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## TEST REPORT

Fish Short Term Reproduction Assay of perfluorohexanoic acid, ammonium salt or sodium salt  
in Medaka

December, 2015

Chemicals Evaluation and Research Institute, Japan, Kurume

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## 1. Title

Fish Short Term Reproduction Assay of perfluorohexanoic acid, ammonium salt or sodium 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 test is to investigate the potential effects to fish reproduction of perfluorohexanoic acid, ammonium salt or sodium salt, and its differences by kind of salts.

## 5. Test method

This test was carried out referring to the OECD Guidelines for Testing of Chemicals, No.229, October 2, 2012, "Fish Short Term Reproduction Assay". However, the secondary sex characteristics or gonad histology were not observed.

## 6. Dates

Study initiation date September 17, 2015

Experimental starting date October 2, 2015

Experimental completion date October 23, 2015

Study completion date December 25, 2015

## 7. Personnel

Study Director Ryuta Adachi (Section 4)

Study personnel (Biological study) Takeshi Matsuura

Naohiro Mizoguchi

Study personnel (Analytical chemistry) Mika Ono

## 8. Summary

Test itemPFHxA-NH<sub>4</sub> or PFHxA-NaObjective

The objective of this test is to investigate the potential effects to fish reproduction of perfluorohexanoic acid, ammonium salt or sodium salt, and its differences by kind of salts.

Test method

This test was carried out referring to the OECD Guidelines for Testing of Chemicals, No.229, October 2, 2012, "Fish Short Term Reproduction Assay". However, the secondary sex characteristics or gonad histology were not observed.

Test conditions

Test organism	Medaka ( <i>Oryzias latipes</i> )
Dilution water	Dechlorinated tap water
Test levels	PFHxA-NH <sub>4</sub> : 100, 10 mg/L (a geometric series with a factor of 10) PFHxA-Na: 100, 10 mg/L (a geometric series with a factor of 10) and a control
Preparation of test solution	Test sample was diluted in dechlorinated tap water to prepare the x20 stock solution of each test concentration. A certain amount of the stock solution and dilution water were mixed into a mixing container to prepare the test solution continuously.
Type of test	Flow-through system
Exposure duration	21 days
Replicate	3 replicate/test level
Number of organism	9 males and 9 females/test level (3 males and 3 females/test vessel)
Volume of test solution	Approximately 5.4 L/test level (Approximately 1.8 L/test vessel)
Temperature of test solution	24.8-25.1°C
Aeration	No aeration
Irradiation condition	Room light, 16-hour light/8-hour dark
Feeding	Fish was fed ad libitum with live <i>Artemia</i> nauplii three times a day.
Analysis of concentration of test item in test solution	HPLC analysis (four times repetitions of weekly measurement from the start of exposure)

Results

## LOEC and NOEC of each endpoint

Endpoint	PFHxA-NH <sub>4</sub>		PFHxA-Na	
	LOEC (mg/L)	NOEC (mg/L)	LOEC (mg/L)	NOEC (mg/L)
Fecundity	> 100	≥ 100	> 100	≥ 100
Fertility	> 100	≥ 100	> 100	≥ 100
Vitellogenin	> 100	≥ 100	> 100	≥ 100

The LOEC and NOEC of PFHxA-NH<sub>4</sub> and PFHxA-Na in the short term reproduction assay were evaluated as > 100 and ≥ 100 mg/L, respectively.

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

## 9. Test materials

9.1 Test item (perfluorohexanoic acid, ammonium salt: PFHxA-NH<sub>4</sub>)

## a) Chemical name etc.

Chemical name	2,2,3,3,4,4,5,5,6,6,6-undecafluorohexanoic acid, ammonium salt
Another name	PFHxA-NH <sub>4</sub>
CAS number	21615-47-4

## b) Chemical structure etc.

Rational formula	CF <sub>3</sub> CF <sub>2</sub> CF <sub>2</sub> CF <sub>2</sub> CF <sub>2</sub> COONH <sub>4</sub>
Molecular formula	C <sub>6</sub> F <sub>11</sub> H <sub>4</sub> NO <sub>2</sub>
Molecular weight	331.08

## c) Test sample

Name	PFHxA-NH <sub>4</sub> -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 item (perfluorohexanoic acid, sodium salt: PFHxA-Na)

