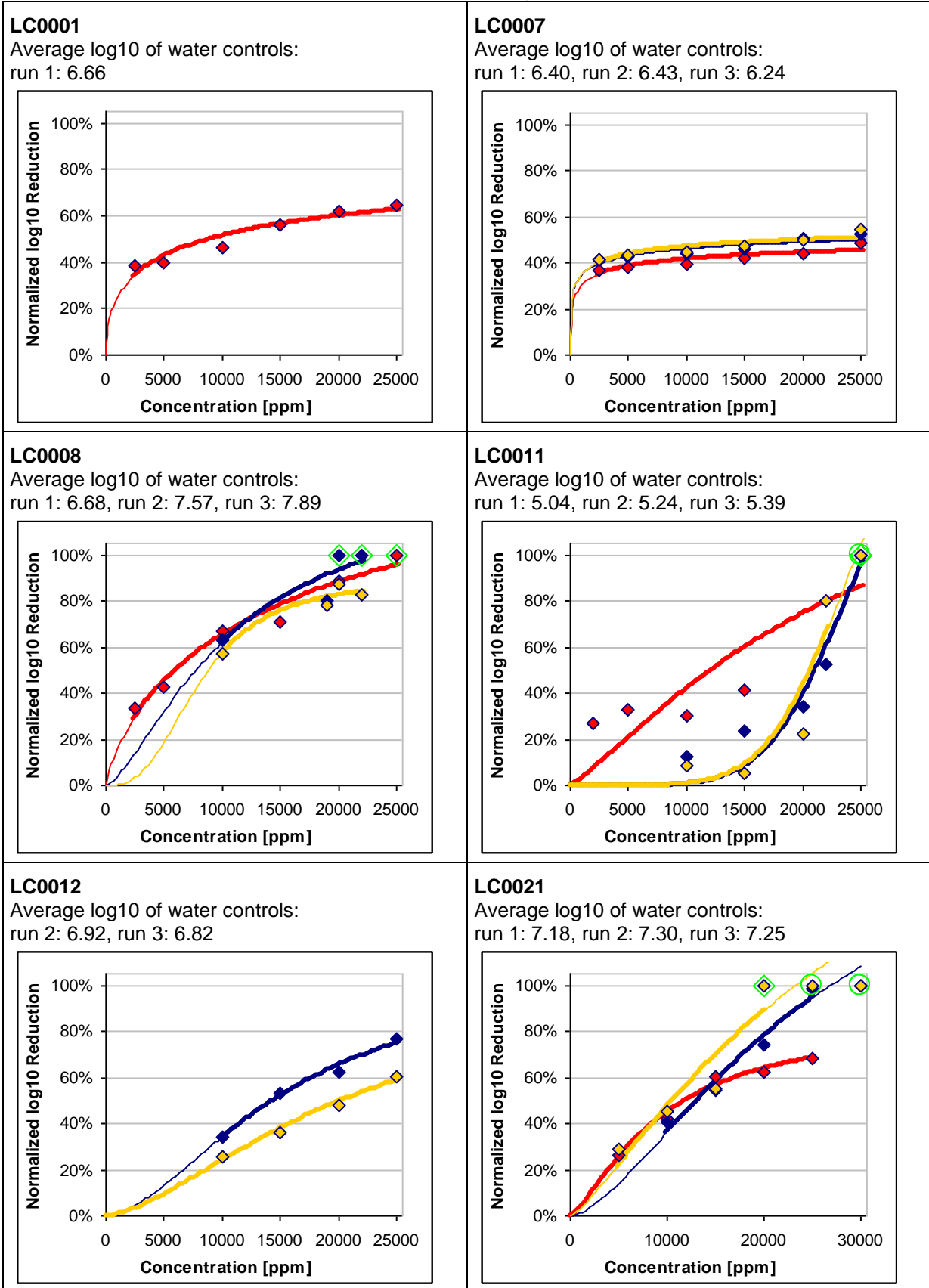
**Figure 134: Normalized run-specific kill curves for *Aspergillus niger* – peracetic acid**  
 (red = 1st run, blue = 2nd run, yellow = 3rd run)



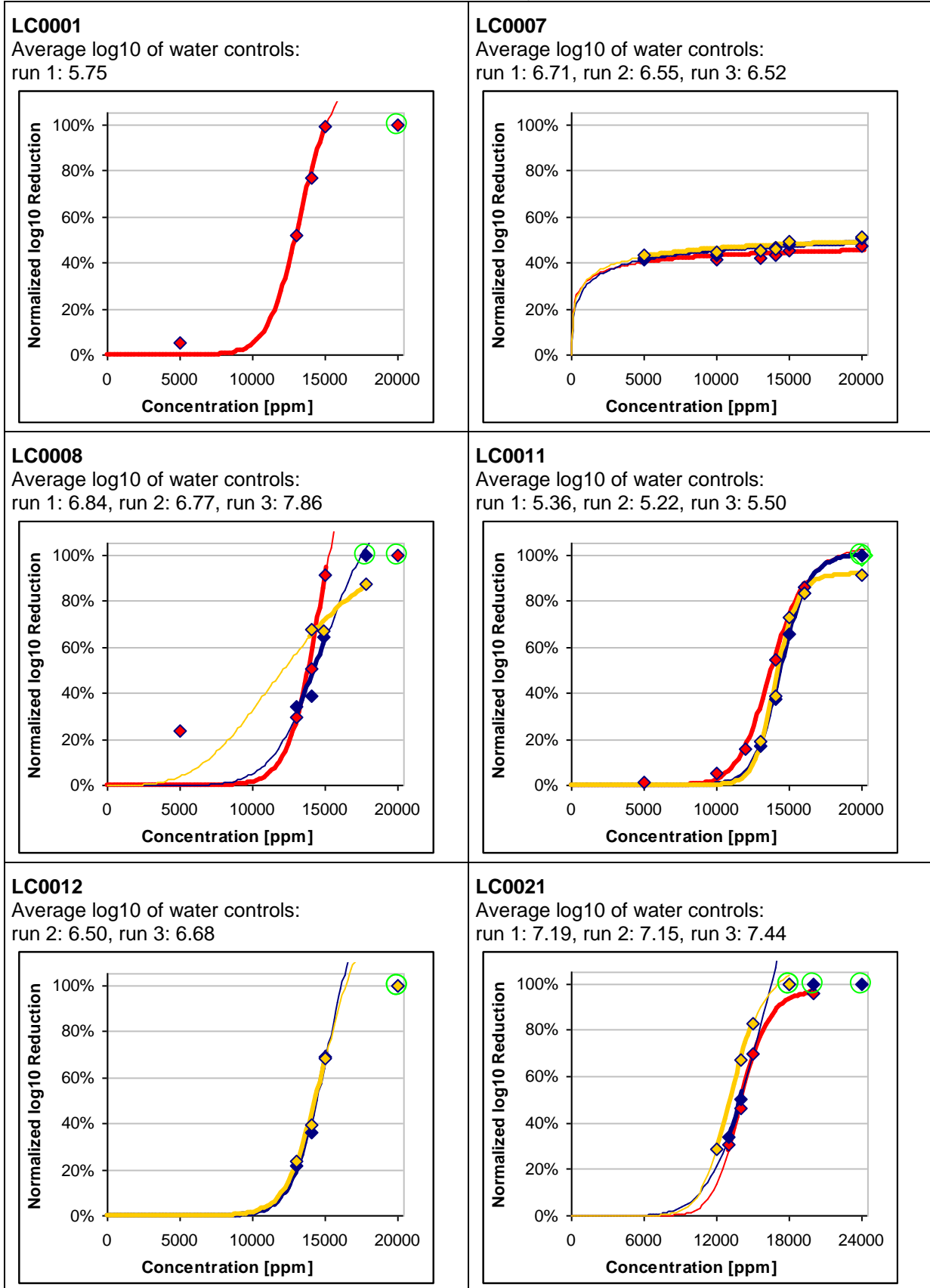
**Figure 135: Normalized run-specific kill curves for *Bacillus subtilis* – peracetic acid (red = 1st run, blue = 2nd run, yellow = 3rd run)**



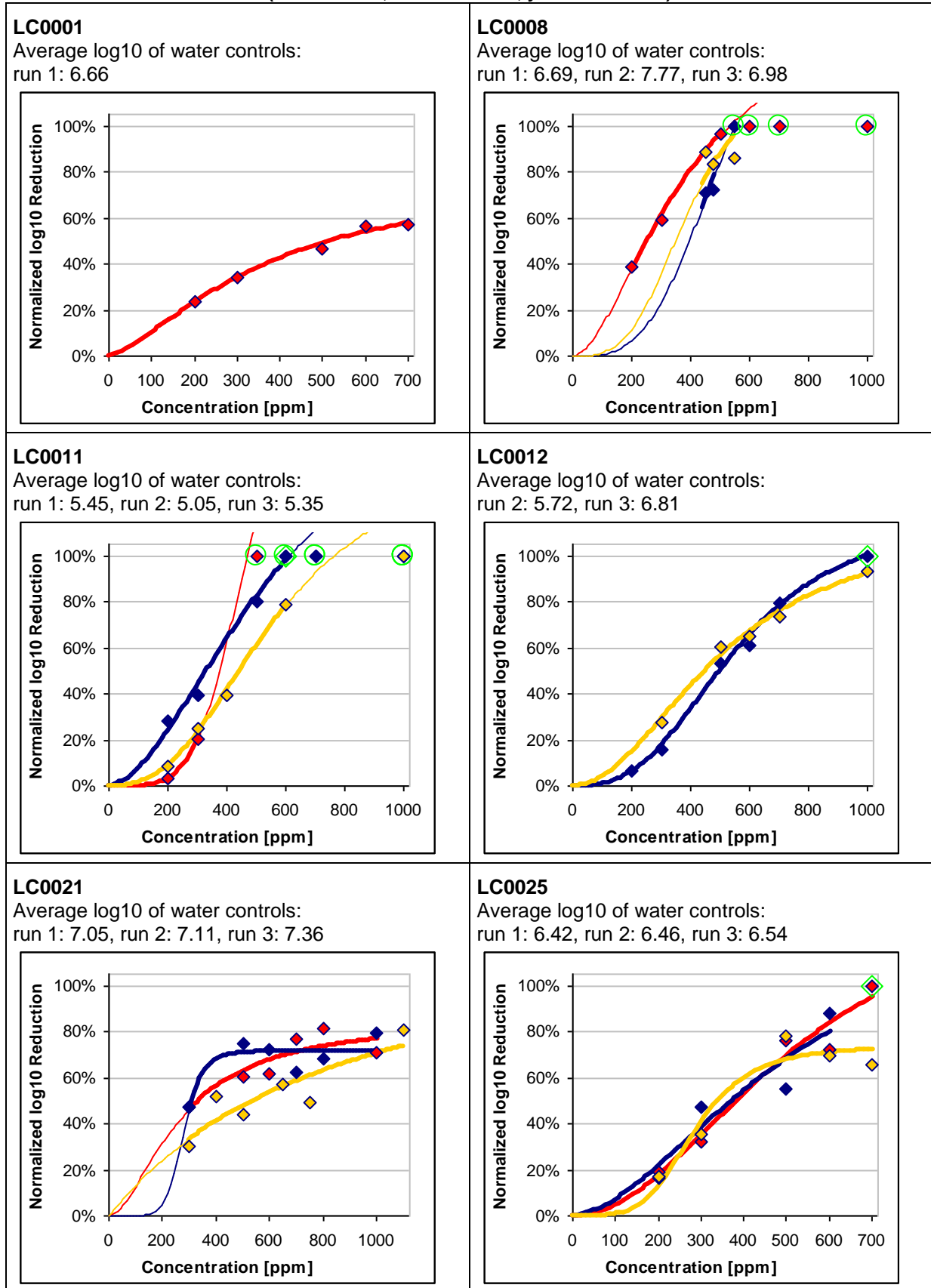
**Figure 136: Normalized run-specific kill curves for *Mycobacterium terrae* – glutaraldehyde**  
 (red = 1st run, blue = 2nd run, yellow = 3rd run)



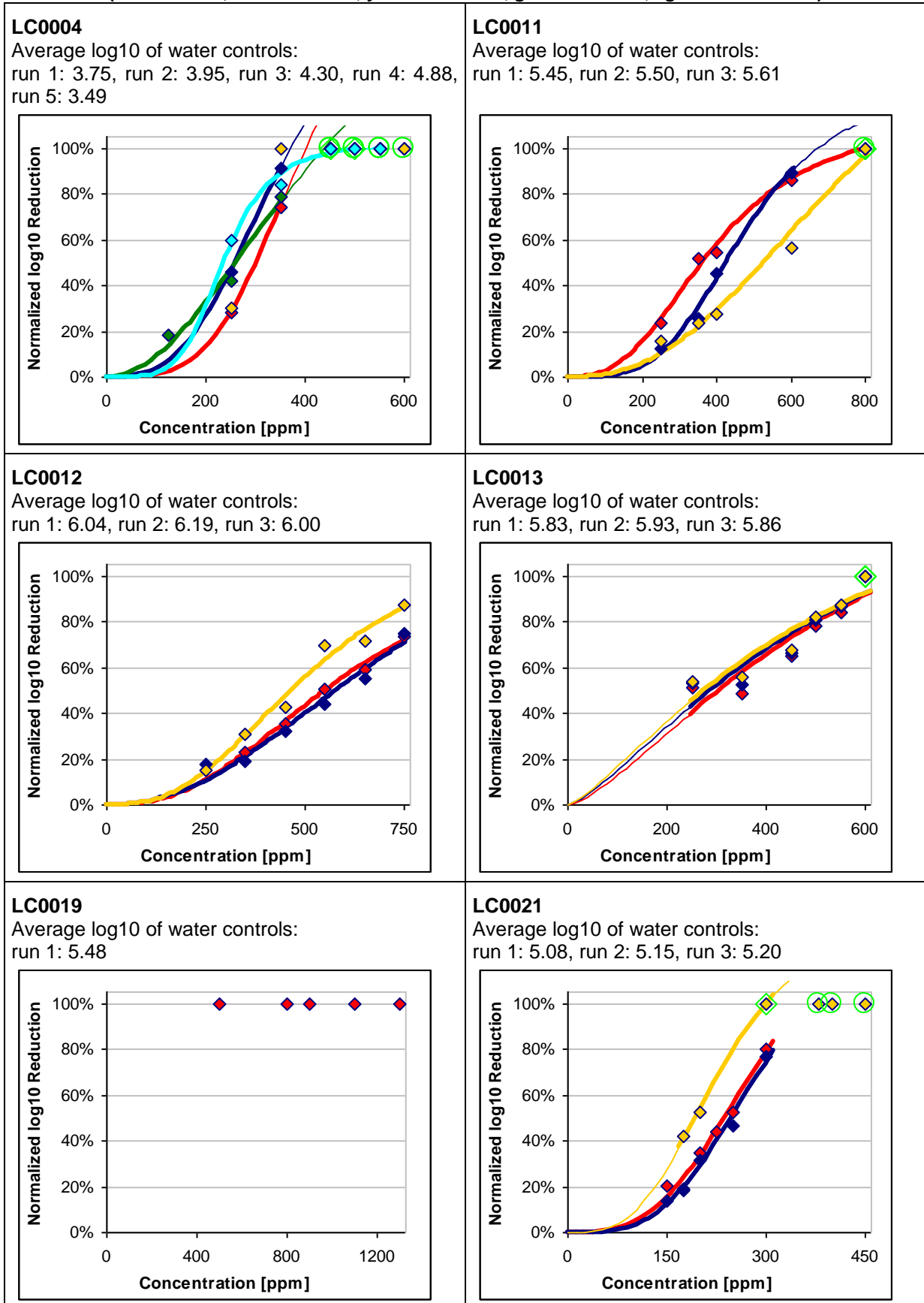
**Figure 137: Normalized run-specific kill curves for *Mycobacterium terrae* – phenol**  
 (red = 1st run, blue = 2nd run, yellow = 3rd run)



**Figure 138: Normalized run-specific kill curves for *Mycobacterium terrae* – peracetic acid (red = 1st run, blue = 2nd run, yellow = 3rd run)**

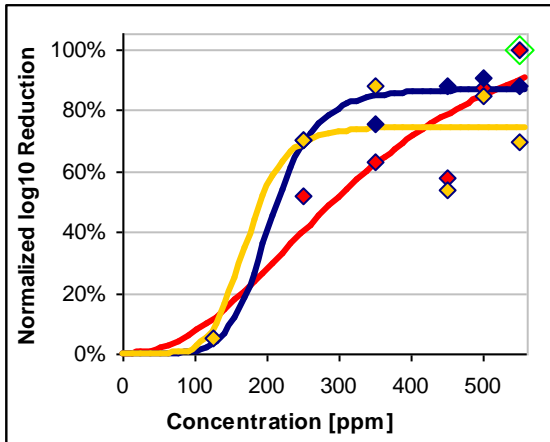


**Figure 139: Normalized run-specific kill curves for *Pseudomonas aeruginosa* – glutaraldehyde**  
 (red = 1st run, blue = 2nd run, yellow = 3rd run, green = 4th run, light blue = 5th run)



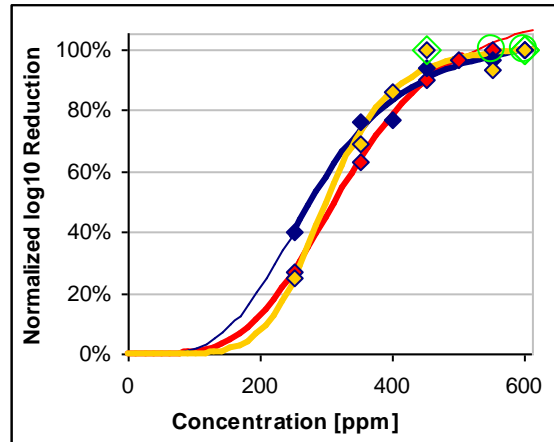
**LC0026**

Average log<sub>10</sub> of water controls:  
run 1: 5.78, run 2: 5.25, run 3: 5.10

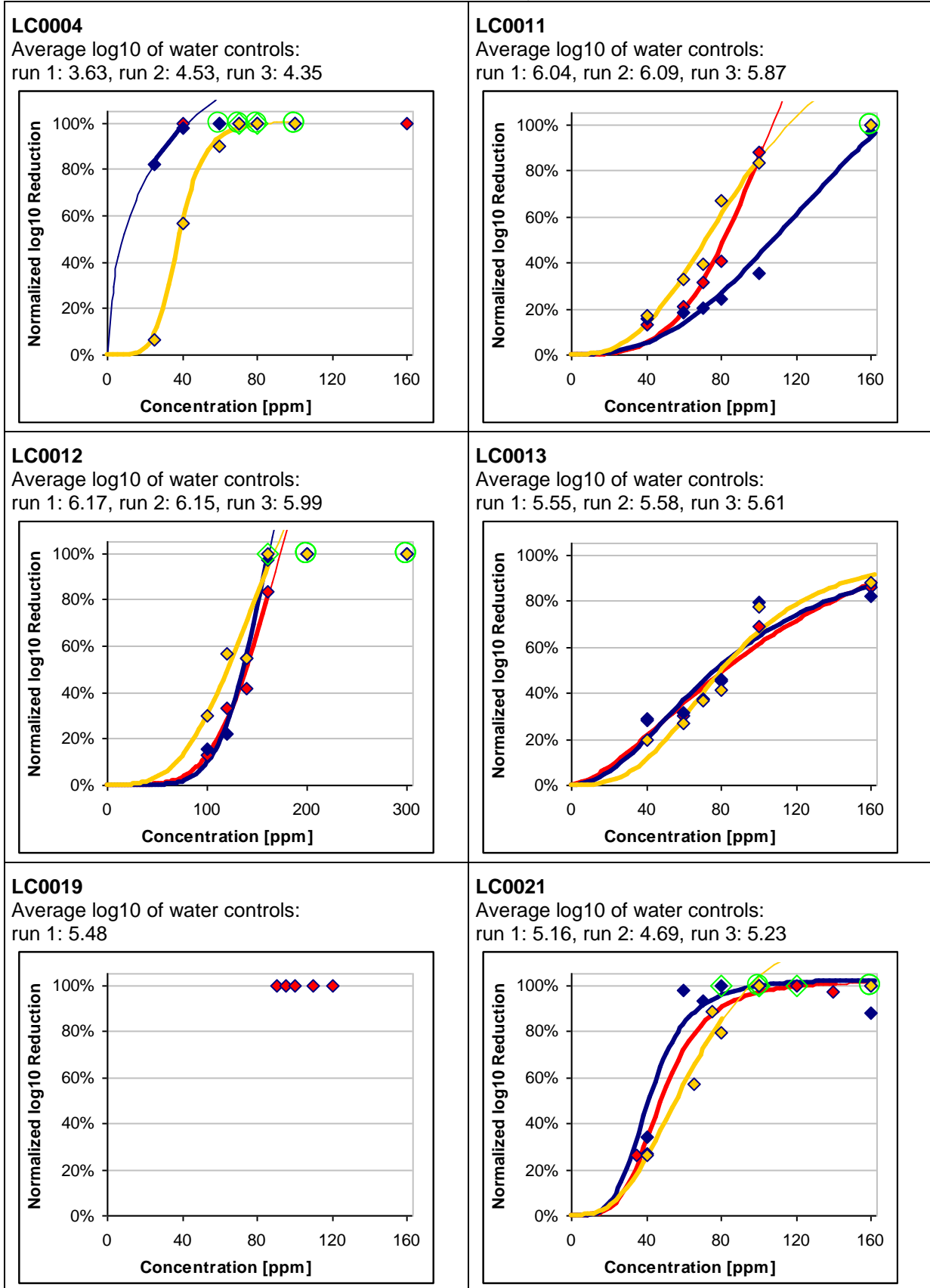


**LC0028**

Average log<sub>10</sub> of water controls:  
run 1: 5.88, run 2: 5.58, run 3: 5.86

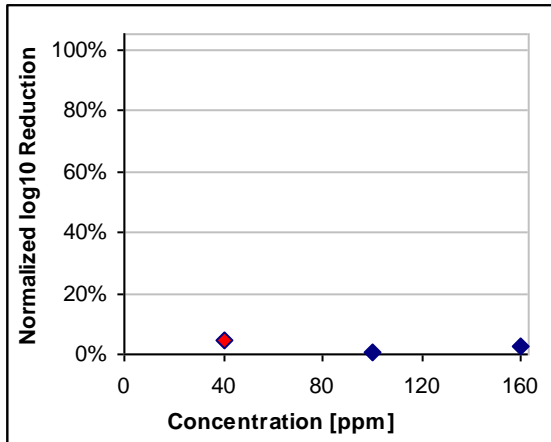


**Figure 140: Normalized run-specific kill curves for *Pseudomonas aeruginosa* – peracetic acid (red = 1st run, blue = 2nd run, yellow = 3rd run)**



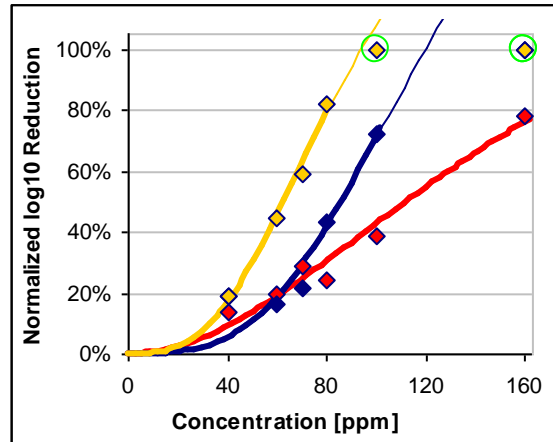
**LC0026**

Average log<sub>10</sub> of water controls:  
run 1: 5.23, run 2: 5.43

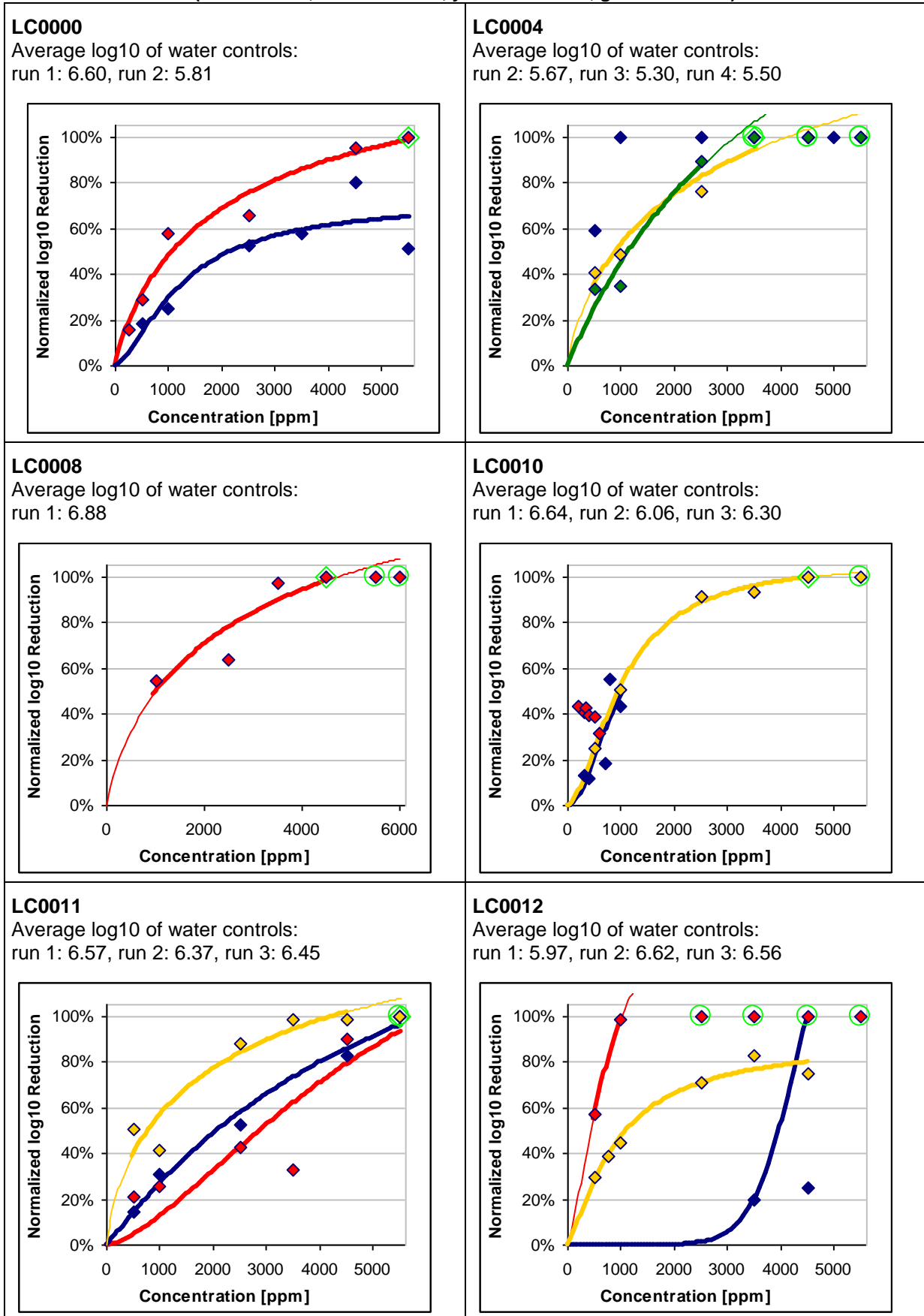


**LC0028**

Average log<sub>10</sub> of water controls:  
run 1: 5.39, run 2: 6.15, run 3: 6.04

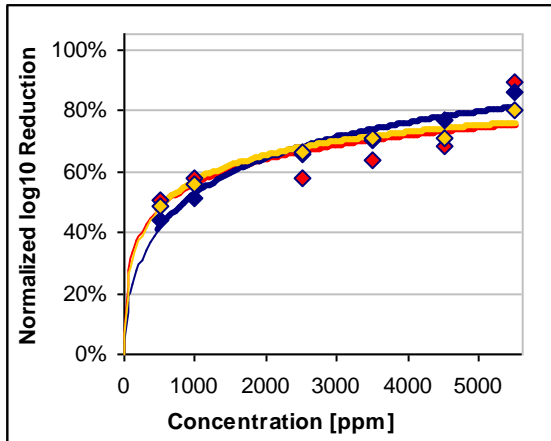


**Figure 141: Normalized run-specific kill curves for *Staphylococcus aureus* – benzalkonium chloride**  
 (red = 1st run, blue = 2nd run, yellow = 3rd run, green = 4th run)



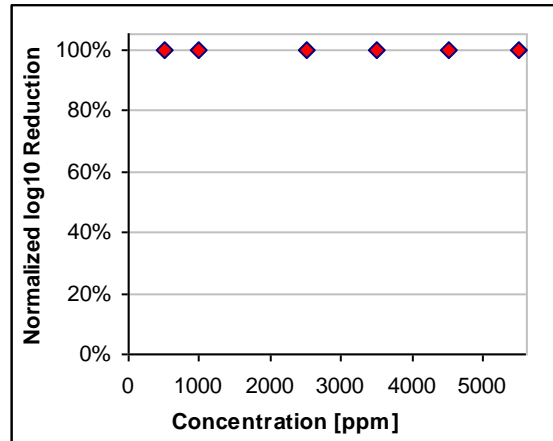
**LC0013**

Average log<sub>10</sub> of water controls:  
run 1: 6.56, run 2: 6.98, run 3: 6.96



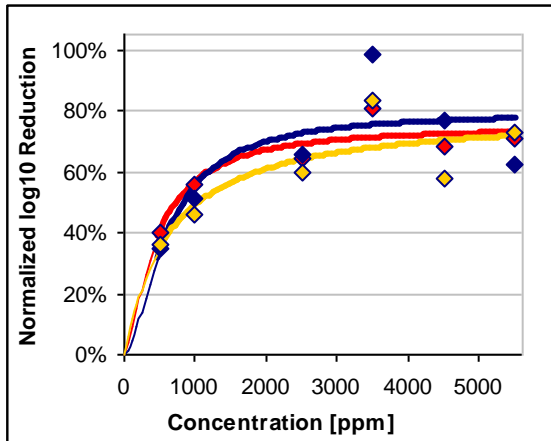
**LC0019**

Average log<sub>10</sub> of water controls:  
run 1: 5.18



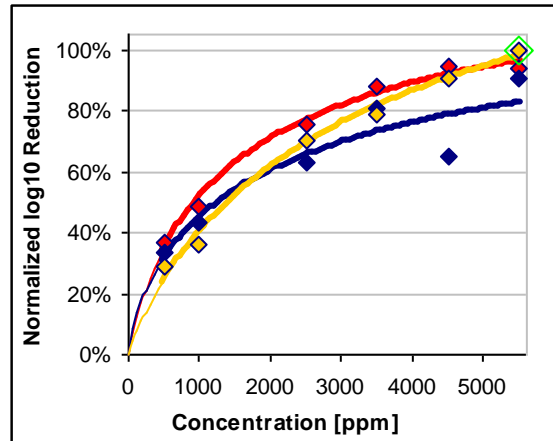
**LC0020**

Average log<sub>10</sub> of water controls:  
run 1: 6.36, run 2: 5.98, run 3: 5.86



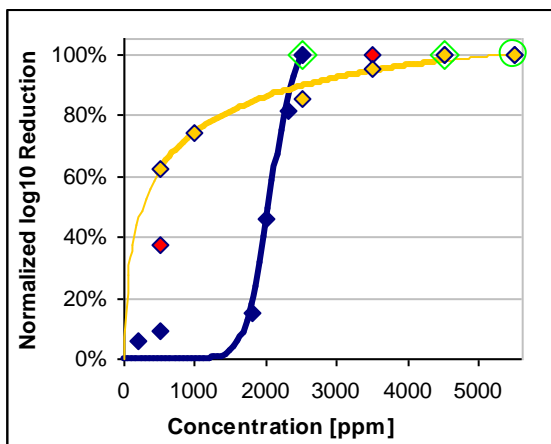
**LC0021**

Average log<sub>10</sub> of water controls:  
run 1: 7.43, run 2: 7.94, run 3: 7.50



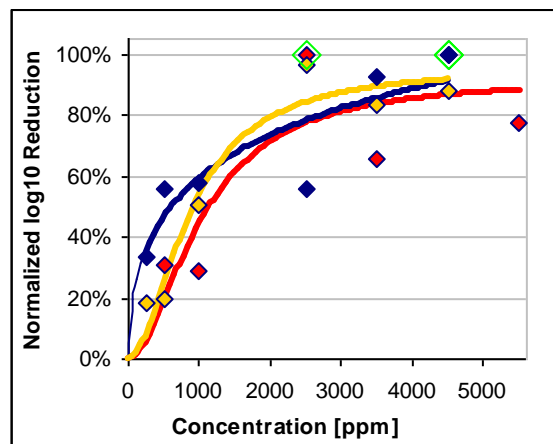
**LC0022**

Average log<sub>10</sub> of water controls:  
run 1: 6.26, run 2: 5.79, run 3: 5.99



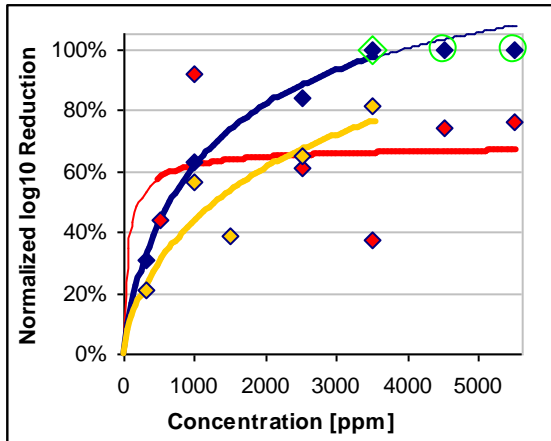
**LC0026**

Average log<sub>10</sub> of water controls:  
run 1: 6.15, run 2: 6.59, run 3: 7.08

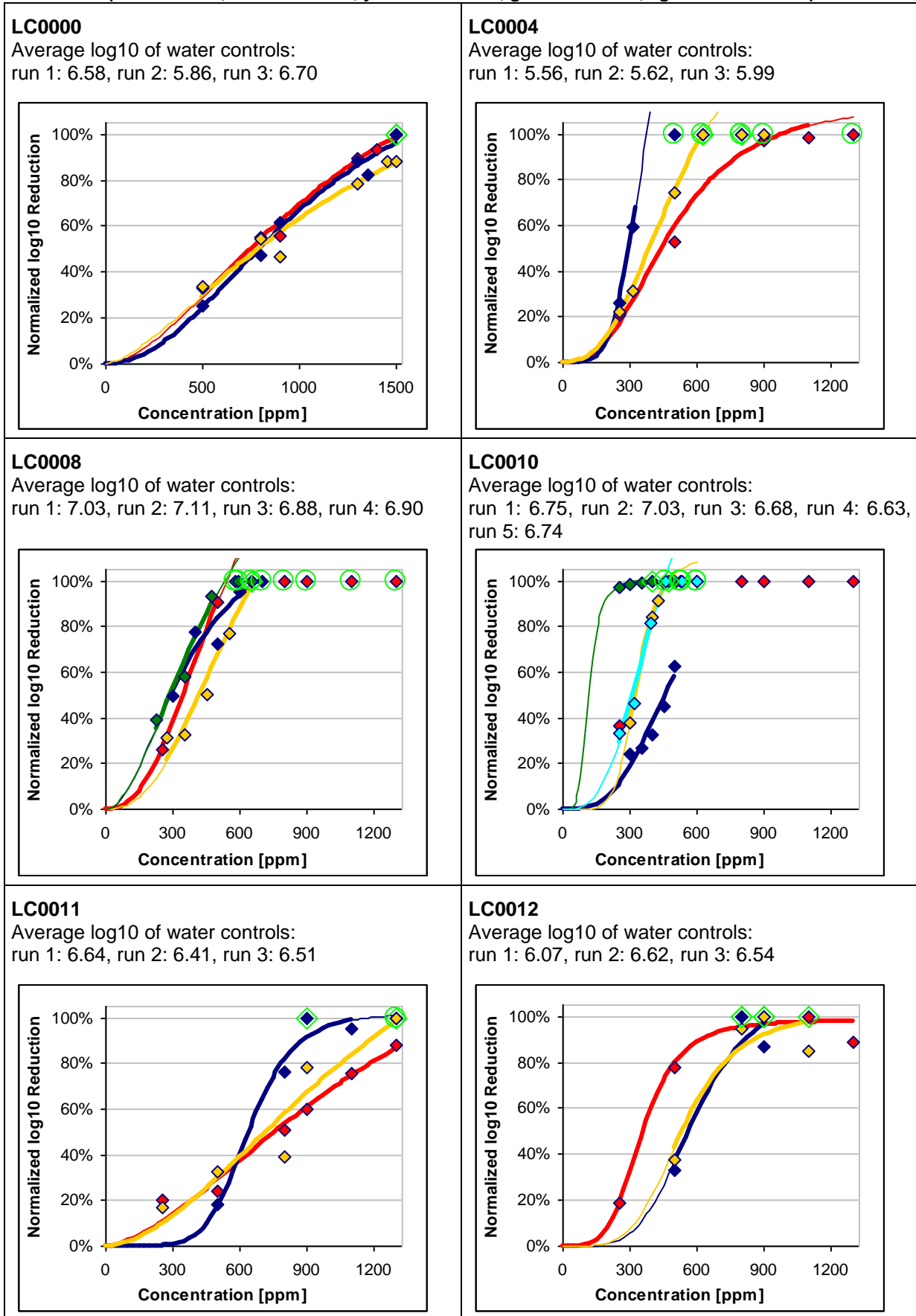


**LC0028**

Average log<sub>10</sub> of water controls:  
run 1: 5.90, run 2: 5.75, run 3: 6.03

