CERI

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TEST REPORT

A 96-hour Acute Toxicity Study of perfluorohexanoic acid, ammonium salt in Medaka

March, 2016

Chemicals Evaluation and Research Institute, Japan, Kurume

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1. Title

A 96-hour Acute Toxicity Study of perfluorohexanoic acid, ammonium salt in Medaka

2. Sponsor

Name	DAIKIN INDUSTRIES, LTD.
Address	1-1, Nishi-Hitotsuya, Settsu-shi, Osaka 566-8585, Japan

3. Test facility

Name	Chemicals Evaluation and Research Institute, Japan, Kurume (CERI Kurume)
Address	3-2-7 Miyanojin, Kurume-shi, Fukuoka 839-0801, Japan

4. Objective

The objective of this study is to determine the 96-hour median lethal concentration (LC_{50}) by conducting an acute toxicity study of the perfluorohexanoic acid, ammonium salt in Medaka..

5. Test method

This test was carried out referring to the OECD Guidelines for Testing of Chemicals, No.203, July 17, 1992, "Fish, Acute Toxicity Test".

6. Dates

Study initiation date	February 26, 2016
Experimental starting date	February 29, 2016
Experimental completion date	March 4, 2016
Study completion date	March 24, 2016

7. Approval of final report

Date

Study Director

8. Summary

Test item

Perfluorohexanoic acid, ammonium salt

Objective

The objective of this study is to determine the 96-hour median lethal concentration (LC_{50}) by conducting an acute toxicity study of the perfluorohexanoic acid, ammonium salt in Medaka.

Test method

This test was carried out referring to the OECD Guidelines for Testing of Chemicals, No.203, July 17, 1992, "Fish, Acute Toxicity Test".

Test conditions

Test organism	Medaka (*Oryzias latipes*)
Dilution water	Dechlorinated tap water
Test concentration	3500, 2500, 1790, 1280, 911 mg/L as nominal concentrations (a geometric series with a factor of 1.4) and a control
Preparation of test solution	Required amount of the test sample and dilution water were mixed and stirred in test vessel to prepare the test solution.
Type of test	Static regime
Exposure duration	96 hours
Replicate	1 replicate/test level
Number of organism	7 fish/test level
Volume of test solution	Approximately 2.0 L/test level
Temperature of test solution	23.5-24.3°C
Aeration	Conducted gently
Lighting condition	Room light, 16-hour light/8-hour dark
Feeding	No feeding
Analysis of concentration of test item in test solution	HPLC analysis (at the start and end of exposure or at the time that all test organisms were confirmed to be dead)

Results

96-hour LC_{50}	1850 mg/L
The minimum concentration causing 100% mortality at 96 hours	2500 mg/L
The maximum concentration causing 0% mortality at 96 hours	1280 mg/L

(The above-mentioned concentrations are based on nominal concentrations.)

9. Test materials

9.1 Test item

a) Chemical name etc.

Chemical name	2,2,3,3,4,4,5,5,6,6,6-undecafluorohexanoic acid, ammonium salt
Another name	Perfluorohexanoic acid, ammonium salt (PFHxA-NH_4)
CAS number	21615-47-4

b) Chemical structure etc.

Rational fomula	$CF_3CF_2CF_2CF_2CF_2COONH_4$
Molecular formula	$C_6F_{11}H_4NO_2$
Molecular weight	331.08

c) Test sample

Name	PFHxA-NH_4-50
Purity of test item	50 mass%
Impurity	Water 50 mass%
Supplier	DAIKIN INDUSTRIES, LTD.
Lot number	C150E57002

The test sample was treated with correcting by the purity of the test item.

d) Physicochemical properties

Water solubility	>500 g/L
Appearance	Colorless and clear liquid

e) Storage condition

The test sample was stored in a dark storage place at room temperature.

f) Safety and handling

In order to avoid inhalation and contact with the skin and eyes, chemically resistant gloves, mask, safety glasses, and white coats were worn when handling test sample.

9.2 Test organisms

Species	Medaka (*Oryzias latipes*)
Reason for selection of species	Species recommended in the test guideline
Supplier	CERI Kurume (in-laboratory production)
Size	Total length 2.0±1.0 cm
Allocation	Medaka was allocated at random to each test group.

Acclimation

Hatching date	October 14, 2015 (age at the start of exposure; four-month-old)
Water	Dechlorinated tap water
Acclimation type	Flow-through regime
Dissolved oxygen concentration	Not less than 80% of air saturation value
Temperature	24±1°C
Photoperiod	16-hour light/8-hour dark with room light
Feed	Feed of Medaka for education (KYORIN)
Feeding amount and frequency	Amount corresponding to 3% of body weight was fed every day.
Use of medicament for external disinfection	None

Duration of acclimation	24 days (February 5, 2016 to February 29, 2016)
Mortality of test groups	0 % (during 7 days before the start of exposure)
Feed withdrawal	24 hours before the start of exposure

10. Test methods

10.1 Dilution water

Dechlorinated tap water, aerated sufficiently and temperature-controlled, was used. Some chemical characteristics of the dilution water measured regularly are listed in Appendix 1. The result of chemical characteristics of the dilution water filled the standard that provided with the standard operating procedure of this laboratory.

10.2 Test apparatus and equipment

Test vessel	3 L glass tank (diameter: 16 cm, depth: 16 cm)
Cover on test vessel	Transparent plastic lid
Water bath	Plastic tank
	Warming/cooling unit; Type HCA 250 (Sato craft)

10.3 Preparation of test solution

Required amount of the test sample and dilution water were mixed and stirred in test vessel to prepare the test solution.