## a) Chemical name etc.

Chemical name	2,2,3,3,4,4,5,5,6,6,6-undecafluorohexanoic acid, sodium salt
Another name	PFHxA-Na
CAS number	2923-26-4

## b) Chemical structure etc.

Rational formula	CF <sub>3</sub> CF <sub>2</sub> CF <sub>2</sub> CF <sub>2</sub> CF <sub>2</sub> COONa
Molecular formula	C <sub>6</sub> F <sub>11</sub> O <sub>2</sub> Na
Molecular weight	336.04

## c) Test sample

Name	PFHxA-Na-50
Purity of test item	50 mass%
Impurity	Water 50 mass%
Supplier	DAIKIN INDUSTRIES, LTD.
Lot number	C150S57001

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.

## 9.3 Test organisms

Species	Medaka ( <i>Oryzias latipes</i> )
Reason for selection of species	Species recommended in the test guideline
Supplier	CERI Kurume (in-laboratory production)
Fish age	After 18 weeks hatching medaka with sexually dimorphic adult fish from a laboratory supply of reproductively mature animal and actively spawning was used.
Allocation	Medaka was allocated at random to each vessel.
Acclimation	
Water	Dechlorinated tap water
Temperature	25±2°C
Photoperiod	16-hour light/8-hour dark with room light
Feed	<i>Artemia</i> nauplii
Feeding amount and frequency	<i>Ad libitum</i> , three times a day
Use of medicament for external disinfection	None
Duration of acclimation	14 days (September 18, 2015 to October 2, 2015)
Mortality of test groups	< 5 % (during 7 days 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: 17 cm) The tank had an overflow tube set at approximately 1.8 L level.
Water bath	Plastic tank Warming/cooling unit RX-401HPN-Z (IWAKI)
Flow-through equipment	
Diluter system	Apparatus to deliver the stock solution of the test sample and dilution water, which were adjusted to a constant flow rate with metering pump and capillary tube respectively, into a mixing container, mix with a magnetic stirrer, and supply the mixed solution continuously to the test vessel as test solution after dividing it into 3 vessels.
Metering pump	Micro volume metering glass pump GMW-A (TOKYO RIKAKIKAI)

### 10.3 Preparation of test solution

Required test sample was diluted in dechlorinated tap water to prepare the x20 stock solution of each test concentration. A certain amount of the stock solution adjusted by a metering pump and dilution water adjusted by diluter system was admixed to prepare the each test solution, continuously. The flow rates were checked at regular intervals and their variation throughout exposure duration resulted in less than 10%. In addition, the each stock solution was prepared every 3 days.

### 10.4 Test conditions

Type of test	Flow-through system (renewal rate: approximately 16 times/day)
Exposure duration	21 days
Test concentration	PFHxA-NH <sub>4</sub> : 100, 10 mg/L (a geometric series with a factor of 10) PFHxA-Na: 100, 10 mg/L (a geometric series with a factor of 10)
Control	Dilution water without the test item
Replicate	3 replicates/test level
Number of organisms	9 males and 9 females/test level (3 males and 3 females/test vessel)
Volume of test solution	Approximately 5.4 L/test level (approx. 1.8 L/test vessel)
Temperature of test solution	25±2°C
Aeration	No aeration
pH adjustment	No adjustment
Lighting condition	Room light, 16-hour light/8-hour dark
Feeding	Fish was fed <i>ad libitum</i> during the exposure with <i>Artemia</i> nauplii three times a day. Feed was not fed within 12 hours of necropsy.

### 10.5 Observation and measurements

#### a) Observation of test organisms

Mortality and visible abnormality were observed daily. 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) Body weight of test organism

Body weight of 5 males and 5 females of the same lot used for the exposure were measured at the start of exposure.