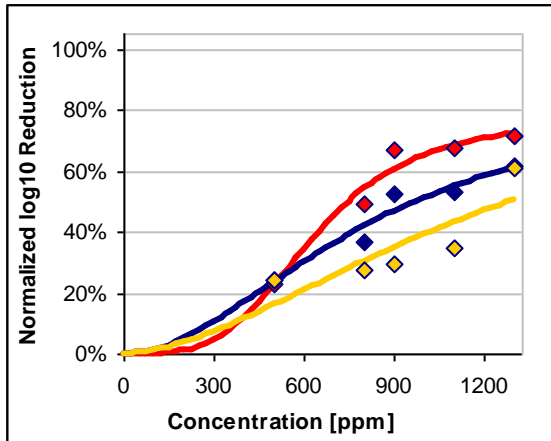


**Figure 142: Normalized run-specific kill curves for *Staphylococcus aureus* – glutaraldehyde**  
 (red = 1st run, blue = 2nd run, yellow = 3rd run, green = 4th run, light blue = 5th run)



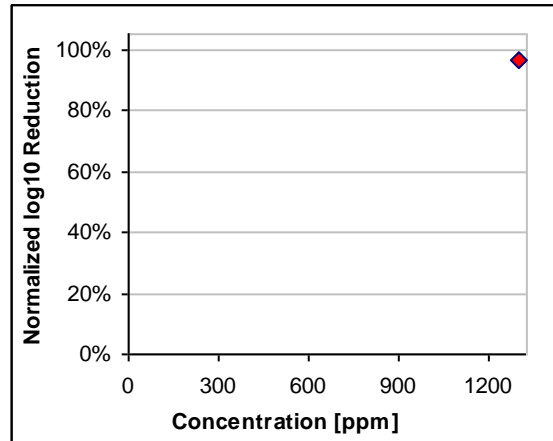
**LC0013**

Average log<sub>10</sub> of water controls:  
run 1: 6.93, run 2: 6.88, run 3: 6.92



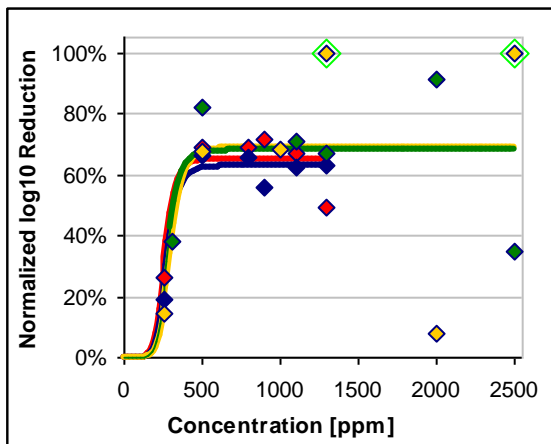
**LC0019**

Average log<sub>10</sub> of water controls:  
run 1: 5.18



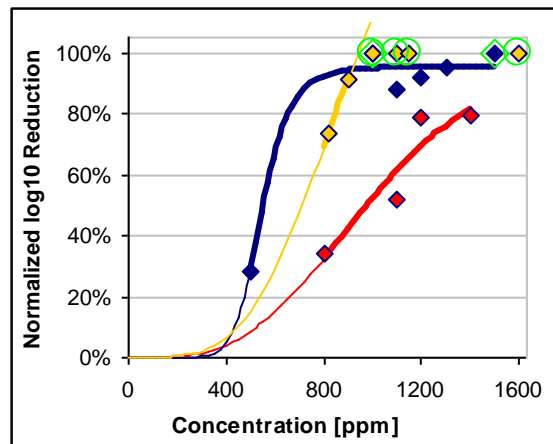
**LC0020**

Average log<sub>10</sub> of water controls:  
run 1: 5.80, run 2: 5.96, run 3: 5.90, run 4: 6.12



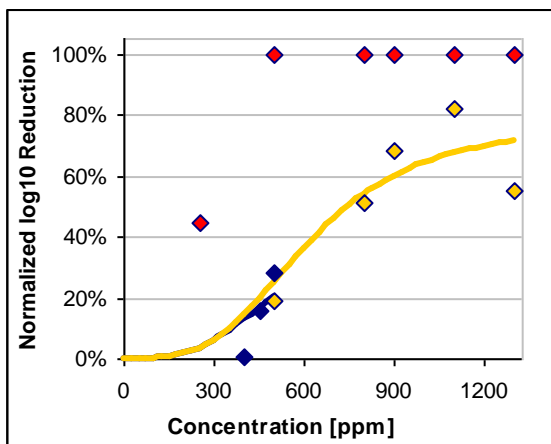
**LC0021**

Average log<sub>10</sub> of water controls:  
run 1: 7.28, run 2: 7.11, run 3: 7.62



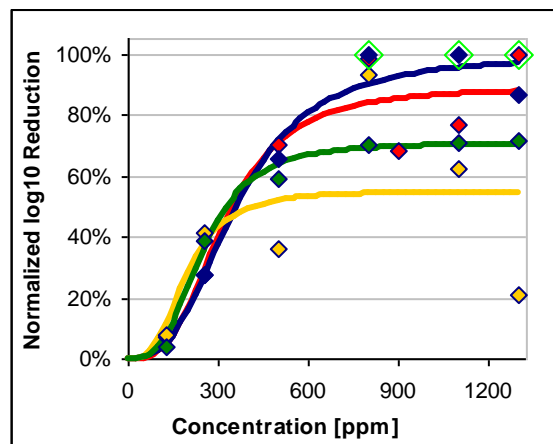
**LC0022**

Average log<sub>10</sub> of water controls:  
run 1: 6.13, run 2: 5.43, run 3: 6.01



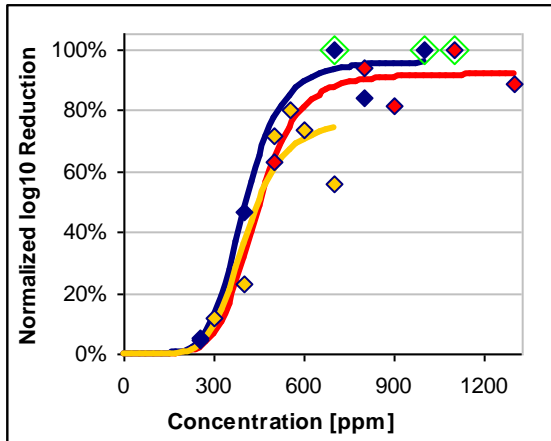
**LC0026**

Average log<sub>10</sub> of water controls:  
run 1: 7.06, run 2: 7.05, run 3: 6.63, run 4: 7.15

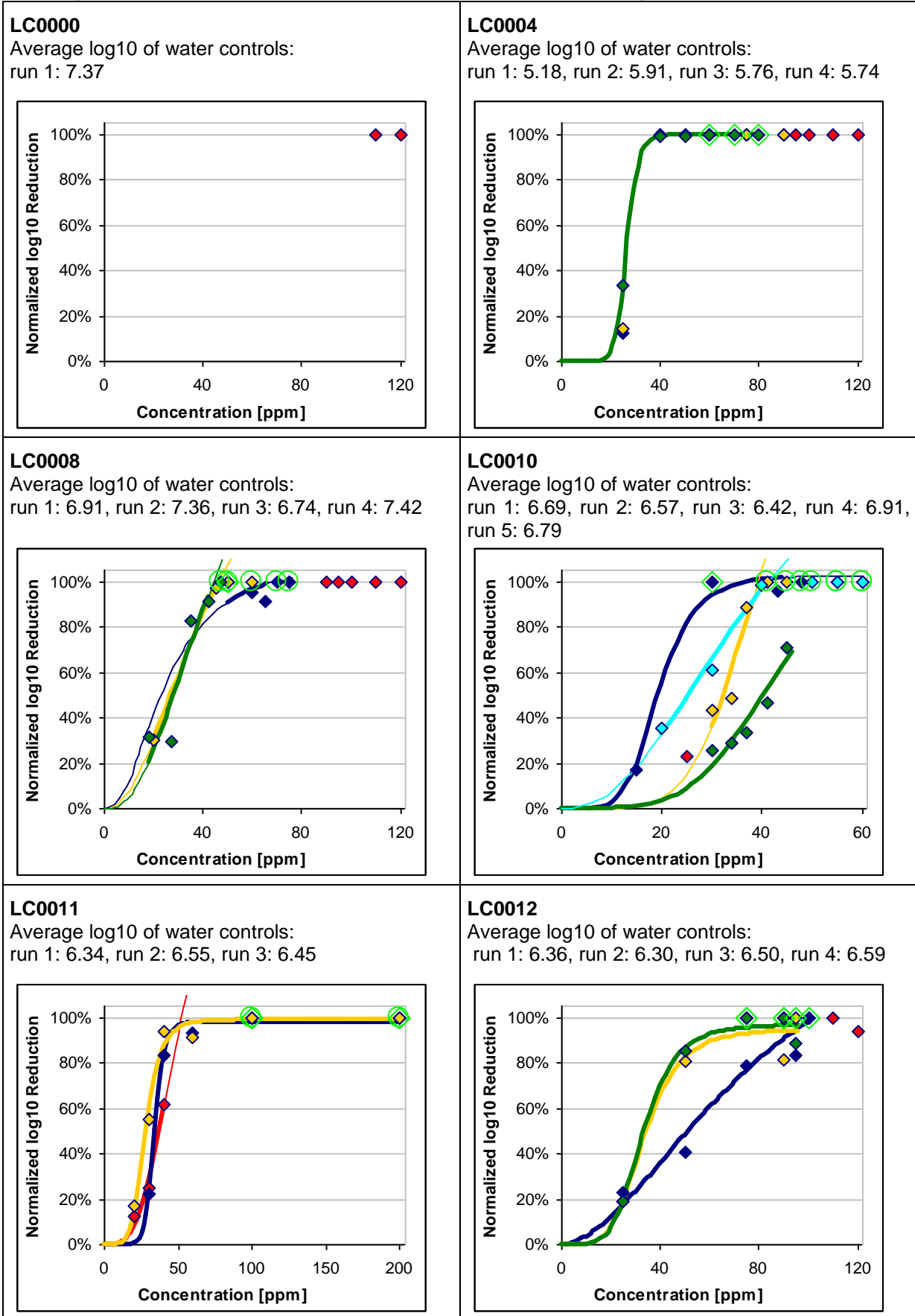


**LC0028**

Average log<sub>10</sub> of water controls:  
run 1: 5.70, run 2: 5.53, run 3: 5.54

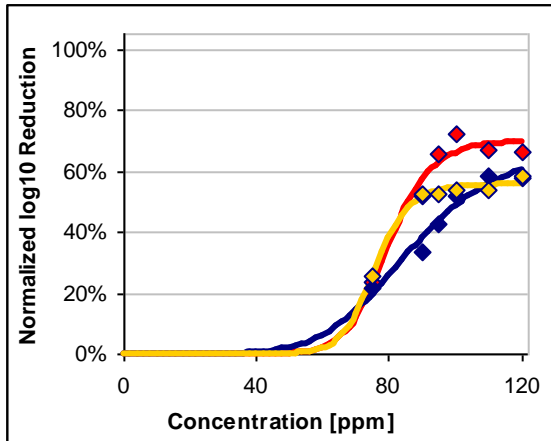


**Figure 143: Normalized run-specific kill curves for *Staphylococcus aureus* – peracetic acid**  
 (light blue = 1st run, middle blue = 2nd run, dark blue = 3rd run, orange = 4th run, red = 5th run)



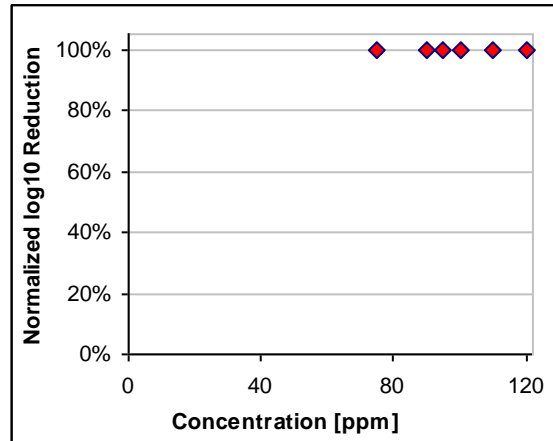
**LC0013**

Average log<sub>10</sub> of water controls:  
run 1: 6.96, run 2: 6.93, run 3: 6.95



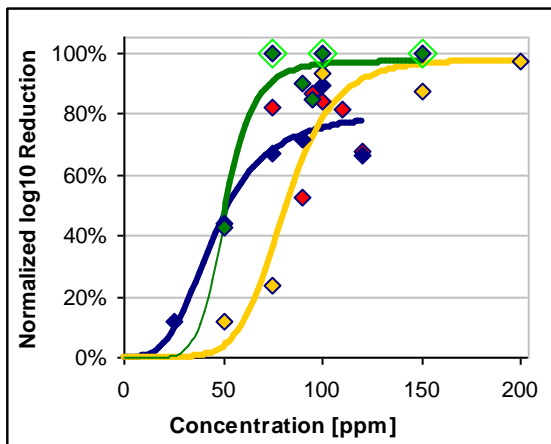
**LC0019**

Average log<sub>10</sub> of water controls:  
run 1: 5.30



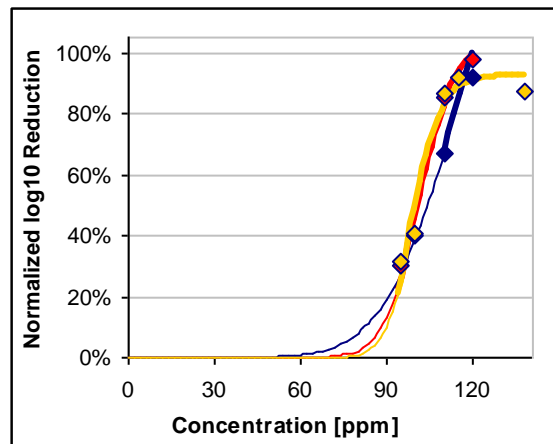
**LC0020**

Average log<sub>10</sub> of water controls:  
run 1: 6.01, run 2: 5.97, run 3: 6.08, run 4: 5.95



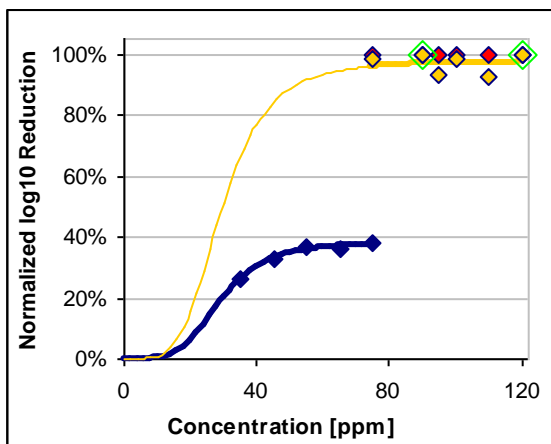
**LC0021**

Average log<sub>10</sub> of water controls:  
run 1: 7.41, run 2: 7.30, run 3: 7.53



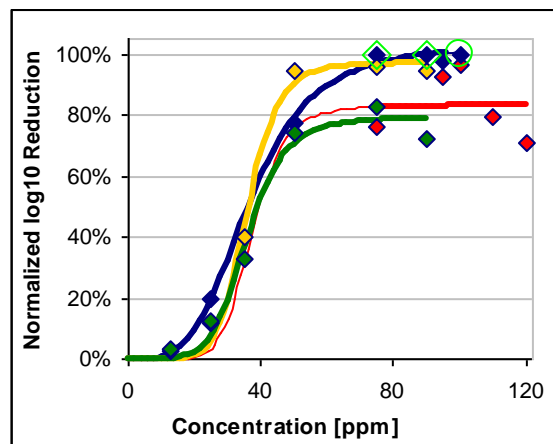
**LC0022**

Average log<sub>10</sub> of water controls:  
run 1: 6.14, run 2: 5.80, run 3: 5.96



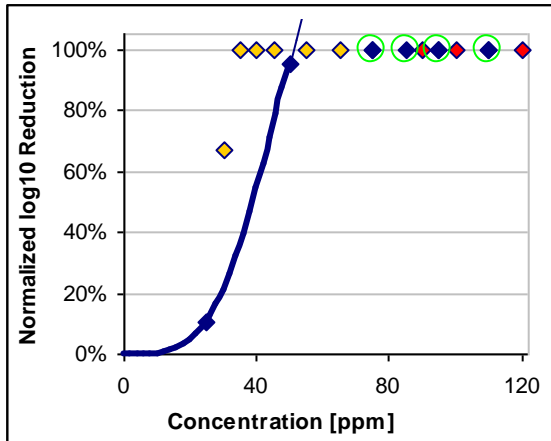
**LC0026**

Average log<sub>10</sub> of water controls:  
run 1: 7.15, run 2: 7.11, run 3: 6.69, run 4: 6.87



**LC0028**

Average log<sub>10</sub> of water controls:  
run 1: 5.81, run 2: 5.68, run 3: 5.65



**Results of phase 1 according to the standard approach**

223. The minimum requirements are the same as for the normalized approach.

**Statistical outlier identification with regard to the single log<sub>10</sub> CFU values of each carrier**

224. The following table summarizes all identified outliers of the single log<sub>10</sub> CFU log<sub>10</sub> CFU values of each carrier. These outlier carriers have been eliminated and do not contribute to any further statistical analysis.

225. For *Adenovirus type 5* (well count) and *Pseudomonas aeruginosa* no outliers could be identified for either substance. For *Aspergillus niger* no outliers could be identified for the measurements with glutaraldehyde.

**Table 47: Outliers with regard to the single log<sub>10</sub> CFU values of each carrier**

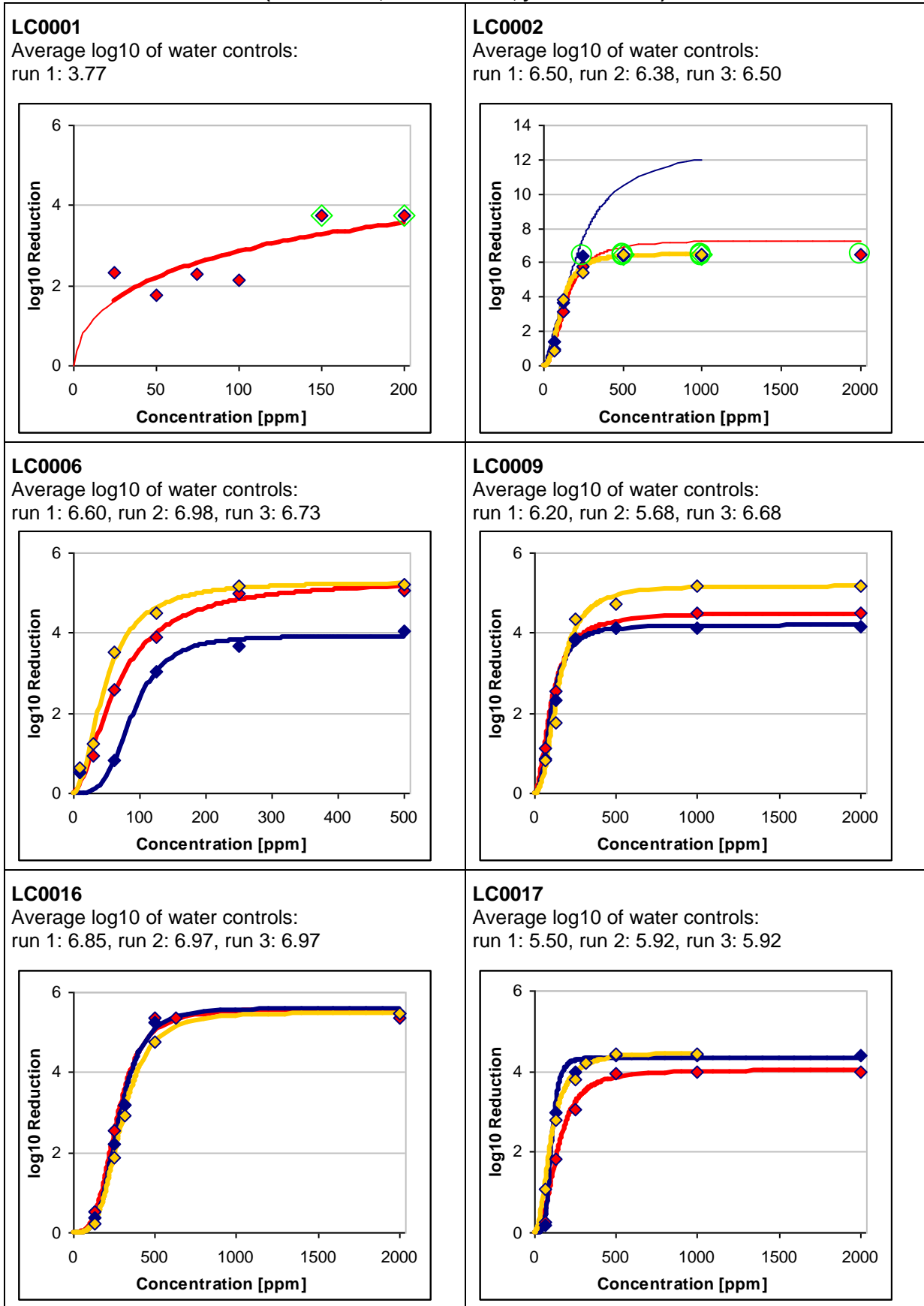
Organism	Substance	Lab Code	Run	Outlier Carrier	Concentration	
<i>Adenovirus type 5</i> (TCID <sub>50</sub> )	peracetic acid	LC0033	3	11	75 ppm	
	glutaraldehyde	LC0006	3	6	250 ppm	
<i>Aspergillus niger</i>	peracetic acid	LC0011	1	4	400 ppm	
		LC0022	1	16	25 ppm	
<i>Bacillus subtilis</i>	peracetic acid	LC0011	1	5	1200 ppm	
<i>Mycobacterium terrae</i>	phenol	LC0012	2	1	20000 ppm	
		peracetic acid	LC0011	1	4	700 ppm
			LC0011	2	6	700 ppm
	glutaraldehyde	LC0011	2	10	500 ppm	
		LC0008	2	5	20000 ppm	
		LC0011	1	3	25000 ppm	
<i>Staphylococcus aureus</i>	peracetic acid	LC0011	1	13	20 ppm	
		LC0028	1	6	110 ppm	
	benzalkonium chloride	LC0004	2	15	1000 ppm	
		LC0008	1	15	2500 ppm	
		LC0012	1	12	2500 ppm	

**Run-specific inactivation curves**

226. In the following, for each combination of organism and substance the run-specific inactivation curves and the mean log<sub>10</sub> reductions at each concentration level are shown separately for each laboratory. All available data is being displayed, i.e. also not-regarded runs and outlier runs. Extrapolated run-specific inactivation curves are shown as thin continuations of the non-extrapolated curve. This applies to cases where the mean log<sub>10</sub> 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.

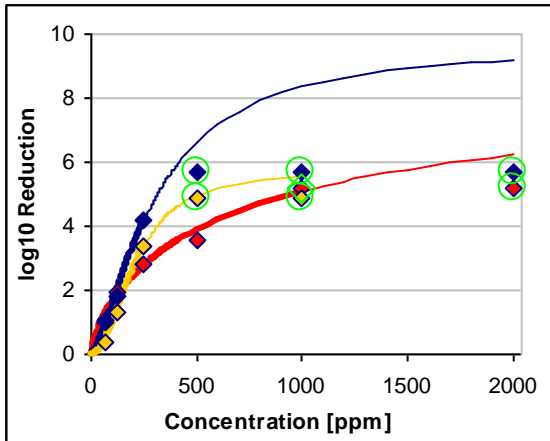
Values of the average log<sub>10</sub> of the water controls that have been included in the final estimation of the run-specific curve are marked by green diamonds, values that have not been included in the final estimation of the run-specific curve are marked by green circles.

**Figure 144: Standard run-specific kill curves for *Adenovirus type 5* (TCID<sub>50</sub>) – glutaraldehyde (red = 1st run, blue = 2nd run, yellow = 3rd run)**



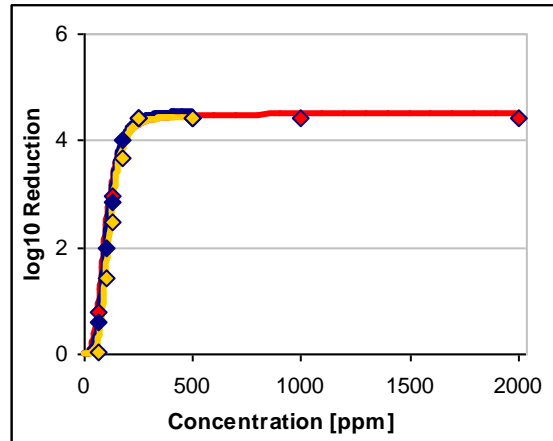
**LC0022**

Average log<sub>10</sub> of water controls:  
run 1: 5.22, run 2: 5.69, run 3: 4.91



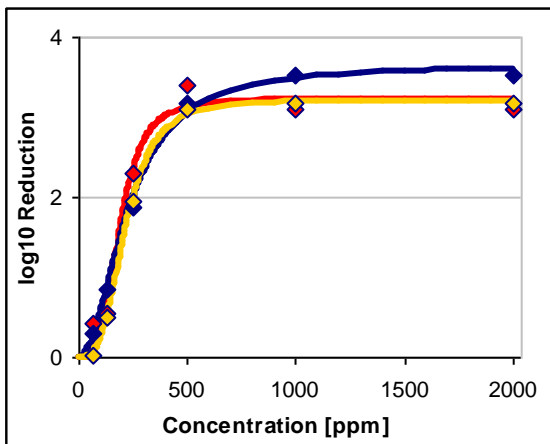
**LC0029**

Average log<sub>10</sub> of water controls:  
run 1: 5.94, run 2: 5.94, run 3: 5.91



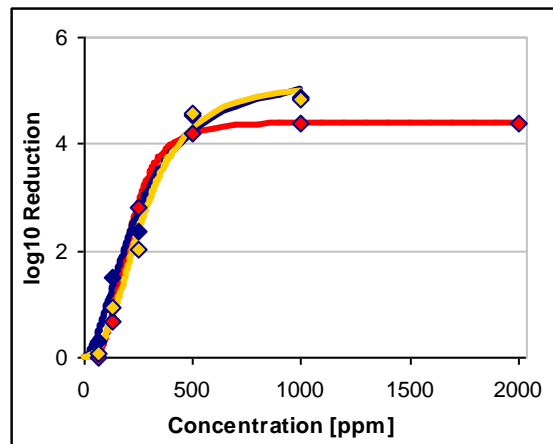
**LC0031**

Average log<sub>10</sub> of water controls:  
run 1: 6.25, run 2: 5.64, run 3: 5.86

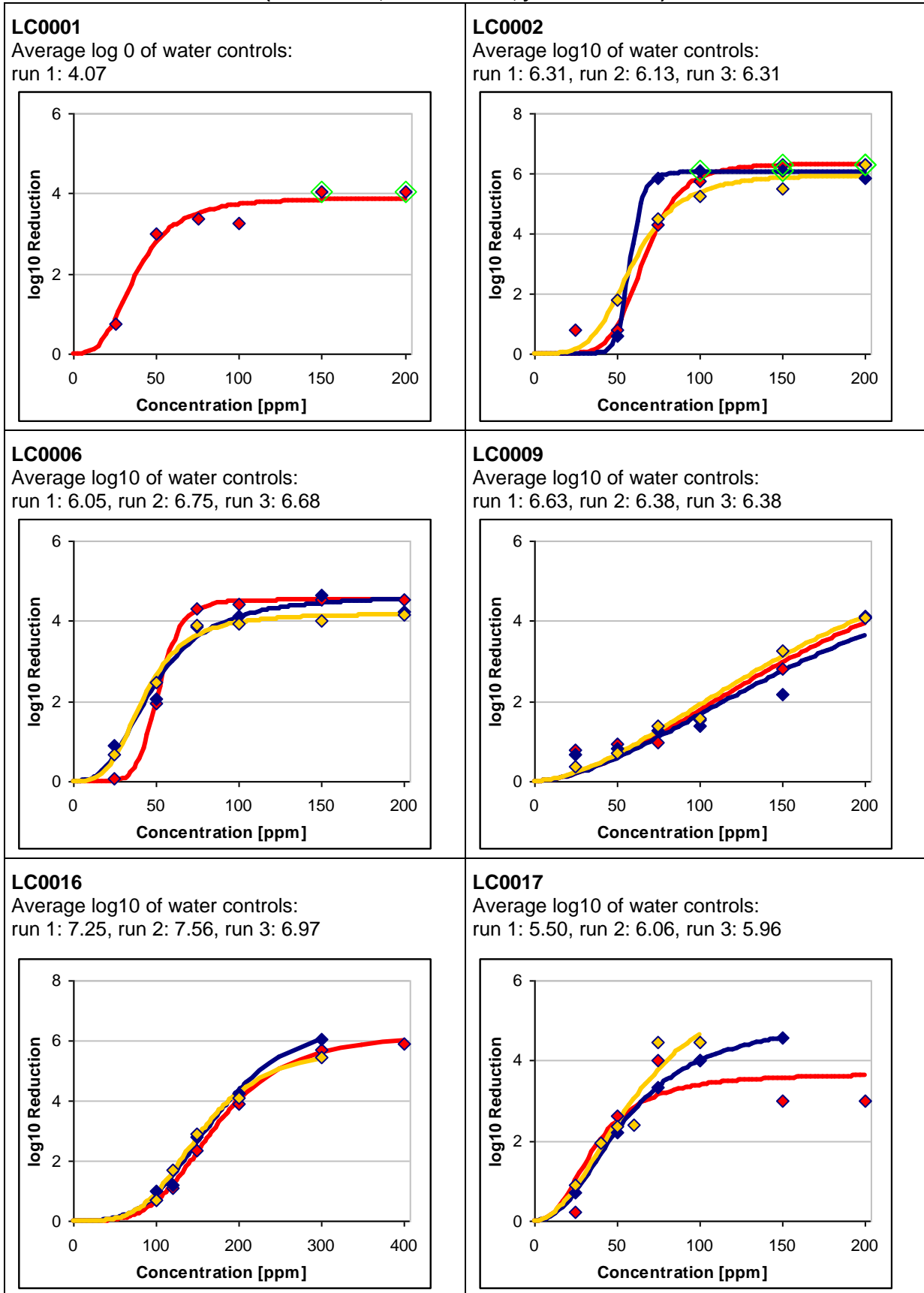


**LC0033**

Average log<sub>10</sub> of water controls:  
run 1: 4.89, run 2: 5.47, run 3: 5.54

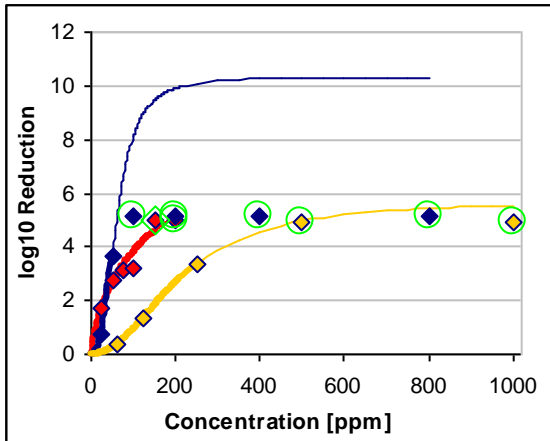


**Figure 145: Standard run-specific kill curves for *Adenovirus type 5* (TCID<sub>50</sub>) – peracetic acid (red = 1st run, blue = 2nd run, yellow = 3rd run)**