10.4 Test conditions

Type of test	Static regime (no renewal of test solution)
Exposure duration	96 hours
Test concentration	3500, 2500, 1790, 1280, 911 mg/L as nominal concentration (a geometric series with a factor of 1.4)
Control	Dilution water without the test item
Replicate	1 replicate/test level
Number of organisms	7 fish/test level
Volume of test solution	Approximately 2.0 L/test level
Temperature of test solution	24±1°C
Aeration	Conducted gently
pH adjustment	No adjustment
Lighting condition	Room light, 16-hour light/8-hour dark
Feeding	No feeding

10.5 Observation and measurements

a) Observation of test organisms

Mortality and visible abnormality were observed at 3, 24, 48, 72 and 96 hours after the start of exposure. A fish was considered as dead if the observable motion (motion of mouth and opercula etc.) were not observed and touching of the caudal peduncle with glass rod produced no reaction. The dead test organisms were removed immediately.

b) Total length and body weight of test organism

The test organisms in the control group were used for measuring total length and body weight after the end of exposure.

c) Appearance of test solution

Appearance of the test solutions was observed at the start and end of exposure or at the time that all test organisms were confirmed to be dead.

d) Condition of test solutions

Item of measurement Dissolved oxygen concentration, pH and temperature

Frequency of measurement

At the start and end of exposure or at the time that all test organisms were confirmed to be dead

Method for measurement

The test solution for measurement was taken out from the test vessels.

Instrument Dissolved oxygen meter YSI Model 58 (YSI Nanotech Japan)
pH meter Model HM-21P (DKK-TOA)
Thermometer of glass stick type

e) Test item concentration in test solution

Frequency of measurement

At the start and end of exposure or at the time that all test organisms were confirmed to be dead

Sampling for measurement

The test solution for analysis was taken out from the middle layer of each test vessel.

Sampling volume Approximately 10 mL (all test levels)

Analytical condition Shown in Appendix 2

10.6 Calculating method of LC_{50}

The LC_{50} values were calculated by Binomial test.

The LC_{50} was estimated using Computer Program (running on Microsoft software "Excel") developed by our laboratory.

The results of this study were estimated based on nominal concentrations since the measured concentration of test item in test solution of all exposure levels were maintained within the range of ±20% of the nominal concentrations during exposure.

10.7 Validity of test

a) The mortality in the control should not exceed one fish.

b) Dissolved oxygen concentration must be at least 60% of the air saturation value at the water temperature throughout exposure duration.

10.8 Treatment of numerical values

Values were rounded off in accordance with JIS Z 8401 rule B, 1999.
(JIS; Japanese Industrial Standards)

11. Results and discussion

11.1 Mortality

Cumulative mortality of each observation time and concentration-cumulative mortality curve are shown in Table 1 and Figure 1.

100% mortality was confirmed at 24 hours after start of exposure in 2500 and 3500 mg/L.

Minimum concentration causing 100% mortality at 96 hours was 2500 mg/L. Maximum concentration causing no mortality at 96 hours was 1280 mg/L. Number of dead fish in the control at the end of exposure was 0, which met the criterion for the validity of the test (i.e. not exceed one fish).

11.2 Observed performance status etc.

The abnormal responses observed during the exposure are shown in Table 2.

No abnormal response was obtained in the control.

The following results of observation were based on the comparison with the control organisms. Observed abnormal responses during exposure were at the water surface, partial loss of equilibrium and reduced activity.

11.3 Size of test organism

[Mean ± Standard deviation (n=7)]

Total length 2.6±0.3 cm

Body weight 0.15±0.06 g

11.4 Observation and measurement of test solution

a) Appearance of test solution

The test solutions in all exposure levels were colorless and clear at the start of exposure, and a little bubbles were observed. The test solutions in exposure levels of 2500 and 3500 mg/L were colorless and clear at the time that all test organisms were confirmed to be dead, and a little bubbles were also observed. The test solutions in other exposure levels were slightly white suspended at the end of exposure.

The test solution in the control was colorless and clear at the start and end of exposure.

b) Condition of test solutions

Condition of the test solutions are shown in Tables 3-1, 3-2 and 3-3.

The measured values of dissolved oxygen concentration, pH and temperature during exposure ranged from 7.4 to 8.2 mg/L, 7.4 to 7.8 and 23.5 to 24.3°C, respectively. The measured values of dissolved oxygen concentration met the criterion for the study validity (at least 60% or more of saturate concentration* at the water temperature).

* Saturated dissolved oxygen concentration (23 - 25°C): 8.39 - 8.11 mg/L (JIS K 0102, 2013)

c) Concentration of test item in test solution

The results of the measured concentrations of the test item are shown in Appendix 2. Calibration curve and chromatogram are shown in Appendix 3. The measured concentrations of the test item in the test solutions were 945-3610 mg/L (102-106% of the nominal concentrations) at the start of exposure and 1010-3680 mg/L (104-111% of the nominal concentrations) at the end of exposure (including the time that all test organisms were confirmed to be dead), and kept within ± 20% of the nominal concentrations.

11.5 LC_{50}

The LC_{50}s at every 24 hours are shown in Table 4.

The 48 and 96-hour LC_{50}s of the test item for Medaka were 2020 mg/L and 1850 mg/L.

11.6 Discussion

This study was conducted in order to confirm the effect of the test item on the test organisms below the solubility of the test item in dilution water. As a result, 96-hour LC_{50} was 1850 mg/L. The test item concentrations in the test solution were maintained within ±20% of the nominal concentrations and the environmental conditions were within the suitable range, therefore, it was concluded that this study complied with the applied test guideline.

