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COMPANY SANITIZED

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MR # 306255

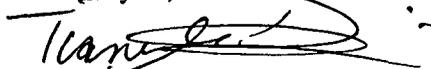
FYI Submission for Peracetic Acid

To Whom It May Concern:

Degussa Corporation is submitting the enclosed final report for Peracetic Acid as an FYI submission. The report identifies potential adverse effects of Peracetic acid in an Early-Life Stage Toxicity Test with Zebrafish. This report is also being submitted to the EPA under FIFRA 6(a)(2).

This report is being submitted to TSCA as Confidential Business Information. A copy of the Final Report along with a Sanitized copy are enclosed.

Thank you,



Tiana M. Rosamilia



Peracetic acid 15%

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OECD CRIC

Early-Life Stage Toxicity Test with Zebrafish ^{2007 AUG -6 AM 6:41}
(*Danio rerio*) under Flow-Through Conditions

Test Guideline

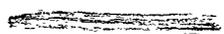
OECD Guideline for Testing of Chemicals No. 210 (1992)

Company Sanitized

Sponsor



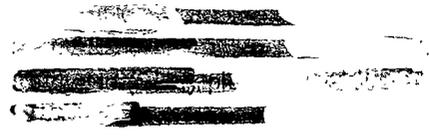
Author



Test Facility



Laboratory Project ID



Page 1 of 70

Date

09. Juli 2007

Statement of GLP Compliance

Title Peracetic acid
Early-Life Stage Toxicity Test with Zebrafish (*Danio rerio*)
under Flow-Through Conditions

Guideline OECD Guideline 210, adopted July 17, 1992

Test Item Peracetic acid ()

Testing Facility

[Redacted]

We declare that this study was conducted and reported in compliance with the present EC and German principles of Good Laboratory Practice.

09.07.07
(Date)

[Redacted]

09.07.07
(Date)

[Redacted]

9.7.07
(Date)

[Redacted]

Statement of the Quality Assurance Unit

Title Peracetic acid
Early-Life Stage Toxicity Test with Fathead Minnow
under Flow-Through Conditions

Guidelines OECD 210 for Testing of Chemicals (1992)

Test Item Peracetic acid

The study was verified as follows:

Inspection	dates	date of report
study plan	2006-03-30	2006-03-30
	2006-07-10	2006-07-10
	2006-07-12	2006-07-12
study based	2006-07-31	2006-07-31
	2006-08-15	2006-08-15
	2006-08-21	2006-08-21
	2006-08-24	2006-08-24
report	2006-12-07	2006-12-08
	2006-12-08	2006-12-08
	2006-12-10	2007-01-23
	2007-01-09	2007-01-23
	2007-01-17	2007-01-23
	2007-01-18	2007-01-23
	2007-01-22	2007-01-23
	2007-01-23	2007-01-23
	2007-01-26	2007-01-26
2007-05-23	2007-05-23	

The reported results accurately and completely reflect the raw data of the study. Also methods, procedures, and observations are accurately and completely described in the report.

The accordance of the study with its study plan and the principles of Good Laboratory Practice is guaranteed.

9.7.07



Table of Content

		Page
	Title Page	1
	Statement of GLP Compliance	2
	Statement of Quality Assurance Unit	3
	Personnel Involved	4
	Table of Content	5
	List of Tables	7
	List of Figures	8
1	Summary	9
2	Characterisation Data of the Test Item	11
3	Method	12
4	Results	23
4.1	Chemical and Physical Results	23
4.1.1	Nominal Test Concentrations	23
4.1.2	Mean Measured Concentration	23
4.1.2.1	Stock Solutions	23
4.1.2.2	Test Media	23
4.1.3	Dissolved Oxygen	23
4.1.4	pH-Value	23
4.1.5	Water Temperature	24
4.1.6	Total Hardness	24
4.1.7	Alkalinity/Acid Capacity	24
4.1.8	Residual Chlorine	24
4.1.9	Total Organic Carbon (TOC)	24
4.1.10	Flow Rate	24
4.2	Biological Results	25
4.2.1	Range Finding Test	25
4.2.2	Definitive Test	25
4.2.2.1	Egg Fertilization Rate	25
4.2.2.2	Egg Hatch and Definition of Post Hatch Day 0	25
4.2.2.3	Swim-up	25
4.2.2.4	Fry Survival (Post Hatch Success)	25
4.2.2.5	Overall Survival	25
4.2.2.6	Fry Growth	26
4.2.2.7	Biomass Loading	26
4.2.2.8	Morphological and Behavioural Effects	26

4.3	Tables	27
5	Validity Criteria	40
6	Conclusions	40
7	Literature / References	40
8	Specific Analysis of Peracetic Acid (LC-MS/MS)	41
8.1	Method	41
8.2	Method Validation	44
8.2.1*	Linearity	44
8.2.2	Repeatability of Injections	45
8.2.3	Limit of Quantification (LOQ)	46
8.2.4	Accuracy, Precision and Specificity	46
8.3	Results	47
8.3.1	Stock Solutions	47
8.3.2	Concentrations of Peracetic Acid in Water	48
8.4	Conclusions	49
8.5	Calibration Curves	49
8.6	Chromatograms	51
9	Spectrophotometric Analysis of Hydrogen Peroxide	56
9.1	Method	56
9.2	Method Validation	58
9.2.1	Linearity	58
9.2.2	Repeatability	59
9.2.3	Limit of Quantification (LOQ)	59
9.2.4	Accuracy, Precision and Specificity	60
9.3	Results	61
9.4	Conclusions	62
9.5	Calibration Curve	62
10	Raw Data	63
11	Certificate of Test Item Analysis	69
12	GLP Certificate of DR.U.NOACK-LABORATORIEN	70

List of Tables

		Page
Table 1:	Fry Survival, Egg Hatch, Time to Hatch, Time to Swim-up, Length, Growth, Morphological Effects: NOEC, LOEC	10
Table 2:	Dilution Table	14
Table 3:	Nominal Water Parameters	15
Table 4:	Mortality (Range Finding Test)	27
Table 5:	Egg Hatch (Range Finding Test)	28
Table 6:	Water Temperature (Continuous Measuring) in the Dilution Water	28
Table 7:	Dissolved Oxygen in Percent Air Saturation Value	29
Table 8:	pH Values in the Test Media	30
Table 9:	Water Temperature in the Test Media	31
Table 10:	Total Hardness in the Dilution Water	32
Table 11:	Alkalinity/Acid Capacity in the Test Media	33
Table 12:	Residual Chlorine of the Dilution Water	33
Table 13:	Total Organic Carbon (TOC)	34
Table 14:	Flow Rates	34
Table 15:	Egg Hatch / Hatching Time	35
Table 16:	Percent Swim-up of Hatched Fry	36
Table 17:	Post Hatch Survival on Study Day 33 (PHD 26)	37
Table 18:	Overall Survival on Study Day 33 (PHD 26)	38
Table 19:	Fry Growth: Length, Wet Weight and Dry Weight on Day 33	39
Table 20:	Gradient Table	42
Table 21:	Dilution Steps	43
Table 22:	Repeatability of Injections of the Standard	45
Table 23:	Recovery Rates of Fortified Samples of Peracetic Acid	46
Table 24:	Concentrations and Recovery Rates of Peracetic Acid in the Stock Solutions	47
Table 25:	Concentrations of Peracetic Acid in Water	48
Table 26:	Dilution Steps of the Stock Solutions	57
Table 27:	Repeatability	59
Table 28:	Recovery Rates of Fortified Samples of Hydrogen Peroxide	60
Table 29:	Concentrations and Recovery Rates of Hydrogen Peroxide in Stock Solutions	61
Table 30:	Mortality Raw Data of Peracetic acid 15% from Study Days 0 to 11	63
Table 31:	Mortality Raw Data of Peracetic acid 15% from Study Days 12 to 22	64
Table 32:	Mortality Raw Data of Peracetic acid 15% from Study Days 23 to 33	65
Table 33:	Length Raw Data from Study Day 33	66
Table 34:	Dry Weight Raw Data from Study Day 33	67
Table 35:	Wet Weight Raw Data from Study Day 33 of the Control	68

List of Figures

	Page
Figure 1: Calibration Curve of the Standard (Range 4.9 to 98 µg/L MTSO)	49
Figure 2: Calibration Curve of the Standard (Range 73.5 to 3136 µg/L MTSO)	50
Figure 3: Chromatogram of the Standard (MTSO)	51
Figure 4: Chromatogram of the Test Item (Stock Solution, d 20)	52
Figure 5: Chromatogram of the Control (d 20)	53
Figure 6: Chromatogram of the Test Item (Mixing Chamber with Fish, d 20)	54
Figure 7: Chromatogram of the Test Item (150 µg/L with Fish, d 20)	55
Figure 8: Calibration Curve of the Standard	62

1 Summary

The effects of the test item Peracetic acid to the early-life stage of fish (zebrafish *Danio rerio*) were determined according to OECD guideline 210 from 2006-07-19 to 2006-08-21 at [redacted]. Peracetic acid contained [redacted] Peracetic acid and [redacted] Hydrogen peroxide as active ingredients.

A test was conducted under flow-through conditions with the nominal concentrations 1.5 - 5 - 15 - 50 - 150 µg/L selected on the basis of a preliminary range finding test. The dosage level of 150 µg/L was set up twice. The second series was run without introduction of fish eggs in order to determine the behaviour of the test item without contact to biological surfaces.

The test was started by placing fertilised eggs in the test vessels and lasted 33 days (26 days post-hatch). 60 eggs of *Danio rerio* were exposed to the test concentrations and the control (4 replicates with 15 eggs each).

The stock solutions were analytically verified. Both active ingredients were quantified: Peracetic acid via LC-MS/MS and hydrogen peroxide via spectrophotometry. Additionally, the highest tested concentration level of 150 µg/L was analysed on Peracetic acid (for details see parts 8.3 and 9.3). With regard to the low nominal concentrations further analysis was not possible.

Water quality parameters pH-value, oxygen concentration, temperature, hardness, acid capacity/alkalinity were determined to be within the acceptable limits.

Different toxic endpoints have been determined: egg hatch, time to hatch, time to swim-up, fry growth (expressed as length and weight), morphological and behavioural effects, post hatch survival and fry survival.

Findings and Observations:

Based on the observation and statistical analysis of egg hatch, time to hatch, time to swim-up, growth (expressed as weight and length), morphological and behavioural effects, post hatch survival and fry survival, the test revealed the following results:

Table 1: **Fry Survival, Egg Hatch, Time to Hatch, Time to Swim-up, Length, Growth, Morphological Effects: NOEC, LOEC**
(Based on the nominal concentrations of Peracetic acid

	NOEC [$\mu\text{g/L}$]	LOEC [$\mu\text{g/L}$]
Egg Hatch (study day 7):	150	> 150
Time to Hatch (study day 4):	150	> 150
Time to Swim-up (study day 5):	150	> 150
Length (study day 33):	150	> 150
Weight (study day 33):	150	> 150
Morphological and behavioural effects	150	> 150
Post Hatch Survival (study day 33):	15	50
Overall Survival (study day 33):	15	50

Conclusion:

In this study, Peracetic acid caused no effects on hatch (egg hatch and time to hatch), growth (length and weight of juveniles) and morphological and behavioural effects. Significant but slight effects were observed for mortality (post hatch survival and overall survival) at the test concentrations 50 and 150 $\mu\text{g/L}$. However, these effects occurred during the time window of post hatch day 6 to 15, when the yolk sac was consumed and external food was applied. Despite of some slight changes, no further reduction of the survival rate was observed in the last third of the study. There was no significant differentiation of the mortality data between the two highest tested concentrations of 50 and 150 $\mu\text{g/L}$, i.e. no dose relation was observed for these concentrations. Based on these findings, Peracetic acid is considered not to have cumulative toxic properties. This result seems to be in accordance with the chemical properties of the active ingredients. Based on the toxic endpoints egg hatch, time to hatch, time to swim-up, fry growth (expressed as length and weight), morphological and behavioural effects, post hatch survival and overall survival, the overall NOEC (0-33 d) was 15 μg Peracetic acid product/L.