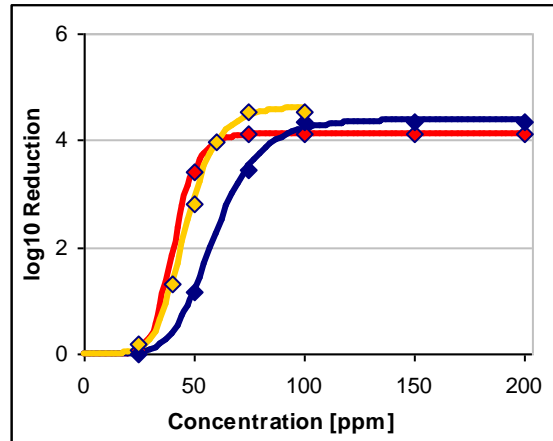
**LC0022**

Average log10 of water controls:  
run 1: 4.97, run 2: 5.16, run 3: 4.91



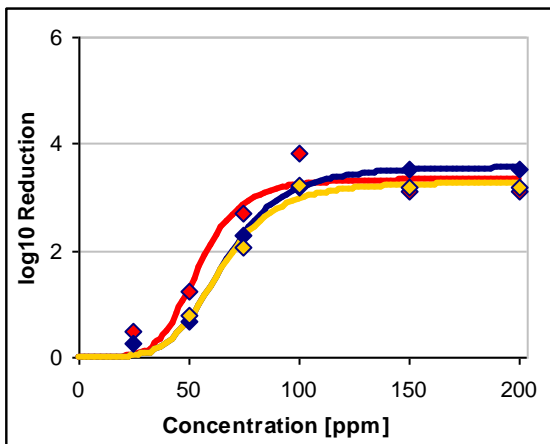
**LC0029**

Average log10 of water controls:  
run 1: 5.63, run 2: 5.85, run 3: 6.03



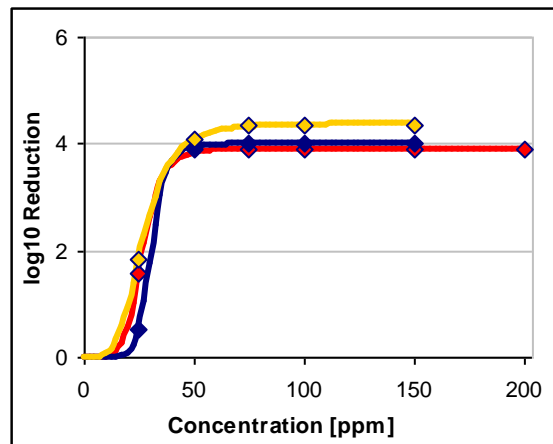
**LC0031**

Average log10 of water controls:  
run 1: 4.16, run 2: 4.57, run 3: 4.22

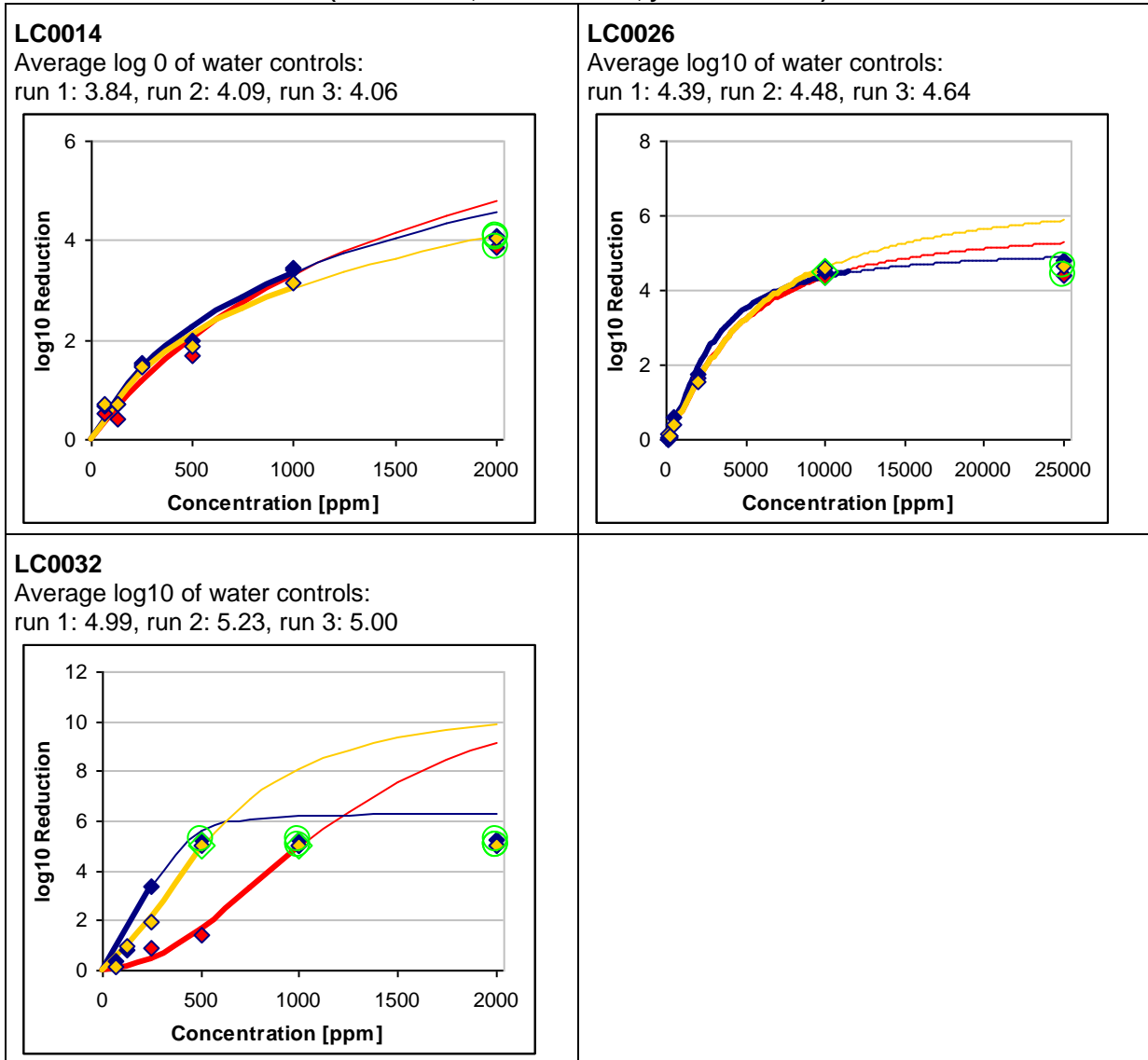


**LC0033**

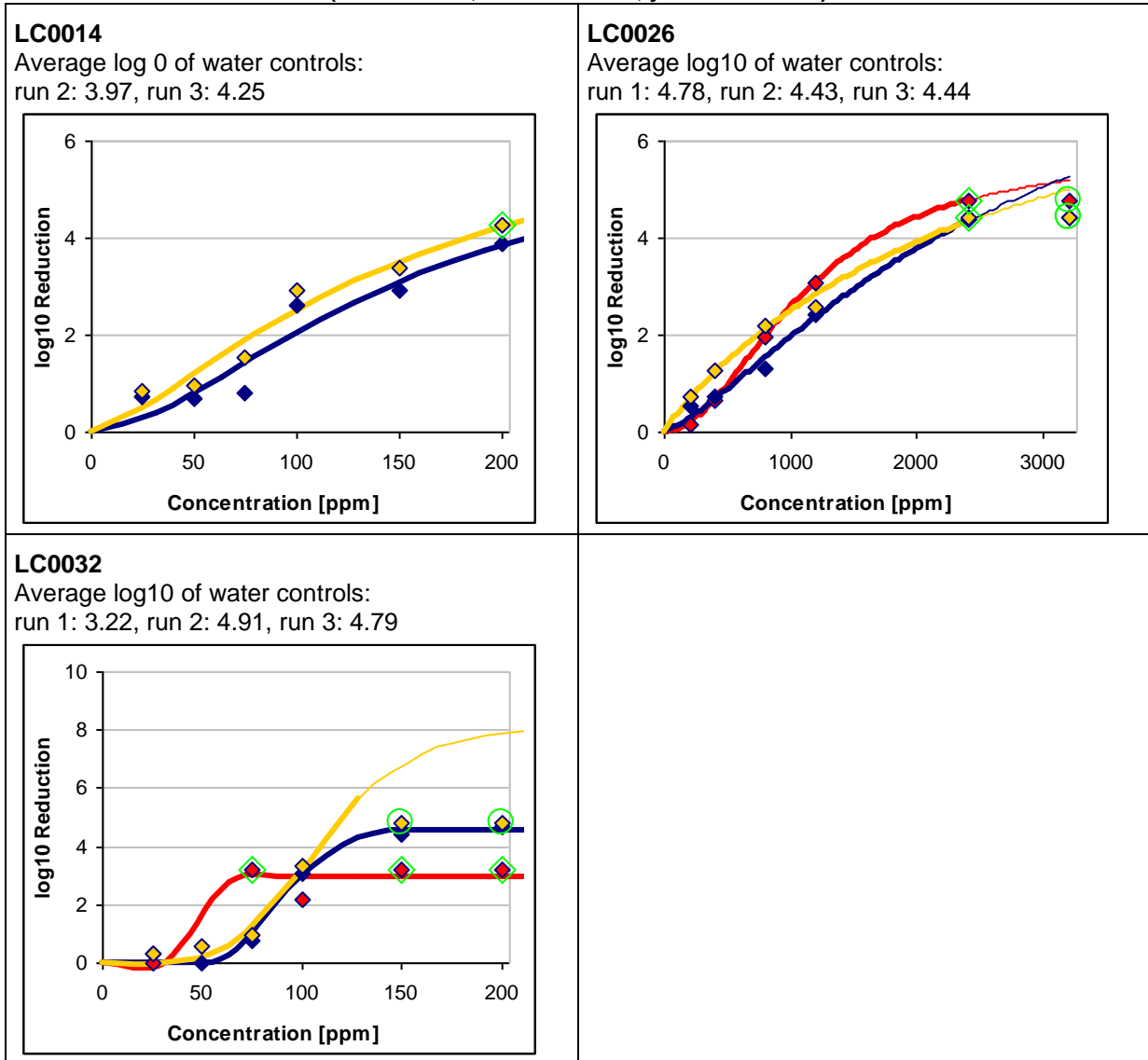
Average log10 of water controls:  
run 1: 5.39, run 2: 5.50, run 3: 5.86



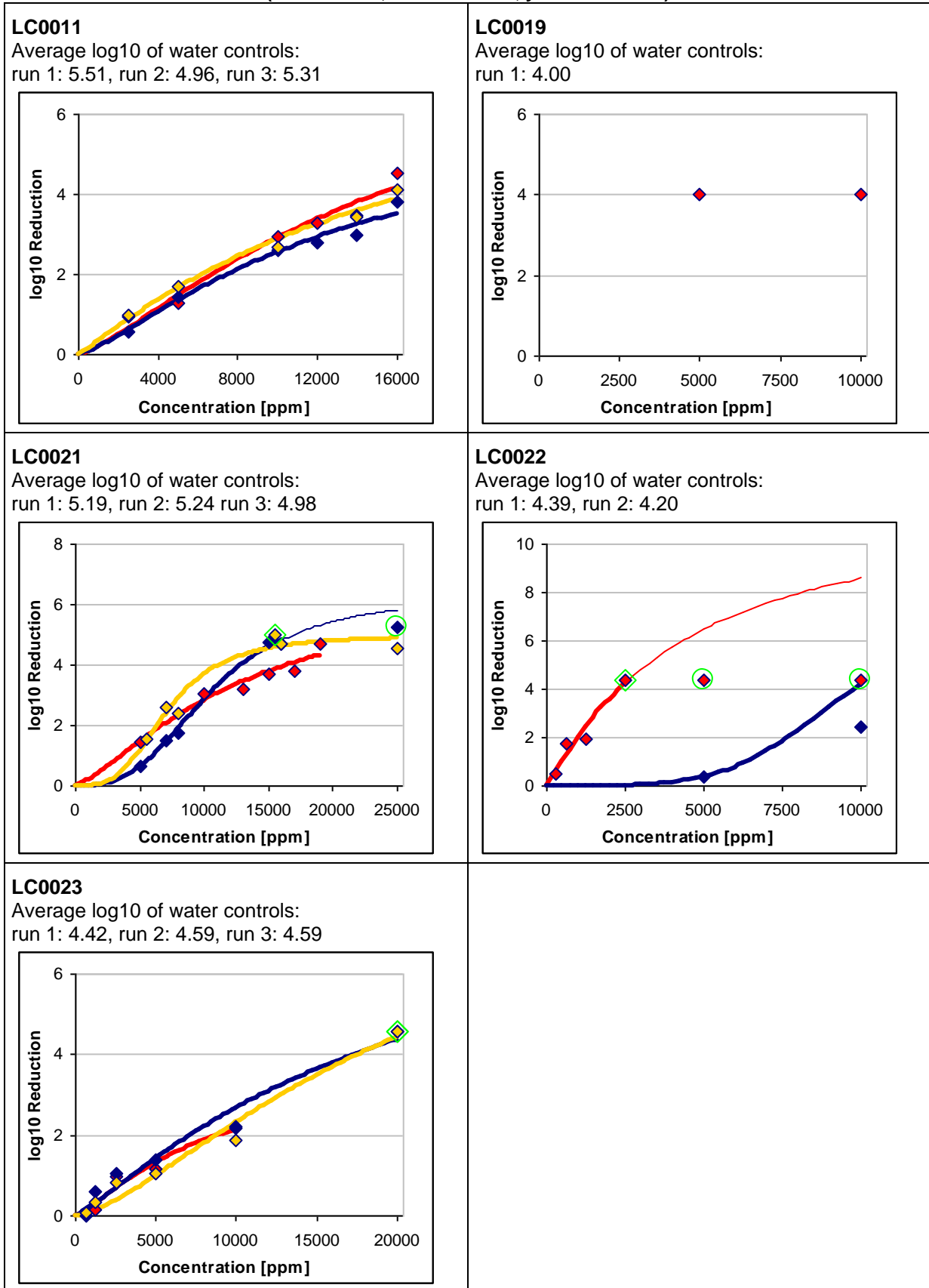
**Figure 146: Standard run-specific kill curves for *Adenovirus type 5* (well count) – glutaraldehyde**  
 (red = 1st run, blue = 2nd run, yellow = 3rd run)



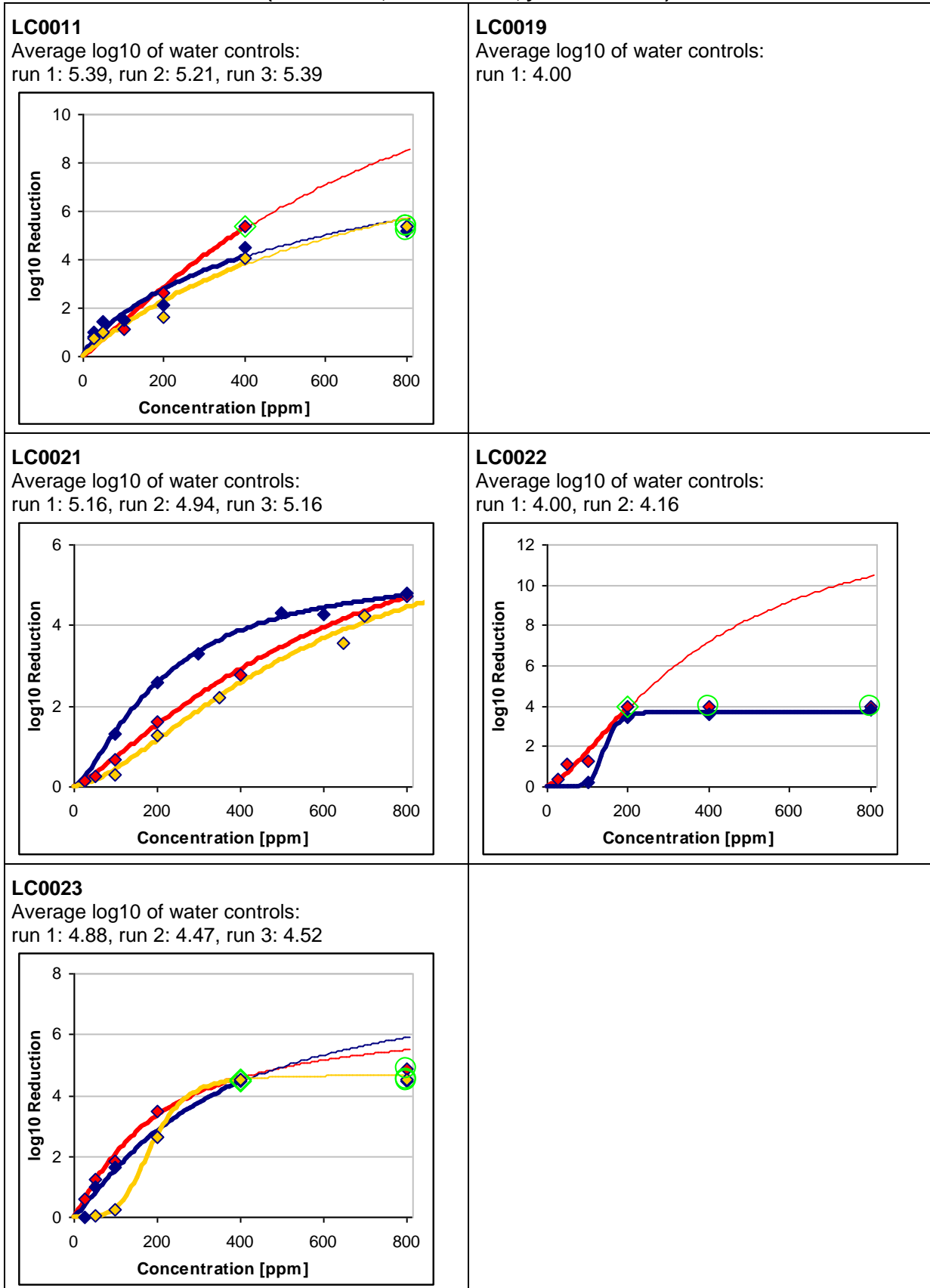
**Figure 147: Standard run-specific kill curves for *Adenovirus type 5* (well count) – peracetic acid (red = 1st run, blue = 2nd run, yellow = 3rd run)**



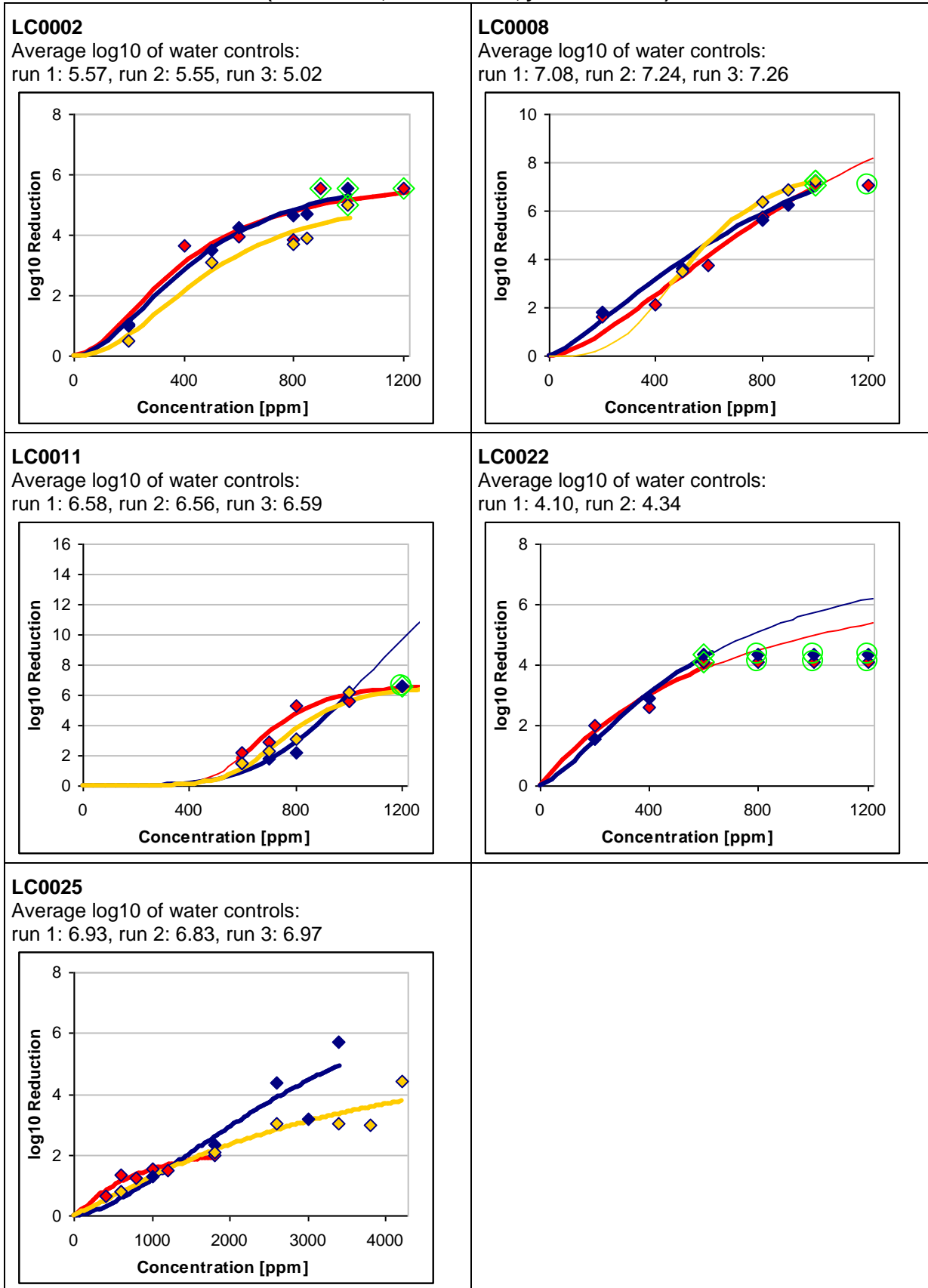
**Figure 148: Standard run-specific kill curves for *Aspergillus niger* – glutaraldehyde**  
 (red = 1st run, blue = 2nd run, yellow = 3rd run)



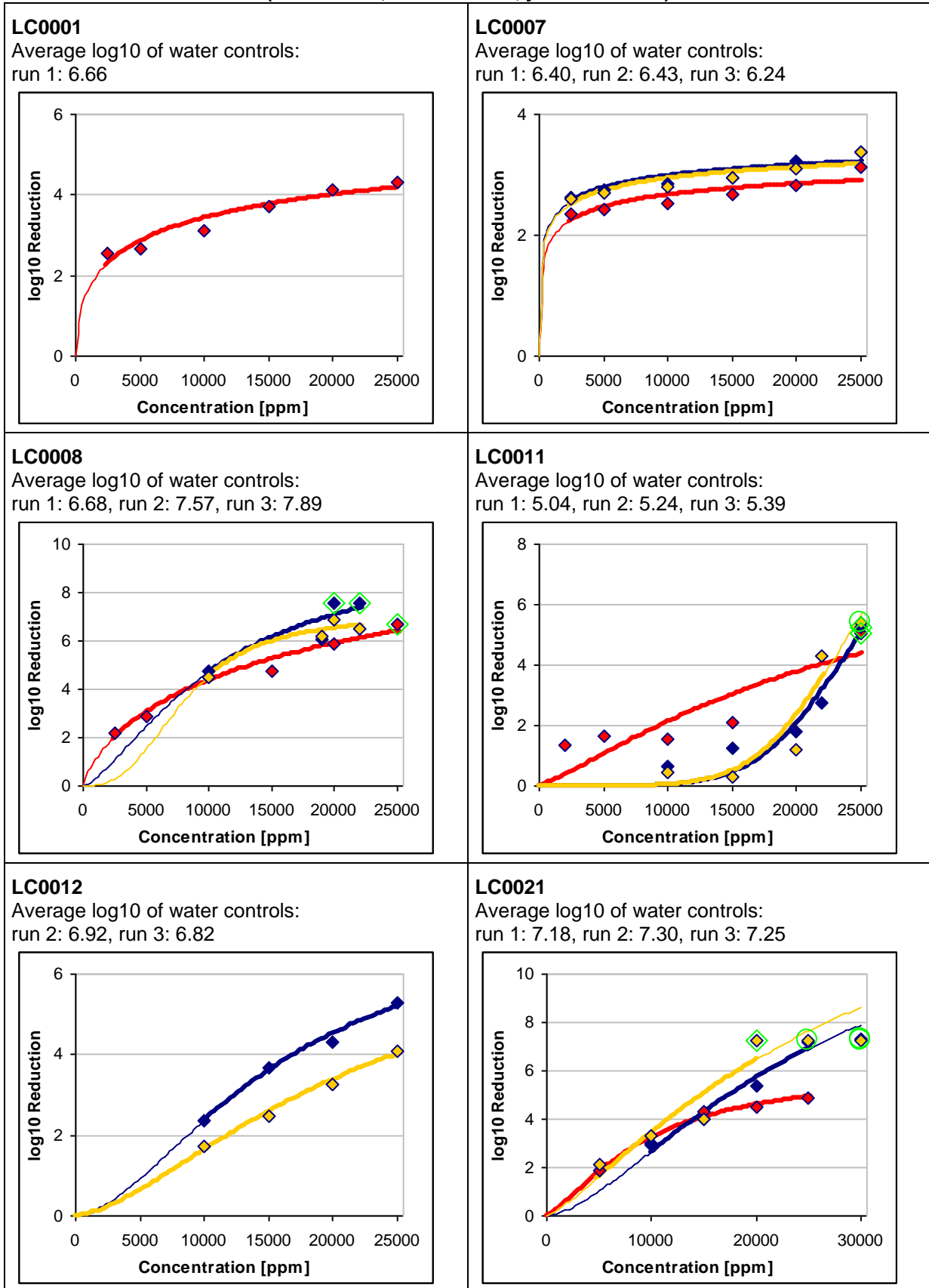
**Figure 149: Standard run-specific kill curves for *Aspergillus niger* – peracetic acid**  
 (red = 1st run, blue = 2nd run, yellow = 3rd run)



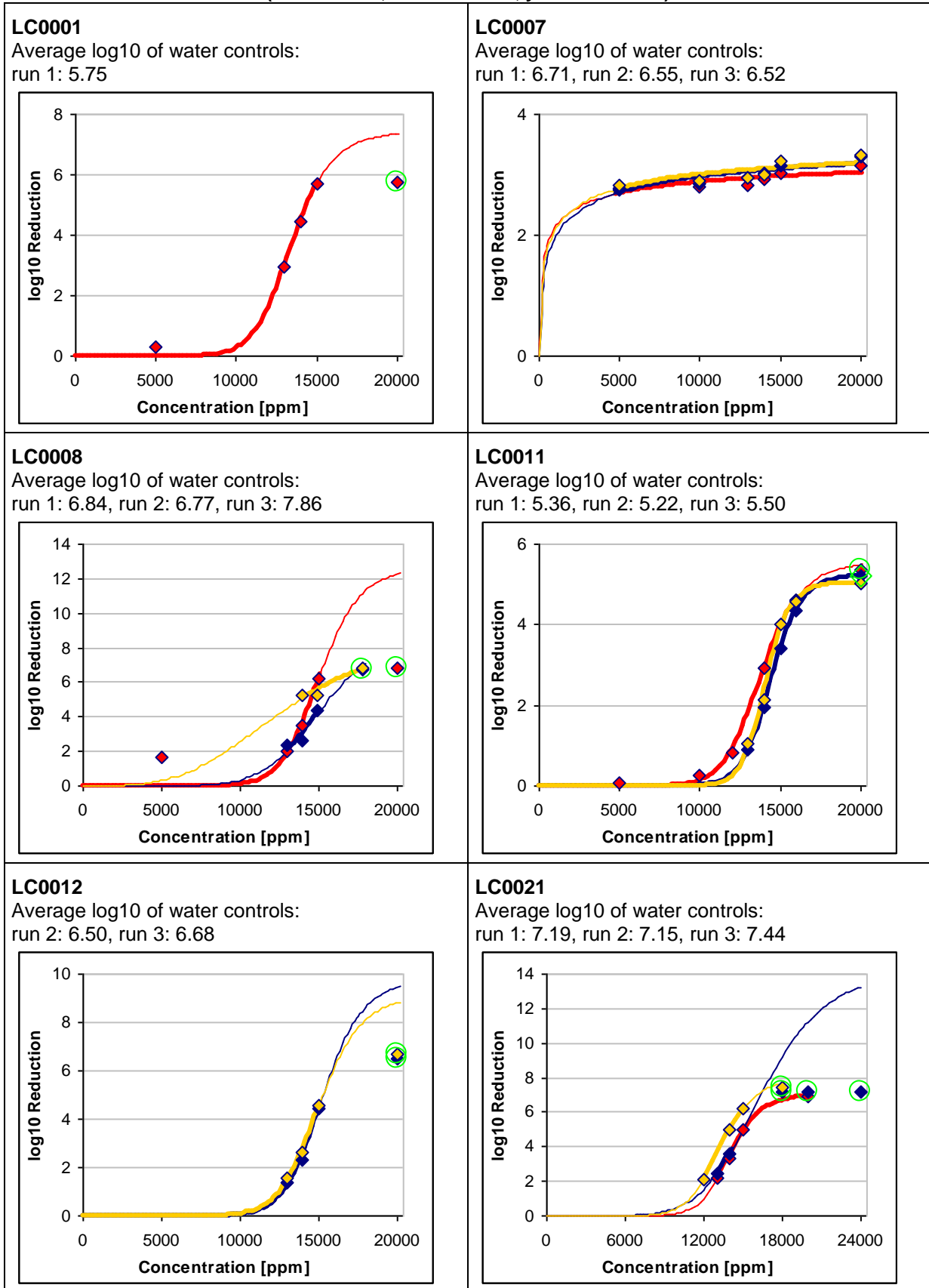
**Figure 150: Standard run-specific kill curves for *Bacillus subtilis* – peracetic acid**  
 (red = 1st run, blue = 2nd run, yellow = 3rd run)



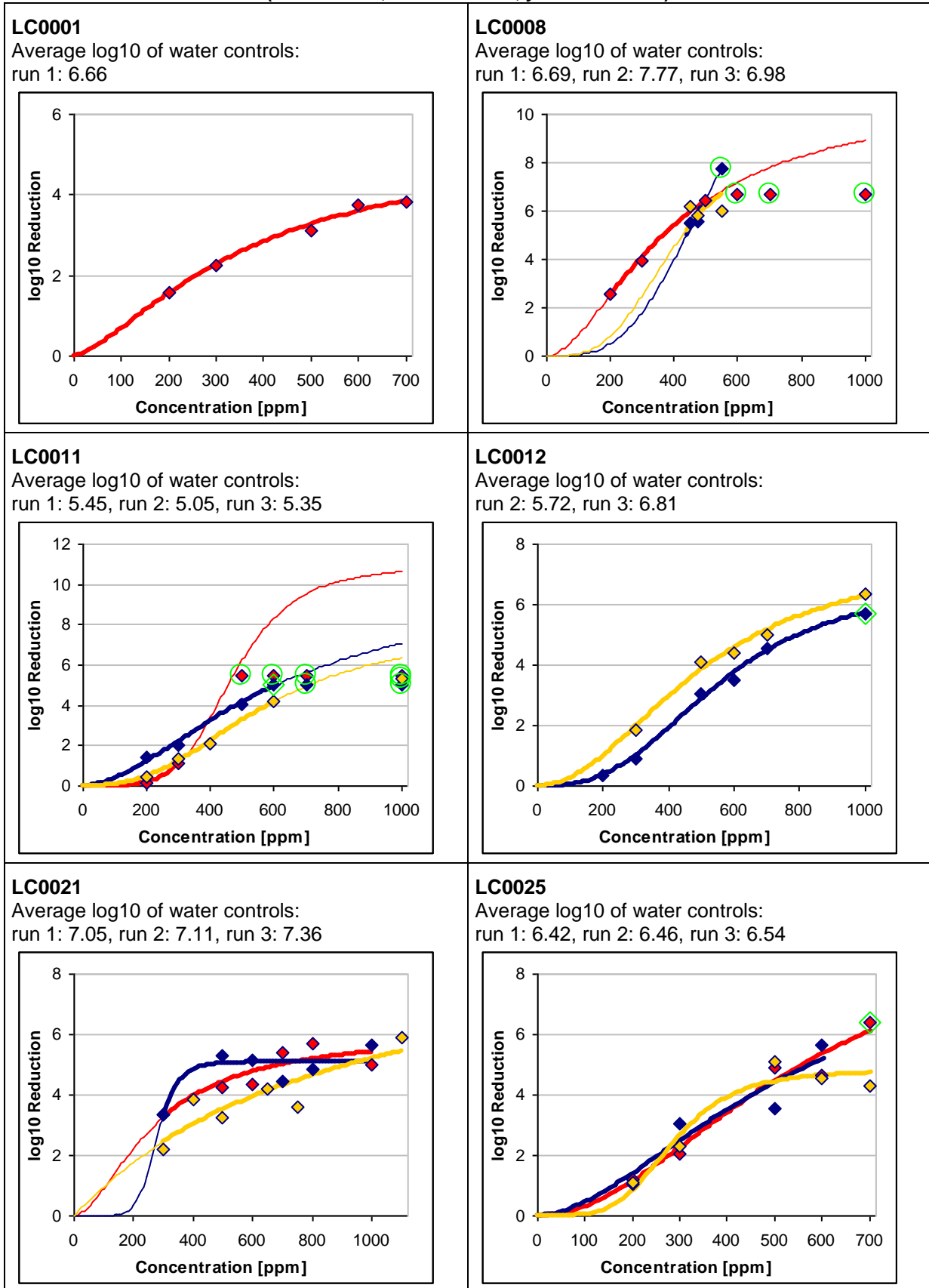
**Figure 151: Standard run-specific kill curves for *Mycobacterium terrae* – glutaraldehyde**  
 (red = 1st run, blue = 2nd run, yellow = 3rd run)



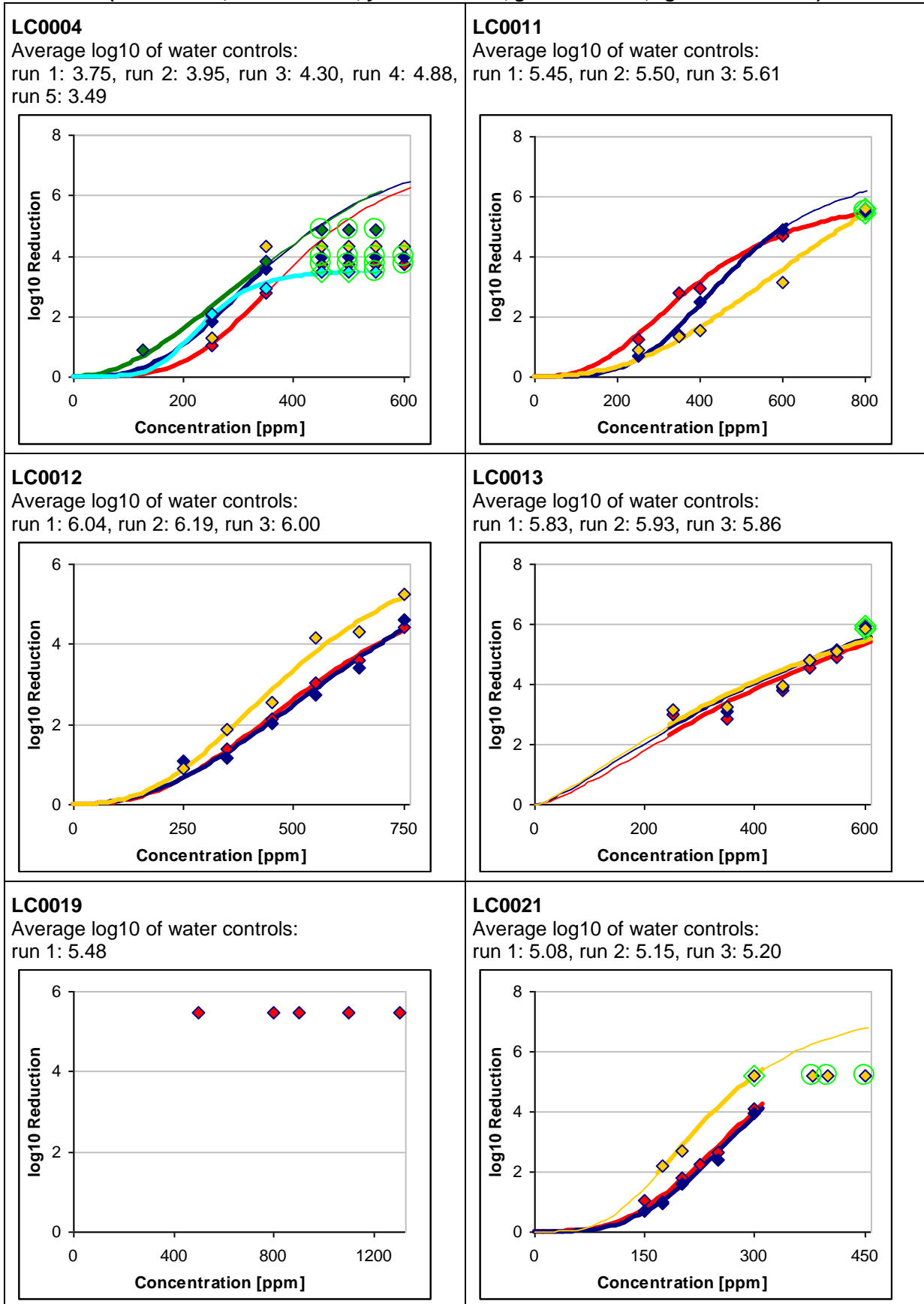
**Figure 152: Standard run-specific kill curves for *Mycobacterium terrae* – phenol**  
 (red = 1st run, blue = 2nd run, yellow = 3rd run)



**Figure 153: Standard run-specific kill curves for *Mycobacterium terrae* – peracetic acid (red = 1st run, blue = 2nd run, yellow = 3rd run)**

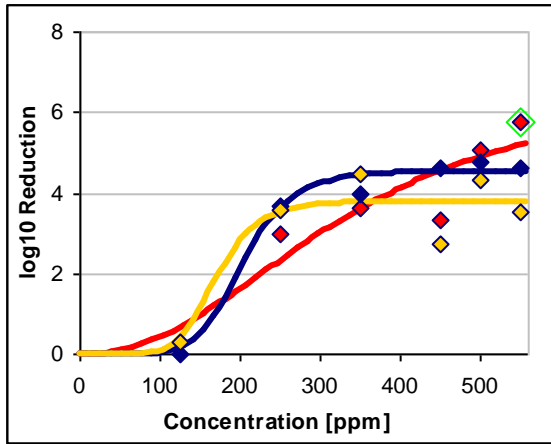


**Figure 154: Standard run-specific kill curves for *Pseudomonas aeruginosa* – glutaraldehyde**  
 (red = 1st run, blue = 2nd run, yellow = 3rd run, green = 4th run, light blue = 5th run)