#### c) Fecundity and fertility

The one-week pre-exposure was performed with medaka placed in vessels similar to the actual test before the exposure. Egg production was assessed during last four days of pre-exposure by counting the number of eggs and fertility per day. Then the respective number of eggs and fertility per vessel were calculated, and the vessels were assigned to each test level as there were no statistically significant differences among test levels on their number of eggs and fertility rate. As well as pre-exposure, egg production was assessed four days a week to calculate total number of eggs and fertility per test vessel during exposure.



d) Excision and pretreatment of liver and measurement of hepatic vitellogenin (VTG) concentration of fish  
 After 21 days exposure, all living fishes were euthanized in ice water and dissected for the excision of their liver. Excised liver was stored at  $-80^{\circ}\text{C}$  until pretreatment of analysis for vitellogenin. As the pretreatment, the liver was homogenized with 50  $\mu\text{L}$  of ELIZA dilution buffer per mg liver weight, and centrifuged at  $13000\times g$  for 10 minute at  $5^{\circ}\text{C}$  (High-speed refrigerated centrifuge, CR21GII, Hitachi Koki). Then the supernatant was collected and stored at  $-80^{\circ}\text{C}$  until analysis for vitellogenin. VTG concentration was measured by using Medaka Vitellogenin ELISA kit (EnBio Medaka Vitellogenin ELISA system, Fujikura Kasei).

e) Appearance of test solution

Appearance of the test solutions was observed at the start and end of the exposure.

f) Condition of test solutions

i) Dissolved oxygen concentration (DO), pH and temperature

Frequency of measurement

DO, pH, temperature: Once a week (including at the start and end of exposure)

Continuous temperature: Once an hour intervals

Measuring point DO, pH, temperature: All test vessels

Continuous temperature: One test vessel of control

Instrument Oxygen meter YSI Model 58 (YSI Nanotech Japan)

pH meter Model HM-21P (DKK-TOA)

Thermometer of glass stick type

Thermo recorder RT-11 (TABAI ESPEC)

ii) Total hardness and alkalinity

Frequency of measurement Once a week (include at the start and end of exposure)

Measuring point One test vessel of control and highest test concentration

g) Test item concentration in test solution

Frequency of measurement

Four times during exposure (including at the start and end of exposure)

Measuring point All test levels

Sampling for measurement

The mixed solution taken out with equal volume from the middle layer of each test vessel

Analytical condition Shown in Appendix 2

## 10.6 Treatment of the results and statistical analysis

The results of the study were estimated with the nominal concentration since the measured concentration of test item in test solution was maintained within  $\pm 20\%$  of nominal concentration during exposure.

Statistical analysis on the results of mortality, total number of eggs and fertility were assessed on the basis of replicate vessel, and vitellogenin concentration was assessed on the basis of individual fish. Statistical analysis on the results of mortality, total number of eggs and fertility were performed by Dunnett's multiple comparison test. Statistical analysis on the results of vitellogenin concentration was performed by the Mann-Whitney U test. All statistical treatments were performed with IBM® SPSS® version 22 (International Business Machines Corporation), and statistical difference was considered to be significant at  $p < 0.05$ .

## 10.7 Validity of test

- a) The mortality in the control should not exceed 10% at the end of the exposure period.
- b) The dissolved oxygen concentration should be at least 60% of the air saturation value throughout the exposure period.
- c) The water temperature should not differ by more than  $\pm 1.5^{\circ}\text{C}$  between test vessels at any one time during the exposure period and be maintained within a range of  $25 \pm 2^{\circ}\text{C}$ .
- d) The concentrations of the test substance in solution have been satisfactorily maintained within  $\pm 20\%$  of the mean measured values.
- e) Fish are actively spawning in all replicates prior to initiating chemical exposure and in control replicates during the test.

## 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 during exposure was shown in Table 1.

During exposure, one male fish died in the 10 mg/L exposure level of PFHxA-NH<sub>4</sub> and 100 mg/L exposure level of PFHxA-Na, respectively. Dead fish in the other exposure levels was not confirmed during exposure.

Number of dead fish in the control at the end of exposure was 0, which meets the criterion for the validity of the test (i.e. not exceed 10%).

### 11.2 Observed performance status etc.

The performance status observed during the exposure are shown in Table 2.

The abnormal response was observed in neither the control nor exposure levels.

### 11.3 Body weight of test organism at the start of exposure

[Mean  $\pm$  Standard deviation (n=5)]

Male 313 $\pm$ 26 mg

Female 351 $\pm$ 53 mg

### 11.4 Observation and measurement of test solution

#### a) Appearance of test solution

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

#### b) Condition of test solutions

Dissolved oxygen, pH, and temperature of the test solutions are shown in Tables 3, 4 and 5. Total hardness and alkalinity of the test solutions are shown in Table 6 and 7.