2 Characterisation Data of the Test Item

TEST ITEM	Peracetic acid
Batch Number	
Active ingredients	1. Peracetic acid 2. Hydrogen peroxide
Purity	1.  2. 
CAS RN	1. 79-21-0 2. 7722-84-1
Density	Ca. 1.15 g/mL (20 °C)
Water solubility	Completely miscible
Stability in water	Hydrolyses: at pH 4 ca. 50 % in 7 days at pH 7 and 9 ca. 50 % in 1 day.
pH value	Ca. 0.4 (20 °C)
Appearance	Liquid, clear, colourless
<hr/>	
Expiry date	Min. 6 months, 2006-12-06
Recommended storage	Room temperature (20 °C)
Storage at testing facility	Room temperature, protected from moisture and light
Retention	At least 1 g has been retained.
Identification parameter at testing facility	Name, batch number, state, colour and turbidity

The test item and the information concerning the test item were provided by the sponsor.

TEST ITEM**Peracetic acid****Test design**

A flow-through exposure design was carried out. Crystallisation dishes (days 0 to 18) and aquaria (days 19 to 33) respectively were used. Membrane piston pumps provided the water flow-through. The dilution water was splitted in 5 separate streams. Precision syringe pumps introduced appropriate volumes of stock solutions with different concentrations. Flow-splitting cells divided the water streams after the introduction of stock solutions and after passing mixing chambers into four aliquots per test concentration before being delivered to replicate test chambers. The accuracy of the test solution splits was checked prior to the test initiation. Water exchange was 10 times per day. Since the analytical methods did not allow the quantification at all dosage levels, a separate test concentration (150 µg Peracetic acid /L) was set up under identical conditions but without introduction of fish eggs.

Test concentrations

A range finding test (NON-GLP) was carried with the nominal test item concentrations 10 – 100 – 1000 – 10000 µg/L. Four replicates with each 15 eggs were tested per concentration. Mortality, egg hatch and swim up were observed. Results are given in Table 4 and Table 5. Based on the results of this test 5 concentrations were tested: 1.5 – 5.0 – 15 – 50 – 150 µg/L. The dosage level of 150 µg/L was set up twice. The second series run without introduction of fish eggs in order to determine the behaviour of the test item without contact to biological surfaces.

Stock solution

Stock solutions were prepared daily with demineralised water. Syringes were filled daily. Details are given in Table 2.

Table 2: Dilution Table
 Values given for Crystallisation Dishes (volume 0.7 L) / Aquaria (volume 3.5 L)

Nominal Test conc. [µg/L]	Flow of dilution water per dosage level [L/h]	Flow of stock solution per dosage level [mL/h]	Concentration of stock solution [mg/L]	Volume of stock solution [mL]	Amount of test item [mg]
Control	1.16 / 5.83	---	---	---	---
1.5	1.16 / 5.83	2	0.875 / 4.38	100	0.0875 / 0.438
5.0	1.16 / 5.83	2	2.92 / 14.58	100	0.292 / 1.458
15	1.16 / 5.83	2	8.75 / 43.75	100	0.875 / 4.38
50	1.16 / 5.83	2	29.2 / 145.8	100	2.92 / 14.85
150	1.16 / 5.83	2	87.5 / 437.5	100	8.75 / 43.75
150 (without fish)	1.16 / 5.83	2	87.5 / 437.5	100	8.75 / 43.75

CONTROL

Dilution water (without test item).

REFERENCE ITEM

No reference item is recommended for this test according to the guideline.

TEST METHOD

Test duration

33 days (26 days post hatch)

Replicates, number of eggs

Four replicates per test and control group with 15 eggs each.

Test vessels

Days 0 to 18:

Crystallisation dishes provided with mesh coated fittings allowing flow-through of test media (inner diameter 13.5 cm, water height about 5 cm) were used. The volume of the test media in the dishes was about 700 mL.

Days 19 to 33:

Glass aquaria provided with mesh coated fittings allowing flow-through of test media (approximately 12.5 x 14 x 21.5 cm with a volume of 3.5 L) were used.

Cleaning The test vessels were siphoned as needed to remove excess fecal material and uneaten food, also to minimize microbial growth and biodegradation of the test compound. Furthermore the mesh coated fittings were cleaned daily.

Aeration No additional aeration was provided.

Dilution water Tap water was used for testing. The water was filtered on activated charcoal to remove potential residual chlorine. The water is analysed biannually for the parameters given in Table 3.

Table 3: Nominal Water Parameters

Item	Maximum concentration
Particulate matter	< 20.0 mg/L
Total organic carbon (TOC)	< 2.0 mg/L
Residual chlorine	< 0.01 mg/L
Un-ionised ammonia	< 0.001 mg/L
Total organophosphorus pesticides	< 0.050 µg/L
Total organochlorine pesticides + PCBs	< 0.050 µg/L
organic chlorine	< 0.025 µg/L
pH-value	6.0 - 8.0
Total hardness (± 10 %)	40 - 180 mg/L CaCO ₃

Equilibration period The flow through of the test solution and dilution water was started 6 days prior to the initiation of the exposure. Actual test concentrations were determined first on day -1 of the study.

Spawning Adult zebrafish were kept in a separate aquarium (15 male and 15 female). About 15 minutes before start of artificial dawning (1 h) 2 glass dishes (26 cm x 14 cm x 6 cm), covered with a stainless steel mesh and provided with artificial plants, were introduced into the aquarium for one hour. At the end of dawning the glass dishes were gently removed and at least 400 eggs were immediately transferred to prepared test and control media. Oocysts were discarded.

Fertilization check After 2 h the eggs were checked for fertilization. Under a stereo microscope every embryo was checked for its blastomer phase. Eggs with only a 2 cell blastomer were regarded not to be fertilised. These eggs as well as coagulated eggs were discarded.

Introduction of eggs Only eggs with more than a 2 cell-stage were introduced in the test vessels. 15 eggs were introduced per replicate.

Feeding of test fish

Feeding of the juveniles started 5 days after spawning. Larvae were first fed with the ciliate *Tetrahymena pyriformis* at least 5 times daily for 4 days. 2 days after start of feeding brine shrimp nauplii (24 h old) were additionally fed (2-3 times daily). All food was given ad libitum.

Tetrahymena origin, breeding conditions:

Tetrahymena pyriformis strain CCAP 1630/1W was purchased from CCAP Culture Collection of Alga and Protozoa, CEH Windermere, the Ferry House, Far Sawrey Ambleside, Cumbria, LA 22 0LP, United Kingdom.

Fresh cultures were bred in PPY medium (proteose peptone 20 g, yeast extract 2.5 g, demineralised water 1 L) for 3 – 6 days at 20 – 26 °C. The cultures were centrifuged, washed and resuspended in sterile dechlorinated tap water. At least 1 mL of this suspension was applied per replicate at each feeding time.

Brine shrimp nauplii origin, breeding conditions:

Artemia salina eggs (Silverstar Artemia Brine Shrimp Ex) were purchased from INTER RYBA GMBH Germany. Fresh cultures were prepared with salt water (NaCl 40 g/L, 2 g eggs to 1 L salt water, gentle aeration). 24 h old brine shrimp nauplii were harvested and washed with a stainless steel mesh and resuspended in dechlorinated tap water.

Transfer of juveniles

On study day 19 juveniles were individually transferred to larger aquaria with a stainless steel spoon.

**ENVIRONMENTAL
CONDITIONS**

Water temperature

25 ± 2 °C

Dissolved oxygen
concentration

Not less than 60 % of air saturation value.

pH-value

6 – 8

Photoperiod

16 h photoperiod occurred during the test.

Light intensity

600 - 900 lx

**TYPE AND FREQUENCY
OF MEASUREMENT AND
OBSERVATIONS**

Biological parameters	Observations were made daily:
Hatched eggs	The number of hatched eggs was determined daily until study day 8.
Post hatch period	On day 7 of the study 98 % of all fertilized and living embryos in the control groups have hatched. Day 7 was defined to be post hatch day 0. (Per definition the post hatch period starts when at least 90 % (better 95 %) of all fertilized and living embryos in the control groups have hatched).
Mortality	<p>Criteria for mortality vary according to life stage:</p> <p><u>Egg mortality</u> as discerned by a distinct change in coloration or a marked loss of translucency and change in coloration, caused by coagulation and/or precipitation of protein, leading to a white opaque appearance, was checked daily. Dead eggs were discarded.</p> <p><u>For embryos</u>: Absence of body movement, change in coloration. Dead embryos were discarded.</p> <p><u>For larvae and juvenile fish</u>: Immobility and/or absence of respiratory movement and/or absence of heart-beat (as far as visible) and/or lack of reaction to mechanical stimulus.</p>
Further effects	<p>Abnormal appearance and behaviour was checked. The number of larvae or fish showing abnormality of body form were recorded. Abnormal animals were removed from the test vessels only on death.</p> <p>Abnormalities, e.g. hyperventilation, uncoordinated swimming, swim-up behaviour, atypical quiescence, and atypical feeding behaviour were also checked by visually inspecting each replicate.</p>
Measurement of fish size	At the end of exposure (after 33 days) the total length of all survivors was measured to the nearest 0.5 mm. Fish were killed in a Benzocaine solution before measurement.

Measurement of
wet body weight

At the end of exposure the wet body weight of the control fish was measured to enable determination of fish biomass loading. Fish was plotted on paper towels to remove excess moisture prior to weighing.

Measurement of
dry body weight

Each single fish was dried at 60 °C for 72 h. Dry biomass weight was measured to the nearest 0.1 mg.

Chemical parameters

Water quality
measurements

Temperature was measured working daily in one replicate of each group and continuously (every 60 min) in the dilution water with computer data storage.
Oxygen saturation was measured working daily in the dilution water and in one replicate of each group. The pH-value was measured weekly in one replicate of each group.
Total hardness and acid capacity/alkalinity were measured weekly in one replicate of control, the lowest, middle and top test item concentration.
Residual chlorine and TOC were measured weekly in the dilution water.

Flow rates

The flow rates of the test media were checked working daily and did not vary more than 10 % throughout the test duration.

Equipment

Oxygen: Oxygen meter "Oxi 197 - S", WTW
pH and water temperature: Multilab 340i, WTW
Water temperature: Datalogger. VOLTcraft
Total hardness: Spectrophotometer, CADAS 100 LPG 158, DR. LANGE
Residual chlorine: Aquaquant 1.14434, MERCK
DOC-analyser: "Multi N/C 3000" ANALYTIK JENA AG
Climate chamber "KF550", VISSMANN
Filter of activated charcoal, BERKEFELD FILTER
Precision syringe pumps, HARVARD APPARATUS
Polypropylene syringes: vol. 60 mL, HSW
Membrane piston pumps, PROMINENT
Thermostate, LAUDA
Digital Lux Meter: Roline, ROTH
Balance LA230-S, SARTORIUS
Balance PJ Precisa junior 2000 C, DIGITANA

**TEST ITEM
CONTROL ANALYSIS**

Peracetic acid was determined via LC-MS/MS. Hydrogen peroxide was determined via spectrophotometry. For details see parts 8 and 9.

Sampling regime Samples of the highest test concentration (150 µg/L) and control (mixing chamber and alternate replicates) were taken on days -1, 0 and at least weekly thereafter until end of exposure. Also, sampling and analysis of the additional test concentration of 150 µg/L (without introduction of fish eggs) was sampled and analysed. These samples were analysed for peracetic acid. Stock solutions were sampled and analysed at least weekly for peracetic acid and hydrogen peroxide.

Sample pre-treatment and storage Samples of stock solutions were acidified with acetic acid (100 %, about 50 µL/50 mL). No pre-treatment was carried out for test solution samples. For storage conditions of the samples see part 8 and 9.

EVALUATION

Data for the replicates of the test and control groups were grouped together for analysis. Replicate means were used for statistical analysis since each test vessel is the experimental unit based on the design of the test system. For each parameter analysed, survival (mortality), egg-hatch, time to percent swim-up, dry weight and length data and abnormal appearance if applicable, the following statistical tests were conducted:

Statistical significance One way analysis of variance (ANOVA) and DUNNETT's test were used for NOEC/LOEC calculations. When running a one way analysis of variance a normality test and an equal variance test were done first.

Data which did not fit a normal distribution nor after transformation were analysed by the non-parametric Kruskal-Wallis one way analysis of variance or the BONFERRONI test to determine if there was a significant difference between the treatment and control groups. These statistical analyses were conducted with conclusions of statistical significance based on a 95 % confidence level. The α -value (acceptable probability of incorrectly concluding that there is a difference) is 0.05.