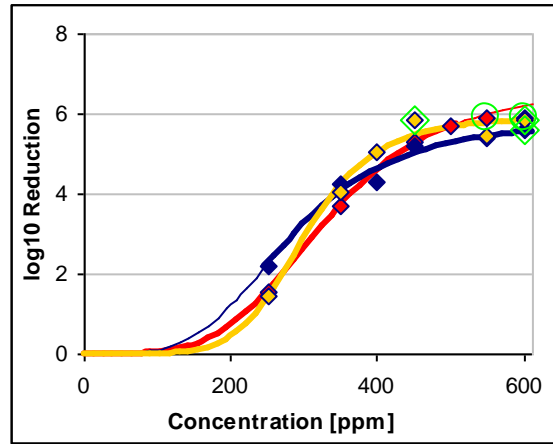
**LC0026**

Average log10 of water controls:  
run 1: 5.78, run 2: 5.25, run 3: 5.10

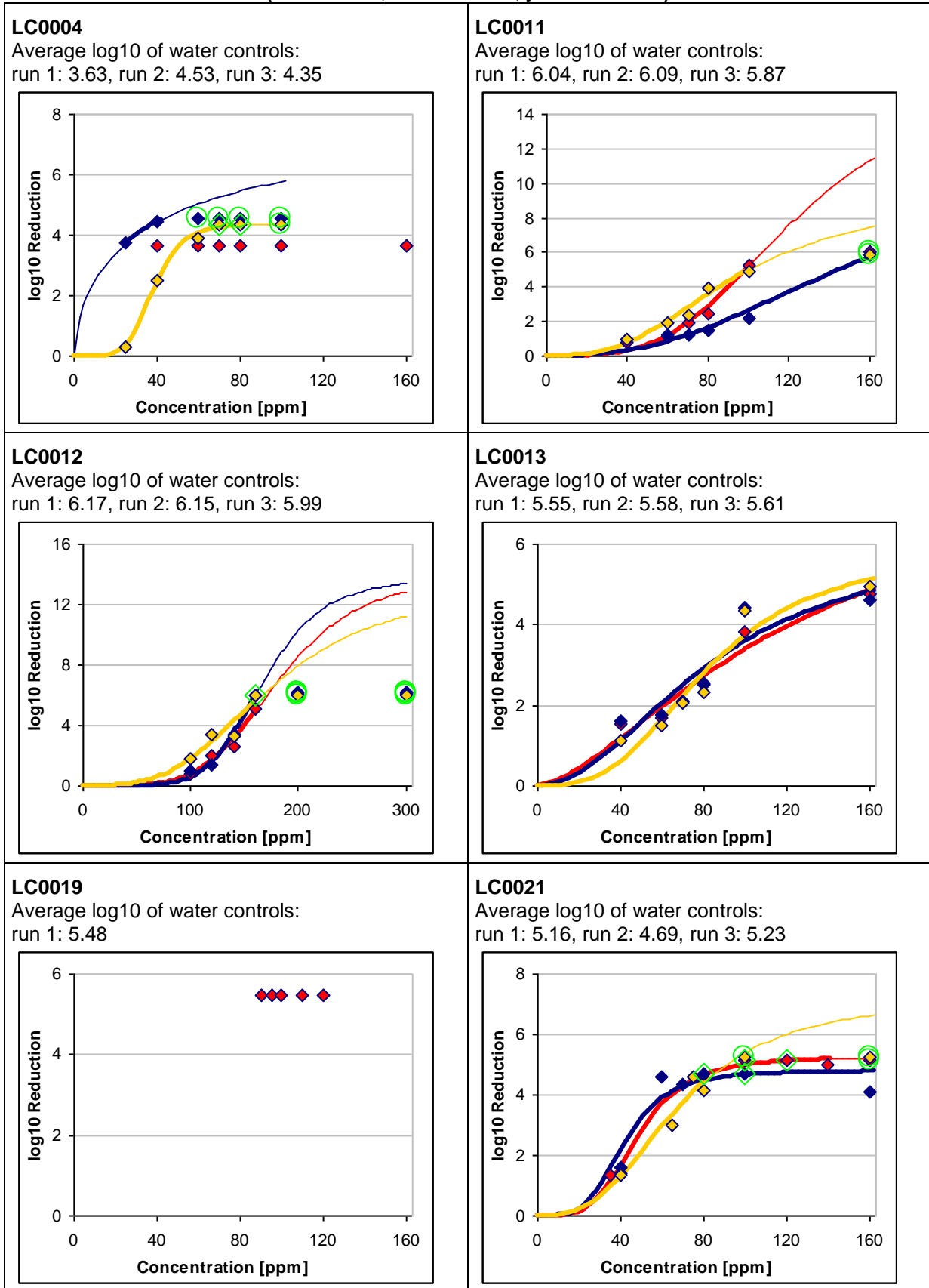


**LC0028**

Average log10 of water controls:  
run 1: 5.88, run 2: 5.58, run 3: 5.86

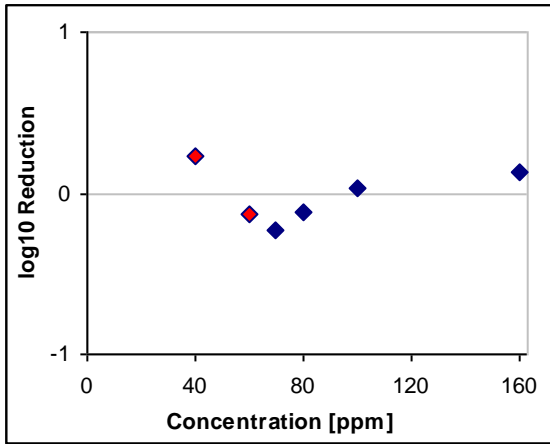


**Figure 155: Standard run-specific kill curves for *Pseudomonas aeruginosa* – peracetic acid (red = 1st run, blue = 2nd run, yellow = 3rd run)**



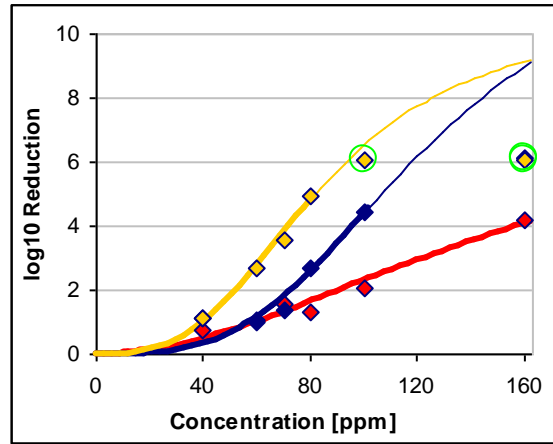
**LC0026**

Average log10 of water controls:  
run 1: 5.23, run 2: 5.43

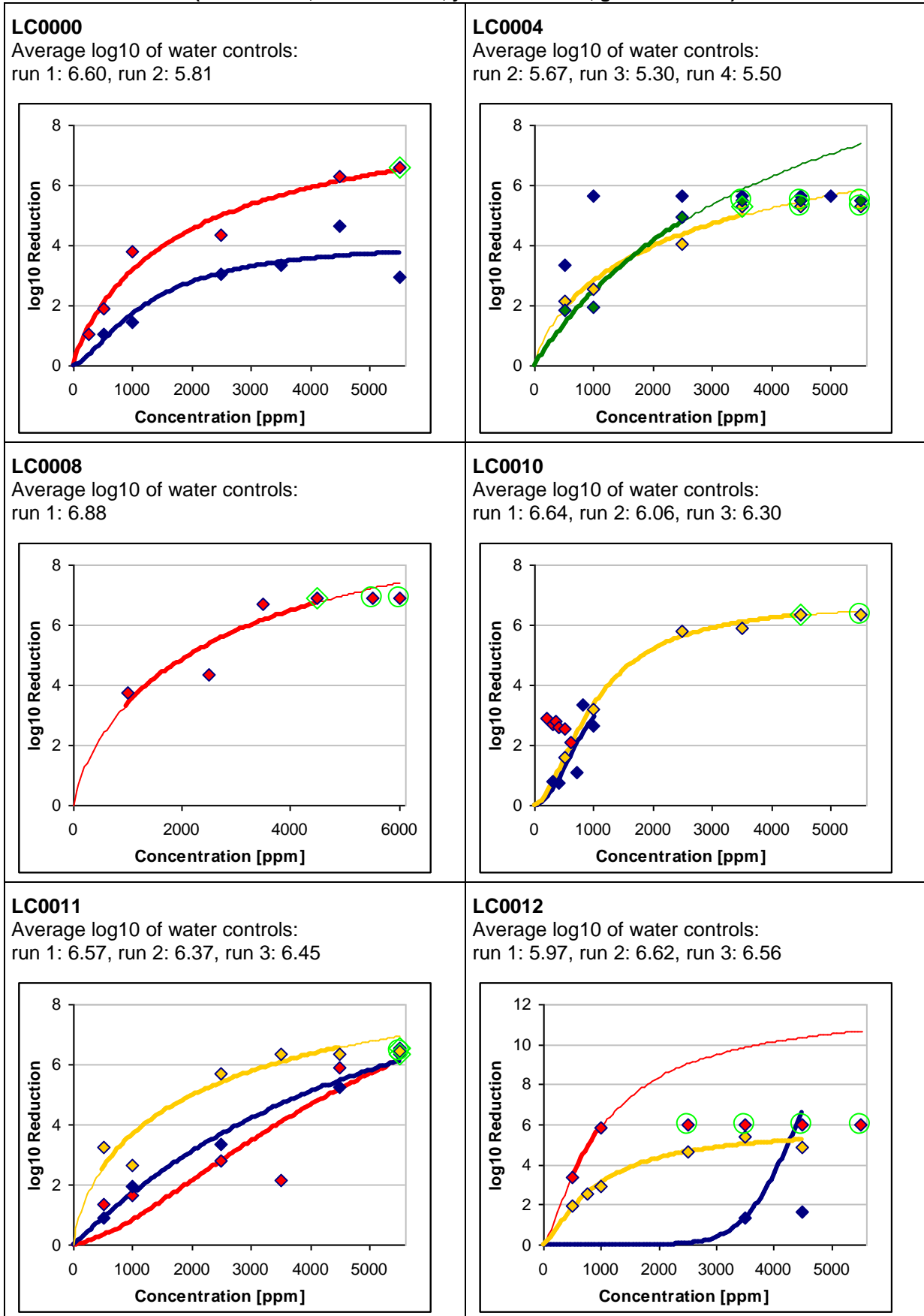


**LC0028**

Average log10 of water controls:  
run 1: 5.39, run 2: 6.15, run 3: 6.04

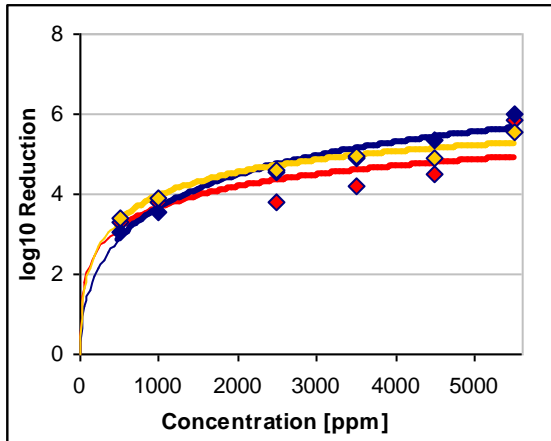


**Figure 156: Standard run-specific kill curves for *Staphylococcus aureus* – benzalkonium chloride**  
 (red = 1st run, blue = 2nd run, yellow = 3rd run, green = 4th run)



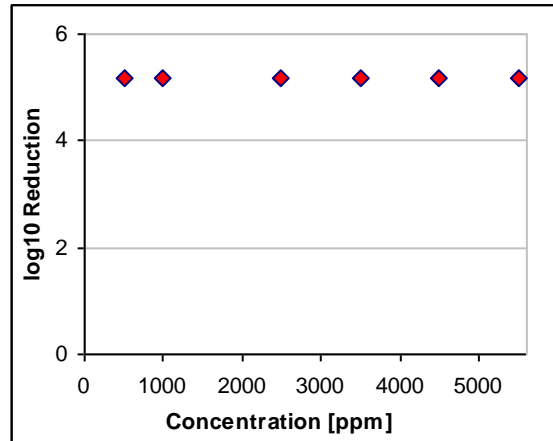
**LC0013**

Average log10 of water controls:  
run 1: 6.56, run 2: 6.98, run 3: 6.96



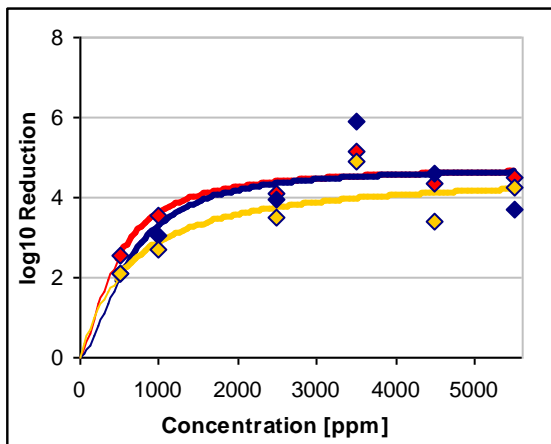
**LC0019**

Average log10 of water controls:  
run 1: 5.18



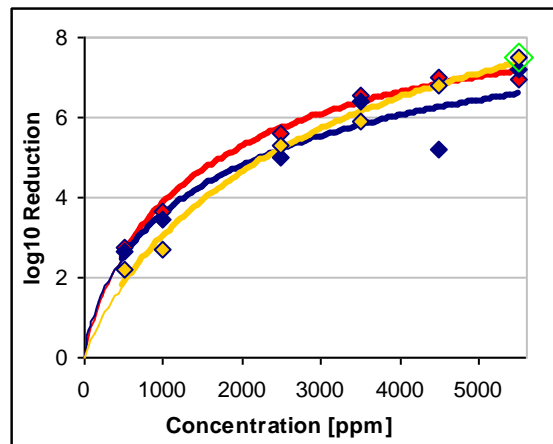
**LC0020**

Average log10 of water controls:  
run 1: 6.36, run 2: 5.98, run 3: 5.86



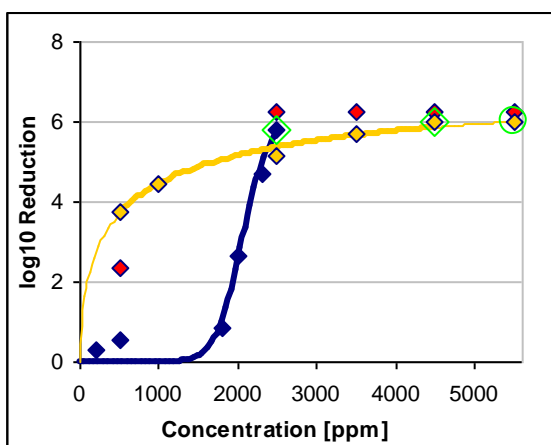
**LC0021**

Average log10 of water controls:  
run 1: 7.43, run 2: 7.94, run 3: 7.50



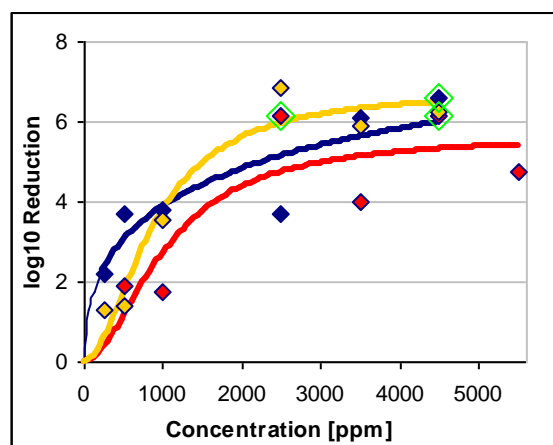
**LC0022**

Average log10 of water controls:  
run 1: 6.26, run 2: 5.79, run 3: 5.99



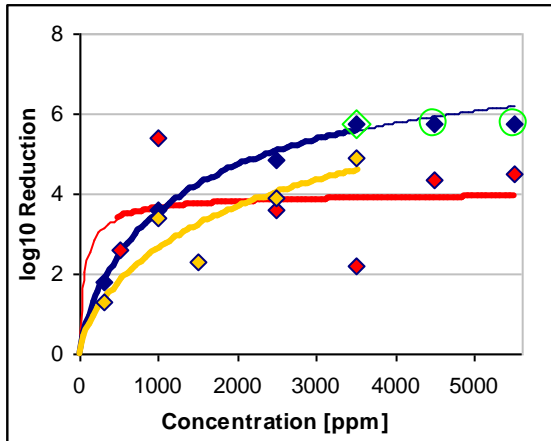
**LC0026**

Average log10 of water controls:  
run 1: 6.15, run 2: 6.59, run 3: 7.08

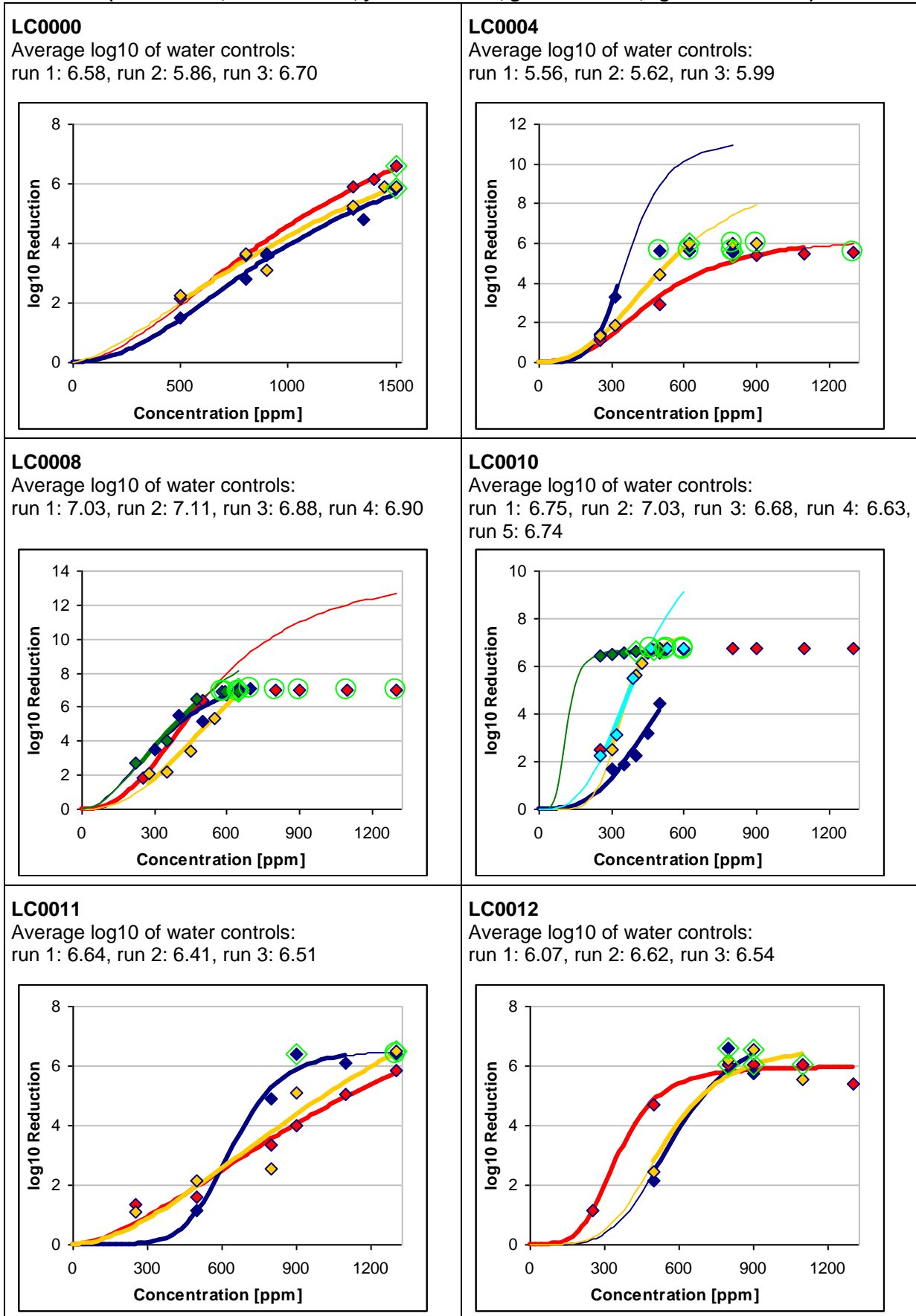


**LC0028**

Average log<sub>10</sub> of water controls:  
run 1: 5.90, run 2: 5.75, run 3: 6.03

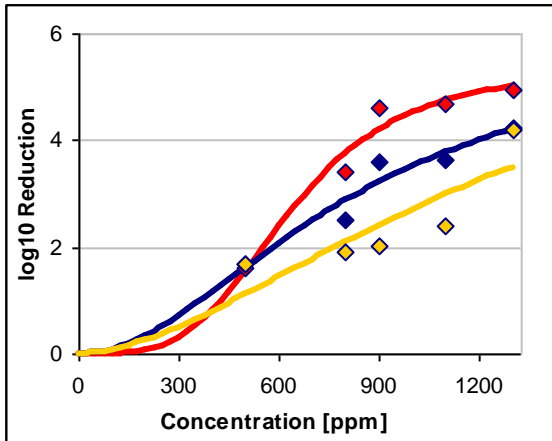


**Figure 157: Standard run-specific kill curves for *Staphylococcus aureus* – glutaraldehyde**  
 (red = 1st run, blue = 2nd run, yellow = 3rd run, green = 4th run, light blue = 5th run)



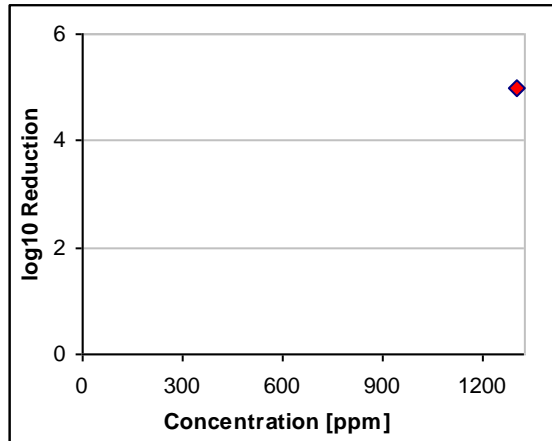
**LC0013**

Average log10 of water controls:  
run 1: 6.93, run 2: 6.88, run 3: 6.92



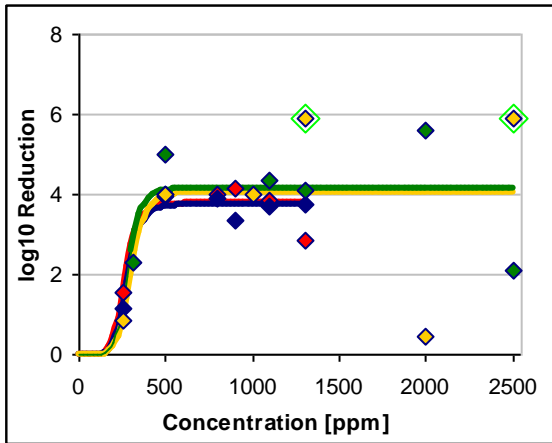
**LC0019**

Average log10 of water controls:  
run 1: 5.18



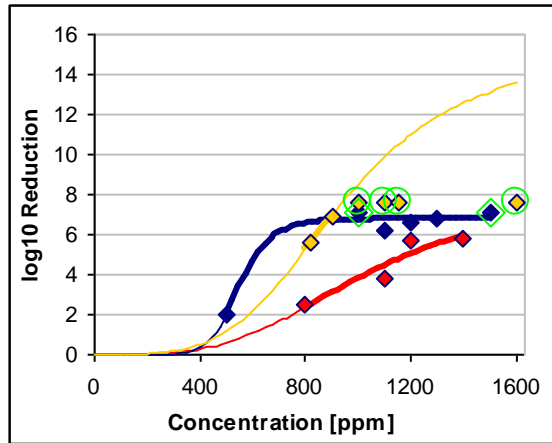
**LC0020**

Average log10 of water controls:  
run 1: 5.80, run 2: 5.96, run 3: 5.90, run 4: 6.12



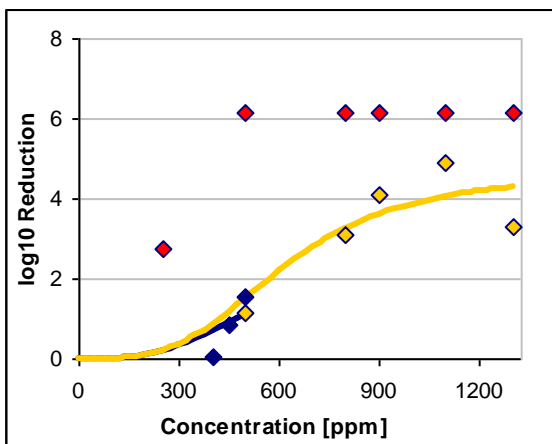
**LC0021**

Average log10 of water controls:  
run 1: 7.28, run 2: 7.11, run 3: 7.62



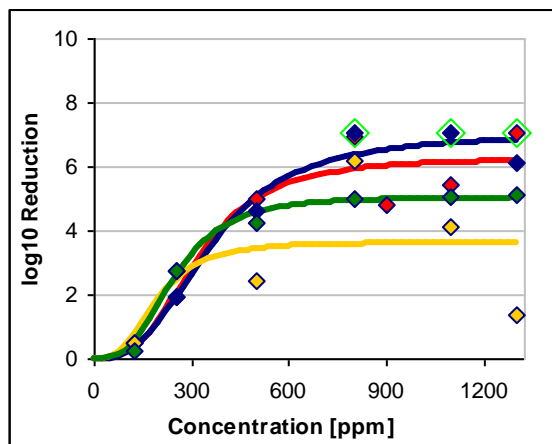
**LC0022**

Average log10 of water controls:  
run 1: 6.13, run 2: 5.43, run 3: 6.01



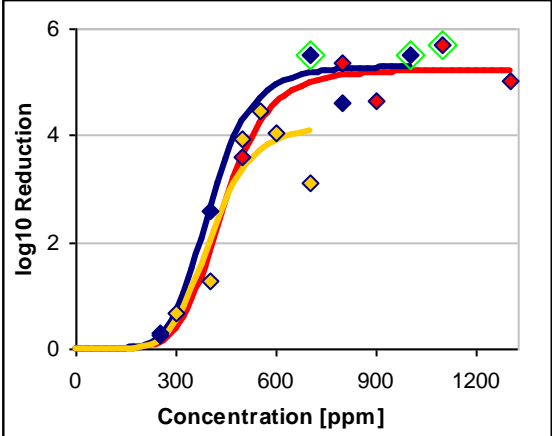
**LC0026**

Average log10 of water controls:  
run 1: 7.06, run 2: 7.05, run 3: 6.63, run 4: 7.15

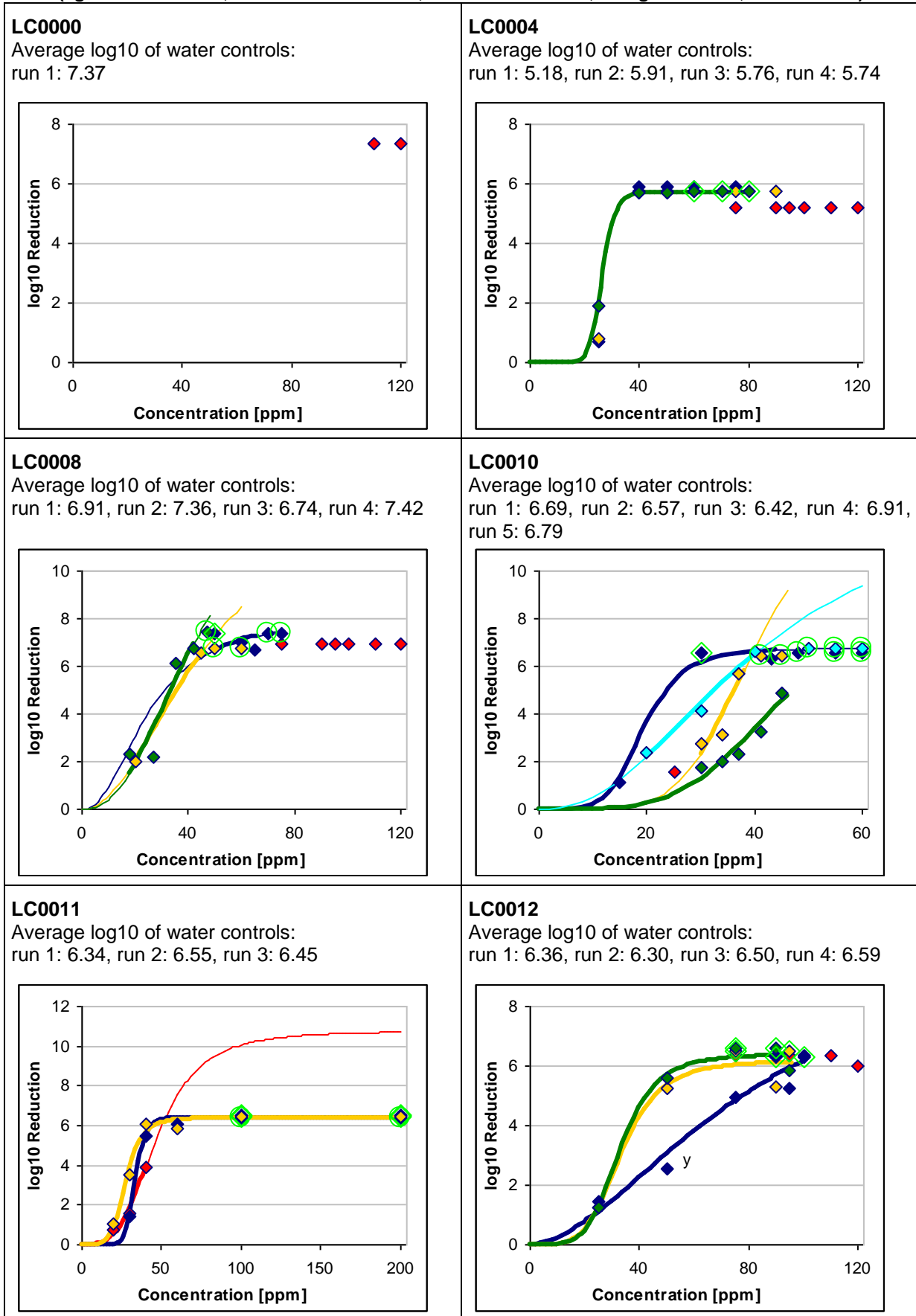


**LC0028**

Average log10 of water controls:  
run 1: 5.70, run 2: 5.53, run 3: 5.54

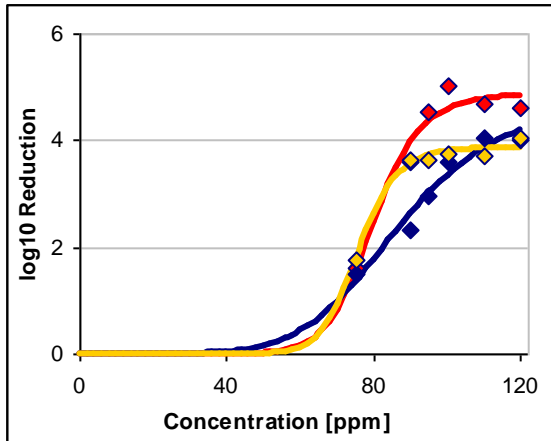


**Figure 158: Standard run-specific kill curves for *Staphylococcus aureus* – peracetic acid**  
 (light blue = 1st run, middle blue = 2nd run, dark blue = 3rd run, orange = 4th run, red = 5th run)



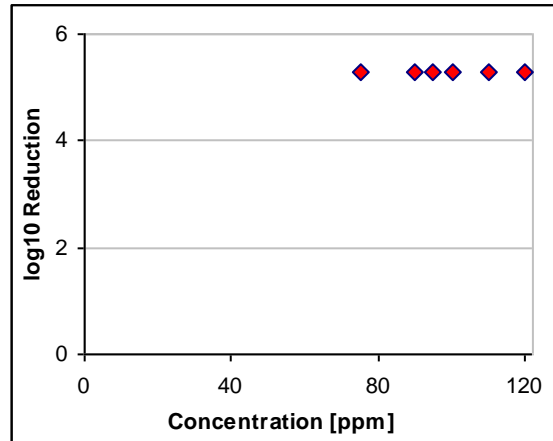
**LC0013**

Average log10 of water controls:  
run 1: 6.96, run 2: 6.93, run 3: 6.95



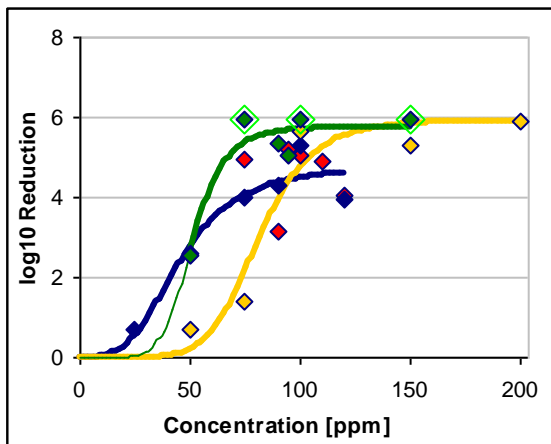
**LC0019**

Average log10 of water controls:  
run 1: 5.30



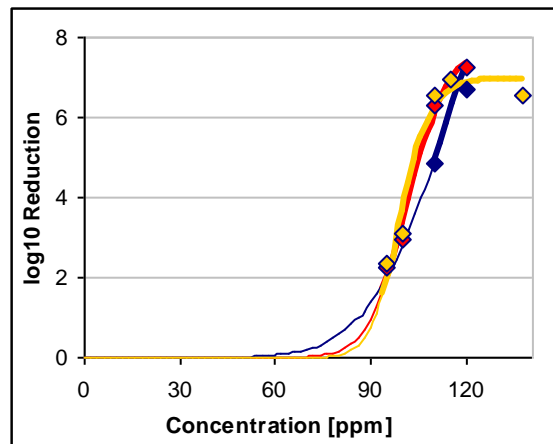
**LC0020**

Average log10 of water controls:  
run 1: 6.01, run 2: 5.97, run 3: 6.08, run 4: 5.95



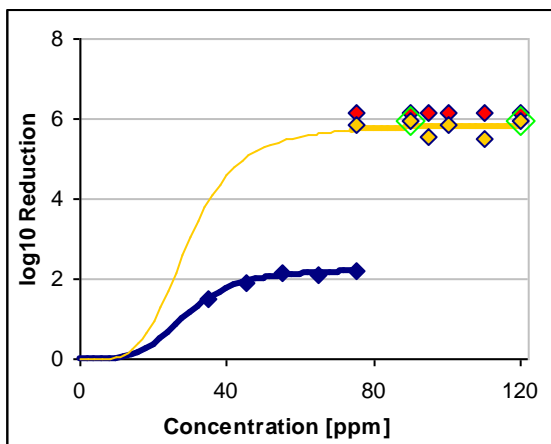
**LC0021**

Average log10 of water controls:  
run 1: 7.41, run 2: 7.30, run 3: 7.53



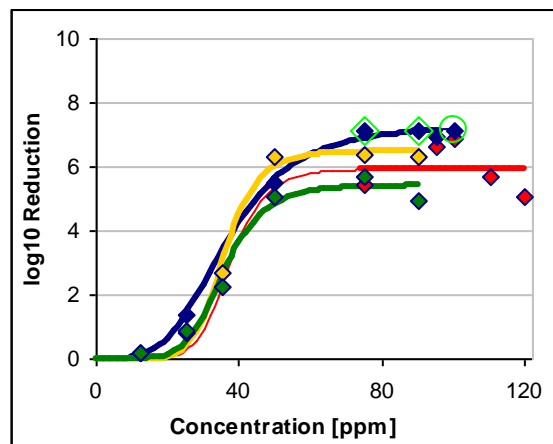
**LC0022**

Average log10 of water controls:  
run 1: 6.14, run 2: 5.80, run 3: 5.96



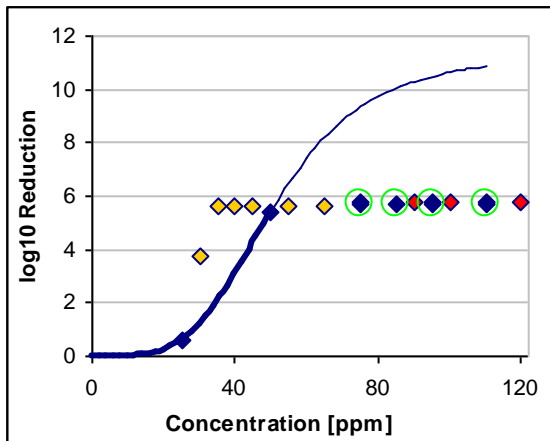
**LC0026**

Average log10 of water controls:  
run 1: 7.15, run 2: 7.11, run 3: 6.69, run 4: 6.87



**LC0028**

Average log<sub>10</sub> of water controls:  
run 1: 5.81, run 2: 5.68, run 3: 5.65

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

227. The following table summarizes the results of the three outlier tests with regard to the run-specific inactivation curves. These outlier laboratories do not contribute to any further statistical analysis.
228. Since for *Adenovirus type 5* (well count) data of only three laboratories is available, no outlier tests are carried out.
229. The Grubbs test with regard to the mean concentration at a normalized log reduction of 50% never yielded in any significant result.
230. For *Mycobacterium terrae* and all substances as well as for *Pseudomonas aeruginosa*, *Aspergillus niger* and *Bacillus subtilis* – each with peracetic acid – no outlier laboratories (or runs) could be identified at all.
231. It has to be noted that the values of laboratory LC0007 for *Mycobacterium terrae* and phenol had to be excluded from the dataset as well. The respective values exhibit a very slow increase (see also Figure 137) and including these values would cause a bias of the results. No outlier test identified the runs as outlier runs, because the standard deviation is very small and the mean value at a log<sub>10</sub> reduction of 3 does not differ significantly from the mean values of the other laboratories. If, however, a different log<sub>10</sub> reduction had been assumed as test level (e.g. 4), the runs would clearly have been identified as outlier runs.