The measured values of dissolved oxygen concentration, pH and temperature during exposure were ranged from 7.5 to 8.1 mg/L, 7.6 to 7.8 and 24.8 to 25.1 $^{\circ}\text{C}$ , respectively. The values of temperature measured by thermo recorder (RT-11) every an hour were ranged from 24.0 to 25.3 $^{\circ}\text{C}$  (measured number: n=498). Total hardness and alkalinity of the test solutions ranged from 42.1 to 45.1 and 37.0 to 39.5, 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). The measured values of temperature also met the criterion for the study validity ( $\pm 1.5^{\circ}\text{C}$  between test vessels at any one time during the exposure period and be maintained within a range of  $25\pm 2^{\circ}\text{C}$ ).

\* Saturated dissolved oxygen concentration (24 to  $26^{\circ}\text{C}$ ): 8.25 to 7.99 mg/L (JIS K 0102, 2008)

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 PFHxA-NH<sub>4</sub> in the test solutions were 9.99 to 105 mg/L which were 99.9 to 105% of the nominal concentration during exposure. The measured concentrations of the PFHxA-Na in the test solutions were 10.2 to 102 mg/L which were 97.1 to 105% of the nominal concentration during exposure. The concentration of test items in test solution were kept within  $\pm 20\%$  of the nominal concentration, therefore, it met the criterion for the study validity (the concentrations of the test substance in solution have been satisfactorily maintained within  $\pm 20\%$  of the mean measured values).

### 11.5 Fecundity and fertility

Fecundity as total number of the eggs per vessel during exposure is shown in Table 8 and Figure 1. Fertility per vessel during exposure is shown in Table 9 and Figure 2. The graph of cumulative number of eggs per female is shown in Figure 3.

Mean total number of the eggs in each test levels were ranged from 563 to 615 during exposure. On the graph of cumulative number of eggs per female, fecundity of exposure levels was the same tendency as the control. The control fishes were actively spawning in all replicates, therefore, it met the criterion for the study validity. Means of fertility in each test levels were ranged from 90.8 to 95.6% during exposure. In fecundity and fertility, there was no drastic change caused by test items compared with the control. In addition, no significant difference was observed in these endpoints.

### 11.6 VTG concentration

Hepatic VTG concentrations at the end of exposure are shown in Table 10, 11 and Figure 4.

VTG concentration of males were below the determination limit ( $< 1.00$  ng/mg liver weight) in all test levels. VTG concentrations of female were ranged 197 to 641 ng/mg liver weight in the mean value of individual fish. There was no concentration-response dependent manner between test item concentration and the VTG concentration. In addition, there was no significant difference between VTG concentration of control and that of exposure levels.

### 11.7 LOEC and NOEC

The results of statistical analysis of significant difference are shown in Table 13. The LOEC and NOEC of test items for the fecundity, fertility and VTG concentration are shown in Table 14.

As the results of statistical analysis, there were no statistically significant difference in any exposure level for fecundity, fertility and VTG concentration. Therefore, the LOEC and NOEC of PFHxA-NH<sub>4</sub> and PFHxA-Na for reproduction of medaka were  $> 100$  mg/L and  $\geq 100$  mg/L, respectively.

## 11.8 Discussion

As a results of this study, NOEC of PFHxA-NH<sub>4</sub> and PFHxA-Na for the reproduction of medaka were both  $\geq 100$  mg/L. Though each one fish died in the 10 mg/L PFHxA-NH<sub>4</sub> exposure level and 100 mg/L PFHxA-Na exposure level during exposure, there was no clear concentration-response dependent manner. Therefore, it was considered that the decease of fish was not caused by the toxicity of test items. The spawning in the exposure levels was active the same as control, and decreasing trend was not observed compared with the control. Although the fertility tended to decrease in 100 mg/L PFHxA-NH<sub>4</sub> and PFHxA-Na exposure levels, it kept more than 90% and there was no significant difference. As for the results of VTG concentration, VTG production was not detected in any male fish. Therefore, it was indicated that PFHxA-NH<sub>4</sub> or PFHxA-Na has no estrogenic activity. Although female VTG concentration in all exposure levels showed higher than that of the control, there was no concentration-response dependent manner. In addition, statistically significant differences were not detected between the control and the exposure levels. Therefore, it was determined that the difference of female VTG concentrations was not caused by the effect of the test items on the endocrine system.