Table 1 Cumulative mortality

Nominal concentration (mg/L)	Cumulative mortality (%)				
	3 hours	24 hours	48 hours	72 hours	96 hours
Control	0	0	0	0	0
911	0	0	0	0	0
1280	0	0	0	0	0
1790	0	0	14	29	43
2500	14	100	100	100	100
3500	14	100	100	100	100

Table 2 Observed abnormal response

Nominal concentration (mg/L)	Result of observation (Left column: Number of affected fish/Total survival number, Right column: Symptom detail)									
	3 hours		24 hours		48 hours		72 hours		96 hours	
Control	0/7	N	0/7	N	0/7	N	0/7	N	0/7	N
911	0/7	N	0/7	N	0/7	N	0/7	N	0/7	N
1280	0/7	N	0/7	N	1/7	AS(1) PLE(1)	1/7	AS(1) PLE(1)	1/7	AS(1) PLE(1)
1790	0/7	N	0/7	N	5/6	AS(1) RA(1)	0/5	N	0/4	N
2500	2/6	AS(2)	-	-	-	-	-	-	-	-
3500	4/6	AS(4)	-	-	-	-	-	-	-	-

N: Normal (No abnormal response)

- : No observation due to 100% mortality

Value in parentheses expresses the number of individuals that showed the symptom.

Abbreviation of symptoms

AS : At the surface

PLE : Partial loss of equilibrium

RA : Reduced activity

Table 3-1 Dissolved oxygen concentration of test solutions

Nominal concentration (mg/L)	At the start	24 hours	48 hours	72 hours	At the end
Control	8.1	7.9	7.7	8.0	8.2
911	8.0	7.9	7.8	8.0	8.1
1280	8.0	7.8	7.7	8.0	8.1
1790	8.0	7.9	7.4	8.0	8.1
2500	8.0	7.9	-	-	-
3500	8.0	7.7	-	-	-

Unit: mg/L

- : No measurement due to 100% mortality

Table 3-2 pH of test solutions

Nominal concentration (mg/L)	At the start	24 hours	48 hours	72 hours	At the end
Control	7.8	7.6	7.6	7.6	7.7
911	7.7	7.7	7.6	7.7	7.6
1280	7.6	7.7	7.6	7.7	7.6
1790	7.4	7.6	7.5	7.6	7.6
2500	7.4	7.7	-	-	-
3500	7.4	7.6	-	-	-

- : No measurement due to 100% mortality

Table 3-3 Temperature of test solutions

Nominal concentration (mg/L)	At the start	24 hours	48 hours	72 hours	At the end
Control	24.0	24.0	24.3	24.2	23.5
911	24.0	24.0	24.3	24.2	23.5
1280	24.0	24.0	24.3	24.2	23.5
1790	24.0	24.0	24.3	24.2	23.5
2500	24.0	24.0	-	-	-
3500	24.0	24.0	-	-	-

Unit: °C

- : No measurement due to 100% mortality

Table 4 LC_{50} to Medaka

Exposure duration	LC_{50} (mg/L)	95% confidence limits (mg/L)	Statistical procedure used for determination of LC_{50}
24-hour	2120		Binomial test
48-hour	2020		Binomial test
72-hour	1940		Binomial test
96-hour	1850		Binomial test

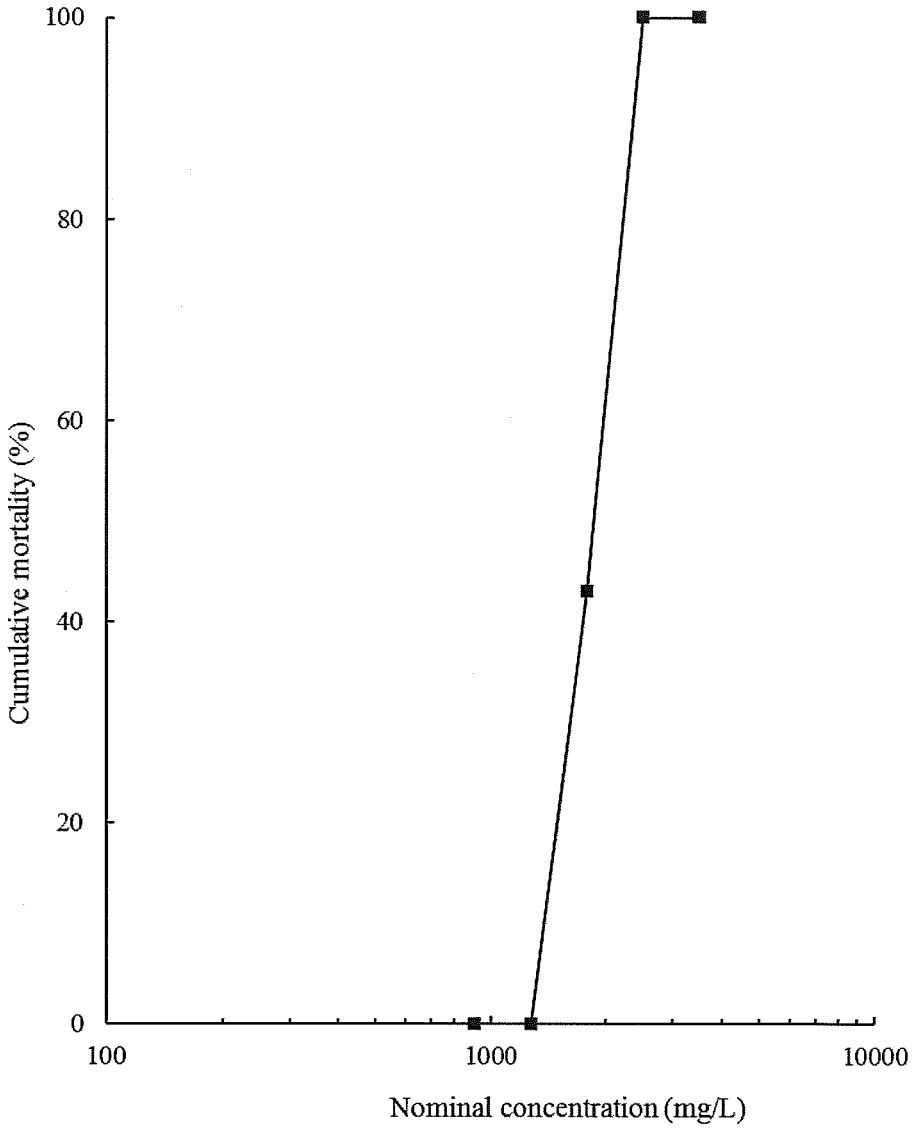


Figure 1 Concentration-cumulative mortality curve at 96 hours.