For the parameters survival (mortality), egg-hatch time to hatch, time to percent swim-up, dry weight and length the following statistical tests were conducted.

Hatching data of days 4-6 were analysed with ANOVA (SigmaStat).

Hatching data of day 7 were analysed with ANOVA after being transformed ($Y = 1/Y$) with GraphPad Prism4.

Swim up data of days 5 and 7 were analysed with ANOVA (SigmaStat).

Swim up data on day 6, 8 and 9 were analysed with ANOVA after being transformed ($Y = 1/Y$) with GraphPad Prism4.

Dry weight was analysed with ANOVA (SIGMASTAT).

Length was analysed with ANOVA and DUNNETT's test (SIGMASTAT).

Mortality data of day 32 were analysed with DUNNETT's test (SIGMASTAT).

These statistical analyses were conducted with conclusions of statistical significance based on a 95 % confidence level. The α -value (acceptable probability of incorrectly concluding that there is a difference) was 0.05.

Software

Calculations was carried out using software

- Microsoft Excel rel. 2000 (2000), MICROSOFT CORPORATION
- SigmaStat rel. 2.03 (1992-1997), SPSS INC.
- GraphPad Prism4 (2005), GRAPHPAD SOFTWARE, INC.

DEFINITIONS

NOEC

The NOEC (No Observed Effect Concentration) is the highest tested concentration of a test item at which the test item is observed to have no significant effect (at $p = 0.05$) when compared with the control.

LOEC

The LOEC (Lowest Observed Effect Concentration) is the lowest tested concentration of a test item at which the test item is observed to have a significant effect (at $p = 0.05$) when compared with the control. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC.

CHRONOLOGICAL TEST DESCRIPTION

Day -6	Start of the equilibration phase
Day -1	Control analysis
Day 0	Spawning of eggs, exposure, control analysis at initiation of exposure
Day 2	Start of hatching period
Day 6	Period of swim-up, start feeding with brine shrimp nauplii and <i>Tetrahymena pyriformis</i> Definition of post-hatch date
Day 7, 14	Control analysis
Day 19	Transfer of juveniles
Day 21, 28	Control analysis
Day 33	End of exposure, determination of weight and length (no feeding 24 h before end of exposure)
Continuously	Temperature in the dilution water (every 60 min).
Daily	Observation on biological parameters, feeding if applicable
Working daily	Determination of <ul style="list-style-type: none">- Dissolved oxygen in the dilution water- Oxygen and temperature in one replicate of each group- Check of water/test item delivery system
Weekly	<ul style="list-style-type: none">- Total hardness, acid capacity/alkalinity in one replicate of control, the lowest, middle and top test item concentration- pH-value in each replicate of each group- TOC in the mixing chamber of the control group- Chlorine from dilution water

VALIDITY CRITERIA

- Dissolved oxygen saturation must be between 60 and 100 % of air saturation value.
- Water temperature must not differ by more than ± 1.5 °C between test vessels or between successive days at any time during the test, and was in the given range.
- Post-hatch success in the controls ≥ 70 %.
- Control analysis: the concentrations of the test item in the test solution should be maintained within ± 20 % of the mean measured values. With respect to the test item properties higher deviations did not necessarily invalidate the test.

DATES

Study initiation	2006-07-06
Experimental starting	2006-07-13
Experimental completion	2006-09-19
Study completion	Please refer to page 1

**DEVIATIONS FROM
THE GUIDELINE**

None

**DEVIATIONS FROM
THE STUDY PLAN**

The equilibration period was shortened to 6 days since at starting day the number of eggs found in the spawning tank was sufficient for a test start. This deviation is considered to have no impact on quality and integrity of the study.

ARCHIVING

The following will be retained in the archive of the test facility for the period as specified in the operative national GLP regulations:

- all raw data
- study plan
- final report
- all records performed by the quality assurance programme including master schedules
- sample of the test item

Carsten, 2006-09-19, Carsten

4 Results

4.1 Chemical and Physical Results

4.1.1 Nominal Test Concentrations

The definitive study was conducted with the nominal test concentrations of 1.5 - 5 - 15 - 50 - 150 µg/L.

4.1.2 Mean Measured Concentration

4.1.2.1 Stock Solutions

The major part of recovery rates of PAA in the stock solutions was in a range of 80 to 120 %. The minimum value was 45 % and the maximum value was 145 % (see Table 24). Hydrogen Peroxide concentrations in the stock solutions mainly were in the expected range (80 - 120 % of nominal). The minimum value was 72 % and the maximum value was 236 % (see Table 29).

4.1.2.2 Test Media

Recovery rates of PAA in mixing chambers and replicates were < LOQ in most of the samples. After transfer of juveniles in larger aquaria recovery rates of PAA in mixing chambers increased due to the higher flow rates. High reactivity of PAA is considered the reason for the low recoveries.

Analysis of Hydrogen Peroxide in the mixing chambers and the replicates could not be carried out due to the low sensitivity of the analytical method.

4.1.3 Dissolved Oxygen

The dissolved oxygen concentrations in the control and test item groups, expressed in percent saturation, were in the mean of 94 to 96 % and ranged from 84 to 100 % during the total test period (see Table 7).

4.1.4 pH-Value

The pH-values in the control and test item groups were in the mean of 7.36 to 7.42 and ranged from 7.26 to 7.55 during the total test period (see Table 8).

4.1.5 Water Temperature

The temperature of control replicate 1 was measured automatically every 60 minutes throughout the test. The mean temperature \pm mean standard deviation was 25.9 ± 0.46 °C (Table 6). The maximum temperature was 27.0, the minimum temperature was 25.0. The mean water temperature measured working daily during the total test period in the replicates of the control was 25.8 °C and ranged from a minimum temperature of 25.2 °C to a maximum temperature of 26.4 °C (see Table 9).

4.1.6 Total Hardness

The mean total hardness ranged from 82 to 90 mg CaCO₃/L in all test and control groups (for details see Table 10).

4.1.7 Alkalinity/Acid Capacity

The alkalinity (K_B) in all test and control groups ranged from 0.04 to 0.11 mmol/L. The acid capacity (K_S) in all test and control groups ranged from 0.54 to 0.81 mmol/L (for details see Table 11).

4.1.8 Residual Chlorine

Residual chlorine, measured of the dilution water on study days 2, 9, 16, 23 and 30 was < 0.01 mg/L (see Table 12).

4.1.9 Total Organic Carbon (TOC)

The mean total organic carbon, measured of the dilution water on study days 2, 9, 16, 23 and 30 was 1.9 mg/L throughout the study (see Table 13).

4.1.10 Flow Rate

The mean flow rate in the crystallisation dishes in all test and control groups was 1.15 ± 0.05 L/h and ranged from 1.05 to 1.27 L/h. In the aquaria the mean flow rates were 5.61 ± 0.19 L/h and ranged from 5.28 to 6.12 L/h (see Table 14).

4.2 Biological Results

4.2.1 Range Finding Test

Four concentrations were tested in the range finding test. In the highest tested concentration of 10 mg/L complete mortality was observed after one day. Significant mortality was found at the dosage levels of 100 and 1000 µg/L, whereas no effects were found at 10 µg/L until study day 11 (see Table 4 and Table 5).

4.2.2 Definitive Test

4.2.2.1 Egg Fertilization Rate

The egg fertilization rate, determined on day 0 (test start) was > 90 %.

4.2.2.2 Egg Hatch and Definition of Post Hatch Day 0

Egg hatch was evaluated on study days 3 - 8. Egg hatch began on study day 3 in the control and all test item concentrations and continued until study day 8. 100 % hatching success was reached on study day 8 (only one embryo not hatched at 15 µg/L, see Table 15).

A statistically significant difference was not found for this parameter.

Study day 7 was determined to be post hatch day 0 with a control hatching rate of 98 %.

4.2.2.3 Swim-up

Swim-up was observed for a 4-day period on study days 5 to 9 (see Table 16). Newly hatched fry began to swim up on study day 5 (post-hatch day -2). No statistically significant differences were found until the end of the swim-up period.

4.2.2.4 Fry Survival (Post Hatch Success)

The post hatch success in all control replicates met the guideline criteria. The fry survival (post hatch success) at the end of the study was 89 % in the control group, 83 % in the 1.5 µg/L group, 88 % in the 5 µg/L group, 82 % in the 15 µg/L group, 66 % in the 50 µg/L group and 64 % in the 150 µg/L group (see Table 17). Compared to the control group, survival was reduced in all dosage levels. At the two highest dosage levels of 50 and 150 µg/L the difference in survival was statistically significant (DUNNETT's test).

4.2.2.5 Overall Survival

Overall survival at the end of the study was 89 % in the control group, 83 % in the 1.5 µg/L group, 87 % in the 5 µg/L group, 80 % in the 15 µg/L group, 65 % in the 50 µg/L group and 64 % in the 150 µg/L group (see Table 18). Compared to the control group, survival was reduced in all dosage levels. At the two highest dosage levels of 50 and 150 µg/L the difference in survival was statistically significant (DUNNETT's test).

4.2.2.6 Fry Growth

The fry growth, expressed as length and dry weight, was measured on study day 33 (post-hatch day 26). Statistical analysis of data showed no significant differences between the controls and the test item concentrations for the dry weight (DUNNETT's test, see Table 19). For the fish length, significant differences were detected for the two highest tested concentrations 50 and 150 µg/L. Higher values were found in these dosage levels. Since, due to higher mortality, the biomass loading in these replicates was lower compared to the control, the growth values of the survivors were higher. This is considered not an effect of a direct impact of the test item. Therefore, the detected significance will not be considered for the determination of the NOEC / LOEC for the parameter fish length and growth.

4.2.2.7 Biomass Loading

The biomass-loading factor for the study was determined from the wet weights of the control fish at study termination (see Table 19). The mean wet weight was 28.2 mg/fish. The biomass-loading based on the 3.5 litre volume of a single growth chamber was 121 mg/L. The biomass loading factor based upon a flow of 35 litres per day through each single test chamber, was 3.1 mg per litre and day. These loads were well within the requirements to ensure adequate dissolved oxygen levels and to avoid crowding of the fish.

4.2.2.8 Morphological and Behavioural Effects

During the post-hatch period, the following morphological and behavioural effects were observed sporadically in the controls and all test levels:

- Delayed development
- Spine distortion

Delayed development was observed in a few cases, but was not reported since length and weight data finally showed these differences to normally developed fish.

No biologically significant morphological and behavioural effects were observed in any tested replicate.

4.3 Tables

Table 4: Mortality (Range Finding Test)

Nominal Concentrations of Peracetic acid [µg/L]	Repl.	Mortality [%] on Day																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Control	1	0	0	0	0	0	0	0	0	0	13	13	13	20	20	20	20	20
	2	0	0	0	0	0	0	0	0	0	13	13	20	20	20	20	20	20
	3	0	0	0	0	0	0	0	0	0	7	7	7	13	13	13	13	13
	4	0	0	0	0	0	0	0	0	0	20	20	20	27	27	27	27	27
	Mean	0	0	0	0	0	0	0	0	0	13	13	15	20	20	20	20	20
10	1	0	0	0	0	0	0	0	0	0	0	0	13					
	2	0	0	0	0	0	0	0	0	0	0	0	33					
	3	0	0	0	0	0	0	0	0	0	7	7	40					
	4	0	0	0	0	0	0	0	0	0	0	0	27					
	Mean	0	0	0	0	0	0	0	0	0	2	2	28*)					
100	1	0	0	0	0	0	0	0	0	13	27	40	53	53	80	80	80	80
	2	0	0	0	0	0	0	0	0	7	7	13	20	27	40	53	53	53
	3	0	0	0	0	0	0	0	0	0	7	40	47	67	73	87	87	87
	4	0	0	0	0	0	0	0	0	0	27	33	33	47	60	80	80	80
	Mean	0	0	0	0	0	0	0	0	0	14	27	33	47	53	72	75	75
1000	1	0	0	0	0	0	0	0	7	7	53	80	80	80	80	100	100	100
	2	0	0	0	0	0	0	0	27	40	60	60	73	87	93	93	93	93
	3	0	0	0	0	0	0	0	0	0	33	33	47	73	73	80	100	100
	4	0	0	0	0	0	0	0	0	27	80	80	80	80	80	87	87	87
	Mean	0	0	0	0	0	0	0	9	19	57	63	70	80	82	90	95	95
10000	1	100																
	2	100																
	3	100																
	4	100																
	Mean	100																