**Table 48: Eliminated outlier laboratories (or runs) due to deviations of the run-specific inactivation curves**

Organism	Substance	RSD (Cochran)	Mean concentration at log10 reduction of 3 (Grubbs)	Standard deviation of concentrations at log10 reduction of 3 (Cochran)
<i>Adenovirus type 5</i> (TCID <sub>50</sub> )	peracetic acid	-	-	LC0022
	glutaraldehyde	LC0001	-	-
<i>Aspergillus niger</i>	glutaraldehyde	LC0022	-	-
<i>Pseudomonas aeruginosa</i>	glutaraldehyde	LC0026	-	-
<i>Staphylococcus aureus</i>	peracetic acid	LC0008	-	-
	glutaraldehyde	LC0020	-	LC0010
	benzalkonium chloride	LC0012	-	-

232. The following figures show the run-specific inactivation curves for each combination of organism and substance. Outlier inactivation curves are marked by different colors.

**Figure 159: Run-specific inactivation curves for *Adenovirus type 5* (TCID<sub>50</sub>) and glutaraldehyde (red: LC0001 – run 1)**

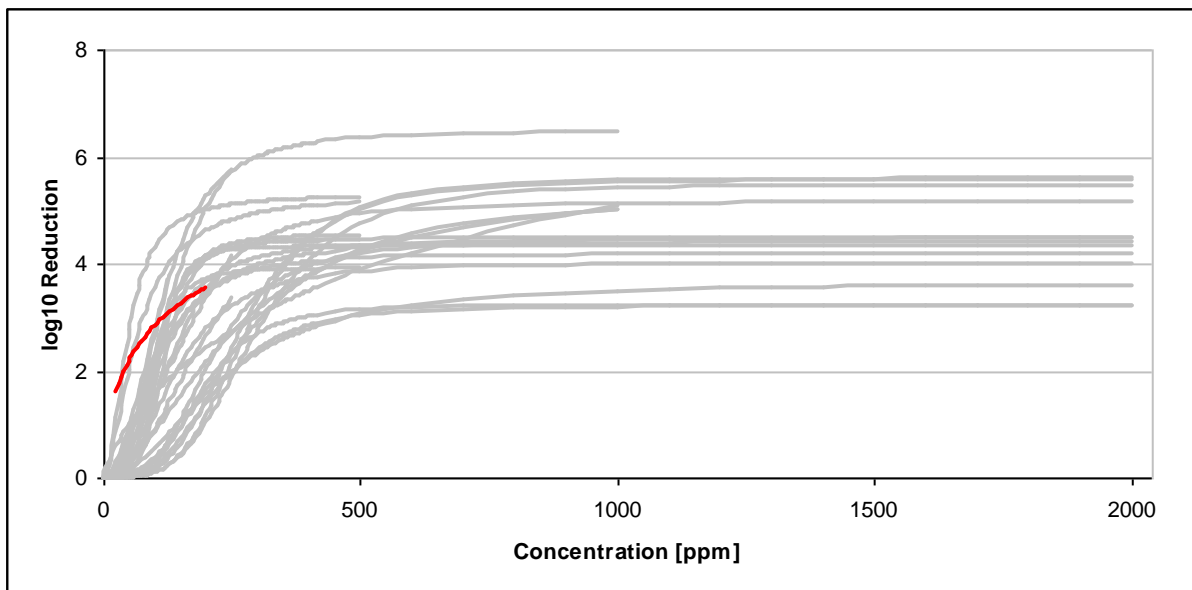


Figure 160: Run-specific inactivation curves for *Adenovirus type 5* (TCID<sub>50</sub>) and peracetic acid (green: LC0022)

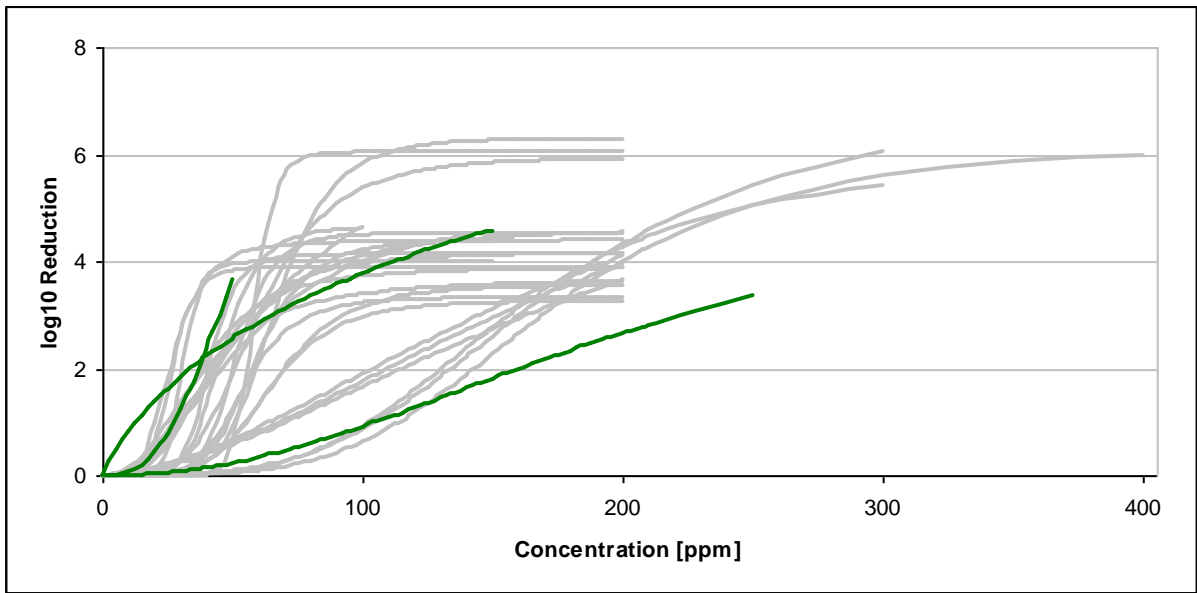


Figure 161: Run-specific inactivation curves for *Aspergillus niger* and glutaraldehyde (green: LC0022)

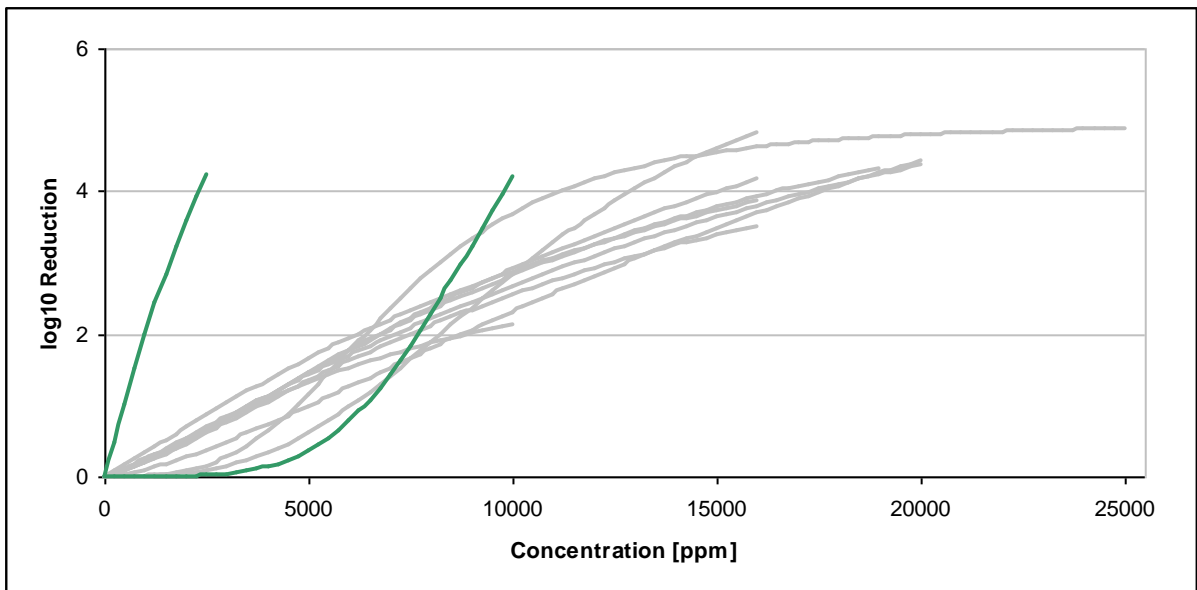


Figure 162: Run-specific inactivation curves for *Aspergillus niger* and peracetic acid (no outliers)

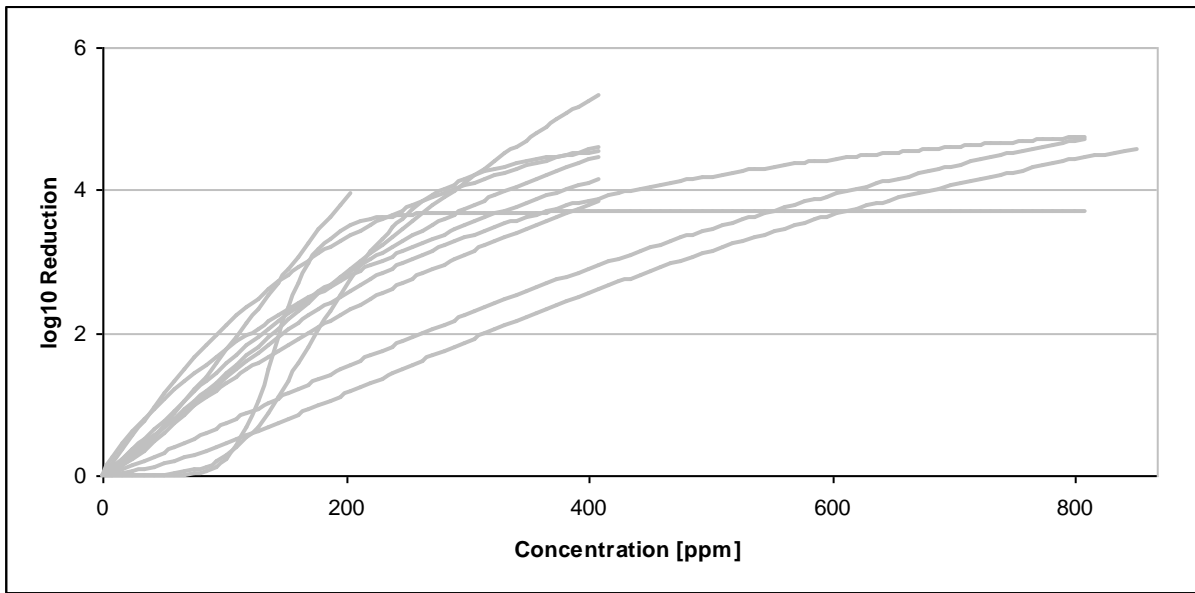
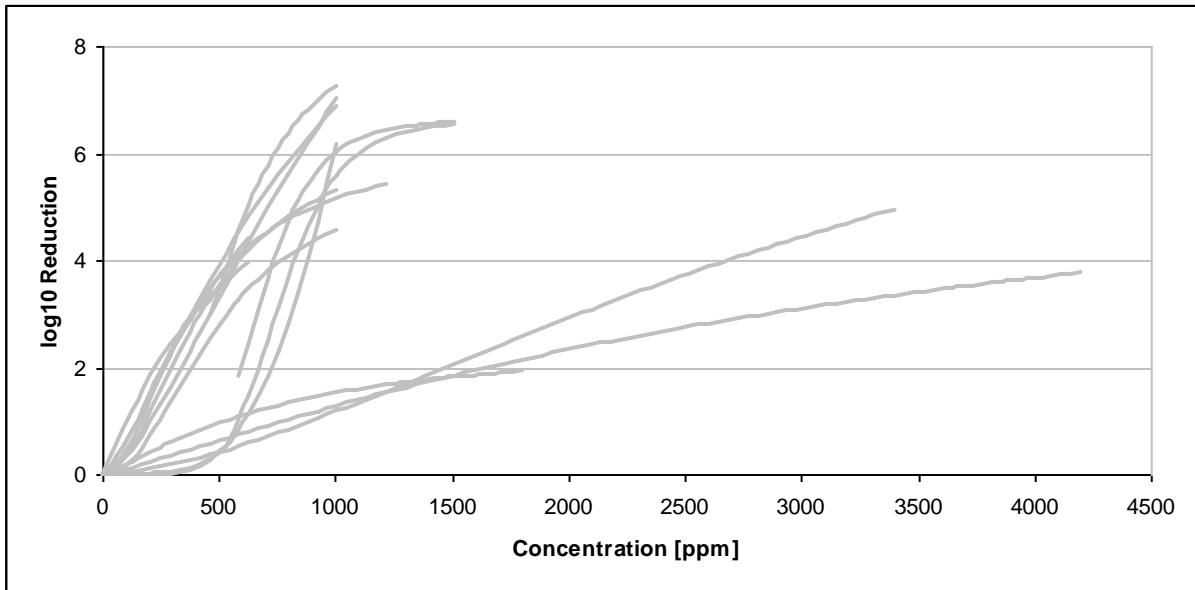
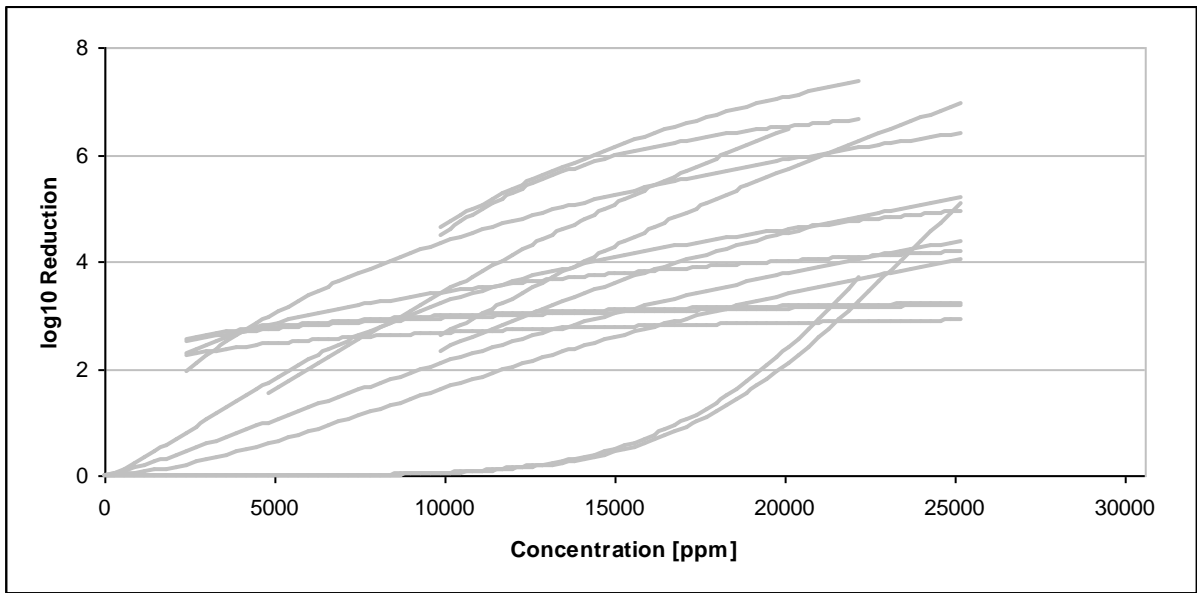


Figure 163: Run-specific inactivation curves for *Bacillus subtilis* and peracetic acid



**Figure 164: Run-specific inactivation curves for *Mycobacterium terrae* and glutaraldehyde (no outliers)**



**Figure 165: Run-specific inactivation curves for *Mycobacterium terrae* and peracetic acid (no outliers)**

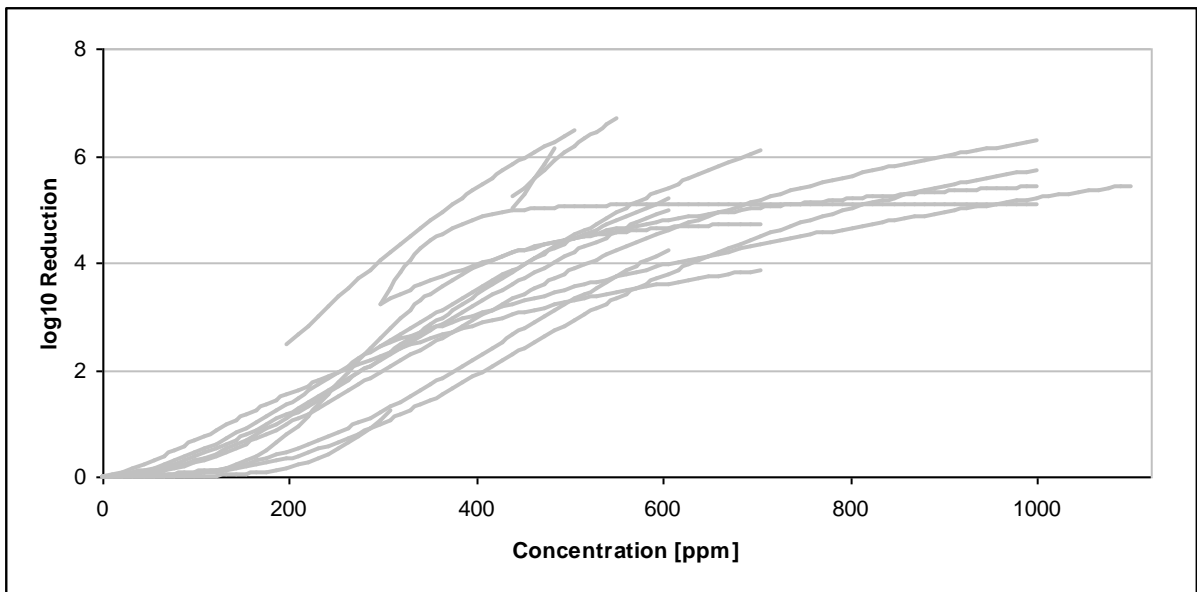


Figure 166: Run-specific inactivation curves for *Mycobacterium terrae* and phenol (blue: LC0007)

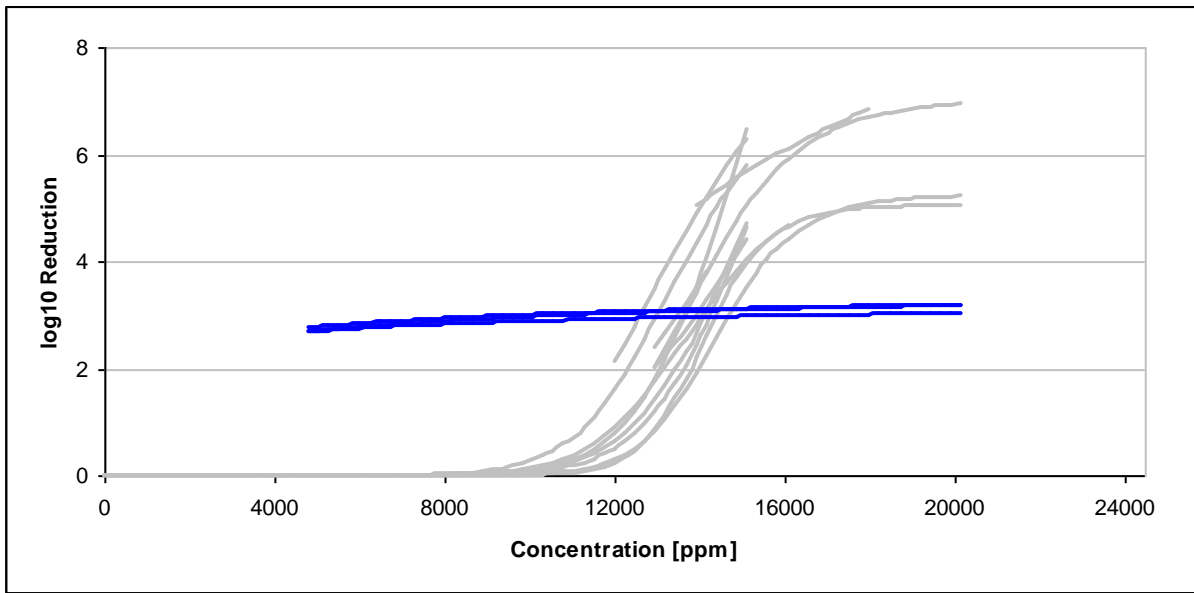
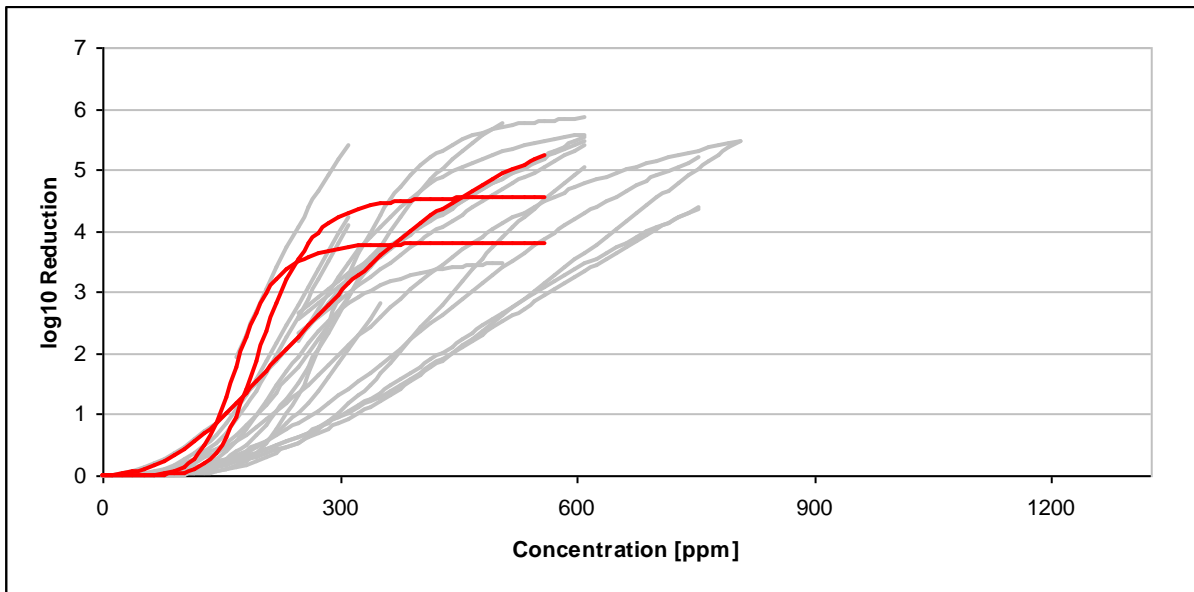
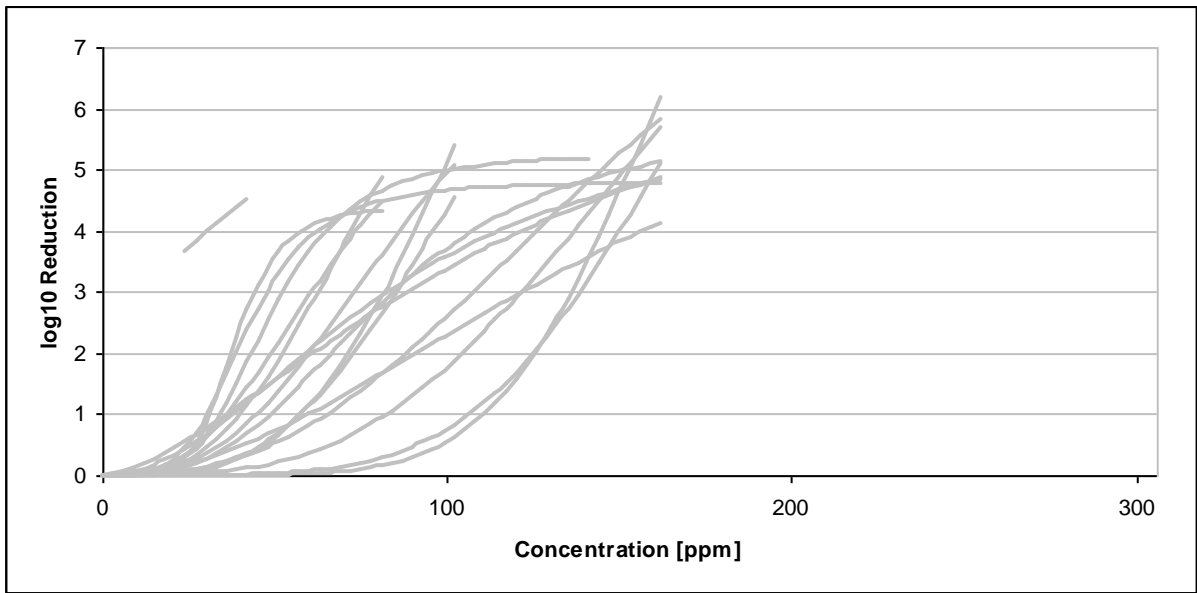


Figure 167: Run-specific inactivation curves for *Pseudomonas aeruginosa* and glutaraldehyde (red: LC0026)



**Figure 168: Run-specific inactivation curves for *Pseudomonas aeruginosa* and peracetic acid (no outliers)**



**Figure 169: Run-specific inactivation curves *Staphylococcus aureus* and benzalkonium chloride (red: LC0012)**

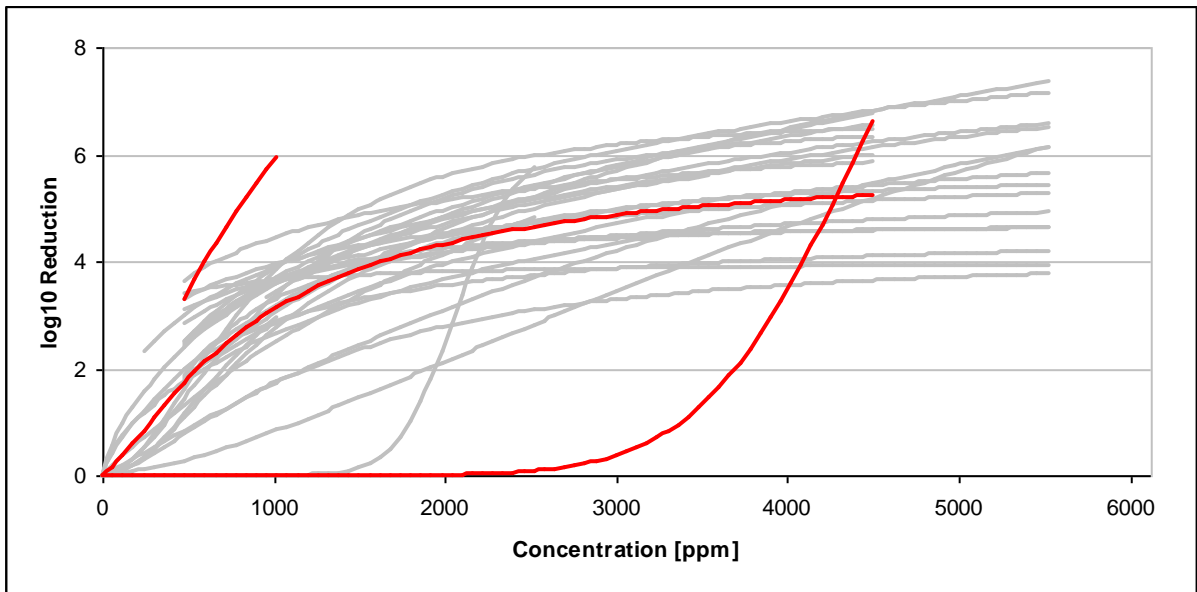


Figure 170: Run-specific inactivation curves *Staphylococcus aureus* and glutaraldehyde (green: LC0010, red: LC0020)

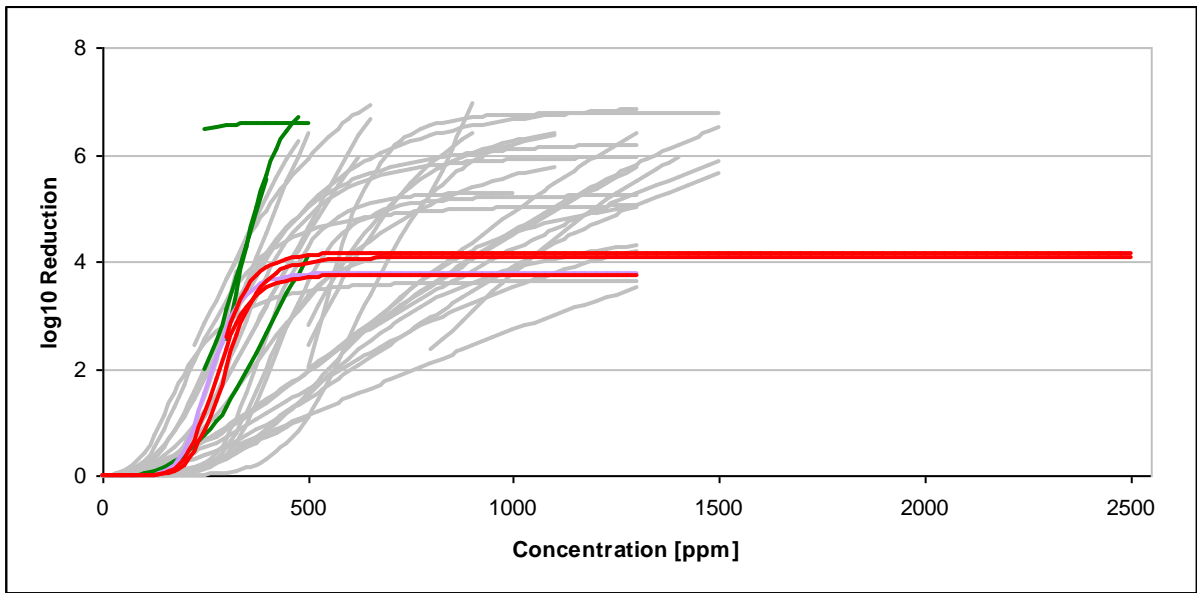
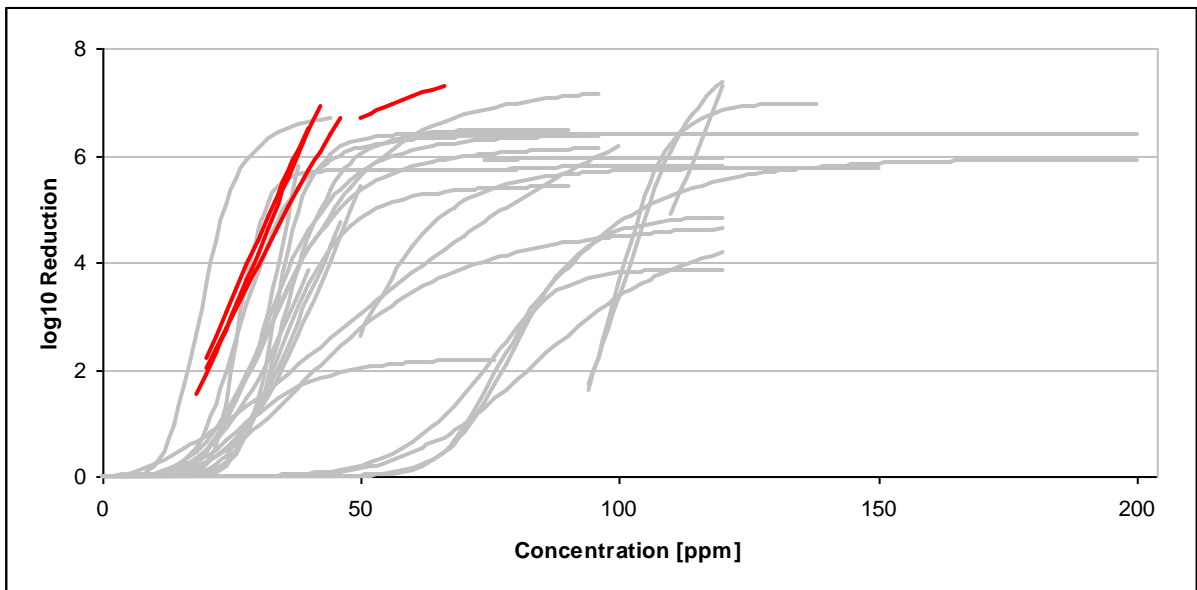


Figure 171: Run-specific inactivation curves *Staphylococcus aureus* and peracetic acid (red: LC0008)

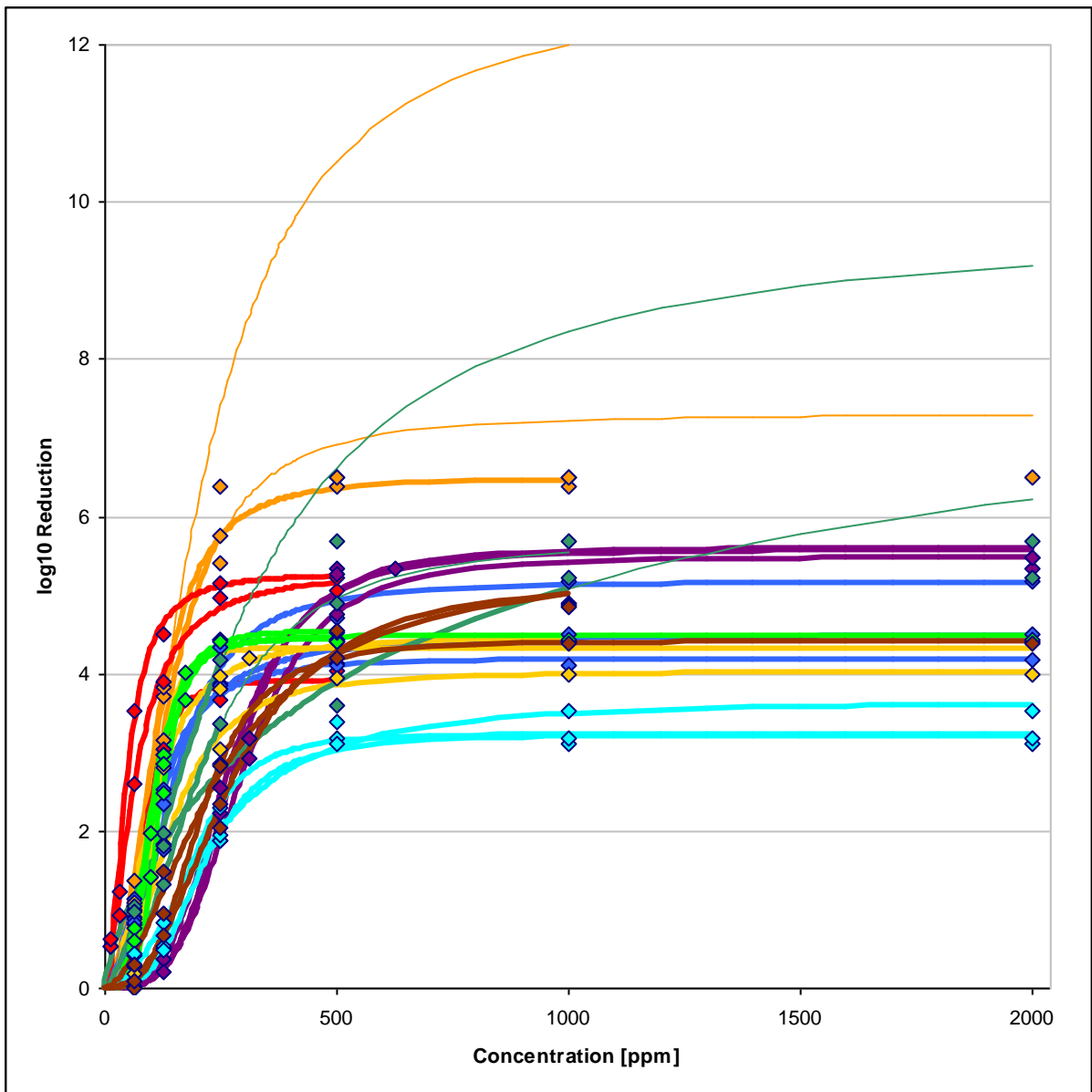


### **Final run-specific inactivation curves**

233. In the following, for each combination of organism and substance, a figure with all final run-specific inactivation curves is shown. In this figure, the mean log<sub>10</sub> reductions at each concentration level are displayed as well. Not regarded runs as well as outlier runs are not shown in the figures.
234. Extrapolated run-specific inactivation curves are shown as thin continuations of the non-extrapolated curve. This applies to cases where the mean log<sub>10</sub> 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.