Appendix 1

Chemical characteristics of dilution water

Chemical characteristics of dilution water (Sampling on January 5, 2016)

Parameter	Unit	Results	Determination limit
Total hardness (as $CaCO_3$)	mg/L	37	1
Suspended solid	mg/L	<1	1
pH	-	7.7 (23.5°C)	-
Total organic carbon	mg/L	<0.5	0.5
Chemical oxygen demand	mg/L	<1	1
Residual chlorine	mg/L	<0.02	0.02
Ammonium ion	mg/L	<0.1	0.1
Total cyanide	mg/L	<0.05	0.05
Alkalinity	mg/L	38	1
Electric conductivity	mS/m	15	0.1
Total mercury	mg/L	<0.0005	0.0005
Cadmium	mg/L	<0.001	0.001
Chromium (VI)	mg/L	<0.01	0.01
Lead	mg/L	<0.001	0.001
Arsenic	mg/L	<0.005	0.005
Iron	mg/L	<0.01	0.01
Copper	mg/L	<0.001	0.001
Cobalt	mg/L	<0.001	0.001
Manganese	mg/L	<0.005	0.005
Aluminum	mg/L	<0.02	0.02
Zinc	mg/L	<0.1	0.1
Nickel	mg/L	<0.001	0.001
Silver	mg/L	<0.0001	0.0001
1,2-dichloropropane	mg/L	<0.002	0.002
Chlorothalonil	mg/L	<0.001	0.001
Propyzamide	mg/L	<0.0008	0.0008
Chlornitrofen	mg/L	<0.0001	0.0001
Simazine	mg/L	<0.0003	0.0003
Thiobencarb	mg/L	<0.001	0.001
Diazinon	mg/L	<0.0005	0.0005
Isoxathion	mg/L	<0.0008	0.0008
Fenitrothion	mg/L	<0.0003	0.0003
EPN	mg/L	<0.0006	0.0006
Dichlorvos	mg/L	<0.001	0.001
Iprobenfos	mg/L	<0.0008	0.0008
PCB	mg/L	<0.0005	0.0005
Boron	mg/L	<0.1	0.1
Fluorine	mg/L	0.5	0.1
Sulfate ion	mg/L	14	0.5
Chloride ion	mg/L	11	0.2
Sodium	mg/L	13	0.2
Potassium	mg/L	3.2	0.2
Calcium	mg/L	11	0.1
Magnesium	mg/L	2.6	0.1

Appendix 2

Analytical method and measured concentration of test item

1. Pretreatment of test solution

The collected test solutions were used as the samples for high-performance liquid chromatography (HPLC) without treatment or after appropriate dilution with dechlorinated tap water.

2. Determination of test item

a) Method of determination

Determination of test item was conducted by absolute calibration curve method using one concentration of standard solution.

The calibration curve was drawn by using four standard solutions of 10.0, 50.0, 100 and 200 mg/L for PFHxA-NH_4 which were prepared in the same way described in c) to confirm the effectiveness of this quantity method. As a result, the effectiveness was confirmed because the regression equation drawn from the relationship between the concentrations and the peak area on the each of chromatograms was confirmed as a straight line from origin. The drawn calibration curve and chromatograms which obtained by analysis of some samples for HPLC are shown in Appendix 3.

The determination limit of the test item in the test solution was the lowest concentration of the standard solution (10.0 mg/L) within the range of the calibration confirmed.

b) Analytical condition

Instrument	High-performance liquid chromatograph
Pump	LC-20AD (Shimadzu)
UV-VIS detector	SPD-20A (Shimadzu)
Column oven	CTO-20A (Shimadzu)
Auto injector	SIL-$20A_{HT}$ (Shimadzu)
System controller	CBM-20A (Shimadzu)
Degasser	DGU-$20A_3$ (Shimadzu)
Column	L-column ODS (150 mm × 4.6 mm I.D., particle size 5 μm, Chemicals Evaluation and Research Institute, Japan)
Column temp.	40°C
Eluent	A (50%) : Acetonitrile B (50%) : Ultra pure water/0.5 mol/L tetra-*n*-butylammonium phosphate solution (100/1 v/v)
Flow rate	1.0 mL/min
Wave length	215 nm
Injection volume	10 μL

c) Preparation of standard solution and calculation of test item concentration

The standard sample for analysis of the test item (50.1 mg) was precisely weighed by an electronic analytical balance and dissolved in ultra pure water to obtain 1000 mg/L solution of the test item. The solution was diluted with dechlorinated tap water to prepare 100 mg/L standard solution.

The concentration of the test item in each sample for HPLC analysis was determined on the basis of a comparison of the peak area on the chromatogram of the sample solution with that of a standard solution.

The standard sample for analysis of the test item ($PFHxA\text{-}NH_4$) (supplied by the sponsor)

Name	$PFHxA\text{-}NH_4$
Purity	99.78 mass%
Lot number	C15FD57002
Storage condition	It was stored in a dark storage place at room temperature in a desiccator.
Appearance	White powder

The standard sample for analysis of the test item was treated with correcting by the purity of the test item.

4. Results of measurement

The results of the measured concentrations of the test item in the test solutions are shown below.