*) no further evaluation possible due to a malfunction of the water supply system

Table 5: Egg Hatch (Range Finding Test)

Nominal Concentrations of Peracetic acid [µg/L]	Repl.	Egg Hatch [%] on Day									
		4	5	6	7	8	9	10	11	12	13
Control	1	0	13	13	53	73	80	87	87	87	87
	2	0	7	47	80	87	100	100	100	100	100
	3	0	20	40	67	93	93	93	93	93	93
	4	0	0	20	53	67	80	80	80	80	80
	Mean	0	10	30	63	80	88	90	90	90	90
10	1	47	60	87	93	100					
	2	40	73	80	87	100					
	3	33	60	80	100	100					
	4	40	53	73	87	100					
	Mean	40	62	80	92	100					
100	1	7	20	40	80	87	93	93	93	93	93
	2	0	20	47	80	87	93	93	93	93	93
	3	0	7	27	53	67	73	93	93	93	93
	4	7	7	40	60	73	80	80	80	80	80
	Mean	4	14	39	68	79	85	90	90	90	90
1000	1	0	0	13	33	40	53	53	53	53	53
	2	0	0	7	7	13	27	40	40	40	40
	3	0	7	7	27	40	47	47	47	47	47
	4	0	0	0	13	13	40	40	40	40	40
	Mean	0	2	7	20	27	42	45	45	45	45

Table 6: Water Temperature (Continuous Measuring) in the Dilution Water

Period of Measurements	July 19 – August 21, 2006
Minimum Temperature [°C]	25.0
Maximum Temperature [°C]	27.0
Mean Temperature ± Standard Deviation [°C]	25.9 ± 0.46

Table 7: Dissolved Oxygen in Percent Air Saturation Value

Study Day	Test Concentrations [$\mu\text{g/L}$]						
	Control	1.5	5	15	50	150	150*
0	97	98	97	97	97	97	97
1	95	96	96	96	96	95	96
2	96	96	95	95	95	95	96
5	96	98	97	97	97	96	94
6	97	96	96	96	98	95	96
7	95	95	93	95	97	96	97
8	91	91	89	92	92	91	92
9	95	93	95	97	96	96	100
12	94	95	96	97	97	97	100
13	95	95	96	97	96	95	100
14	96	97	95	96	97	96	97
15	93	95	95	95	97	96	99
16	94	94	95	95	93	94	98
19	97	98	96	98	97	96	99
20	98	97	96	97	98	97	99
21	97	98	97	96	97	96	97
22	93	93	93	85	84	84	90
23	97	96	96	89	90	91	92
26	95	94	94	93	91	90	91
27	95	95	95	93	94	92	92
28	94	94	95	96	95	93	93
29	95	94	94	92	91	92	93
30	95	94	95	94	95	94	93
Mean	95	95	95	95	95	94	96
SD	1.59	1.80	1.68	2.94	3.26	2.96	3.10
Min.	91	91	89	85	84	84	90
Max.	98	98	97	98	98	97	100

*) = without fish
SD = Standard Deviation
Min. = Minimum measured dissolved oxygen concentration
Max. = Maximum measured dissolved oxygen concentration

Table 8: pH Values in the Test Media

Study Day	pH-value						
	Test Concentrations [µg/L]						
	Control	1.5	5	15	50	150	150*
0	7.29	7.33	7.33	7.38	7.38	7.39	7.40
7	7.42	7.44	7.46	7.47	7.48	7.47	7.55
14	7.26	7.36	7.34	7.34	7.34	7.33	7.35
21	7.43	7.42	7.41	7.39	7.39	7.39	7.39
28	7.42	7.45	7.42	7.40	7.38	7.38	7.40
Mean	7.36	7.40	7.39	7.40	7.39	7.39	7.42
SD	0.07	0.05	0.05	0.04	0.05	0.04	0.07
Min.	7.26	7.33	7.33	7.34	7.34	7.33	7.35
Max.	7.43	7.45	7.46	7.47	7.48	7.47	7.55

*) = without fish
 SD = Standard Deviation
 Min. = Minimum pH value
 Max. = Maximum pH value

Table 9: Water Temperature in the Test Media

Temperature [°C]							
Study Day	Test Concentrations [µg/L]						
	Control	1.5	5	15	50	150	150*
0	26.4	26.5	26.4	26.5	26.4	26.4	26.2
1	26.4	26.4	26.3	26.4	26.0	26.0	26.2
2	26.3	26.4	26.4	26.4	26.4	26.4	26.0
5	25.7	26.0	26.0	25.9	25.8	25.7	26.1
6	25.6	25.8	25.7	25.7	25.6	25.5	25.7
7	25.9	26.2	26.1	26.0	25.6	25.5	25.3
8	25.4	25.8	25.9	26.2	25.4	25.8	25.2
9	25.3	25.4	25.5	25.3	25.3	25.3	25.3
12	25.5	25.6	25.6	25.7	25.5	25.4	25.6
13	25.5	26.0	26.0	26.0	25.8	25.5	25.4
14	25.2	25.6	25.6	25.4	25.3	25.3	25.2
15	25.5	25.8	25.7	25.4	25.3	25.2	25.4
16	25.4	25.6	25.6	25.6	25.4	25.5	25.3
19	25.6	25.6	25.7	25.4	25.5	25.6	25.4
20	25.7	25.9	25.9	26.0	25.4	25.4	25.4
21	25.7	25.8	25.9	25.8	25.5	25.6	25.3
22	25.7	25.8	25.7	25.6	25.4	25.6	25.3
23	25.5	25.8	25.6	25.3	25.3	25.4	25.5
26	26.0	26.0	26.1	26.2	25.7	25.6	25.5
27	26.0	26.1	26.0	25.9	25.7	25.6	25.4
28	26.1	26.1	26.3	26.2	25.8	25.7	25.6
29	26.0	26.3	26.2	26.0	25.8	25.6	25.6
30	26.0	26.1	26.1	26.0	25.9	26.0	25.5
Mean	25.8	25.9	25.9	25.9	25.6	25.6	25.5
SD	0.34	0.29	0.27	0.35	0.31	0.30	0.30
Min.	25.2	25.4	25.5	25.3	25.3	25.2	25.2
Max.	26.4	26.5	26.4	26.5	26.4	26.4	26.2

*) = without fish
 SD = Standard Deviation
 Min. = Minimum pH value
 Max. = Maximum pH value

Table 10: Total Hardness in the Dilution Water

Study Day	Total Hardness [mg CaCO ₃ /L]			
	Control	Test Concentrations [g/L]		
		1.5	15	150
2	87	73	77	76
9	85	86	85	95
16	95	87	120	86
23	77	89	88	81
30	108	75	69	82
Mean	90	82	88	84
SD	10.5	6.63	17.4	6.36
Min.	77	73	69	76
Max.	108	89	120	95

SD = Standard Deviation
 Min. = Minimum measured total hardness
 Max. = Maximum measured total hardness

Table 11: Alkalinity/Acid Capacity in the Test Media

Alkalinity / Acid Capacity [mmol/L]								
Study Day	Test Concentrations [$\mu\text{g/L}$]							
	Control		1.5		15		150	
	K_B	K_S	K_B	K_S	K_B	K_S	K_B	K_S
2	0.10	0.71	0.11	0.71	0.05	0.72	0.06	0.70
9	0.11	0.80	0.07	0.79	0.07	0.81	0.08	0.79
16	0.04	0.80	0.05	0.81	0.05	0.80	0.06	0.81
23	0.06	0.58	0.06	0.58	0.05	0.54	0.09	0.62
30	0.10	0.60	0.06	0.60	0.05	0.60	0.06	0.61
Mean	0.08	0.70	0.07	0.70	0.05	0.69	0.07	0.71
SD	0.03	0.09	0.02	0.09	0.01	0.11	0.01	0.08
Min.	0.04	0.58	0.05	0.58	0.05	0.54	0.06	0.61
Max.	0.11	0.80	0.11	0.81	0.07	0.81	0.09	0.81

SD = Standard Deviation
 Min. = Minimum measured values
 Max. = Maximum measured values

Table 12: Residual Chlorine of the Dilution Water

Date of Measurement	Study Day	Residual Chlorine [mg/L]
2006-07-21	2	< 0.01
2006-07-28	9	< 0.01
2006-08-04	16	< 0.01
2006-08-11	23	< 0.01
2006-08-18	30	< 0.01

Table 13: Total Organic Carbon (TOC)

Date of Measurement	Study Day	TOC [mg/L]
2006-07-21	2	1.9
2006-07-28	9	2.1
2006-08-04	16	1.7
2006-08-11	23	2.0
2006-08-18	30	1.8
	Mean value	1.89

Table 14: Flow Rates

Period of Measurements	July 19, 2006 - August 06, 2006
Minimum Flow Rate in [L/h]	1.05
Maximum Flow Rate in [L/h]	1.27
Overall Mean Flow Rate ± Standard Deviation in [L/h]	1.15 ± 0.05
Period of Measurements	August 07, 2006 - August 21, 2006
Minimum Flow Rate in [L/h]	5.28
Maximum Flow Rate in [L/h]	6.12
Overall Mean Flow Rate ± Standard Deviation in [L/h]	5.61 ± 0.19

Table 15: Egg Hatch / Hatching Time

Nominal Conc. [$\mu\text{g/L}$]	Repl.	PHD -4 (Study Day 3)	PHD -3 (Study Day 4)	PHD -2 (Study Day 5)	PHD -1 (Study Day 6)	PHD 0 (Study Day 7)	PHD 1 (Study Day 8)
		Egg Hatch [%]					
Control	1	20	67	80	93	100	100
	2	7	47	53	87	100	100
	3	7	27	67	87	100	100
	4	0	20	47	60	93	100
	Mean	9	40	62	82	98	100
1.5	1	0	33	93	100	100	100
	2	7	33	100	100	100	100
	3	7	20	80	100	100	100
	4	7	20	67	87	93	100
	Mean	5	27	85	97	98	100
5	1	15	40	80	87	100	100
	2	20	53	73	80	100	100
	3	0	20	80	100	100	100
	4	15	20	53	73	100	100
	Mean	13	33	72	85	100	100
15	1	0	27	67	73	87	100
	2	0	27	60	80	93	93*
	3	7	60	73	93	93	100
	4	40	60	93	93	100	100
	Mean	12	44	73	85	93	98
50	1	0	27	80	87	100	100
	2	0	27	80	93	100	100
	3	7	53	87	100	100	100
	4	15	53	73	73	100	100
	Mean	6	40	80	88	100	100
150	1	7	7	87	93	100	100
	2	7	27	87	93	100	100
	3	20	40	100	100	100	100
	4	20	33	100	100	100	100
	Mean	14	27	94*	97	100	100

Repl. = Replicate
 PHD = Post hatch day
 * = 1/15 eggs dead

Statistical Significance Tests were carried out for days 4, 5, 6, 7.

* = Statistically significant difference from control ($\alpha=0.05$)

Table 16: Percent Swim-up of Hatched Fry

Nominal Concentrations [µg/L]	Repl.	Swim-up [%] on Study Day				
		5	6	7	8	9
Control	1	60	87	100	100	100
	2	40	73	100	100	100
	3	53	73	93	93	93
	4	40	60	80	93	93
	Mean	48	73	93	97	97
1.5	1	47	93	100	100	100
	2	87	93	93	93	93
	3	67	93	100	100	93
	4	20	80	87	87	87
	Mean	55	90	95	95	93
5	1	53	73	100	100	93
	2	47	73	80	87	73
	3	33	93	93	100	100
	4	40	60	80	87	80
	Mean	43	75	88	94	87
15	1	33	53	80	73	73
	2	20	60	73	80	73
	3	33	47	93	100	100
	4	40	87	93	100	100
	Mean	32	62	85	88	87
50	1	47	73	87	93	93
	2	47	80	93	93	100
	3	47	80	93	93	87
	4	33	40	93	87	80
	Mean	44	68	92	92	90
150	1	27	93	93	100	100
	2	40	87	93	93	93
	3	73	100	100	100	100
	4	40	100	93	100	100
	Mean	45	95	95	98	98

Statistical Significance Tests were carried out for days 5, 6, 7, 8, 9.

No significant differences were found.