**Figure 172: Single log<sub>10</sub> reductions and run-specific standard inactivation curves for *Adenovirus type 5* (TCID<sub>50</sub>) and glutaraldehyde**

LC0002	LC0006	LC0009	LC0016	LC0017	LC0022	LC0029	LC0031	LC0033



**Figure 173: Single log<sub>10</sub> reductions and run-specific standard inactivation curves for *Adenovirus type 5* (TCID<sub>50</sub>) and peracetic acid**

LC0001	LC0002	LC0006	LC0009	LC0016	LC0017	LC0029	LC0031	LC0033

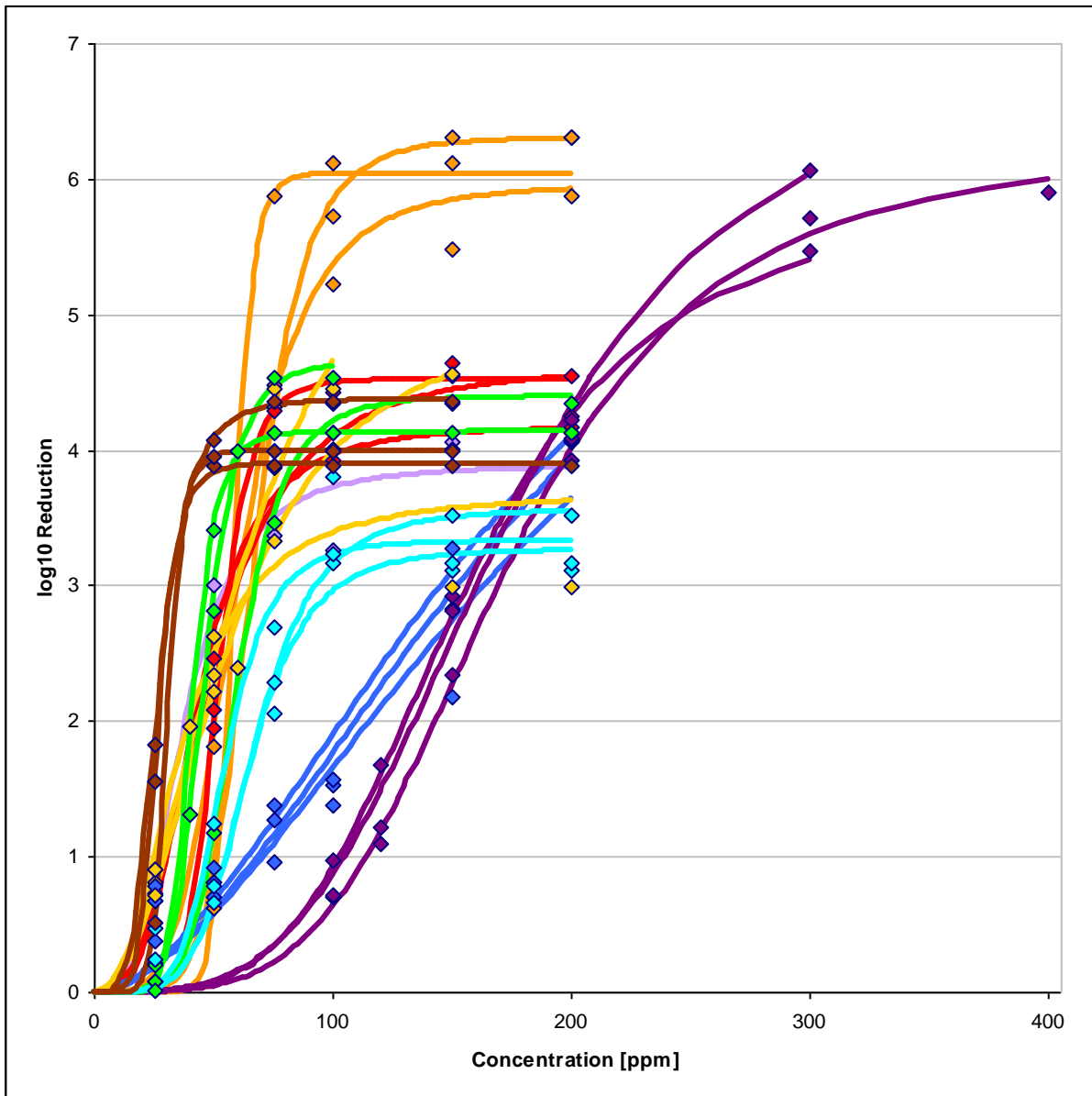


Figure 174: Single log<sub>10</sub> reductions and run-specific standard inactivation curves for *Adenovirus type 5* (well count) and glutaraldehyde

LC0014	LC0026	LC0032
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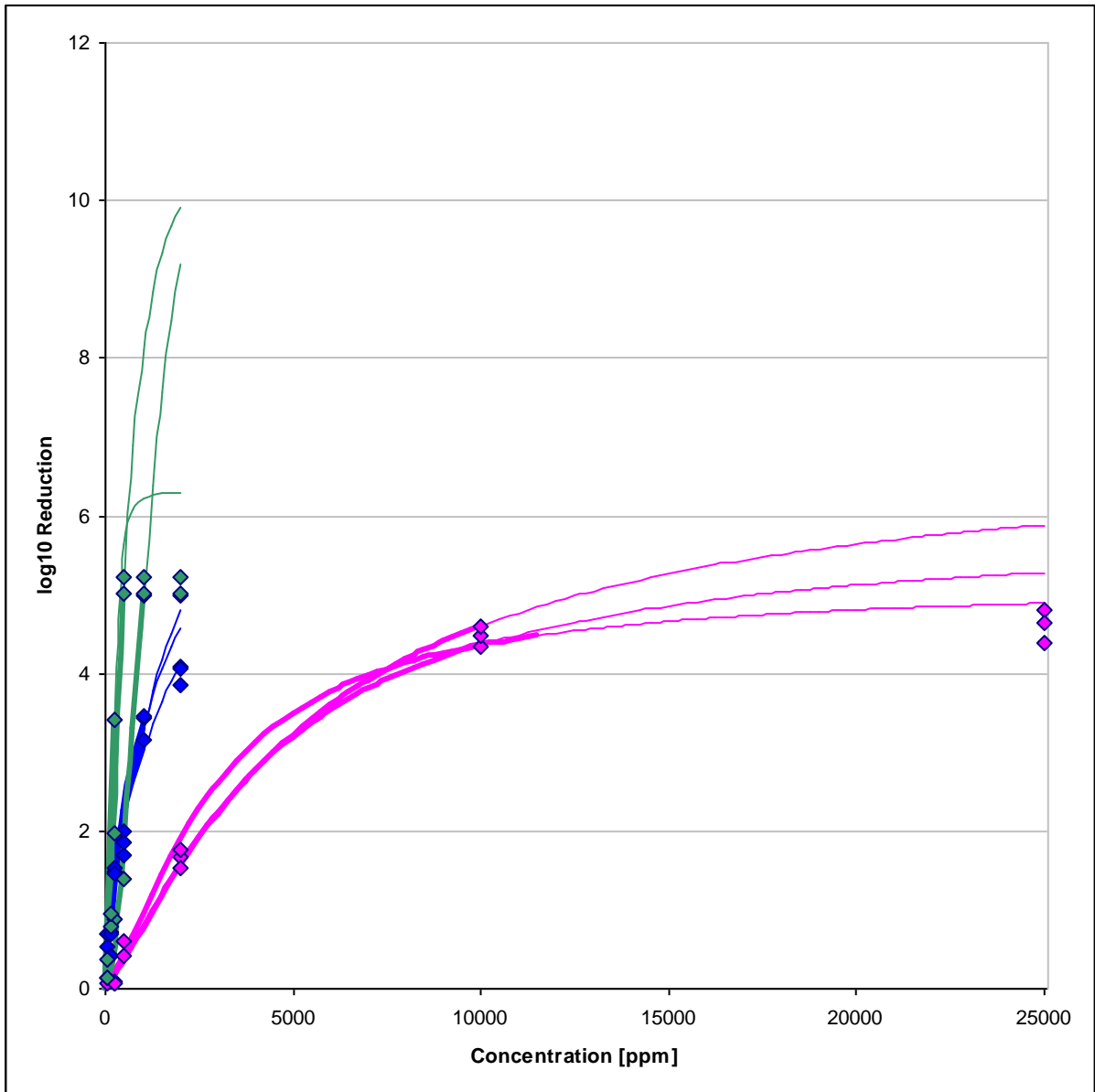


Figure 175: Single log<sub>10</sub> reductions and run-specific standard inactivation curves for *Adenovirus type 5* (well count) and peracetic acid

LC0014	LC0026	LC0032
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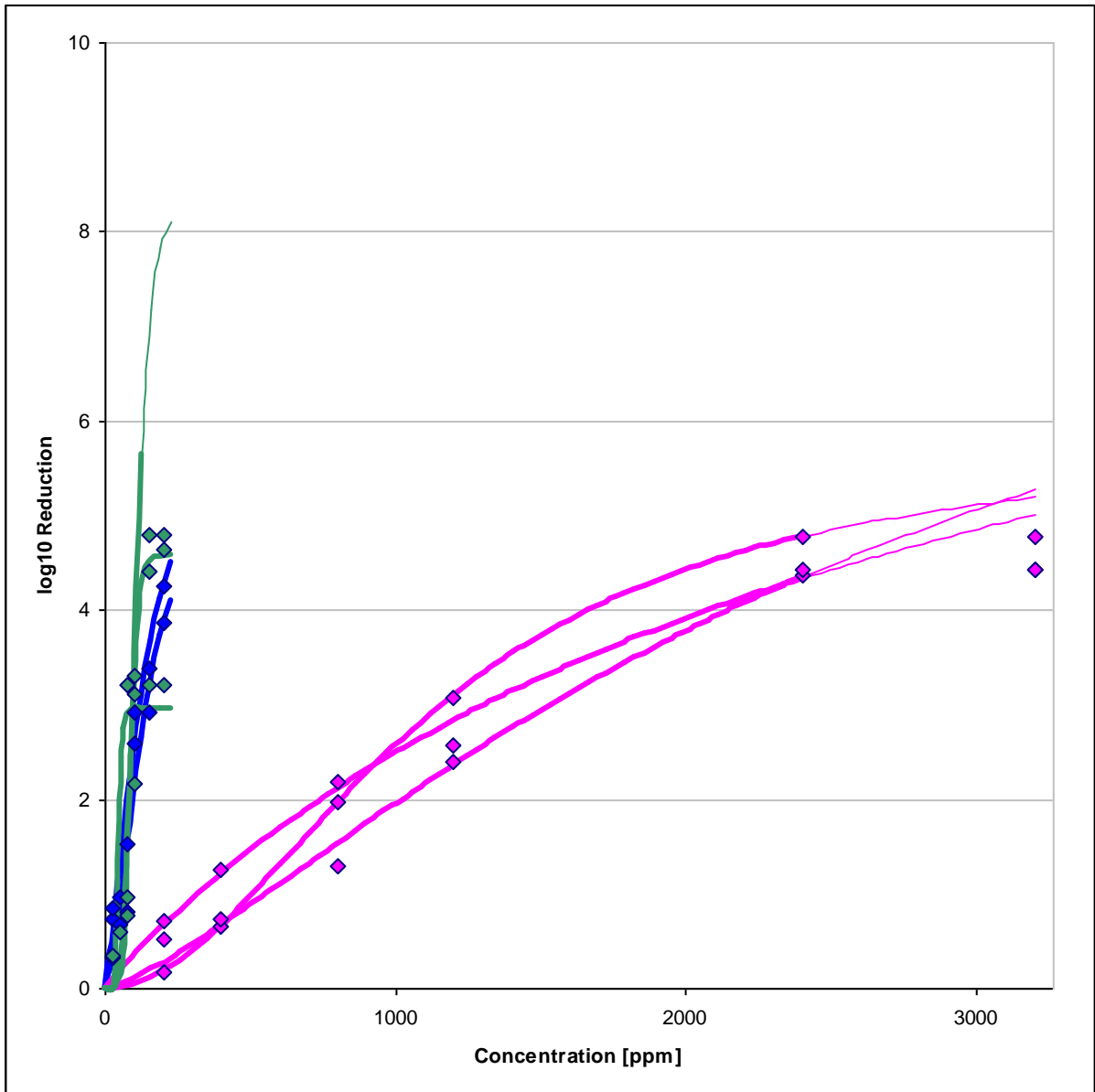
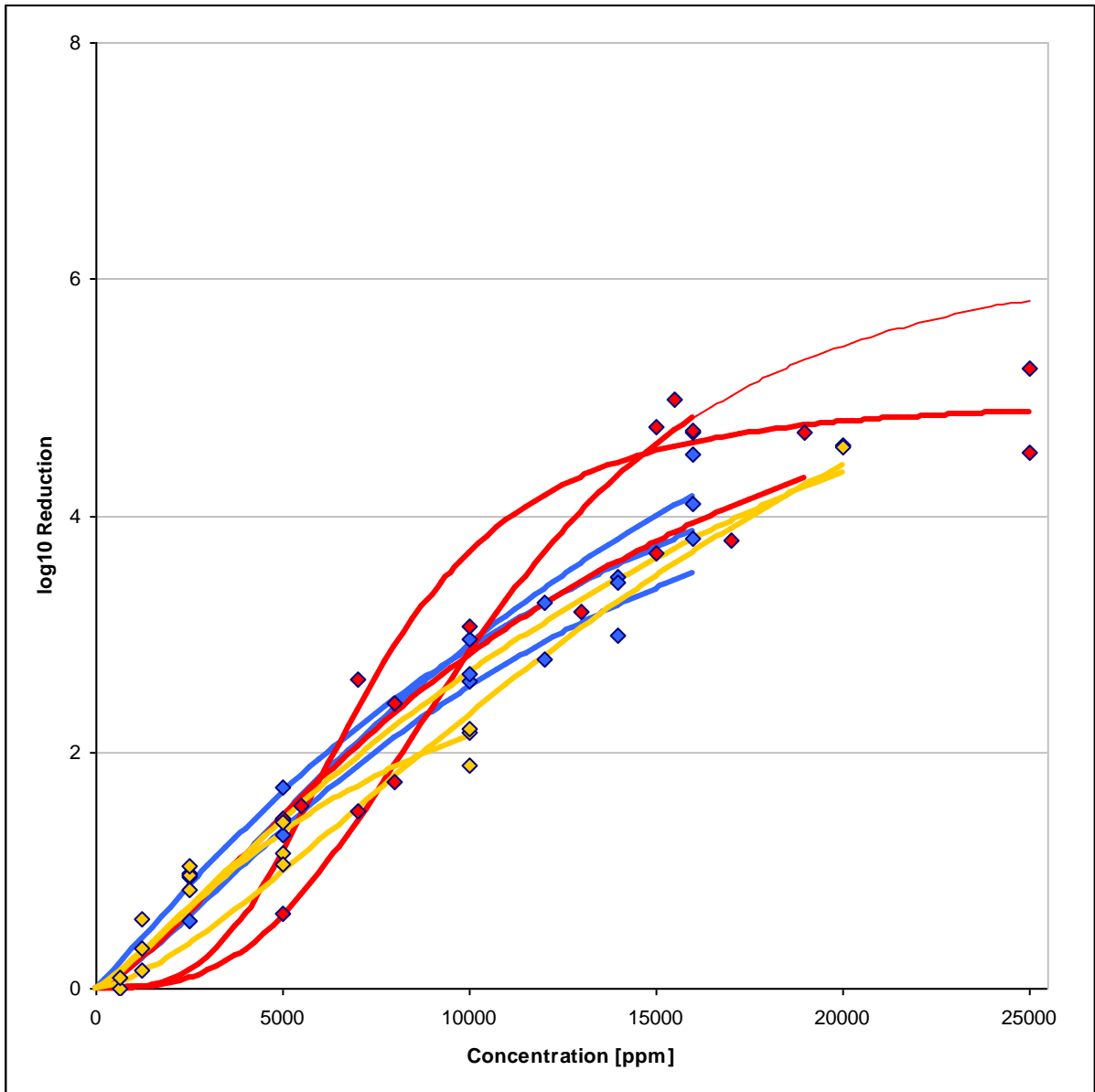


Figure 176: Single log<sub>10</sub> reductions and run-specific standard inactivation curves for *Aspergillus niger* and glutaraldehyde

LC0011	LC0019	LC0021	LC0023



**Figure 177: Single log<sub>10</sub> reductions and run-specific standard inactivation curves for *Aspergillus niger* and peracetic acid**

LC0011	LC0021	LC0022	LC0023

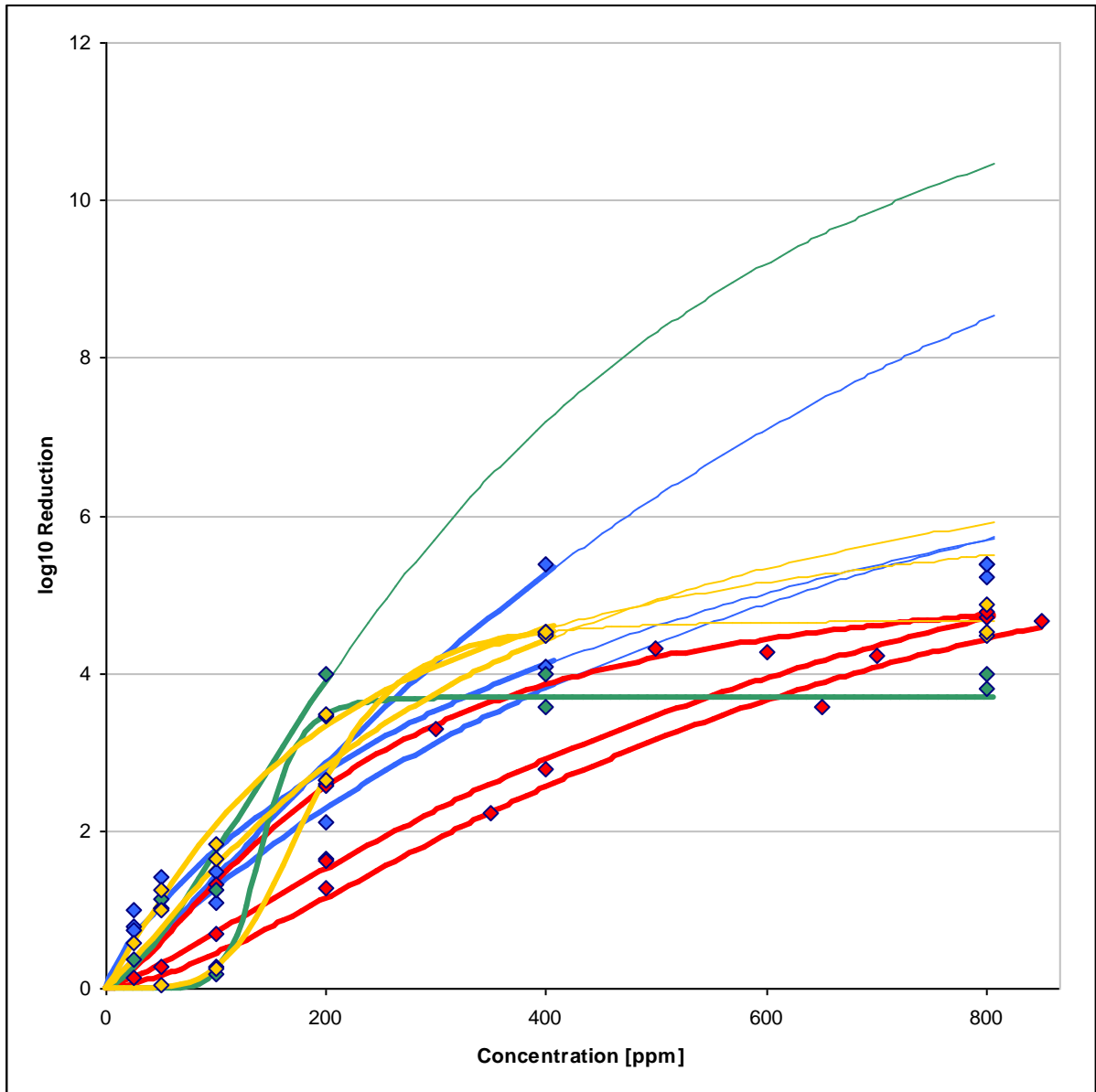


Figure 178: Single log10 reductions and run-specific standard inactivation curves for *Bacillus subtilis* and peracetic acid

LC0002	LC0008	LC0011	LC0022	LC0025

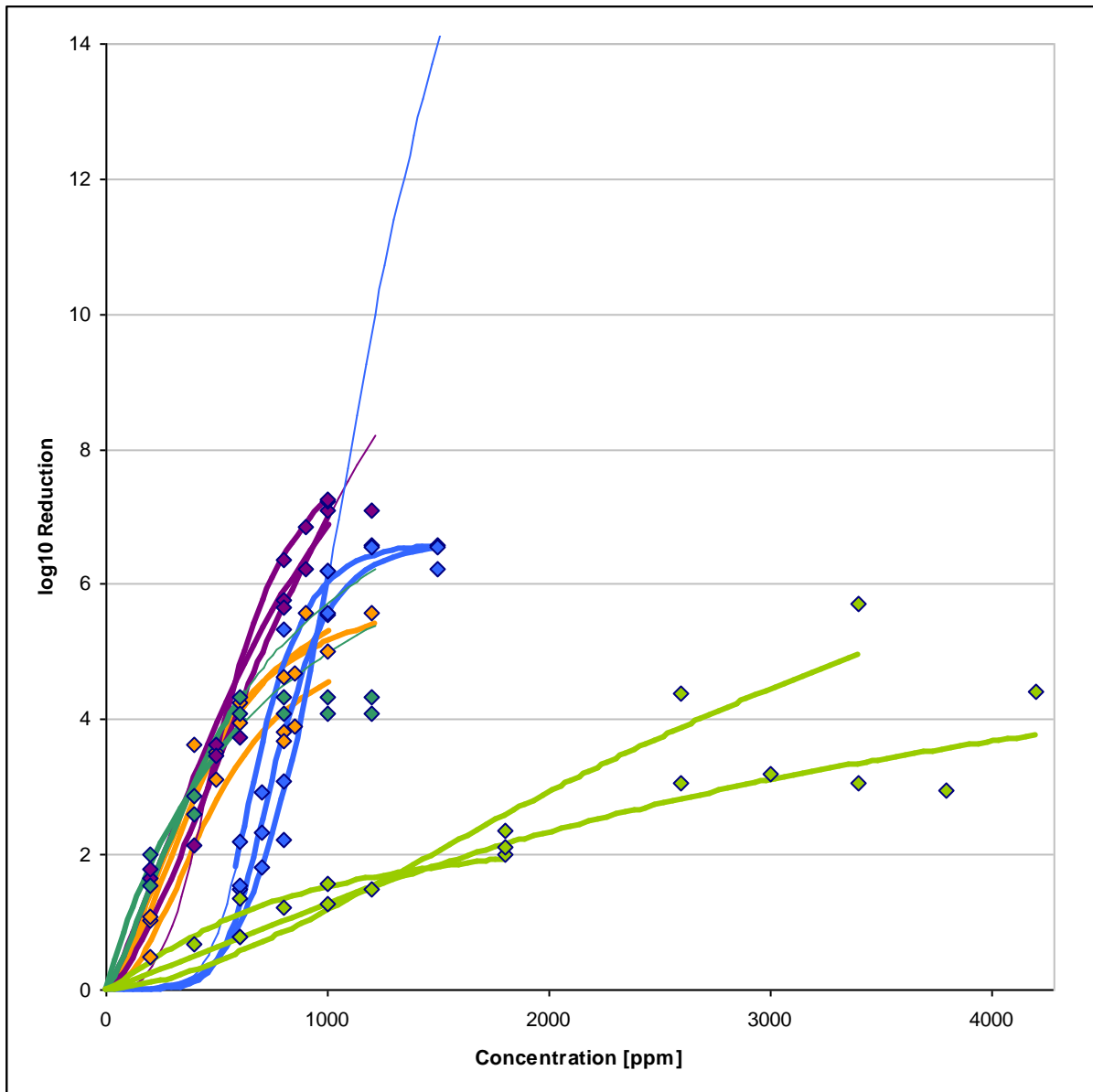


Figure 179: Single log<sub>10</sub> reductions and run-specific standard inactivation curves for *Mycobacterium terrae* and phenol

LC0001	LC0008	LC0011	LC0012	LC0021

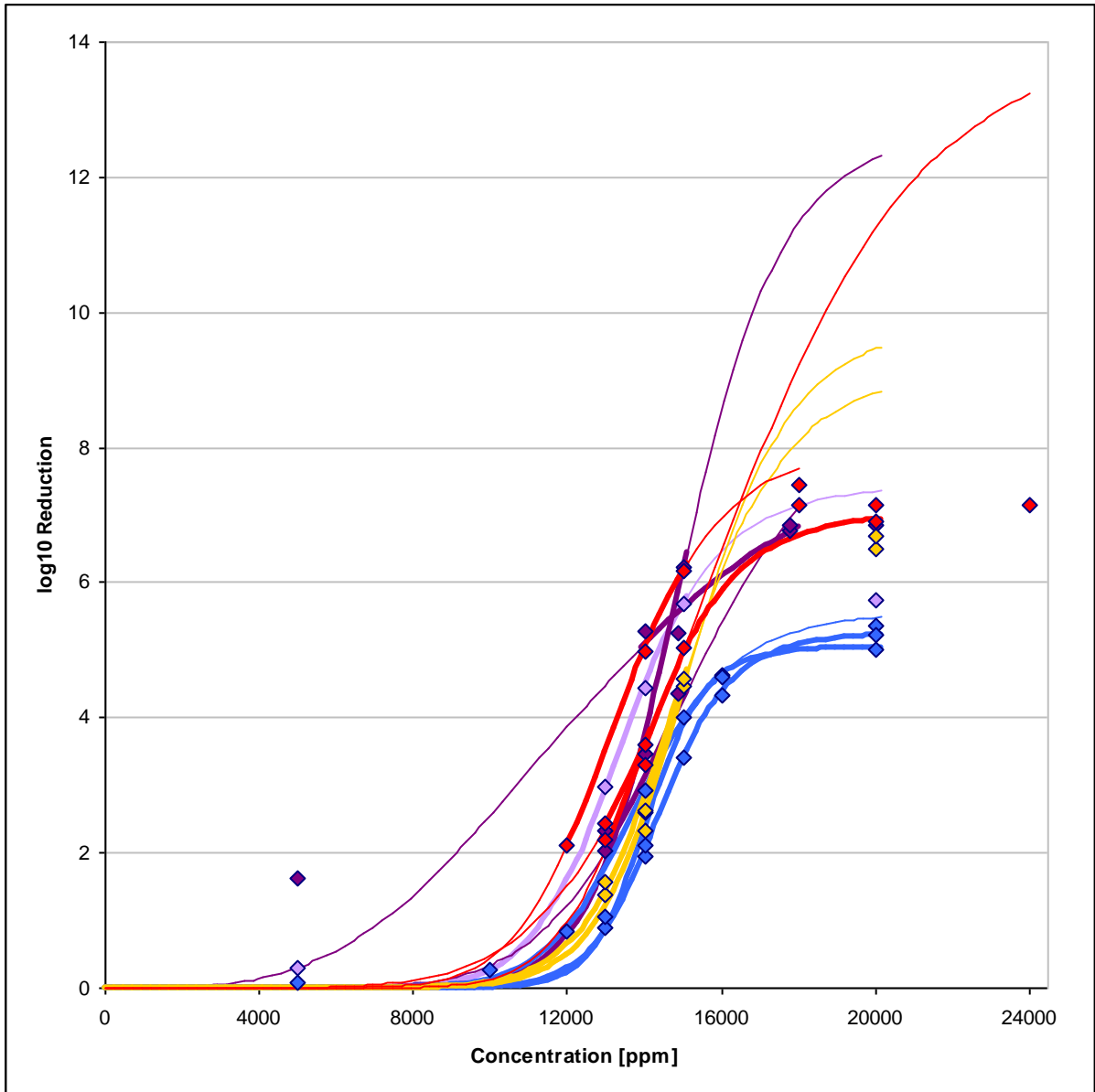


Figure 180: Single log<sub>10</sub> reductions and run-specific standard inactivation curves for *Mycobacterium terrae* and glutaraldehyde

LC0001	LC0007	LC0008	LC0011	LC0012	LC0021

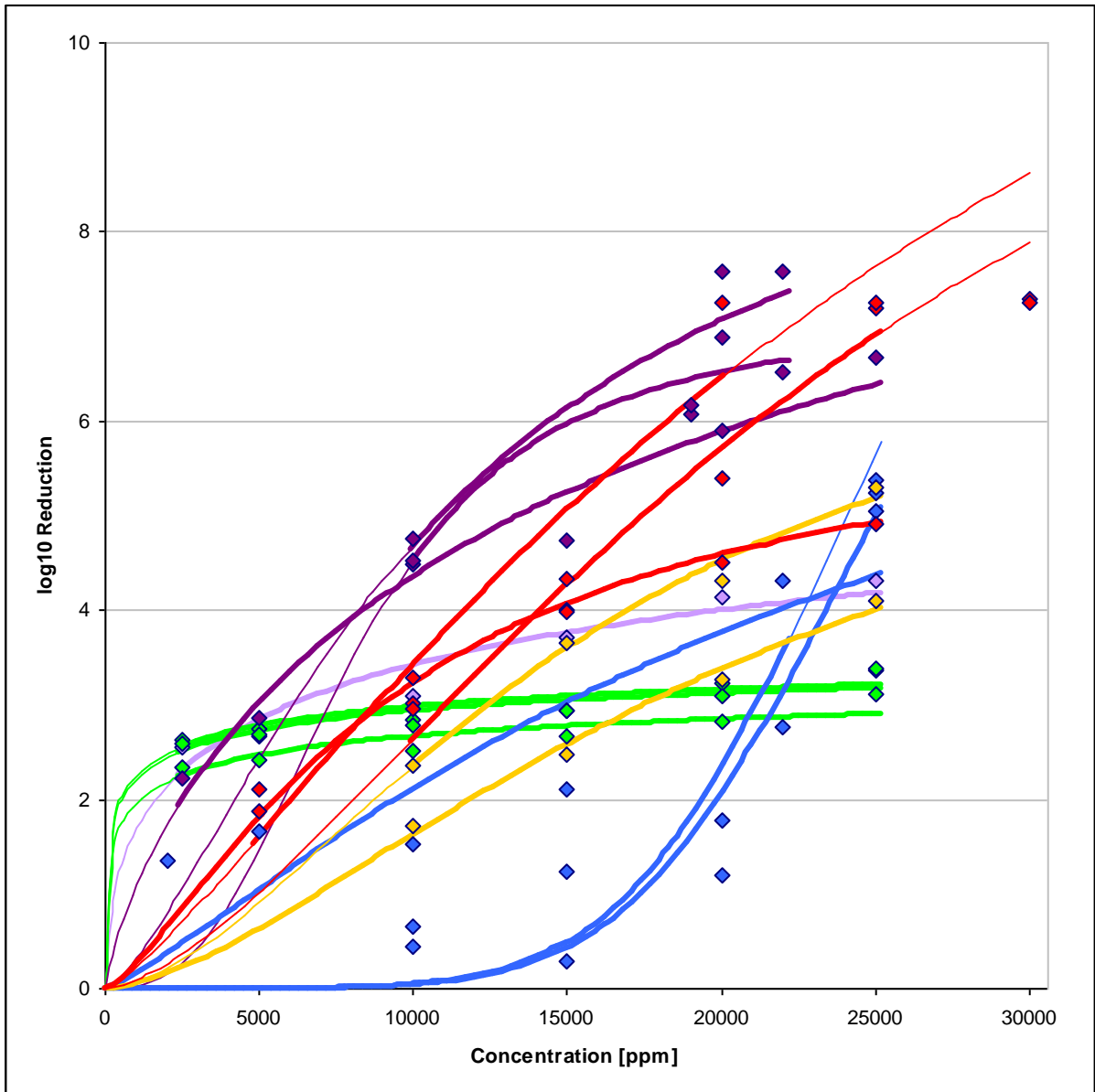


Figure 181: Single log<sub>10</sub> reductions and run-specific standard inactivation curves for *Mycobacterium terrae* and peracetic acid

LC0001	LC0008	LC0011	LC0012	LC0021	LC0025

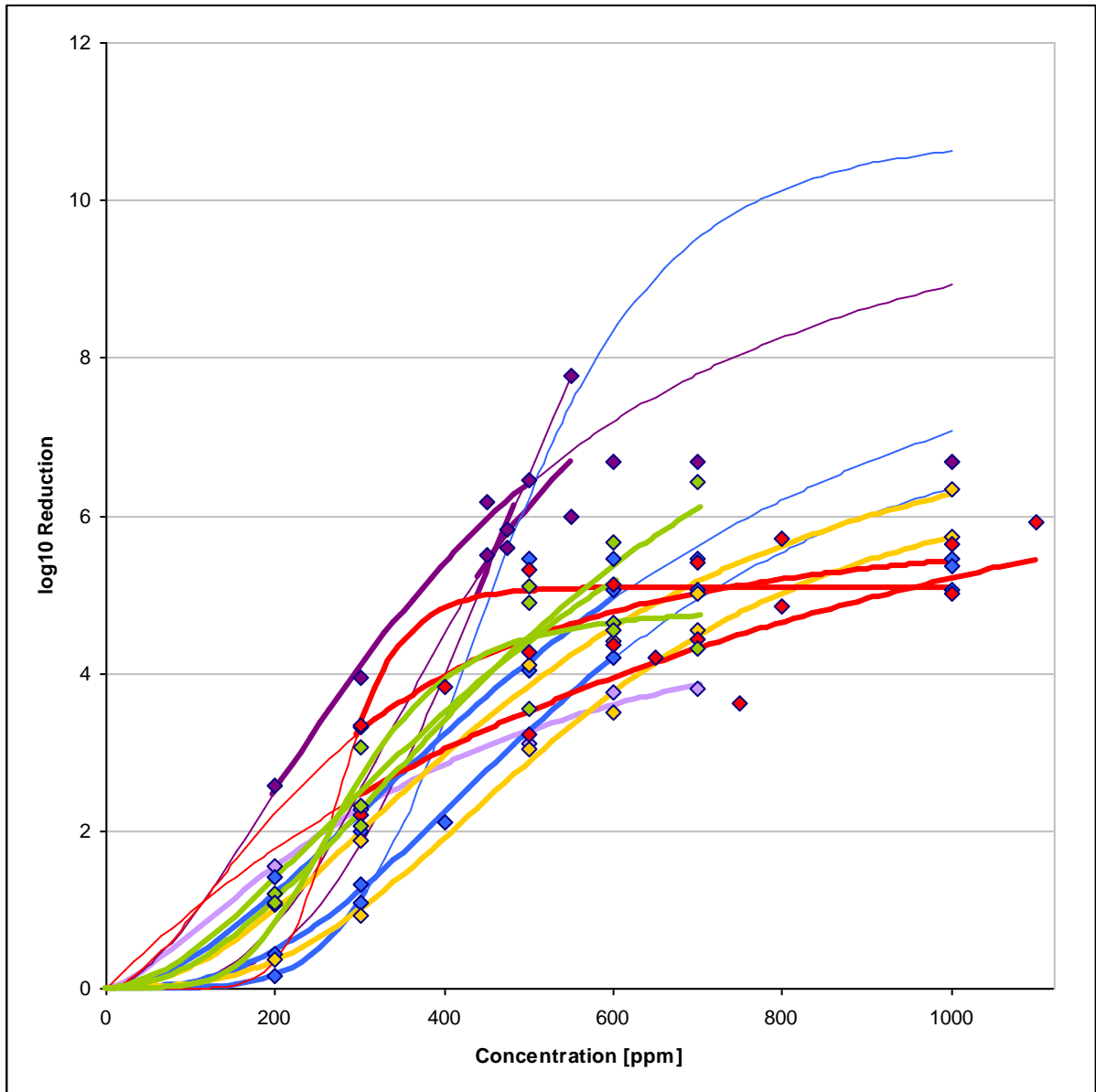


Figure 182: Single log<sub>10</sub> reductions and run-specific standard inactivation curves for *Pseudomonas aeruginosa* and glutaraldehyde