As above results, it was estimated that PFHxA-NH<sub>4</sub> and PFHxA-Na have no estrogenic activity to endocrine of medaka, and have no effect on reproduction of medaka in the test concentration.

Table 1 Cumulative mortality

Nominal concentration (mg/L)	Cumulative mortality (%)																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Total
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10 (PFHxA-NH <sub>4</sub> )	0	0	0	0	0	0	0	0	0	5.6 (1;♂)	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6
100 (PFHxA-NH <sub>4</sub> )	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10 (PFHxA-Na)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
100 (PFHxA-Na)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.6 (1;♂)	5.6

Value in parentheses expresses the number and the sex of the dead fish.

Table 2 Observed abnormal response

Nominal concentration (mg/L)	Observed toxic symptoms on each exposure day																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
Control	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
10 (PFHxA-NH <sub>4</sub> )	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
100 (PFHxA-NH <sub>4</sub> )	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
10 (PFHxA-Na)	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
100 (PFHxA-Na)	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

N: Normal (No abnormal response)

Table 3 Dissolved oxygen concentration of test solutions

Nominal concentration (mg/L)		Measured value (mg/L)			
		0 day	7 days	14 days	21 days
Control	A	8.1	8.1	7.7	8.1
	B	8.1	8.1	7.7	8.0
	C	8.1	8.1	7.6	7.9
10 (PFHxA-NH <sub>4</sub> )	A	8.1	8.1	7.6	8.0
	B	8.1	8.1	7.5	7.8
	C	8.0	8.1	7.5	7.8
100 (PFHxA-NH <sub>4</sub> )	A	8.0	8.1	7.6	7.7
	B	8.0	8.1	7.5	7.7
	C	8.1	8.1	7.5	7.8
10 (PFHxA-Na)	A	8.1	8.1	7.7	7.8
	B	8.1	8.1	7.6	7.9
	C	8.1	8.1	7.7	7.9
100 (PFHxA-Na)	A	8.1	8.1	7.7	7.9
	B	8.1	8.1	7.6	7.9
	C	8.1	8.1	7.5	7.9

Table 4 pH of test solutions

Nominal concentration (mg/L)		Measured value			
		0 day	7 days	14 days	21 days
Control	A	7.8	7.6	7.6	7.6
	B	7.8	7.7	7.6	7.6
	C	7.8	7.6	7.6	7.6
10 (PFHxA-NH <sub>4</sub> )	A	7.8	7.6	7.7	7.7
	B	7.8	7.6	7.6	7.7
	C	7.8	7.6	7.6	7.6
100 (PFHxA-NH <sub>4</sub> )	A	7.8	7.6	7.6	7.7
	B	7.8	7.6	7.6	7.7
	C	7.7	7.6	7.6	7.6
10 (PFHxA-Na)	A	7.8	7.6	7.6	7.6
	B	7.8	7.6	7.6	7.7
	C	7.8	7.6	7.6	7.7
100 (PFHxA-Na)	A	7.8	7.6	7.7	7.7
	B	7.8	7.6	7.7	7.7
	C	7.8	7.6	7.7	7.7

Table 5 Temperature of test solutions measuring by thermometer of glass stick type

Nominal concentration (mg/L)		Measured value (°C)			
		0 day	7 days	14 days	21 days
Control	A	25.0	25.0	24.9	24.8
	B	25.0	24.9	24.9	24.8
	C	25.0	25.0	24.9	24.8
10 (PFHxA-NH <sub>4</sub> )	A	25.0	25.0	25.0	25.0
	B	25.0	25.0	25.0	25.0
	C	25.0	25.0	25.0	25.0
100 (PFHxA-NH <sub>4</sub> )	A	25.1	25.0	25.0	25.0
	B	25.1	25.0	25.0	25.0
	C	25.1	25.0	24.9	25.0
10 (PFHxA-Na)	A	25.1	24.9	25.0	25.0
	B	25.1	24.9	25.0	25.0
	C	25.1	24.9	25.0	25.0
100 (PFHxA-Na)	A	25.1	24.9	25.0	25.0
	B	25.1	24.9	25.0	25.0
	C	25.1	24.9	25.0	25.0