Appendix table 2-1 Measured concentrations of test item in test solutions

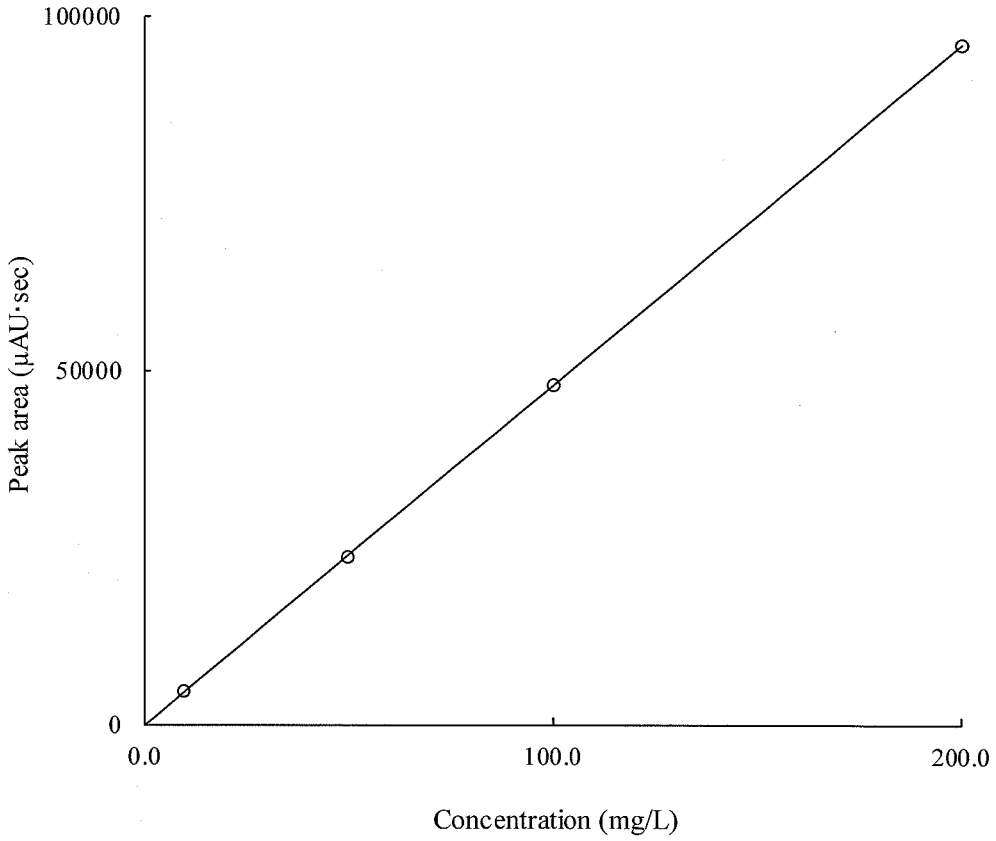
Nominal concentration (mg/L)	Measured concentration (mg/L) (Percentage of measured concentration versus nominal concentration %)		
	At the start	At the end	Geometric mean
Control	n.d.	n.d.	
911	945 (104)	1010 (111)	976 (107)
1280	1360 (106)	1410 (110)	1380 (108)
1790	1840 (103)	1910 (107)	1870 (105)
2500	2560 (102)	2590 [a] (104)	2580 (103)
3500	3610 (103)	3680 [a] (105)	3640 (104)

n.d. : <10.0 mg/L

a It indicates the measured value at the time confirmed all test organisms dead.

Appendix 3

Calibration curve and chromatogram



y = 479x

r = 1.00

Concentration (mg/L)	Peak area (μAU·sec)
10.0	4521
50.0	23505
100	48006
200	95979

Appendix figure 3-1 Calibration curve of test item (PFHxA-NH_4) for analysis by HPLC.

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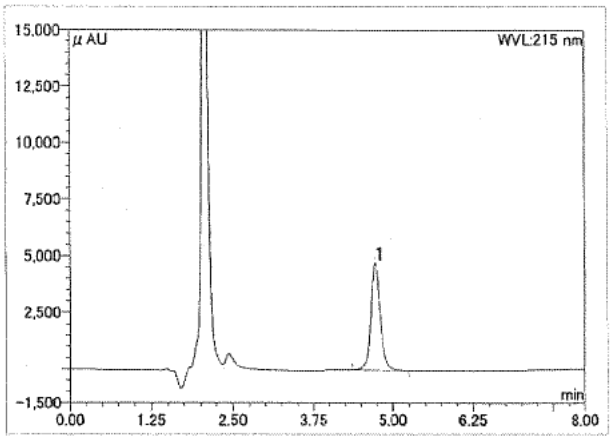
Standard solution 100 mg/L

Operator:	
Operating date:	29/Feb/2016
Sample ID:	97289_160229_S02
Program:	97289NH4_97290Na
Vial No.:	1_1
Channel:	UV_VIS_1



Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	4.72	4733	48697	100.00
Total	-	-	48697	100.00

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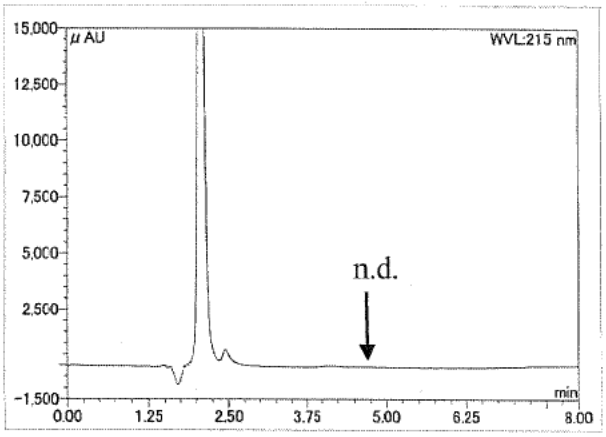
Control

Operator:	
Operating date:	29/Feb/2016
Sample ID:	97289_160229_H0HZ
Program:	97289NH4_97290Na
Vial No.:	1_2
Channel:	UV_VIS_1