LC0004	LC0011	LC0012	LC0013	LC0021	LC0028

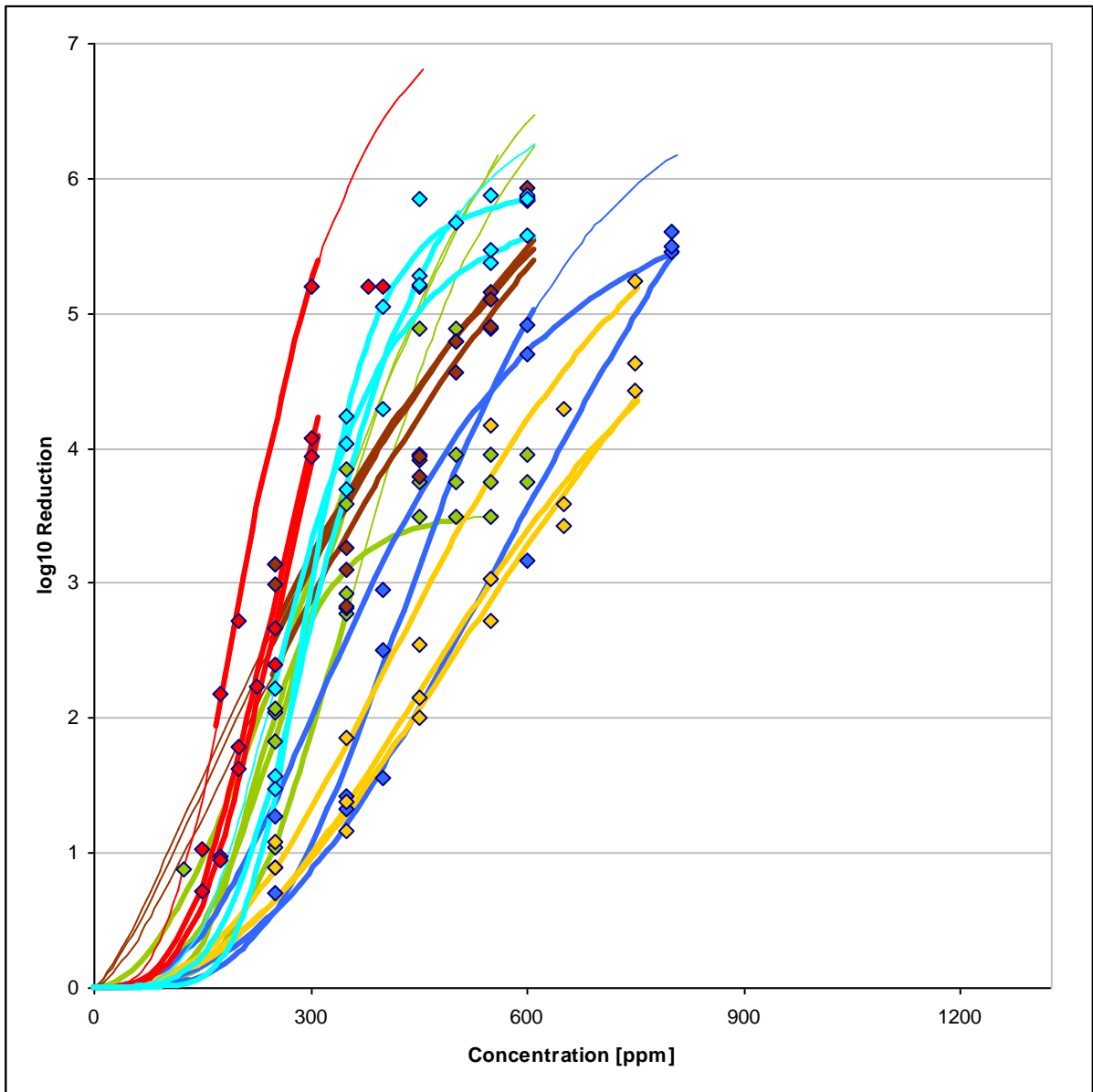
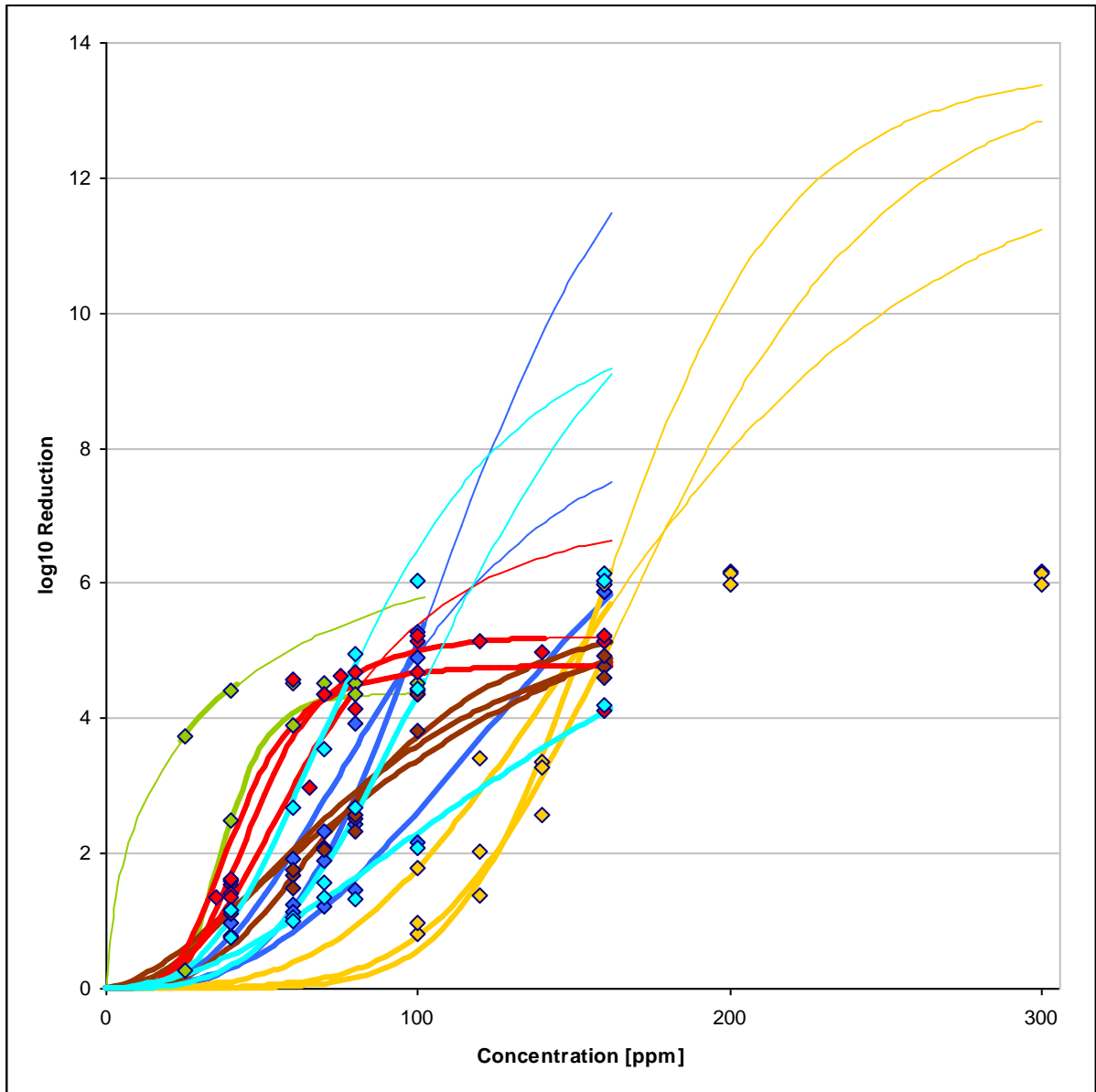


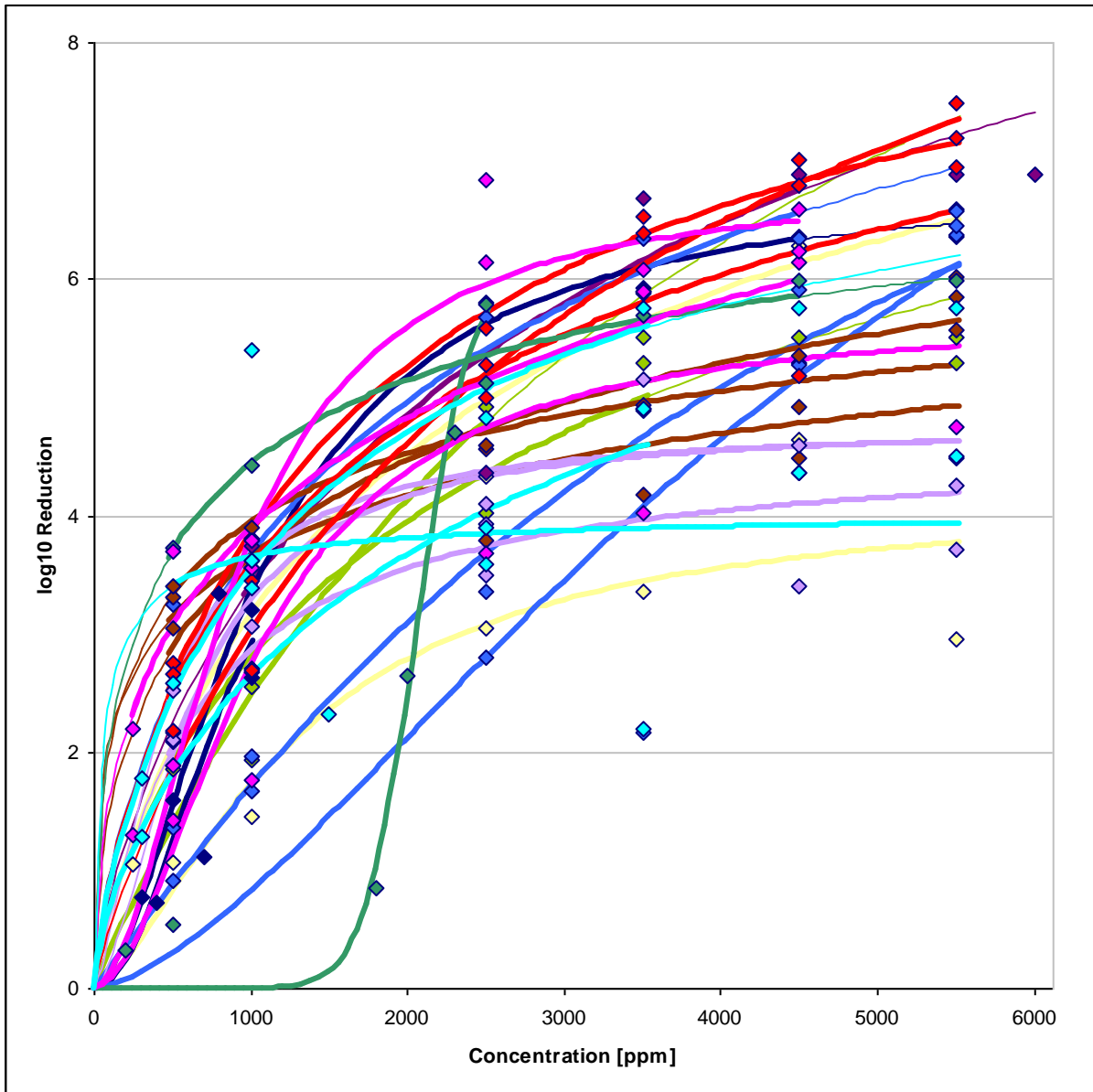
Figure 183: Single log<sub>10</sub> reductions and run-specific standard inactivation curves for *Pseudomonas aeruginosa* and peracetic acid

LC0004	LC0011	LC0012	LC0013	LC0021	LC0028



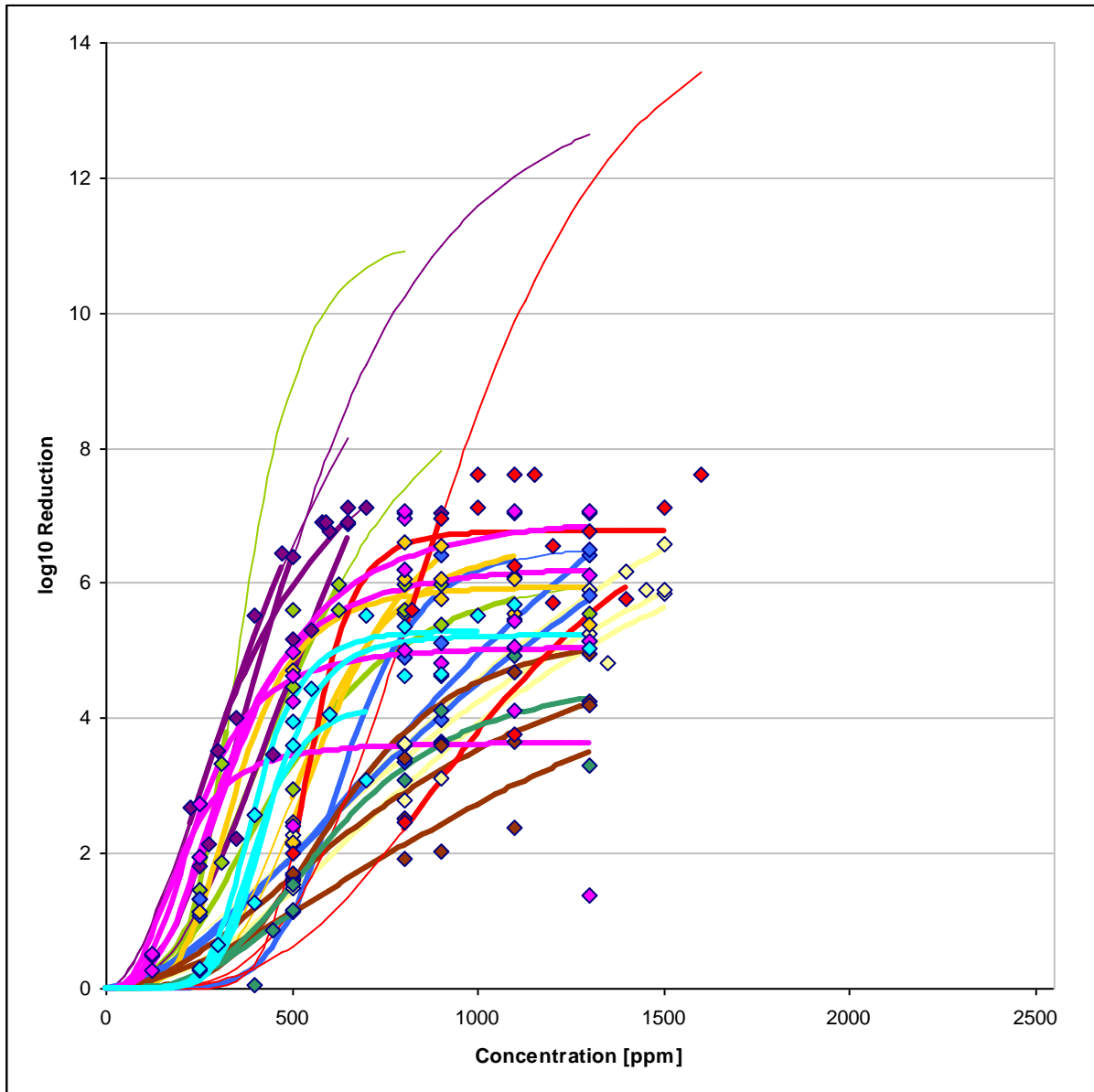
**Figure 184: Single log10 reductions and run-specific standard inactivation curves for *Staphylococcus aureus* and benzalkonium chloride**

LC0000	LC0004	LC0008	LC0010	LC0011	LC0013	LC0019	LC0020	LC0021	LC0022	LC0026	LC0028



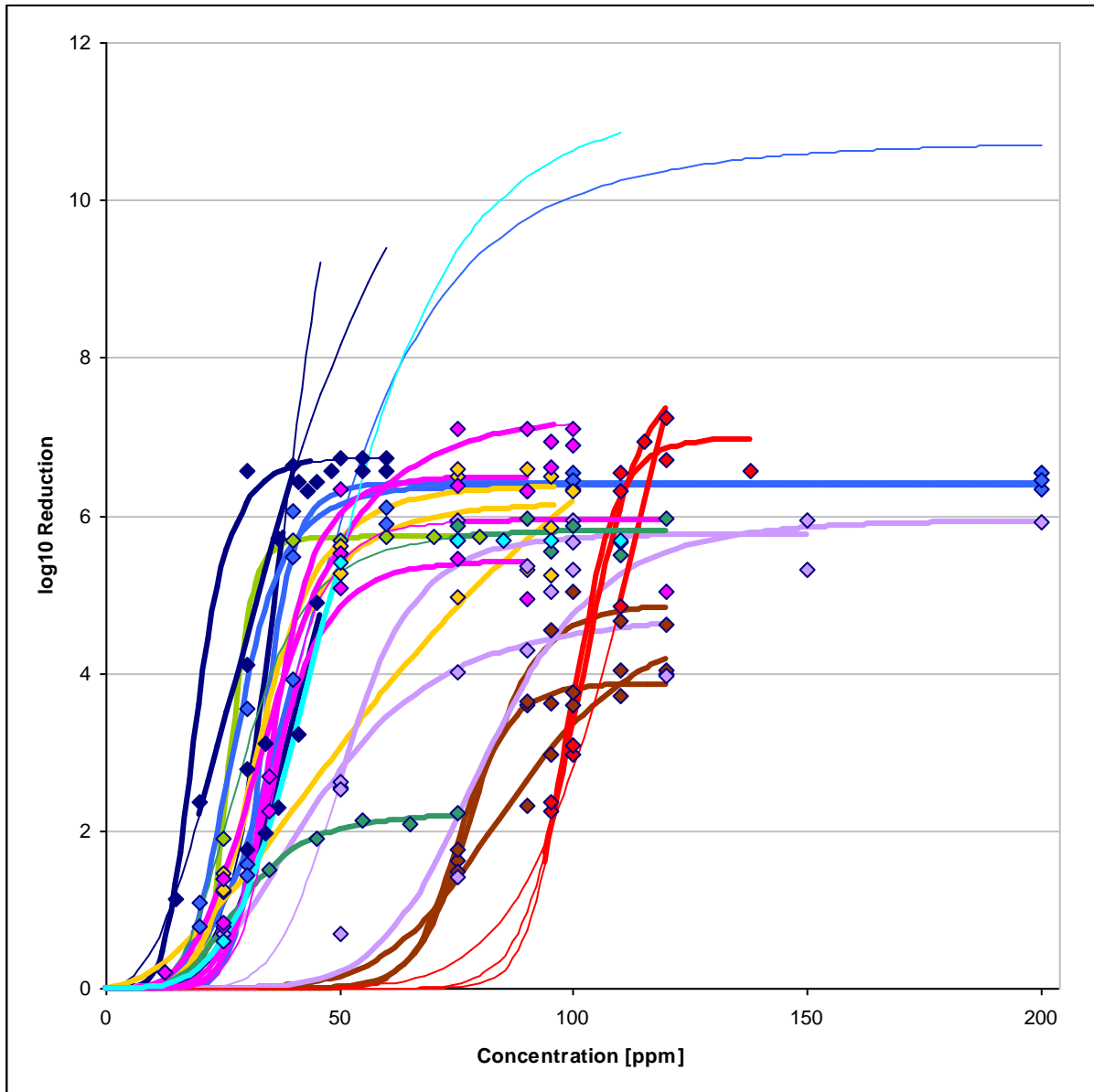
**Figure 185: Single log<sub>10</sub> reductions and run-specific standard inactivation curves for *Staphylococcus aureus* and glutaraldehyde**

LC0000	LC0004	LC0008	LC0010	LC0011	LC0012	LC0013	LC0019	LC0021	LC0022	LC0026	LC0028



**Figure 186: Single log<sub>10</sub> reductions and run-specific standard inactivation curves for *Staphylococcus aureus* and peracetic acid**

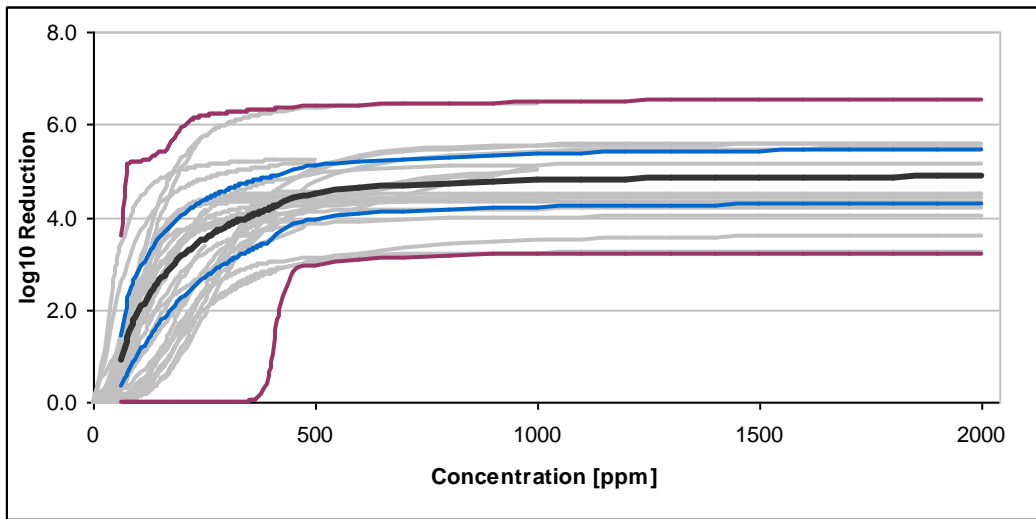
LC0000	LC0004	LC0010	LC0011	LC0012	LC0013	LC0019	LC0020	LC0021	LC0022	LC0026	LC0028



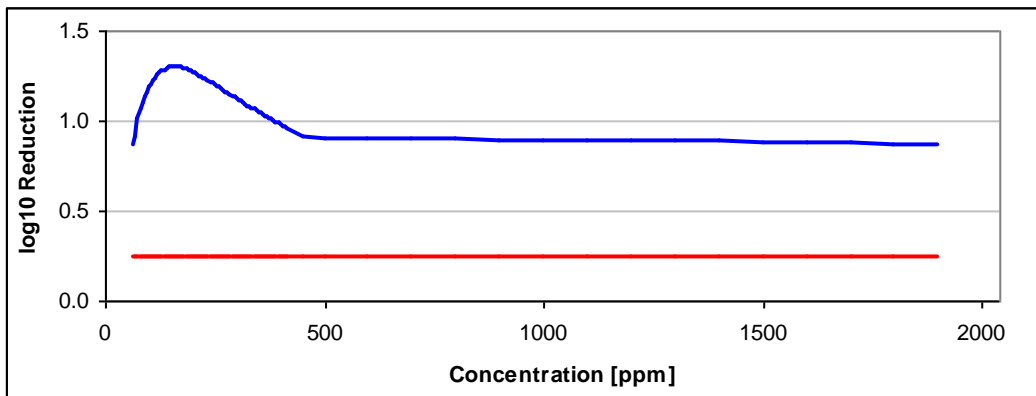
### **Overall (laboratory-comprehensive) inactivation curves and intra- and inter-laboratory reproducibility**

235. In the following figures, for each combination of organism and substance 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) and 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.
236. Each overall inactivation curve is followed by a figure showing the inter-laboratory reproducibility  $s_R$  (blue) and the intra-laboratory reproducibility within one run  $s_r$  (red) in dependence of the concentration. Of course, the intra-laboratory reproducibility within one run  $s_r$  (repeatability) is the smaller than the inter-laboratory reproducibility  $s_R$ .
237. Below each figure of the standard deviations a table is given in which 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  $s_t$  and intra-laboratory reproducibility within one run  $s_r$  are summarized for the six concentration levels as originally suggested to the laboratories. No values can be given if the respective concentration lies not within the concentration range considered for the bootstrap.
238. Since for *Adenovirus type 5* (well count) data of only three laboratories is available, neither the overall inactivation curve nor the intra- and inter-laboratory reproducibility are determined.

**Figure 187: Standard overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Adenovirus type 5* (TCID<sub>50</sub>) and glutaraldehyde**



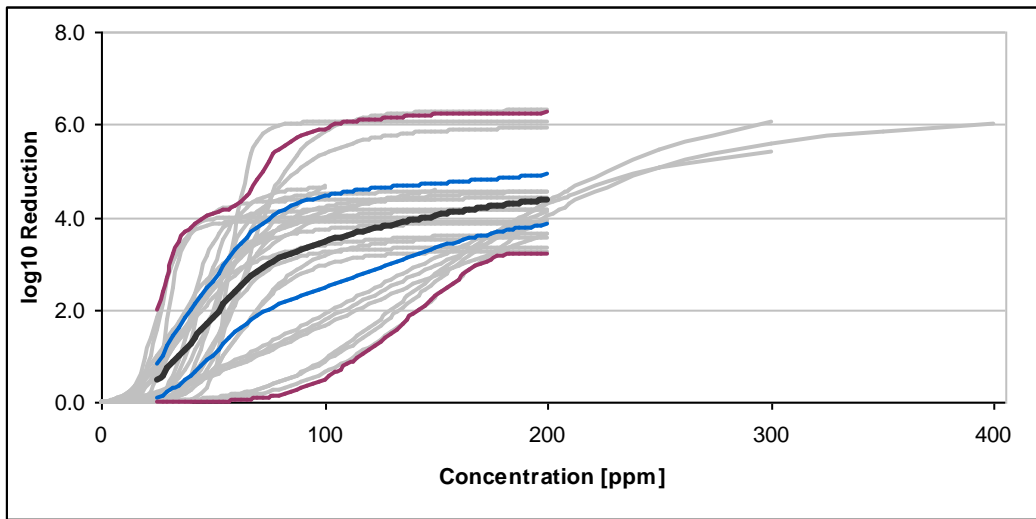
**Figure 188: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Adenovirus type 5* (TCID<sub>50</sub>) and glutaraldehyde**



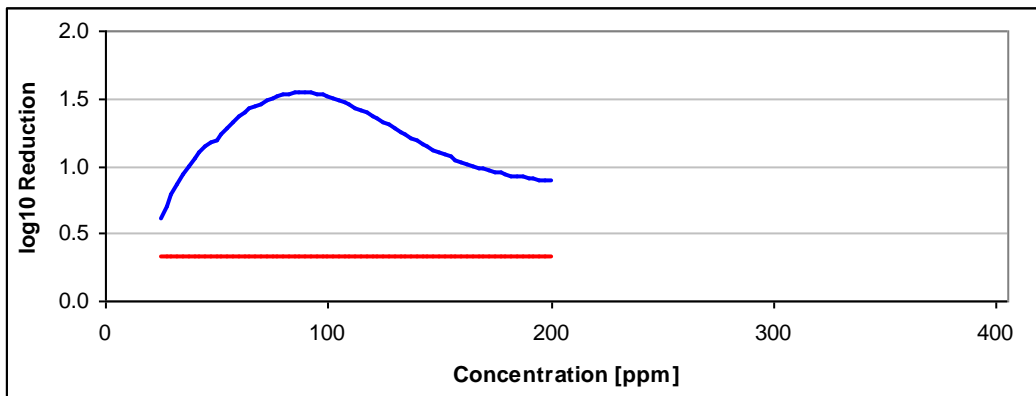
**Table 49: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Adenovirus type 5* (TCID<sub>50</sub>) and glutaraldehyde**

Concentration [ppm]	Overall inactivation curve (log <sub>10</sub> reduction)	Absolute inter-laboratory reproducibility $s_R$	Absolute intra-laboratory reproducibility within one run $s_r$	Absolute intra-laboratory reproducibility over time $s_l$
62.5	0.89	0.87	0.24	0.00
125	2.29	1.26	0.24	0.24
250	3.51	1.20	0.24	0.29
500	4.52	0.90	0.24	0.37
1000	4.78	0.89	0.24	0.35
2000	4.88	0.86	0.24	0.24

**Figure 189: Standard overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Adenovirus type 5* (TCID<sub>50</sub>) and peracetic acid**



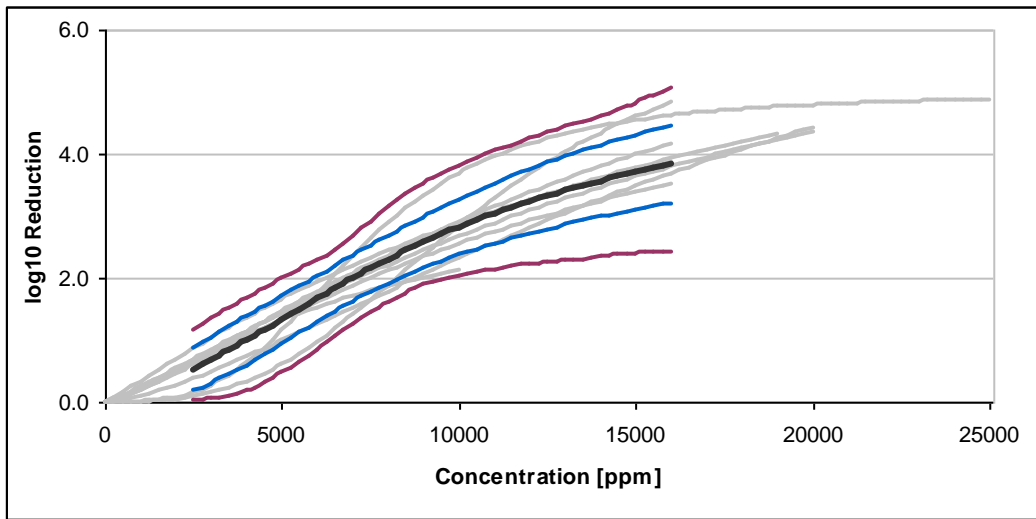
**Figure 190: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Adenovirus type 5* (TCID<sub>50</sub>) and peracetic acid**



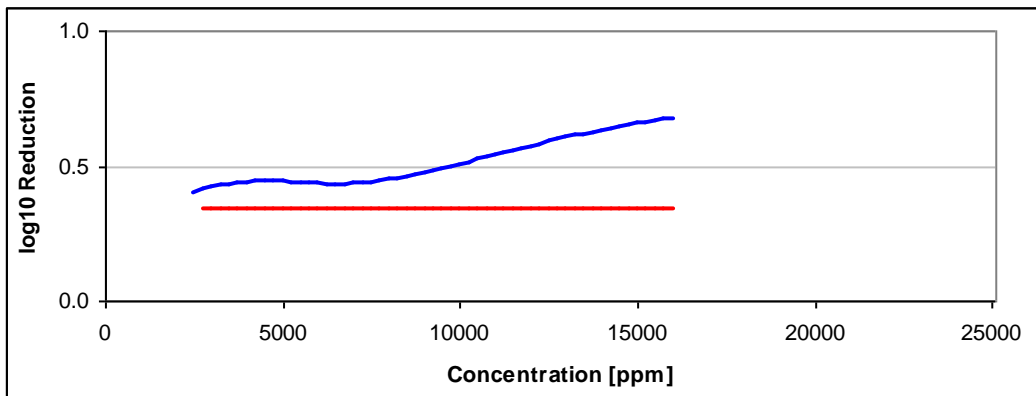
**Table 50: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Adenovirus type 5* (TCID<sub>50</sub>) and peracetic acid**

Concentration [ppm]	Overall inactivation curve (log10 reduction)	Absolute inter-laboratory reproducibility $s_R$	Absolute intra-laboratory reproducibility within one run $s_r$	Absolute intra-laboratory reproducibility over time $s_t$
25	0.46	0.60	0.32	0.36
50	1.81	1.19	0.32	0.37
75	2.97	1.49	0.32	0.37
100	3.46	1.51	0.32	0.40
150	4.02	1.10	0.32	0.46
200	4.37	0.89	0.32	0.48

**Figure 191: Standard 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**



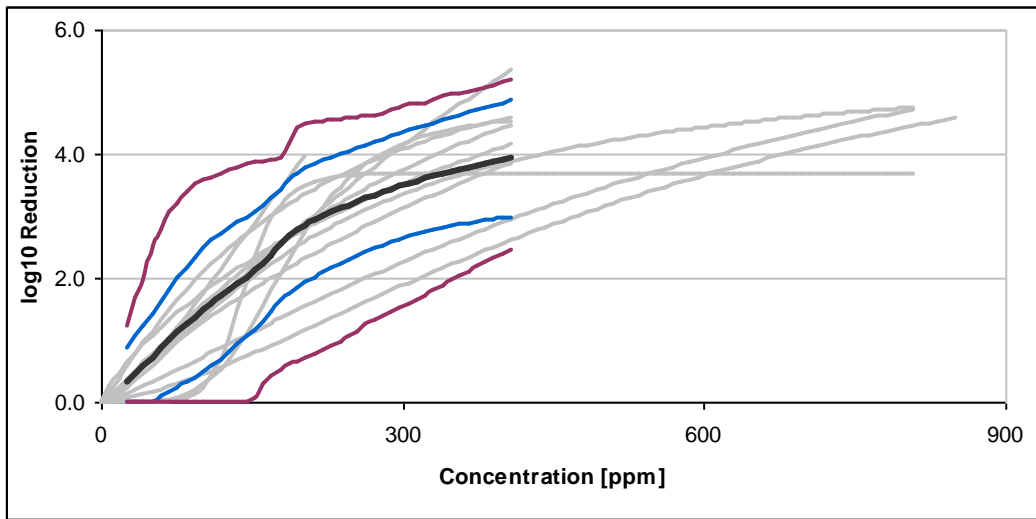
**Figure 192: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Aspergillus niger* and glutaraldehyde**



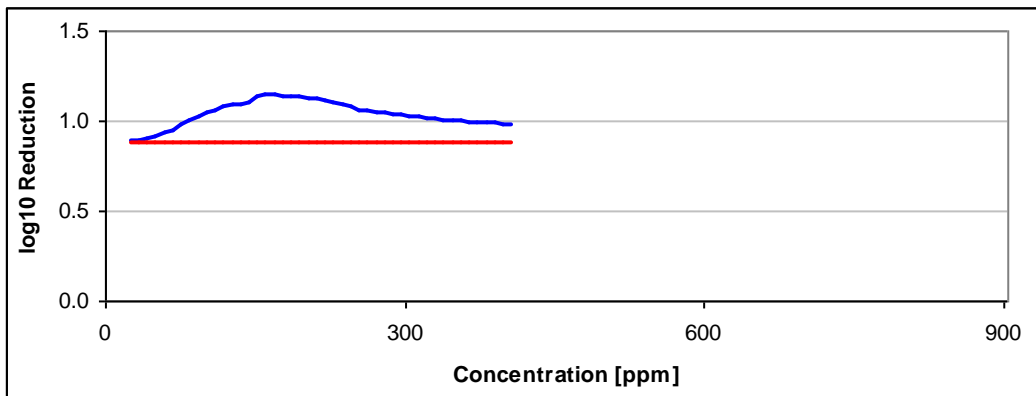
**Table 51: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Aspergillus niger* and glutaraldehyde**

Concentration [ppm]	Overall inactivation curve (log10 reduction)	Absolute inter-laboratory reproducibility $s_R$	Absolute intra-laboratory reproducibility within one run $s_r$	Absolute intra-laboratory reproducibility over time $s_l$
312				
625				
1250				
2500	0.53	0.40	0.34	0.37
5000	1.32	0.44	0.34	0.40
10000	2.82	0.51	0.34	0.43

**Figure 193: Standard 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 peracetic acid**



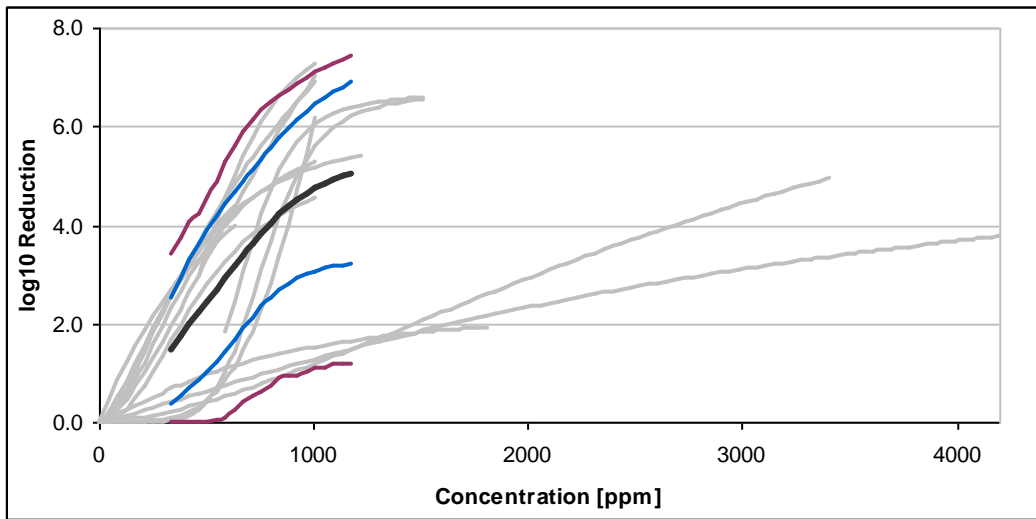
**Figure 194: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Aspergillus niger* and peracetic acid**