Table 6 Total hardness of test solutions

Nominal concentration (mg/L)		Total hardness (mg CaCO <sub>3</sub> /L)			
		0 day	7 days	14 days	21 days
Control	A	44.1	42.1	43.1	43.1
100 (PFHxA-NH <sub>4</sub> )	A	45.1	42.1	45.1	42.5
100 (PFHxA-Na)	A	45.1	42.1	43.1	43.1

Table 7 Alkalinity of test solutions

Nominal concentration (mg/L)		Alkalinity hardness (mg CaCO <sub>3</sub> /L)			
		0 day	7 days	14 days	21 days
Control	A	39.5	37.0	38.0	38.5
100 (PFHxA-NH <sub>4</sub> )	A	39.5	37.0	38.5	38.5
100 (PFHxA-Na)	A	39.5	37.0	38.5	39.0

Table 8 Total number of eggs

Nominal concentration (mg/L)	Total number of eggs			
	Vessel	Mean	S.D.	
Control	A	621	574	41
	B	546		
	C	556		
10 (PFHxA-NH <sub>4</sub> )	A	633	586	73
	B	623		
	C	501		
100 (PFHxA-NH <sub>4</sub> )	A	653	586	64
	B	525		
	C	581		
10 (PFHxA-Na)	A	619	615	8
	B	619		
	C	606		
100 (PFHxA-Na)	A	484	563	101
	B	676		
	C	528		



Table 9 Fertility

Nominal concentration (mg/L)	Fertility (%)			
	Vessel	Mean	S.D.	
Control	A	94.1	94.9	0.7
	B	95.3		
	C	95.2		
10 (PFHxA-NH <sub>4</sub> )	A	98.7	95.6	3.2
	B	92.2		
	C	95.7		
100 (PFHxA-NH <sub>4</sub> )	A	82.6	91.2	7.6
	B	97.1		
	C	93.9		
10 (PFHxA-Na)	A	95.0	94.4	2.6
	B	91.6		
	C	96.7		
100 (PFHxA-Na)	A	96.1	90.8	8.6
	B	80.9		
	C	95.4		

Table 10 VTG concentration of male medaka after 21-d exposure

No.	VTG concentration (ng/mg liver weight)				
	Control	PFHxA-NH <sub>4</sub>		PFHxA-Na	
		10 mg/L	100 mg/L	10 mg/L	100 mg/L
A-1	n.d.	n.d.	n.d.	n.d.	n.d.
A-2	n.d.	n.d.	n.d.	n.d.	n.d.
A-3	n.d.	-	n.d.	n.d.	n.d.
B-1	n.d.	n.d.	n.d.	n.d.	n.d.
B-2	n.d.	n.d.	n.d.	n.d.	n.d.
B-3	n.d.	n.d.	n.d.	n.d.	n.d.
C-1	n.d.	n.d.	n.d.	n.d.	n.d.
C-2	n.d.	n.d.	n.d.	n.d.	n.d.
C-3	n.d.	n.d.	n.d.	n.d.	-
Mean	n.d.	n.d.	n.d.	n.d.	n.d.
S.D.	/	/	/	/	/

The individual number is arbitrary.

-: It shows that the data was not obtained because the fish died.

n.d.: < 1.00 ng/mg liver weight

Table 11 VTG concentration of female medaka after 21-d exposure

No.	VTG concentration (ng/mg liver weight)				
	Control	PFHxA-NH <sub>4</sub>		PFHxA-Na	
		10 mg/L	100 mg/L	10 mg/L	100 mg/L
A-1	44.0	831	333	47.3	36.3
A-2	50.2	601	432	653	383
A-3	48.4	2060	809	663	280
B-1	506	496	496	811	399
B-2	42.7	379	252	49.6	327
B-3	642	27.4	463	294	176
C-1	42.7	424	681	458	425
C-2	345	594	250	433	342
C-3	49.1	355	34.5	433	34.2
Mean	197	641	417	427	267
S.D.	238	575	235	264	150

The individual number is arbitrary.