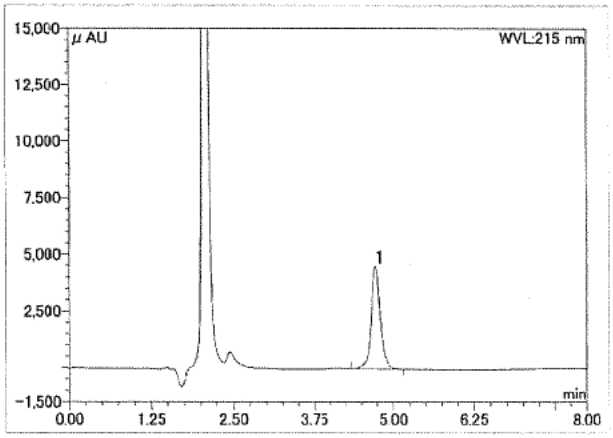


Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
Total	-	-	0	0.00

Study No. 97289

911 mg/L exposure level

Operator:	
Operating date:	29/Feb/2016
Sample ID:	97289_160229_H0HE
Program:	97289NH4_97290Na
Vial No.:	1_3
Channel:	UV_VIS_1



Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	4.72	4508	46000	100.00
Total	-	-	46000	100.00

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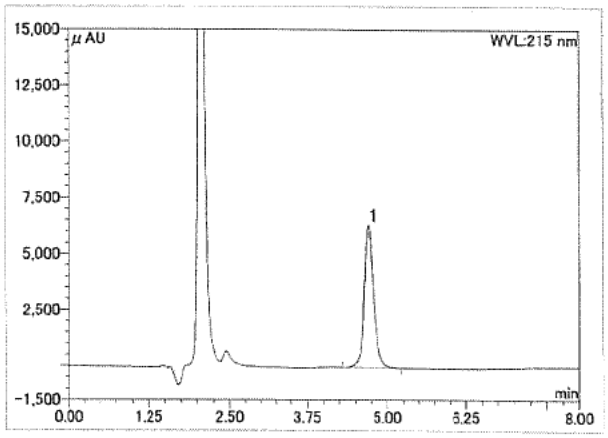
1280 mg/L exposure level

Operator:	
Operating date:	29/Feb/2016
Sample ID:	97289_160229_H0HD
Program:	97289NH4_97290Na
Vial No.:	1_4
Channel:	UV_VIS_1



Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	4.71	6366	65991	100.00
Total	-	-	65991	100.00

Appendix figure 3-2-1 HPLC chromatograms at start of exposure.

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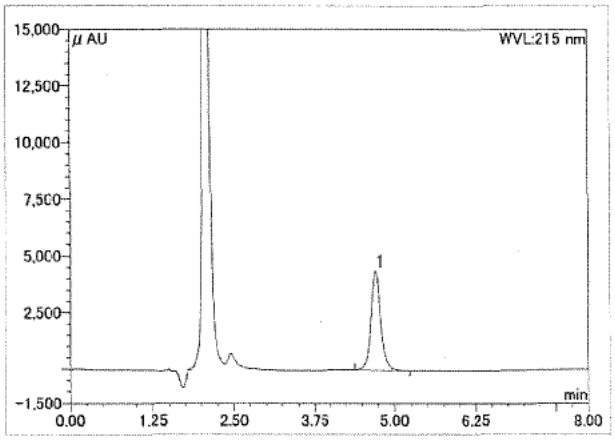
1790 mg/L exposure level

Operator:	
Operating date:	29/Feb/2016
Sample ID:	97289_160229_H0hC
Program:	97289NH4_97290Na
Vial No.:	1_5
Channel:	UV_VIS_1

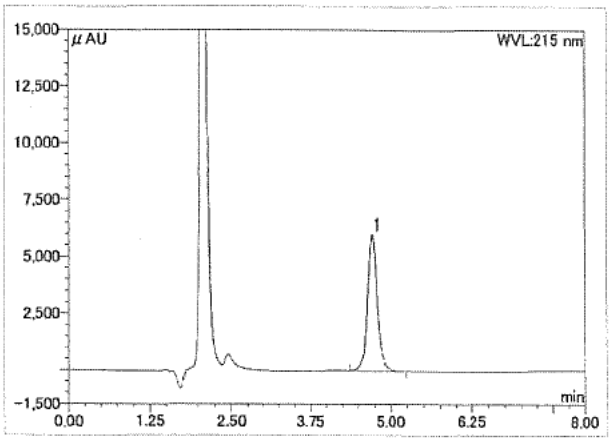


Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	4.71	4387	44695	100.00
Total	–	–	44695	100.00

Study No. 97289

2500 mg/L exposure level

Operator:	
Operating date:	29/Feb/2016
Sample ID:	97289_160229_H0hB
Program:	97289NH4_97290Na
Vial No.:	1_6
Channel:	UV_VIS_1

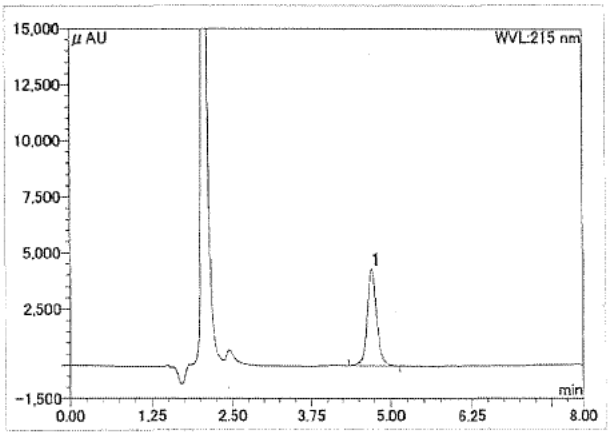


Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	4.71	6028	62348	100.00
Total	–	–	62348	100.00

Study No. 97289

3500 mg/L exposure level

Operator:	
Operating date:	29/Feb/2016
Sample ID:	97289_160229_H0hA
Program:	97289NH4_97290Na
Vial No.:	1_7
Channel:	UV_VIS_1



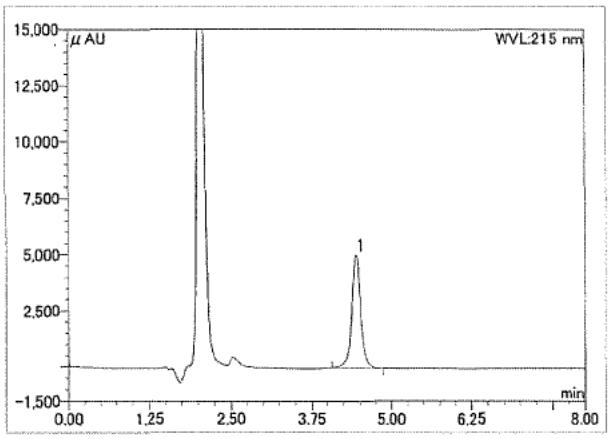
Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	4.71	4328	43918	100.00
Total	–	–	43918	100.00

Appendix figure 3-2-2 HPLC chromatograms at start of exposure.