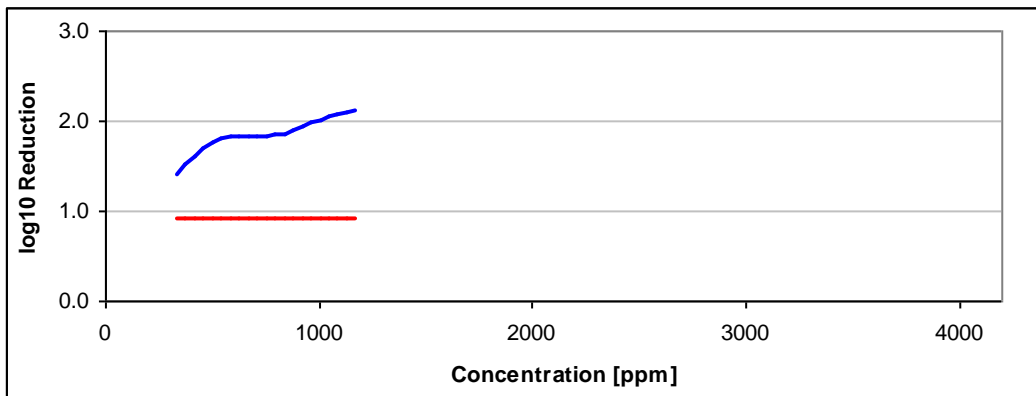
**Table 52: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Aspergillus niger* and peracetic acid**

Concentration [ppm]	Overall inactivation curve (log10 reduction)	Absolute inter-laboratory reproducibility $s_R$	Absolute intra-laboratory reproducibility within one run $s_r$	Absolute intra-laboratory reproducibility over time $s_t$
25	0.31	0.89	0.88	0.88
50	0.72	0.91	0.88	0.88
100	1.48	1.04	0.88	0.88
200	2.85	1.13	0.88	0.93
400	3.89	0.98	0.88	0.88
800				

**Figure 195: Standard overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Bacillus subtilis* and peracetic acid**



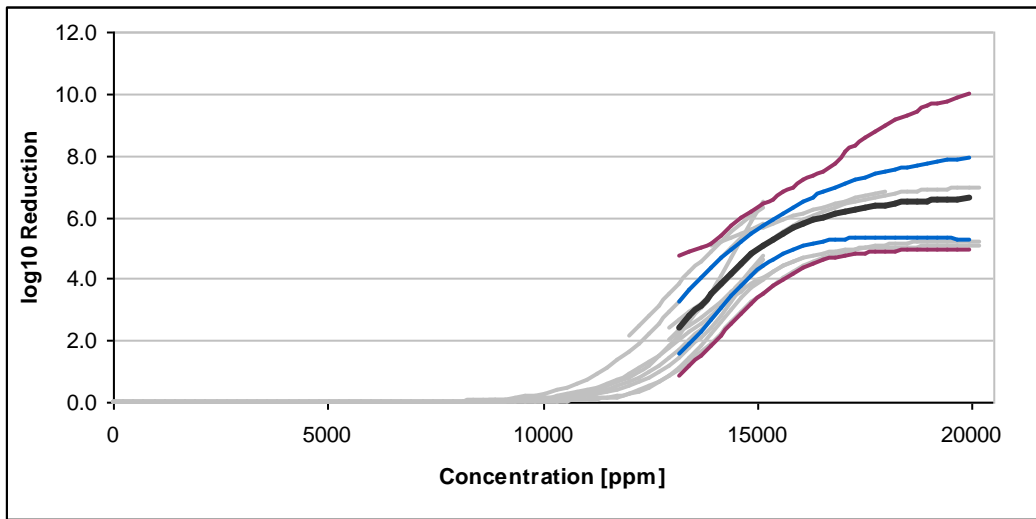
**Figure 196: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Bacillus subtilis* and peracetic acid**



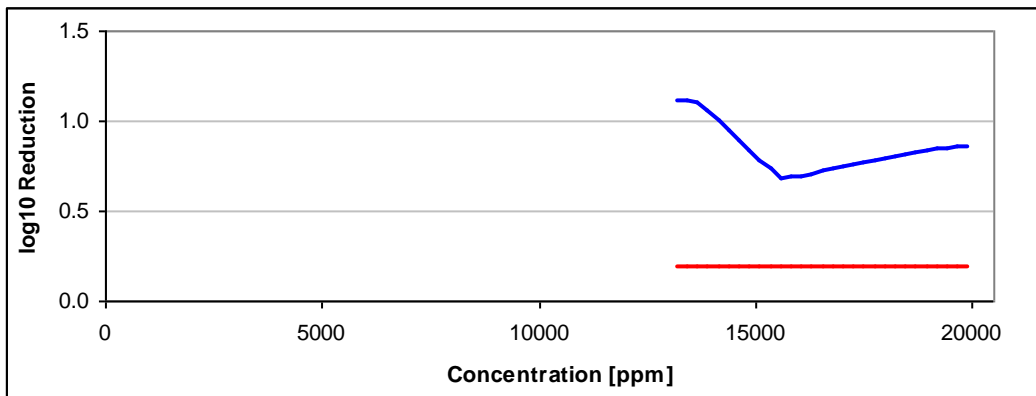
**Table 53: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Bacillus subtilis* and peracetic acid**

Concentration [ppm]	Overall inactivation curve (log10 reduction)	Absolute inter-laboratory reproducibility $s_R$	Absolute intra-laboratory reproducibility within one run $s_r$	Absolute intra-laboratory reproducibility over time $s_t$
200				
400	1.73	1.51	0.92	0.94
600	2.92	1.82	0.92	0.96
800	4.03	1.84	0.92	0.95
1000	4.75	2.01	0.92	0.92
1200	5.05	2.11	0.92	0.92

**Figure 197: Standard overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Mycobacterium terrae* and phenol**



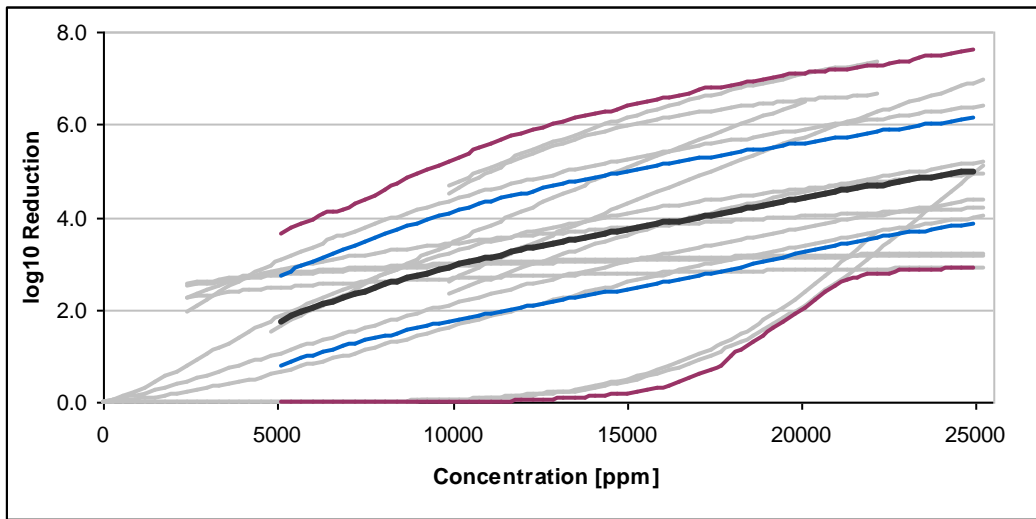
**Figure 198: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Mycobacterium terrae* and phenol**



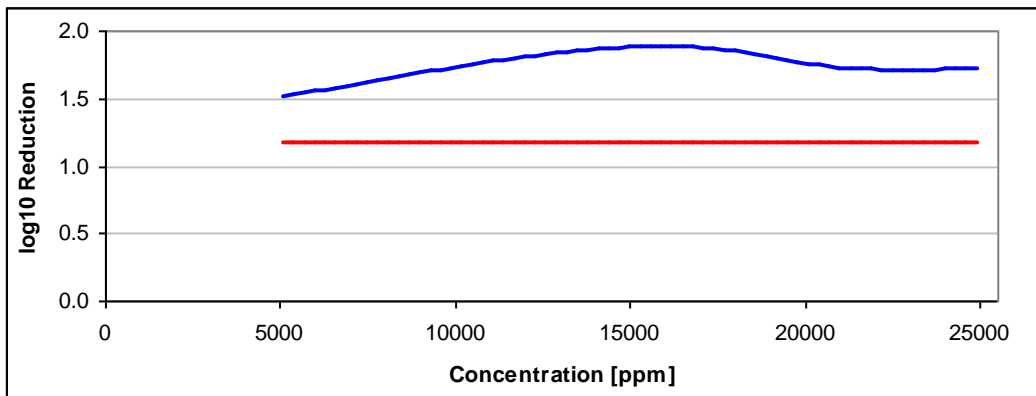
**Table 54: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Mycobacterium terrae* and phenol**

Concentration [ppm]	Overall inactivation curve (log10 reduction)	Absolute inter-laboratory reproducibility $s_R$	Absolute intra-laboratory reproducibility within one run $s_r$	Absolute intra-laboratory reproducibility over time $s_t$
5000				
10000				
13000	2.42	1.11	0.19	0.72
14000	3.49	1.06	0.19	0.59
15000	5.04	0.78	0.19	0.39
20000	6.59	0.86	0.19	0.19

**Figure 199: Standard overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Mycobacterium terrae* and glutaraldehyde**



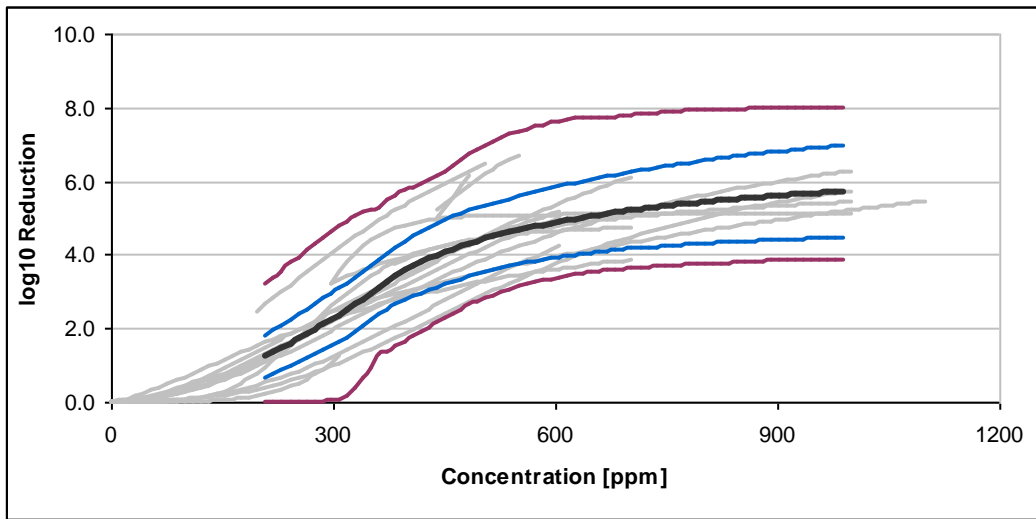
**Figure 200: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Mycobacterium terrae* and glutaraldehyde**



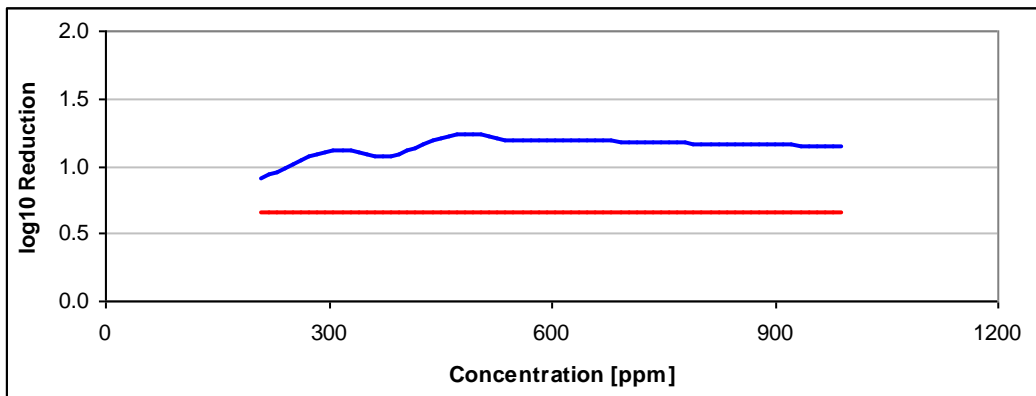
**Table 55: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Mycobacterium terrae* and glutaraldehyde**

Concentration [ppm]	Overall inactivation curve (log10 reduction)	Absolute inter-laboratory reproducibility $s_R$	Absolute intra-laboratory reproducibility within one run $s_r$	Absolute intra-laboratory reproducibility over time $s_t$
2500				
5000	1.74	1.51	1.18	1.21
10000	2.91	1.72	1.18	1.24
15000	3.71	1.88	1.18	1.31
20000	4.42	1.75	1.18	1.27
25000	4.99	1.72	1.18	1.27

**Figure 201: Standard overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Mycobacterium terrae* and peracetic acid**



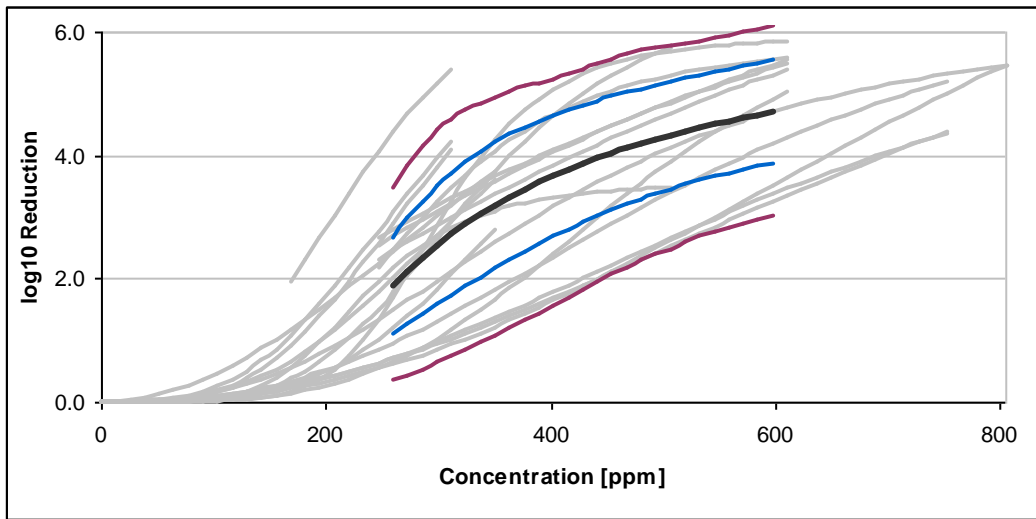
**Figure 202: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Mycobacterium terrae* and peracetic acid**



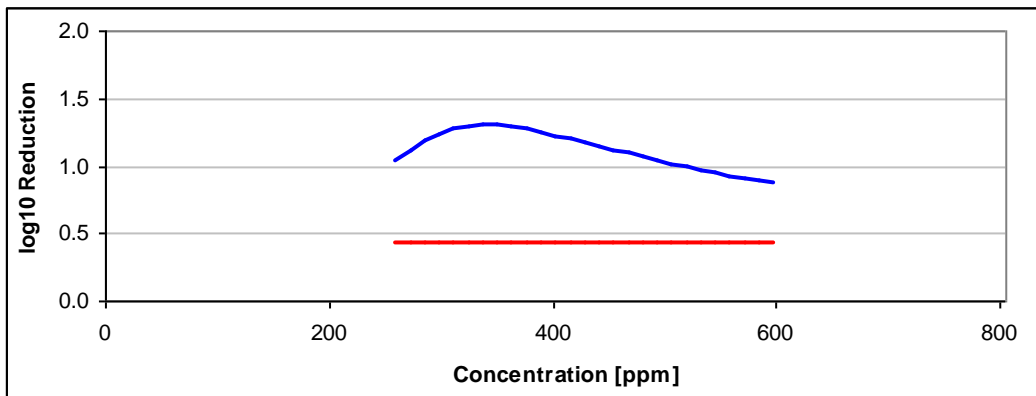
**Table 56: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Mycobacterium terrae* and peracetic acid**

Concentration [ppm]	Overall inactivation curve (log10 reduction)	Absolute inter-laboratory reproducibility $s_R$	Absolute intra-laboratory reproducibility within one run $s_r$	Absolute intra-laboratory reproducibility over time $s_l$
200	1.23	0.90	0.65	0.83
300	2.23	1.10	0.65	0.94
500	4.37	1.23	0.65	0.75
600	4.89	1.19	0.65	0.65
700	5.20	1.18	0.65	0.65
1000	5.71	1.14	0.65	0.65

**Figure 203: Standard overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Pseudomonas aeruginosa* and glutaraldehyde**



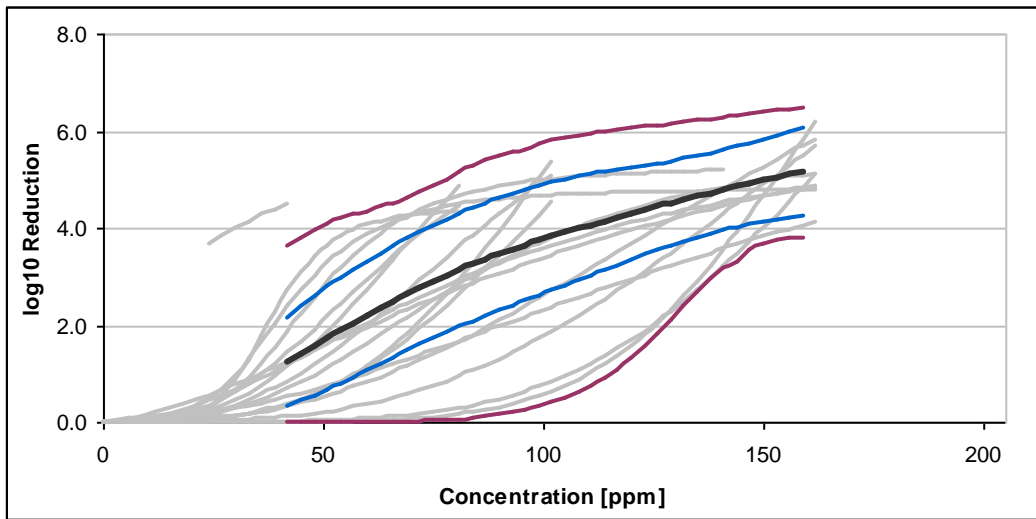
**Figure 204: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Pseudomonas aeruginosa* and glutaraldehyde**



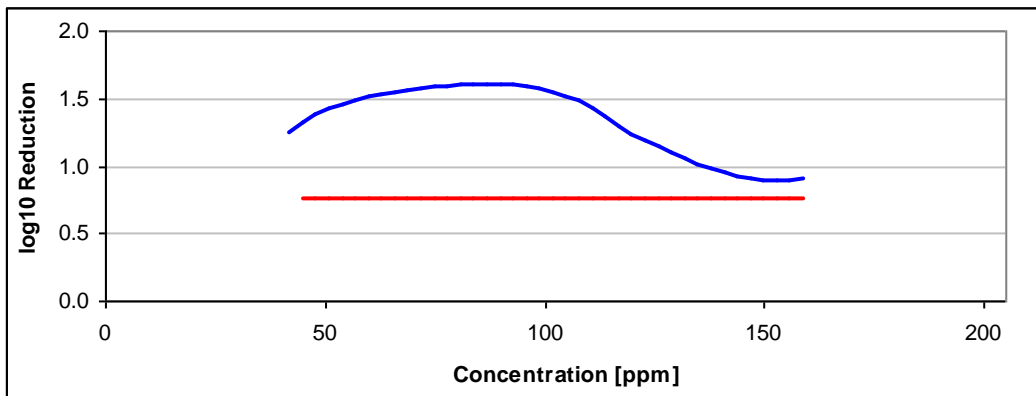
**Table 57: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Pseudomonas aeruginosa* and glutaraldehyde**

Concentration [ppm]	Overall inactivation curve (log10 reduction)	Absolute inter-laboratory reproducibility $s_R$	Absolute intra-laboratory reproducibility within one run $s_r$	Absolute intra-laboratory reproducibility over time $s_l$
250	1.89	1.03	0.42	0.51
350	3.19	1.31	0.42	0.51
450	4.03	1.12	0.42	0.42
500	4.25	1.04	0.42	0.42
550	4.50	0.95	0.42	0.42
600	4.70	0.87	0.42	0.42

**Figure 205: Standard overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Pseudomonas aeruginosa* and peracetic acid**



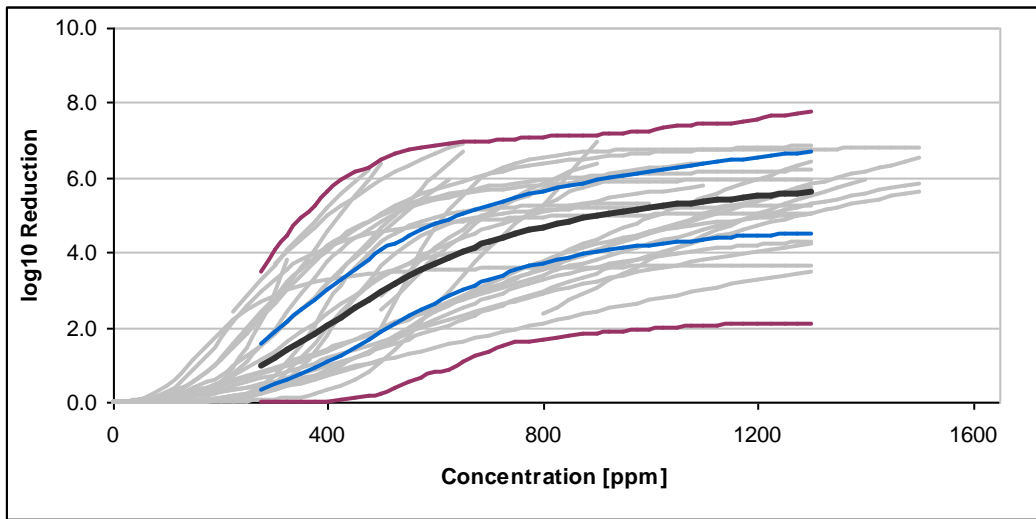
**Figure 206: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Pseudomonas aeruginosa* and peracetic acid**



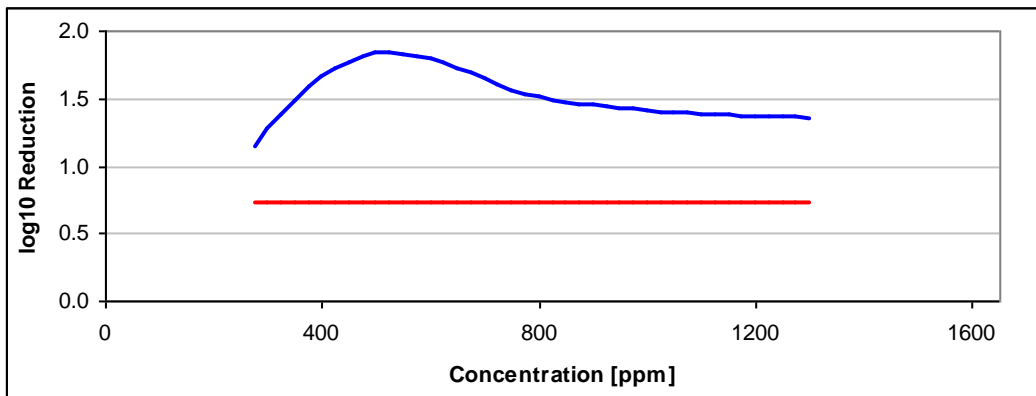
**Table 58: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Pseudomonas aeruginosa* and peracetic acid**

Concentration [ppm]	Overall inactivation curve (log10 reduction)	Absolute inter-laboratory reproducibility $s_R$	Absolute intra-laboratory reproducibility within one run $s_r$	Absolute intra-laboratory reproducibility over time $s_l$
40	1.24	1.25	0.75	0.79
60	2.19	1.51	0.75	0.84
70	2.62	1.56	0.75	0.90
80	3.13	1.60	0.75	0.95
100	3.74	1.57	0.75	0.95
160	5.15	0.90	0.75	0.75

**Figure 207: Standard overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Staphylococcus aureus* and glutaraldehyde**



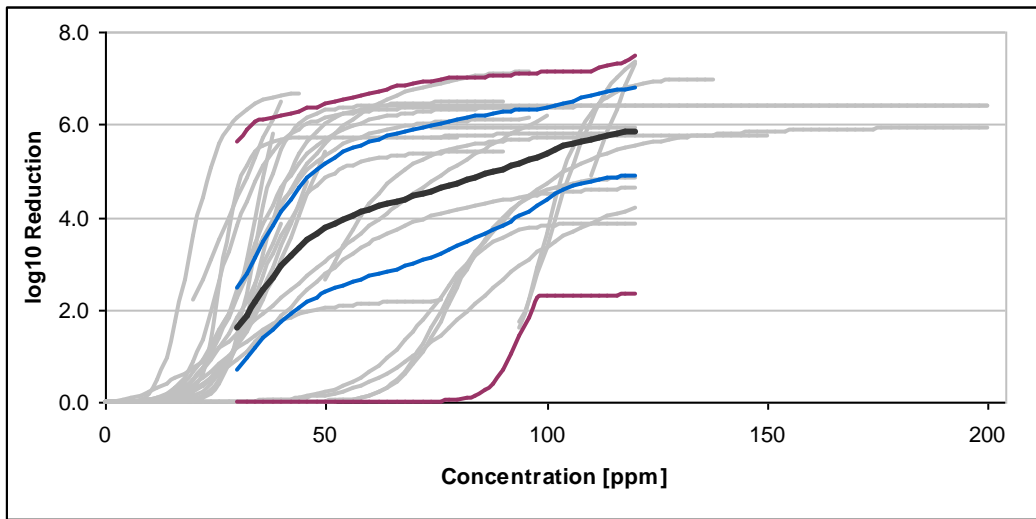
**Figure 208: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Staphylococcus aureus* and glutaraldehyde**



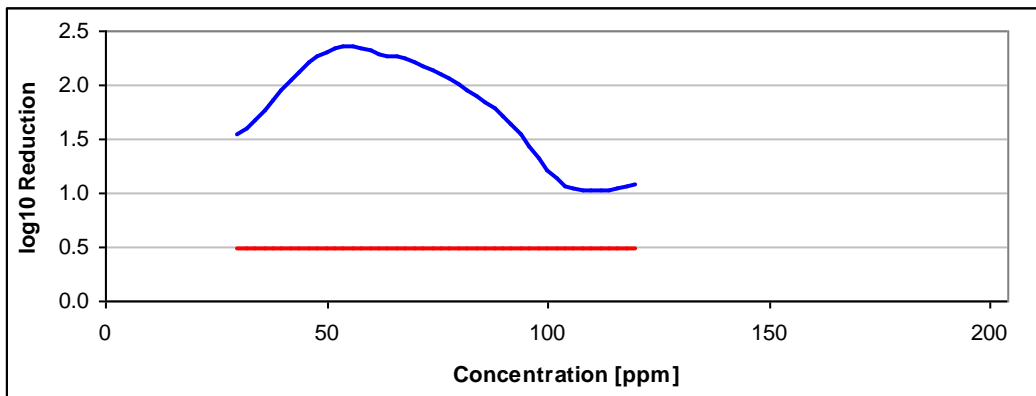
**Table 59: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Staphylococcus aureus* and glutaraldehyde**

Concentration [ppm]	Overall inactivation curve (log10 reduction)	Absolute inter-laboratory reproducibility $s_R$	Absolute intra-laboratory reproducibility within one run $s_r$	Absolute intra-laboratory reproducibility over time $s_t$
250	0.95	1.14	0.73	0.83
500	2.96	1.83	0.73	0.93
800	4.66	1.50	0.73	0.73
900	4.96	1.45	0.73	0.73
1100	5.35	1.38	0.73	0.73
1300	5.60	1.36	0.73	0.73

**Figure 209: Standard overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Staphylococcus aureus* and peracetic acid**



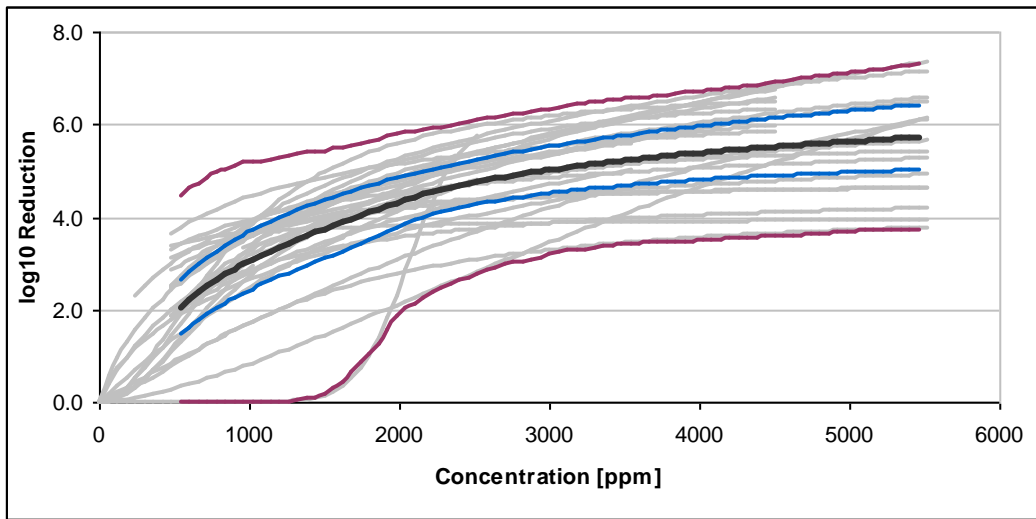
**Figure 210: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Staphylococcus aureus* and peracetic acid**



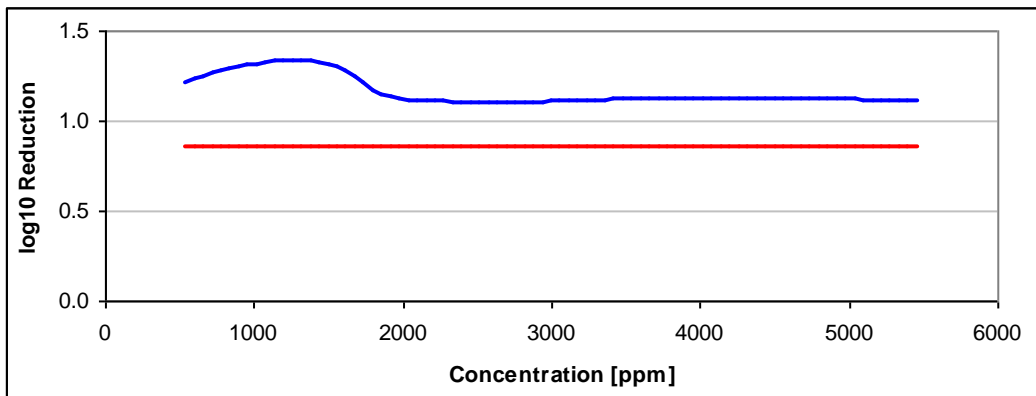
**Table 60: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Staphylococcus aureus* and peracetic acid**

Concentration [ppm]	Overall inactivation curve (log10 reduction)	Absolute inter-laboratory reproducibility $s_R$	Absolute intra-laboratory reproducibility within one run $s_r$	Absolute intra-laboratory reproducibility over time $s_t$
75	4.56	2.14	0.48	0.48
90	5.02	1.71	0.48	0.48
95	5.22	1.42	0.48	0.48
100	5.37	1.21	0.48	0.48
110	5.68	1.01	0.48	0.48
120	5.85	1.07	0.48	0.48

**Figure 211: Standard overall inactivation curve (black), 95%-confidence interval for overall inactivation curve (blue) and 95%-prediction interval for run-specific curves (purple) for *Staphylococcus aureus* and benzalkonium chloride**



**Figure 212: Inter-laboratory reproducibility  $s_R$  (blue) and intra-laboratory reproducibility within one run  $s_r$  (red) depending on concentration for *Staphylococcus aureus* and benzalkonium chloride**



**Table 61: Overall inactivation curve and absolute standard deviations for specified concentration levels for *Staphylococcus aureus* and benzalkonium chloride**

Concentration [ppm]	Overall inactivation curve (log10 reduction)	Absolute inter-laboratory reproducibility $s_R$	Absolute intra-laboratory reproducibility within one run $s_r$	Absolute intra-laboratory reproducibility over time $s_l$
500	2.05	1.22	0.85	1.04
1000	3.07	1.32	0.85	1.12
2500	4.76	1.10	0.85	0.97
3500	5.20	1.12	0.85	0.94
4500	5.51	1.12	0.85	0.91
5500	5.71	1.11	0.85	0.87

**Validation assessment**

239. For assessing whether test methods can be considered as validated, the ratio of the maximum absolute interlaboratory reproducibility and the maximum of the overall curve (both within the concentration range of the bootstrap) may be consulted.

240. A possible rule for the assessment could be: If the average of the ratio over all substances is not exceeding 30%, the method may be considered as validated for the organism tested.

241. The following table summarizes for each combination of organism and substance the number of laboratories and the maximum absolute interlaboratory reproducibility standard deviation within the concentration range of the bootstrap. Combinations are highlighted in yellow if data of at least five laboratories were included in the analysis.

**Table 62: Overview of validation parameters (yellow: combinations for which data from at least 5 laboratories was included in the analysis; red: combinations for which the ratio exceeds 30%)**

Organism	Substance	Number of laboratories included in the analysis	Maximum absolute inter-laboratory reproducibility $s_R$	Maximum of overall inactivation curve	Ratio
<i>Adenovirus type 5</i> (TCID <sub>50</sub> )	peracetic acid	9	1.54	4.37	35%
	glutaraldehyde	9	1.30	4.88	27%
<i>Aspergillus niger</i>	peracetic acid	4	1.15	3.92	29%
	glutaraldehyde	3	0.68	3.83	18%
<i>Bacillus subtilis</i>	peracetic acid	5	2.11	5.05	42%
<i>Mycobacterium terrae</i>	phenol	5	1.11	6.59	17%
	peracetic acid	6	1.23	5.71	22%
	glutaraldehyde	6	1.88	4.99	38%
<i>Pseudomonas aeruginosa</i>	peracetic acid	6	1.60	5.15	31%
	glutaraldehyde	6	1.31	4.70	28%
<i>Staphylococcus aureus</i>	peracetic acid	11	2.34	5.85	40%
	glutaraldehyde	10	1.84	5.60	33%
	benzalkonium chloride	11	1.34	5.71	23%

242. Of special interest is the performance of the methods for concentrations with high log<sub>10</sub> reduction factors to be expected, e.g. 4 for the standard approach. The precision figures for this level are presented in the following Table 63. If the average of the absolute reproducibility standard deviation over all substances is not exceeding 1 (corresponds to “25% of 4”), the method may be considered as validated for the organism tested. Apparently, this criterion leads to a similar assessment as the criterion of the preceding paragraph. This does not necessarily mean that the test methods are not applicable. However, variability is

relatively large, so that assessments based on only one or two laboratories will be not very reliable.

**Table 63: Overview of validation parameters at a concentration level at which a log reduction of 4 is expected**

Organism	Substance	Mean concentration for log <sub>10</sub> red. = 4 [ppm]	Reproducibility s.d. of log reductions	Repeatability s.d. of log reductions	Intermediate s.d. of log reductions
<i>Adenovirus type 5</i> (TCID <sub>50</sub> )	peracetic acid	150	1.10	0.32	0.46
	glutaraldehyde	350	1.05	0.24	0.25
<i>Aspergillus niger</i>	peracetic acid	300 (at log <sub>10</sub> red = 3.5)	1.02	0.88	0.88
	<b>glutaraldehyde</b>	<b>13500</b> (at log <sub>10</sub> red = 3.5)	<b>0.62</b>	<b>0.34</b>	<b>0.48</b>
<i>Bacillus subtilis</i>	peracetic acid	780	1.83	0.92	0.95
<i>Mycobacterium terrae</i>	<b>phenol</b>	<b>14300</b>	<b>0.97</b>	<b>0.19</b>	<b>0.52</b>
	peracetic acid	450	1.19	0.65	0.77
	glutaraldehyde	17100	1.87	0.65	0.77
<i>Pseudomonas aeruginosa</i>	peracetic acid	110	1.47	0.75	0.91
	glutaraldehyde	450	1.13	0.42	0.42
<i>Staphylococcus aureus</i>	peracetic acid	55	2.34	0.48	0.81
	glutaraldehyde	650	1.72	0.73	0.90
	benzalkonium chloride	1700	1.23	0.85	1.01