Table 17: Post Hatch Survival on Study Day 33 (PHD 26)

Live fry on study day 8 were set to 100 % for the calculation of the post hatch survival

Nominal Concentrations [µg/L]	Repl.	Live Fry on Study Day 8	Live Fry on Study Day 33	Post Hatch Survival [%]
Control	1	15	12	80
	2	15	15	100
	3	15	13	87
	4	15	13	87
	Mean	15	13.25	88.5
1.5	1	15	14	93
	2	15	12	80
	3	15	12	80
	4	15	12	80
	Mean	15	12.5	83.25
5	1	15	13	87
	2	14	11	79
	3	15	15	100
	4	15	13	87
	Mean	14.75	13	88.25
15	1	15	13	87
	2	14	11	79
	3	15	11	73
	4	15	13	87
	Mean	14.75	12	82
50	1	15	9	60
	2	15	10	67
	3	15	10	67
	4	14	10	71
	Mean	14.75	9.75	66.25⁺
150	1	15	9	60
	2	15	9	60
	3	15	10	67
	4	15	10	67
	Mean	15	9.50	63.5⁺

⁺ = Statistically significant difference from control ($\alpha=0.05$)

For the evaluation of post hatch success data see part 4.2.2.4.

Table 18: Overall Survival on Study Day 33 (PHD 26)

Nominal Concentrations [µg/L]	Repl.	Live Fry on Study Day 8	Overall Survival [%]	Live Fry on Study Day 33	Overall Survival [%]
Control	1	15	100	12	80
	2	15	100	15	100
	3	15	100	13	87
	4	15	100	13	87
	Mean	15	100	13.25	88.5
1.5	1	15	100	14	93
	2	15	100	12	80
	3	15	100	12	80
	4	15	100	12	80
	Mean	15	100	12.5	83.25
5	1	15	100	13	87
	2	14	93	11	73
	3	15	100	15	100
	4	15	100	13	87
	Mean	14.75	98.25	13	86.75
15	1	15	100	13	87
	2	14	93	11	73
	3	15	100	11	73
	4	15	100	13	87
	Mean	14.75	98.25	12	80
50	1	15	100	9	60
	2	15	100	10	67
	3	15	100	10	67
	4	14	93	10	67
	Mean	14.75	98.25	9.75	65.25⁺
150	1	15	100	9	60
	2	15	100	9	60
	3	15	100	10	67
	4	15	100	10	67
	Mean	15	100	9.50	63.5⁺

⁺ = Statistically significant difference from control ($\alpha=0.05$)

For the evaluation of overall survival data see part 4.2.2.5.

Table 19: Fry Growth: Length, Wet Weight and Dry Weight on Day 33

Nominal Conc. [µg/L]	Repl.	PHD 33 (Study Termination)		
		Length [mm]	Wet Weight [mg]	Dry Weight [mg]
Control	1	16.0	30.8	7.7
	2	15.2	23.7	5.9
	3	15.5	28.8	7.4
	4	15.9	30.4	8.2
	Mean	15.6	28.2	7.3
1.5	1	16.5	/	8.4
	2	16.3		8.7
	3	16.4		8.7
	4	16.8		8.2
	Mean	16.5		8.5
5	1	16.5	/	7.9
	2	16.9		9.3
	3	15.8		8.6
	4	16.1		7.8
	Mean	16.3		8.4
15	1	16.1	/	8.4
	2	16.8		9.3
	3	15.7		8.8
	4	16.0		7.5
	Mean	16.2		8.5
50	1	17.7	/	10.1
	2	17.0		8.6
	3	16.1		7.5
	4	16.8		9.0
	Mean	16.9⁺		8.8
150	1	18.2	/	10.9
	2	16.8		8.2
	3	17.6		9.8
	4	16.2		7.9
	Mean	17.1⁺		9.2

⁺ = Statistically significant difference from control ($\alpha=0.05$)

For the evaluation of length and growth data see part 4.2.2.6.

5 Validity Criteria

The study was performed according to OECD 210 and met the validity criteria:

- Dissolved oxygen saturation was between 84 and 100 % of air saturation value.
- Water temperature did not differ by more than ± 1.5 °C between test vessels or between successive days at any time during the test, and was in the given range
- Post-hatch success in the controls ≥ 70 %.
- Control analysis: Recovery rates of PAA in mixing chambers and replicates were mostly < LOQ. After transfer of juveniles in larger aquaria recovery rates of PAA in mixing chambers increased. High reactivity of PAA is considered the reason for the low recoveries. Hydrogen peroxide was not analysed from the test solutions due to the low sensitivity of the analytical method. All effect levels were based on nominal concentrations.

6 Conclusions

Peracetic acid caused significant effects on chronic toxicity (zebrafish early life stage test, 26 days post hatch) at the nominal dosage levels 50 and 150 $\mu\text{g/L}$. The overall NOEC (0-33 d) was 15 $\mu\text{g Peracetic acid /L}$ based on the toxic endpoints egg hatch, time to hatch, time to swim-up, fry growth (expressed as length and weight), post hatch survival and overall survival.

7 Literature / References

- (1) OECD guideline 210, adopted July 17, 1992
- (2) DIN Guideline 32645: Nachweis- und Bestimmungsgrenze (January, 1990)

8 Specific Analysis of Peracetic Acid (LC-MS/MS)

8.1 Method

PRINCIPLE

Analysis of various concentrations of peracetic acid (PAA), active ingredient of Peracetic acid [^], in dechlorinated tap water were carried out via HPLC-MS/MS. The quantitative reaction of PAA with methyl p-tolyl sulphide (MTS) yields the corresponding sulfoxide (MTSO). To prevent the reaction of the active ingredient hydrogen peroxide (H₂O₂) with MTS, Triphenyl-phosphine (TPP) was added to reduce H₂O₂. Reagent concentrations were at least twice as high as the expected peroxide concentrations. Analytical evaluation of MTSO was carried via LC-MS/MS on a reversed-phase column in gradient mode. Detection was carried out with an electrospray tandem mass spectrometer in positive mode using an external standard.

Equipment	HPLC : 2695 Alliance separation module, WATERS Detection : Mass selective detector, Micromass Quattro Premier™ MS/MS-detector, WATERS Software : Mass Lynx™ 4.1, WATERS
Reagents	Methanol, J.T. BAKER HPLC water, J.T. BAKER Acetic acid, ROTH Acetonitrile, J.T. BAKER Methyl p-tolyl sulphide (MTS), 99.9 %, ALDRICH Triphenylphosphine (TPP), 99.5 %, SIGMA-ALDRICH
Standard	Methyl p-tolyl sulfoxide (MTSO), 98 %, Batch: 02417P2, ALDRICH

CONDITIONS OF ANALYSIS

Pre-Column Nucleosil 50-5 C8 ec, 8 x 3 mm, Serial No.: 2115868,
Batch: 9012, MACHEREY-NAGEL
Column Nucleosil 50-5 C8 ec, 125 x 3 mm, Serial No.: 2075594,
Batch: 9012, MACHEREY-NAGEL
Column temperature 20 °C
Mobile phase A = acetic acid (0.15 %)
B = methanol
Gradient mode, see Table 20:

Table 20: Gradient Table

Time [min]	A [%]	B [%]
0	40	60
0.50	40	60
2.00	15	85
4.00	15	85
5.00	40	60
7.00	40	60

Flow rate 0.5 mL/min
Run time 7 min
Injection volume 20 µL

CONDITIONS OF DETECTION

Ionization mode Electrospray positive
Type Multiple Reaction Mode (MRM)
Mass trace 155.0 → 92.2 (Precursor/Product ion)
Capillary voltage 3.0 kV
Cone voltage 22 V
Source temperature 100 °C
Desolvation gas (N₂) 300 L/h (350 °C)
Cone gas flow (N₂) 20 L/h
Collision gas pressure (Ar) 4.04 x 10⁻³ mbar
Collision energy 22 eV
Dwell time 0.5 sec

PREPARATION OF STANDARD

A stock solution of the standard (100 mg/L) was prepared in acetonitrile, diluted with HPLC water (at least 6 concentrations) and used for calibration. For calibration ranges, see section 8.2.1.

PREPARATION OF SAMPLES

Test medium

To 0.1 mL MTS solution (0.2 mM in acetonitrile) 1.0 mL of the sample were added. After a reaction time of 10 min (MTS will be oxidized to MTSO) 0.1 mL TPP solution (0.1 mM in acetonitrile) were added. After another 10 min (in darkness) the solution was analysed via LC-MS/MS.

Stock solution

A specific volume of the stock solution (see table 20) was filled up to 1.0 mL with HPLC-water and added to 0.1 mL MTS solution (2 mM in acetonitrile). After a reaction time of 10 min, 0.1 mL TPP solution (1 mM in acetonitrile) were added. After another 10 min (in darkness) the solution was analysed via LC-MS/MS.

Table 21: Dilution Steps

Nominal test item concentration [mg/L]	Dilution factor	Sample volume [mL]	Final volume [mL]
0.875	1.2	1.0	1.2
2.92	1.2	1.0	1.2
8.75	1.2	1.0	1.2
29.2	12	0.1	1.2
87.5	12	0.1	1.2
4.375	1.2	1.0	1.2
14.6	12	0.1	1.2
43.75	12	0.1	1.2
146.0	60	0.02	1.2
437.5	60	0.02	1.2

SAMPLE STORAGE

All samples were analysed directly after sampling.

EVALUATION

Quantification of the test item was calculated by peak area of MTSO based on the external standard. The conversion factor to peracetic acid is 2.028 (MTSO 154.23 g/mol, peracetic acid 76.05 g/mol).

METHOD VALIDATION

Following SANCO 3029/99 rev.4 (2000-07-11)

Linearity	Linearity of detector response was checked by analysis of standards and plotting a calibration graph of peak area versus concentration. The coefficient of correlation was calculated.
Repeatability of injections	6 sub-samples of the highest and lowest concentration of the standard prepared from a single homogeneous sample were analysed. Mean values, standard deviations and variations of coefficients were calculated.
LOQ	The limit of quantification (LOQ) for peracetic acid (PAA) in water was defined as 7.54 µg/L (equivalent to 50.6 µg/L Peracetic acid and 15.3 µg/L MTSO) for the analytical method and checked by means of accuracy.
Accuracy (Fortified samples)	Five replicates of dilution water (see part 3) fortified at 1 x LOQ level and two blank samples were prepared and analysed.
Precision	Recovery rates should be between 70 and 110 %. Relative standard deviation should be lower than 20 %.
Specificity	Analysis of a specific mass trace (precursor / product ion) via LC-MS/MS. Blank samples were used to prove specificity and values being < 30 % of the LOQ.

8.2 Method Validation

8.2.1 Linearity

The analytical system gave linear response in the calibration ranges of 4.9 to 98 and 73.5 to 3136 µg MTSO/L, respectively (corresponding to 2.4 to 48.3 and 36.2 to 1546 µg PAA/L). The coefficients of regression (r^2) of the calibration curves were > 0.992. Representative calibration curves and chromatograms are given in Figure 1 – Figure 3.

8.2.2 Repeatability of Injections

The results of the repeatability tests are provided in the table below.

Table 22: **Repeatability of Injections of the Standard**
Peak area [counts]

Serial No.	MTSO	
	4.8 µg/L	3136 µg/L
1	782.54	330212.06
2	678.07	312958.19
3	725.45	315734.38
4	759.72	335096.41
5	730.73	324157.13
6	758.50	327089.06
Mean ± SD	739.17 ± 36.5	324208 ± 8500
CV [%]	4.9	2.6

SD = Standard deviation
CV = Coefficient of variation

8.2.3 Limit of Quantification (LOQ)

The limit of quantification for peracetic acid in dilution water was defined as 7.54 µg/L (equivalent to 50.6 µg/L Peracetic acid and 15.3 µg/L MTSO) for the analytical method and checked by means of accuracy.

8.2.4 Accuracy, Precision and Specificity

Accuracy was determined from fortified samples at LOQ level (Table 23).

Table 23: Recovery Rates of Fortified Samples of Peracetic Acid

Fortified concentration: 7.54 µg/L

Replicate	Peracetic Acid	
	Calc. conc. [µg/L]	RR [%]
1	7.74	103
2	7.35	97
3	7.64	101
4	8.04	107
5	7.99	106
Mean	7.75	103
SD	0.280	4.02
CV [%]	3.61	3.90

Calc. conc. = Calculated concentration (measured as MTSO)
RR = Recovery rate related to the fortified concentration
SD = Standard deviation
CV = Coefficient of variation

Specificity is given by analysing a specific mass trace (precursor / product ion) via LC-MS/MS-detection. Response of dilution water control samples were lower than 30 % of LOQ.