Study No. 97289

Standard solution 100 mg/L

Operator:	
Operating date:	01/Mar/2016
Sample ID:	97289_160301_S02
Program:	97289NH4_97290Na
Vial No.:	1_1
Channel:	UV_VIS_1

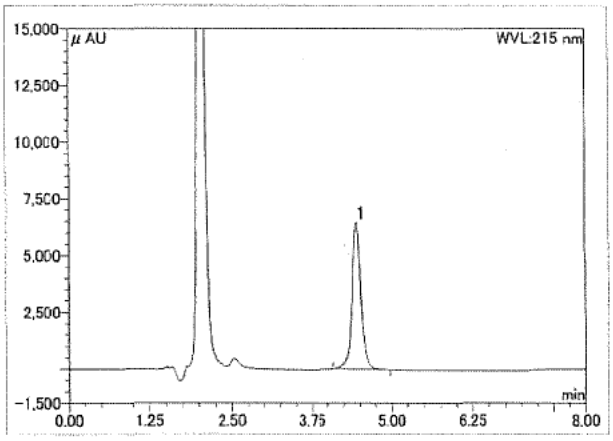


Peak No.	Time (min)	Height (μ AU)	Area (μ AU·sec)	Area (%)
1	4.44	5027	48928	100.00
Total	-	-	48928	100.00

Study No. 97289

2500 mg/L exposure level

Operator:	
Operating date:	01/Mar/2016
Sample ID:	97289_160301_H24hB
Program:	97289NH4_97290Na
Vial No.:	1_2
Channel:	UV_VIS_1

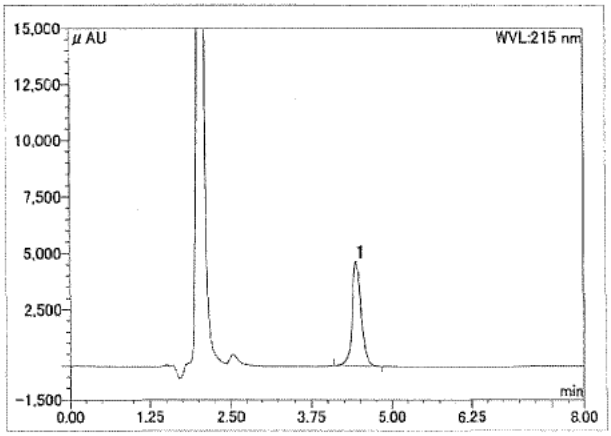


Peak No.	Time (min)	Height (μ AU)	Area (μ AU·sec)	Area (%)
1	4.44	6450	63410	100.00
Total	-	-	63410	100.00

Study No. 97289

3500 mg/L exposure level

Operator:	
Operating date:	01/Mar/2016
Sample ID:	97289_160301_H24hA
Program:	97289NH4_97290Na
Vial No.:	1_3
Channel:	UV_VIS_1



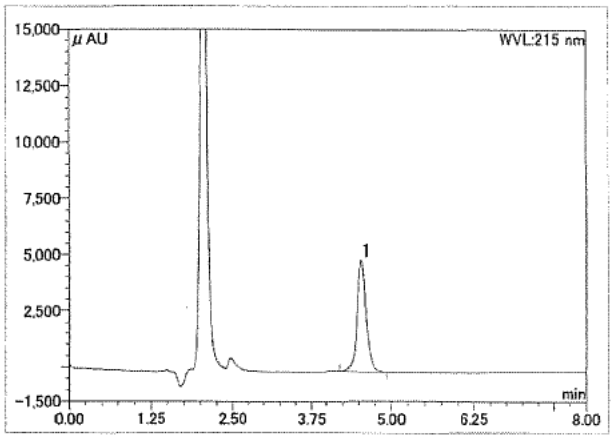
Peak No.	Time (min)	Height (μ AU)	Area (μ AU·sec)	Area (%)
1	4.45	4634	44968	100.00
Total	-	-	44968	100.00

Appendix figure 3-3 HPLC chromatograms at the time that confirmed all test organisms dead (at 24 hours after exposure).

Study No. 97289

Standard solution 100 mg/L

Operator:	
Operating date:	04/Mar/2016
Sample ID:	97289_160304_S02
Program:	97289NH4_97290Na
Vial No.:	1_1
Channel:	UV_VIS_1

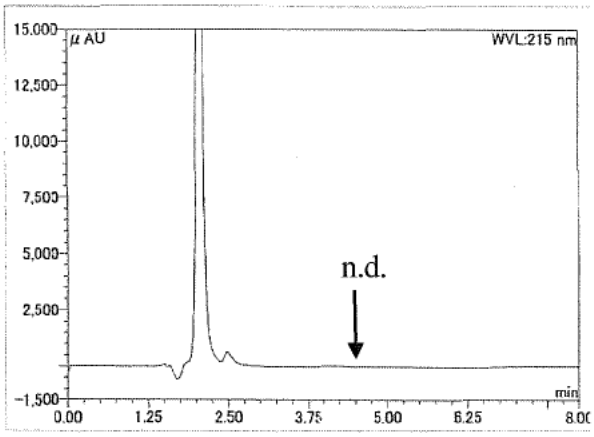


Peak No.	Time (min)	Height (μ AU)	Area (μ AU·sec)	Area (%)
1	4.53	4960	48553	100.00
Total	-	-	48553	100.00