8.3 Results

8.3.1 Stock Solutions

Table 24: Concentrations and Recovery Rates of Peracetic Acid in the Stock Solutions

Nom. conc. [mg/L]	Test item	0.875		2.92		8.75		29.2		87.5	
	Peracetic acid	0.130		0.435		1.30		4.35		13.0	
Study day	Peracetic Acid										
	Calc. conc. [mg/L]	RR [%]									
-2	0.112	86	n.a.	--	1.10	84	n.a.	--	15.8	121	
-1	0.154	118	n.a.	--	1.52	117	n.a.	--	14.3	110	
0	0.119	92	n.a.	--	1.37	105	n.a.	--	12.6	97	
1	0.118	91	0.474	109	1.40	107	5.18	119	14.1	108	
2	0.181	139	0.518	119	1.36	104	5.67	130	14.6	112	
7	0.112	86	0.418	96	1.21	93	4.37	100	13.7	105	
9	0.144	111	0.492	113	1.33	102	4.38	101	13.2	101	
14	0.131	101	0.478	110	1.39	107	4.51	104	12.9	99	
Nom. conc. [mg/L]	Test item	4.38		14.6		43.8		146		438	
	Peracetic acid	0.652		2.18		6.52		21.8		65.2	
19	0.524	80	2.28	105	5.94	91	21.0	97	54.5	84	
20	0.556	85	2.28	105	7.33	112	24.7	113	72.4	111	
21	0.749	115	2.76	127	7.61	117	25.4	117	65.2	100	
27	0.331	51	1.78	82	7.64	117	29.5	135	79.4	122	
29	0.292	45	1.90	87	8.93	137	31.5	145	88.0	135	

Nom. conc. = Nominal concentration

Calc. conc. = Calculated concentration of peracetic acid in stock solutions (measured as MTSO)

RR = Recovery rate related to the nominal concentration

n.a. = not analysed

8.3.2 Concentrations of Peracetic Acid in Water

Table 25: Concentrations of Peracetic Acid in Water

Sample	Mixing chamber 150 µg/L				Replicate 150 µg/L				Control
	(with fish)		(without fish)		with fish		without fish		
Study day	Peracetic Acid								
	Calc. conc. [µg/L]	RR [%]	Calc. conc. [µg/L]	RR [%]	Calc. conc. [µg/L]	RR [%]	Calc. conc. [µg/L]	RR [%]	Calc. conc. [µg/L]
-1	< LOQ	--	< LOQ	--	< LOQ	--	< LOQ	--	n.a.
0	< LOQ	--	< LOQ	--	< LOQ	--	< LOQ	--	n.a.
1	< LOQ	--	< LOQ	--	< LOQ	--	< LOQ	--	(7.94) ¹⁾
7	9.63	69	12.3	81	< LOQ	--	< LOQ	--	< LOQ
9	< LOQ	--	< LOQ	--	< LOQ	--	< LOQ	--	< LOQ
14	< LOQ	--	< LOQ	--	< LOQ	--	< LOQ	--	< LOQ
15	< LOQ	--	< LOQ	--	< LOQ	--	< LOQ	--	< LOQ
20	12.1	54	13.1	58	< LOQ	--	< LOQ	--	< LOQ
21	10.9	49	17.3	77	< LOQ	--	< LOQ	--	< LOQ
27	18.4	82	21.2	95	8.88	40	9.76	44	< LOQ
29	15.6	70	19.3	86	7.50	33	9.17	41	< LOQ

Nom. conc. = Nominal concentration

Calc. conc. = Calculated concentration of peracetic acid in dilution water (measured as MTSO)

RR = Recovery rate related to the nominal concentration

n.a. = not analysed

¹⁾ = derivatizing product was analysed; no reaction product of PAA

8.4 Conclusions

The recovery rates of peracetic acid (PAA) in the stock solutions were mainly in a range of 80 to 120 % (Table 23). Recovery rates of PAA in mixing chambers and replicates were mostly < LOQ. After transfer of juveniles in larger aquaria recovery rates of PAA in mixing chambers increased due to the higher flow rates. High reactivity of PAA is considered the reason for the low recoveries.

8.5 Calibration Curves

Compound name: MTSO
Correlation coefficient: $r = 0.999840$, $r^2 = 0.999680$
Calibration curve: $130.876 * x + -8.51778$
Response type: External Std, Area
Curve type: Linear, Origin: Include, Weighting: 1/x, Axis trans. None

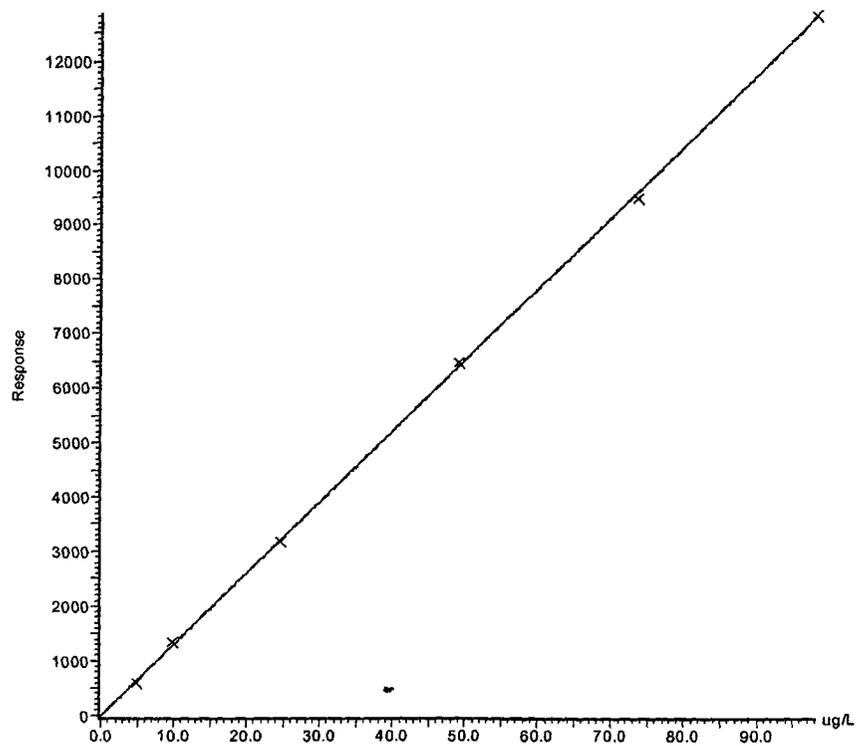


Figure 1: Calibration Curve of the Standard (Range 4.9 to 98 $\mu\text{g/L}$ MTSO) (dated 2006-07-18)

Compound name: MTSO
Correlation coefficient: $r = 0.999933$, $r^2 = 0.999866$
Calibration curve: $114.387 * x + 559.556$
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None

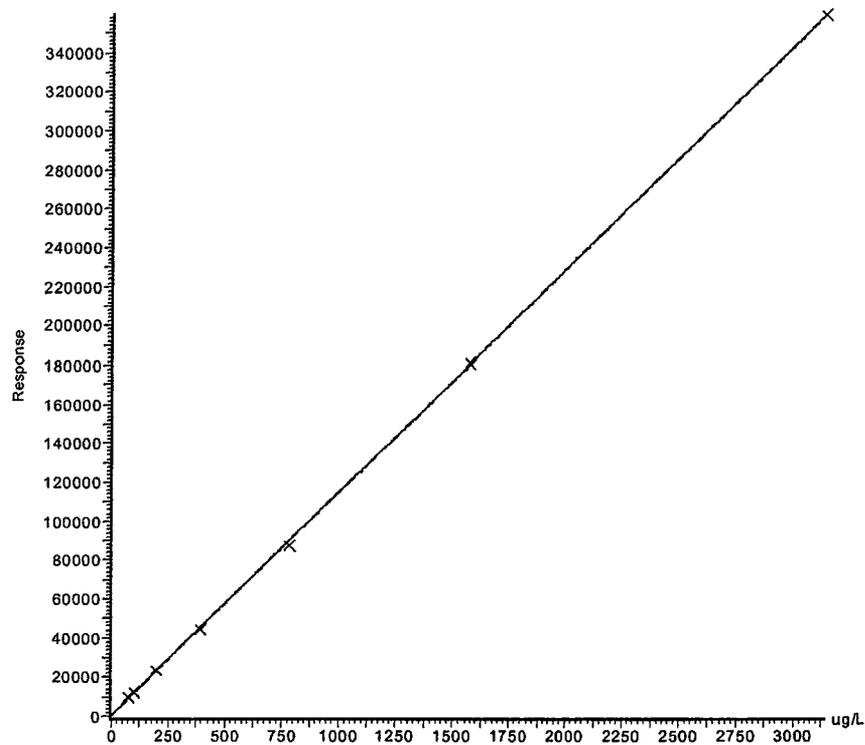


Figure 2: **Calibration Curve of the Standard (Range 73.5 to 3136 $\mu\text{g/L}$ MTSO)**
(dated 2006-07-18)

8.6 Chromatograms

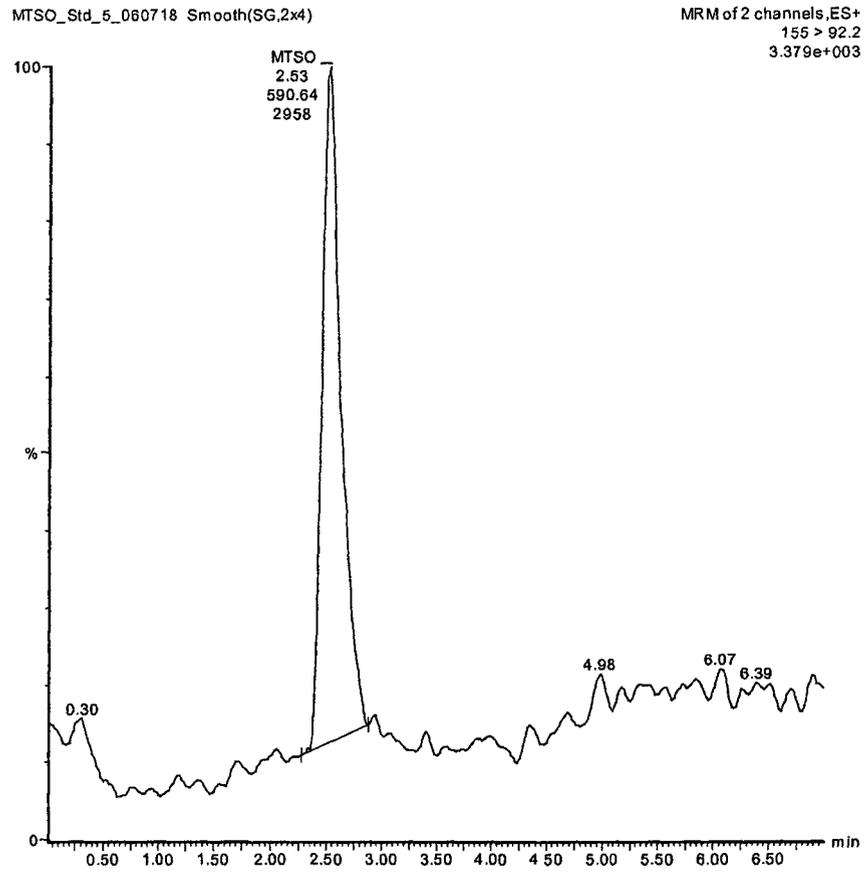


Figure 3: **Chromatogram of the Standard (MTSO)**
4.9 µg/L (dated 2006-07-18)

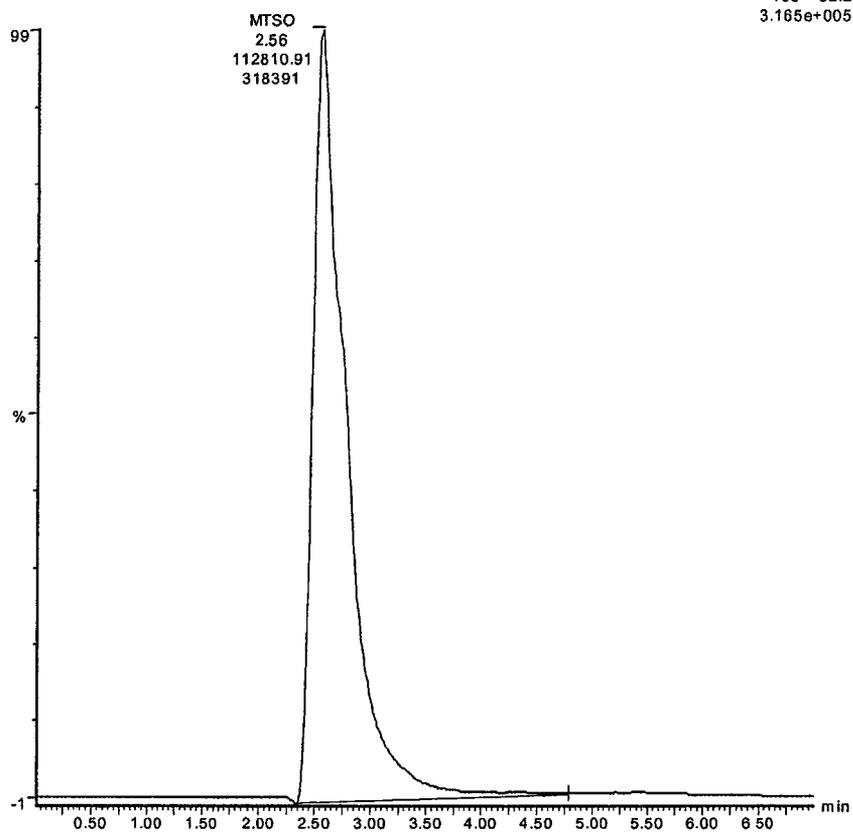


Figure 4: **Chromatogram of the Test Item (Stock Solution, d 20)**
4.38 mg/L (dated 2006-08-08)

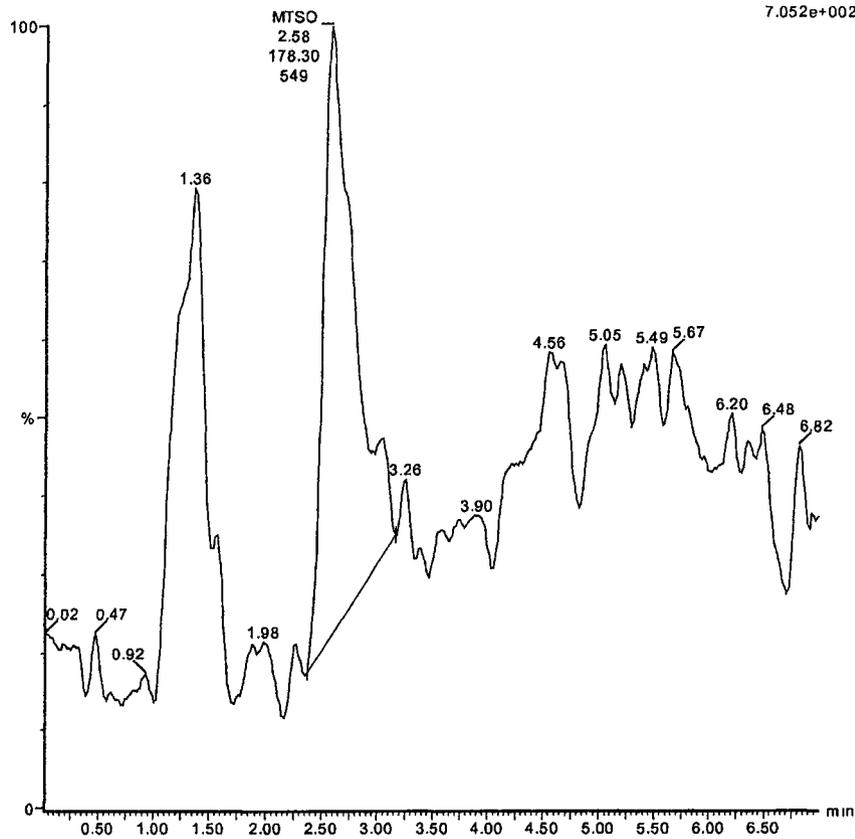


Figure 5: Chromatogram of the Control (d 20)
(dated 2006-08-08)

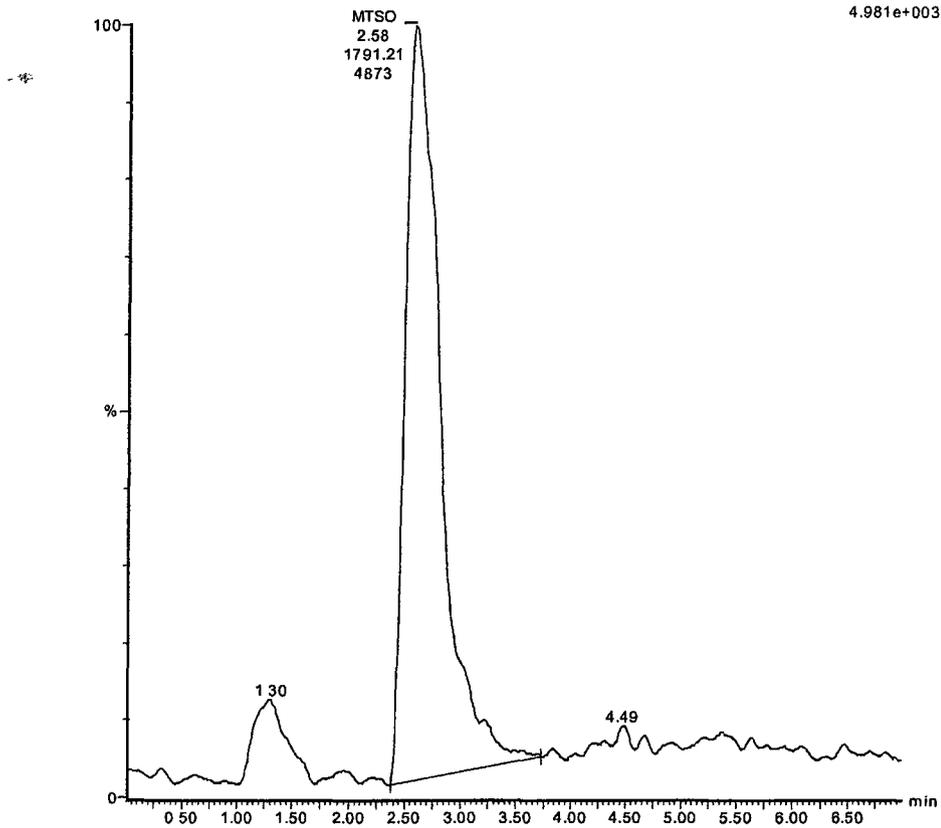


Figure 6: **Chromatogram of the Test Item (Mixing Chamber with Fish, d 20)**
(dated 2006-08-08)

FSZ109021_150ugL_mit Fischen_0.2mM-MTS_d20_060808_inj1 Smooth(SG.2x4) MRM of 2 channels, ES+
155 > 92 2
1.037e+003

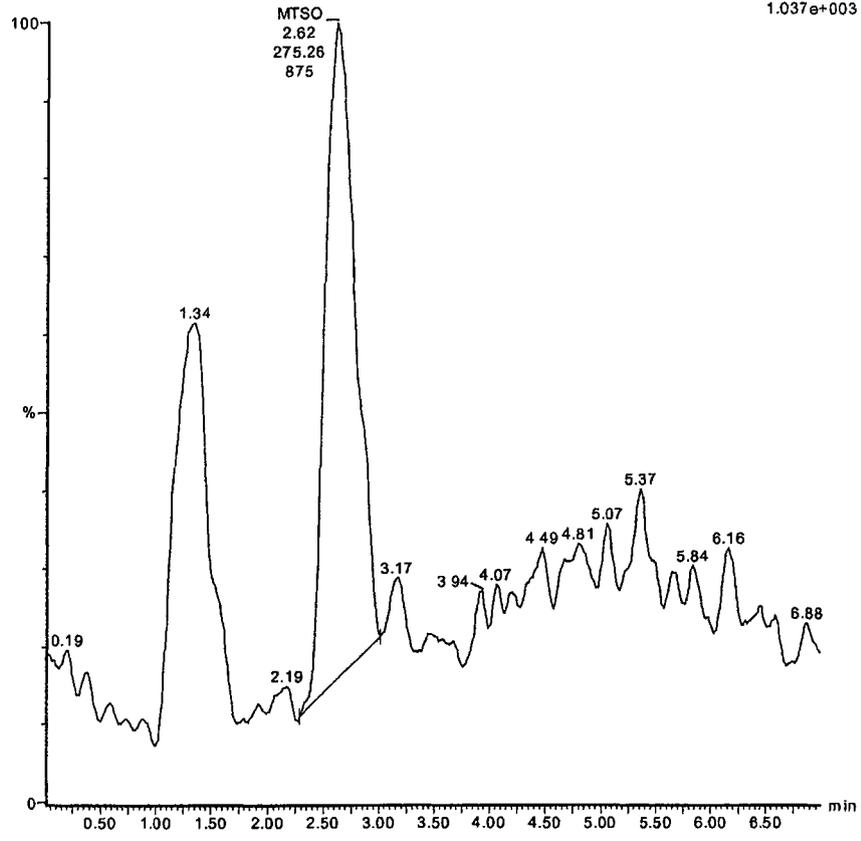


Figure 7: Chromatogram of the Test Item (150 µg/L with Fish, d 20)
(dated 2006-08-08)

9 Spectrophotometric Analysis of Hydrogen Peroxide

9.1 Method

PRINCIPLE

Analysis of various concentrations of peracetic acid (PAA), active ingredient of Peracetic acid in water was carried out. The quantitative reaction of H_2O_2 with potassium bis(oxalato)oxotitanate(IV) yields a yellowish peroxide-titan-complex with a maximum of absorption at 385 nm. Analytical evaluation of the complex was done spectrophotometrically using external standards. Due to the sensitivity of the method only the stock solutions for the test could be analysed.

Equipment

Spectral photometer CADAS 100 LPG 158, DR. LANGE

Reagents

HPLC water, J.T. BAKER
Oxalic acid, RIEDEL DE HAEN

Titan-reagent

Potassium bis(oxalato)oxotitanate(IV), 99.998 %, ALFA AESAR
0.5 g of potassium bis(oxalato)oxotitanate(IV) and 0.75 g of oxalic acid were dissolved in warm HPLC water and filled up to 25 mL (every sampling day).

Standard

Hydrogen peroxide, 31.0 %, density 1.117 g/mL, Batch: 1153294, FLUKA

CONDITIONS OF ANALYSIS

Wavelength

385 nm

PREPARATION OF STANDARD

The standard was diluted 1 : 5000 with HPLC water in two steps to reach a concentration of 69 mg/L (stock solution). To 25 mL of the stock solution 1.25 mL of titan-reagent was added. After a reaction time of at least 5 min. dilutions were carried out with HPLC water (6 concentrations) and used for calibration. For calibration range, see section 9.2.1.

PREPARATION OF STOCK SOLUTIONS

To 25.0 mL of the final volume (Table 25) 1.25 mL titan-reagent was added. After a reaction time of at least 5 min. the solution was spectrophotometrically analysed.

Table 26: Dilution Steps of the Stock Solutions

Nominal concentration [mg/L]	Dilution factor	Sample volume [mL]	Final volume [mL]
0.875	1	25.0	25.0
2.92	1	25.0	25.0
8.75	1	25.0	25.0
29.2	5	5.0	25.0
87.5	10	10.0	25.0
4.38	1	25.0	25.0
14.6	5	5.0	25.0
43.8	5	5.0	25.0
146	20	1.25	25.0
438	20	1.25	25.0

SAMPLE STORAGE

Samples were stored at -20 ± 2 °C until start of analysis, if necessary.

EVALUATION

Quantification of the active ingredient hydrogen peroxide from Peracetic acid was calculated by extinction based on the external standard (Peroxide-Titan-Complex).

METHOD VALIDATION

Following SANCO 3029/99 rev.4 (2000-07-11)

Linearity

Linearity of spectrophotometrical response was checked by analysis of standards and plotting a calibration graph of extinction versus concentration. The coefficient of correlation was calculated.

Repeatability

6 sub-samples of the highest and lowest concentration of the standard prepared from a single homogeneous sample were analysed. Mean values, standard deviations and variations of coefficients were calculated.