Study No. 97289

Control

Operator:	
Operating date:	04/Mar/2016
Sample ID:	97289_160304_H96hZ
Program:	97289NH4_97290Na
Vial No.:	1_2
Channel:	UV_VIS_1

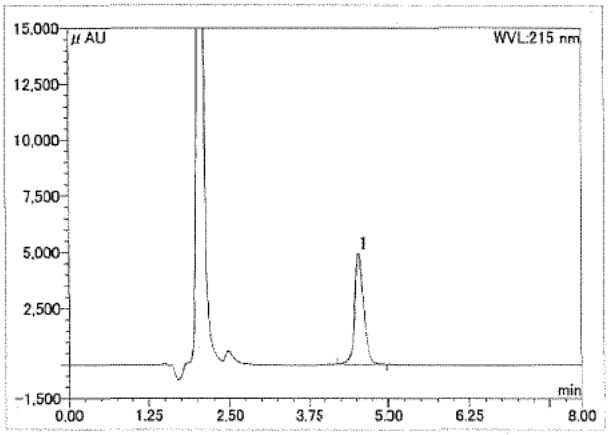


Peak No.	Time (min)	Height (μ AU)	Area (μ AU·sec)	Area (%)
Total	-	-	0	0.00

Study No. 97289

911 mg/L exposure level

Operator:	
Operating date:	04/Mar/2016
Sample ID:	97289_160304_H96hE
Program:	97289NH4_97290Na
Vial No.:	1_3
Channel:	UV_VIS_1

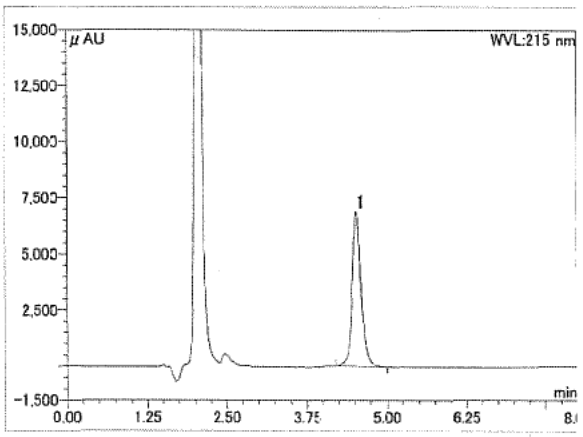


Peak No.	Time (min)	Height (μ AU)	Area (μ AU·sec)	Area (%)
1	4.54	4989	48977	100.00
Total	-	-	48977	100.00

Study No. 9728

1280 mg/L exposure level

Operator:	
Operating date:	04/Mar/2016
Sample ID:	97289_160304_H96hD
Program:	97289NH4_97290Na
Vial No.:	1_4
Channel:	UV_VIS_1



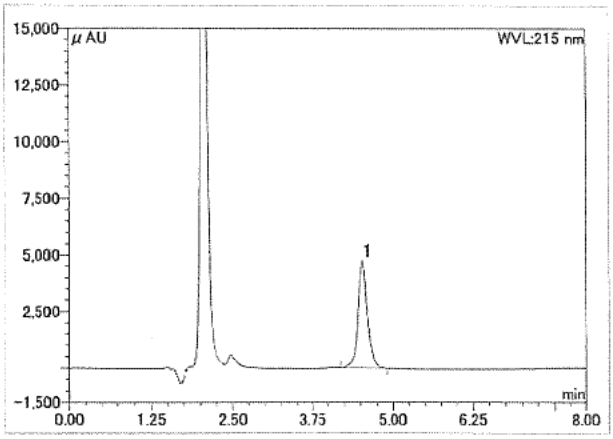
Peak No.	Time (min)	Height (μ AU)	Area (μ AU·sec)	Area (%)
1	4.53	6897	68446	100.00
Total	-	-	68446	100.00

Appendix figure 3-4-1 HPLC chromatograms at end of exposure.

Study No. 97289

1790 mg/L exposure level

Operator:	
Operating date:	04/Mar/2016
Sample ID:	97289_160304_H96hC
Program:	97289NH4_97290Na
Vial No.:	1_5
Channel:	UV_VIS_1



Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	4.54	4757	46439	100.00
Total	-	-	46439	100.00

Appendix figure 3-4-2 HPLC chromatogram at end of exposure.

Additional data

Results of preliminary study

1. Preliminary studies of effect on test organism

Type of test: Static regime

Number of organisms/volume of test solution: 2 fish/1 L

Aeration: Conducted gently

Preparation of test solution: Required amount of test sample and dilution water were mixed and stirred to prepare the test solution.

Analysis: Test item concentration in test solution was measured.

<Result of effect on test organisms>

Nominal concentration (mg/L)	Left column: Cumulative mortality (%) Right column : Existence of abnormal response (abnormalities : *, no abnormalities : N)									
	3 hours		24 hours		48 hours		72 hours		96 hours	
100	0	N	0	N	0	N	0	N	0	N
316	0	N	0	N	0	N	0	N	0	N
1000	0	N	0	N	0	N	0	N	0	N
3160	0	N	50	*	100	-	100	-	100	-
10000	0	*	100	-	100	-	100	-	100	-

- : All test organisms died.

<Measured concentration of test item in test solution>

Nominal concentration (mg/L)	Measured concentration (mg/L) (Percentage of measured concentration versus that at the nominal concentration %)	
	At the start	At the end (after 96 hours)
100	105 (105)	113 (113)
10000	10300 (103)	10400 [a] (104)

a : It indicates the measured value at the time that mortality of all test organisms was confirmed (24 hours after exposure).

2. Condition of definitive study

Test concentration:	3500, 2500, 1790, 1280, 911 mg/L as nominal concentrations (a geometric series with a factor of 1.4) and a control
Type of exposure:	Static regime
Aeration:	Conduct gently