9.3 Results

Table 29: Concentrations and Recovery Rates of Hydrogen Peroxide in Stock Solutions

Nom. conc. [mg/L]	Test item	0.875		2.92		8.75		29.2		87.5	
	Hydrogen peroxide	0.127		0.423		1.27		4.23		12.7	
		Hydrogen Peroxide									
Study day		Meas. conc. [mg/L]	RR [%]								
	-2	0.154	121	n.a.	--	1.40	110	n.a.	--	14.0	110
	-1	0.191	150	n.a.	--	1.49	90	n.a.	--	13.8	72
	0	0.121	95	n.a.	--	1.35	107	n.a.	--	15.1	119
	1	0.104	82	0.455	108	1.30	102	4.63	109	13.6	107
	7*	0.102	80	0.400	95	1.34	106	4.29	101	13.1	103
	9*	0.091	72	1.00	236	1.28	101	4.46	105	13.2	104
	14	0.129	102	0.460	109	1.36	107	4.53	107	13.4	106
	15	0.210	165	0.540	128	1.44	113	4.99	118	14.4	113
Nom. conc. [mg/L]	Test item	4.38		14.6		43.8		146		438	
	Hydrogen peroxide	0.634		2.12		6.34		21.2		63.4	
	19	0.818	129	3.20	151	8.02	126	26.5	125	79.5	125
	20	0.738	116	2.55	120	6.69	106	22.8	108	58.8	93
	21	0.866	137	3.02	143	7.44	117	25.4	120	62.1	98
	27*	0.576	91	2.23	105	6.40	101	21.8	103	64.0	101
	29*	0.544	86	2.60	123	7.15	113	24.0	113	72.5	114

- Nom. conc. = Nominal concentration
 Meas. conc. = Measured concentration of peracetic acid in stock solutions
 RR = Recovery rate related to the nominal concentration
 n.a. = not analysed
 *) = After evaluation these raw data were erroneously destroyed.

9.4 Conclusions

Hydrogen peroxide concentrations in the stock solutions were mainly in the expected range (80 – 120 % of nominal). Analysis of Hydrogen peroxide in the mixing chambers and the replicates could not be carried out due to the low sensitivity of the analytical method.

9.5 Calibration Curve

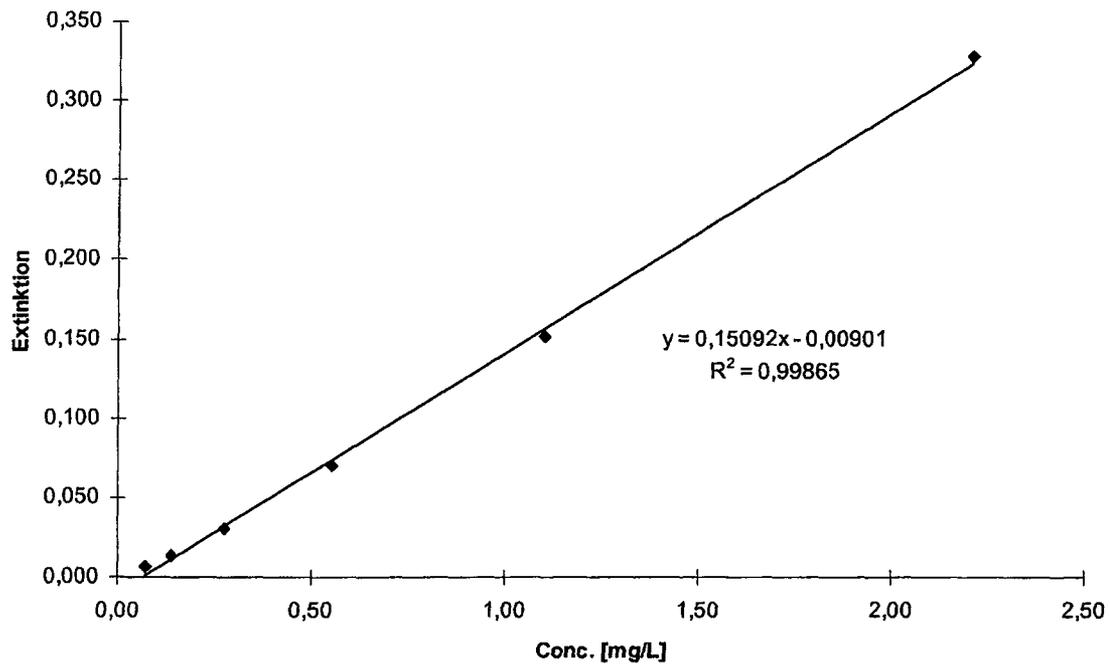


Figure 8: Calibration Curve of the Standard
(dated 2006-07-19)

Table 33: Length Raw Data from Study Day 33

Nominal Concentrations [µg/L]	Repl.	Length of Individual Fish in [mm]														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Control	1	17.0	20.0	12.5	19.0	16.5	14.5	17.0	17.5	17.0	12.5	17.0	11.5	-	-	-
	2	12.5	17.5	17.5	17.5	18.0	17.0	15.0	12.5	16.5	14.0	15.0	11.5	15.0	14.5	14.0
	3	20.0	19.0	14.5	14.5	11.0	19.0	18.0	12.0	11.0	13.0	15.5	15.0	18.5	-	-
	4	22.0	20.0	14.0	14.0	16.0	16.0	13.0	16.0	13.0	16.5	16.0	17.0	12.0	-	-
1.5	1	15.0	15.0	19.0	15.0	18.5	20.0	15.5	14.0	15.0	16.0	14.0	19.0	20.0	15.5	-
	2	20.0	15.0	16.0	16.5	15.5	12.5	18.5	15.0	20.5	13.0	16.0	16.5	-	-	-
	3	12.5	16.5	15.5	16.5	18.5	16.0	21.0	19.5	14.0	14.5	17.0	15.5	-	-	-
	4	16.5	16.5	20.0	16.0	14.0	16.0	18.0	15.0	17.5	19.5	16.0	17.0	-	-	-
5	1	18.0	16.5	20.0	13.0	18.0	15.0	16.5	16.0	11.5	18.0	17.5	16.0	18.5	-	-
	2	17.5	17.0	17.0	17.0	20.5	18.0	13.0	14.0	19.0	17.5	15.5	-	-	-	-
	3	12.0	18.5	15.5	14.5	17.0	15.0	16.5	15.0	15.5	14.0	17.0	17.0	18.0	15.0	16.5
	4	18.0	17.0	13.0	15.5	15.0	16.0	18.0	19.0	16.0	17.0	15.0	16.0	14.0	-	-
15	1	16.5	14.0	14.0	18.0	14.0	18.5	14.0	18.5	17.0	14.0	17.0	16.0	18.0	-	-
	2	21.0	19.0	18.5	19.0	13.5	16.5	16.0	14.0	15.5	17.5	14.5	-	-	-	-
	3	10.0	14.0	20.0	18.0	17.5	12.5	14.5	12.0	17.0	20.0	17.5	-	-	-	-
	4	17.5	17.0	12.5	18.5	15.0	11.5	15.0	14.0	17.0	19.5	14.0	17.0	20.0	-	-
50	1	15.0	16.0	17.5	19.0	19.0	16.0	19.5	18.0	19.0	-	-	-	-	-	-
	2	15.0	17.5	16.0	16.0	17.0	18.0	16.5	18.0	19.0	17.0	-	-	-	-	-
	3	17.5	19.0	15.0	17.0	15.0	12.0	14.5	16.0	18.0	17.0	-	-	-	-	-
	4	13.5	18.0	18.0	16.0	12.0	18.5	19.5	20.0	16.0	16.5	-	-	-	-	-
150	1	19.0	18.0	17.5	20.0	16.0	19.0	18.0	18.0	18.0	-	-	-	-	-	-
	2	17.0	18.0	18.0	15.5	13.0	15.5	19.5	19.0	15.5	-	-	-	-	-	-
	3	15.0	20.0	20.0	17.0	18.0	16.0	15.0	19.0	17.5	18.0	-	-	-	-	-
	4	9.5	18.0	15.5	15.0	14.5	18.0	19.0	13.5	19.0	19.5	-	-	-	-	-

- = Fish died before end of the study

Table 34: Dry Weight Raw Data from Study Day 33

Nominal Concentrations [µg/L]	Repl.	Dry Weight of Individual Fish in [mg]														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Control	1	7.9	16.8	2.9	12.8	7.9	4.2	8.1	9.7	9.4	1.7	9.1	1.8	-	-	-
	2	3.7	10.6	9.1	9.8	11.1	7.5	5.3	2.0	7.1	4.0	4.6	1.6	3.5	4.0	4.2
	3	18.5	13.0	4.5	4.7	3.4	12.2	10.8	2.0	1.7	3.0	5.7	6.1	10.6	-	-
	4	23.1	15.0	3.8	4.9	6.8	6.9	3.0	9.0	2.9	7.7	7.1	11.1	4.5	-	-
1.5	1	5.1	3.8	15.7	4.8	11.3	18.3	5.9	3.4	4.3	6.7	4.8	14.8	13.0	5.8	-
	2	15.2	5.9	15.9	7.3	4.9	2.4	11.2	4.5	17.9	3.9	6.6	8.8	-	-	-
	3	2.9	8.1	6.1	6.9	12.2	6.2	20.7	16.3	3.8	6.0	8.7	6.5	-	-	-
	4	6.7	7.0	17.9	6.3	4.0	7.5	8.6	5.5	8.7	12.8	5.6	8.2	-	-	-
5	1	9.8	6.4	13.4	2.8	11.1	5.0	6.8	5.3	1.3	10.6	10.1	7.1	13.0	-	-
	2	8.6	7.7	7.9	8.2	19.2	12.2	3.9	4.5	13.8	10.1	6.1	-	-	-	-
	3	3.5	12.0	6.9	6.2	8.5	6.4	27.2	6.0	7.4	5.4	7.9	9.3	9.6	5.4	8.0
	4	11.6	9.3	3.9	6.6	6.4	7.4	11.0	12.7	6.1	9.3	6.0	6.9	4.7	-	-
15	1	7.8	5.6	4.5	15.4	4.8	11.4	4.2	13.2	9.6	4.6	10.6	7.6	10.0	-	-
	2	18.7	14.0	12.9	11.6	4.1	7.1	6.5	4.3	7.7	9.5	5.6	-	-	-	-
	3	2.1	6.4	15.9	9.8	9.9	8.6	5.3	2.7	9.4	16.6	10.2	-	-	-	-
	4	7.9	8.6	2.3	11.1	4.7	1.2	4.2	4.0	9.5	15.9	4.3	7.1	16.3	-	-
50	1	5.8	6.0	8.0	12.6	13.9	6.6	13.2	11.8	13.4	-	-	-	-	-	-
	2	4.7	8.8	6.6	7.3	7.6	11.2	6.9	11.0	13.2	8.9	-	-	-	-	-
	3	8.5	12.5	4.9	8.8	5.3	2.3	4.4	6.3	11.8	10.6	-	-	-	-	-
	4	4.6	9.3	10.9	7.2	2.5	11.8	15.1	14.3	7.1	7.7	-	-	-	-	-
150	1	12.2	10.9	9.2	16.7	6.6	11.4	9.4	11.4	10.3	-	-	-	-	-	-
	2	8.1	10.9	10.0	5.0	2.9	5.4	13.4	12.6	5.3	-	-	-	-	-	-
	3	4.6	15.2	15.9	11.4	9.2	6.9	4.8	11.1	8.7	9.8	-	-	-	-	-
	4	0.9	10.4	5.2	4.9	4.5	10.3	10.3	4.2	13.7	14.6	-	-	-	-	-

Table 35: Wet Weight Raw Data from Study Day 33 of the Control

	Repl.	Wet Weight Individual Fish in [mg]														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Control	1	32.4	63.4	10.8	49.5	31.1	17.0	35.3	40.5	39.2	10.1	32.0	8.3	-	-	-
	2	13.6	37.3	38.9	36.7	42.5	30.4	20.8	9.6	30.6	15.5	21.2	9.0	18.6	16.8	14.7
	3	67.2	53.2	17.8	21.0	8.5	47.7	39.6	8.7	7.7	13.8	23.1	22.0	44.5	-	-
	4	82.4	55.7	19.8	20.3	24.3	30.1	12.7	35.0	12.1	28.9	25.1	37.8	-	-	-