Table 12 Total length and body weight after 21-d exposure

Nominal concentration (mg/L)	Total length (cm)		Body weight (mg)	
	Male	Female	Male	Female
Control	2.84 ± 0.07	2.98 ± 0.03	243 ± 36	313 ± 3
10 (PFHxA-NH <sub>4</sub> )	2.92 ± 0.11	2.96 ± 0.02	268 ± 24	297 ± 15
100 (PFHxA-NH <sub>4</sub> )	2.95 ± 0.06	2.97 ± 0.04	280 ± 21	315 ± 28
10 (PFHxA-Na)	2.93 ± 0.08	2.97 ± 0.05	270 ± 38	307 ± 10
100 (PFHxA-Na)	3.05 ± 0.15	2.97 ± 0.04	317 ± 46	296 ± 6

The data is shown as mean value of each vessel ± standard deviation

Table 13 Results of the statistical analysis

Nominal concentration (mg/L)	Mortality	Total number of eggs	Fertility	VTG concentration	
				Male	Female
10 (PFHxA-NH <sub>4</sub> )	n.s.	n.s.	n.s.	n.s.	n.s.
100 (PFHxA-NH <sub>4</sub> )	n.s.	n.s.	n.s.	n.s.	n.s.
10 (PFHxA-Na)	n.s.	n.s.	n.s.	n.s.	n.s.
100 (PFHxA-Na)	n.s.	n.s.	n.s.	n.s.	n.s.

Statistical procedure: Comparison among mean value of vessel (n=3) using Dunnett's multiple comparison test; Comparison among individual fish (n=9) using Mann-Whitney's u-test

n.s.: no significant difference

Table 14 LOEC and NOEC

Endpoint	PFHxA-NH <sub>4</sub>		PFHxA-Na	
	LOEC (mg/L)	NOEC (mg/L)	LOEC (mg/L)	NOEC (mg/L)
Fecundity	> 100	≥ 100	> 100	≥ 100
Fertility	> 100	≥ 100	> 100	≥ 100
Vitellogenin	> 100	≥ 100	> 100	≥ 100

The concentrations are based on nominal concentration.

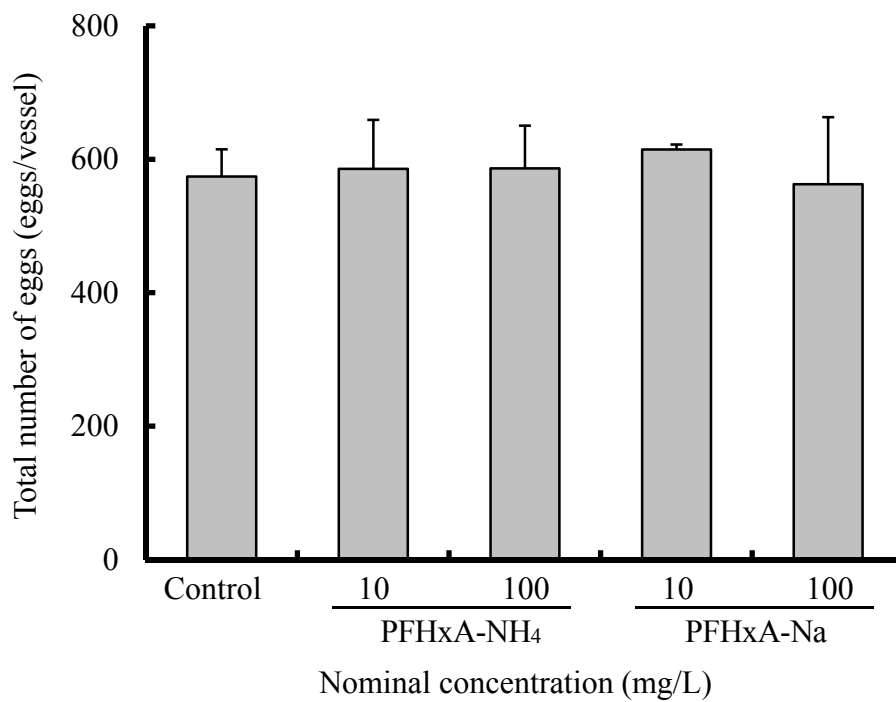


Figure 1 Total number of eggs.

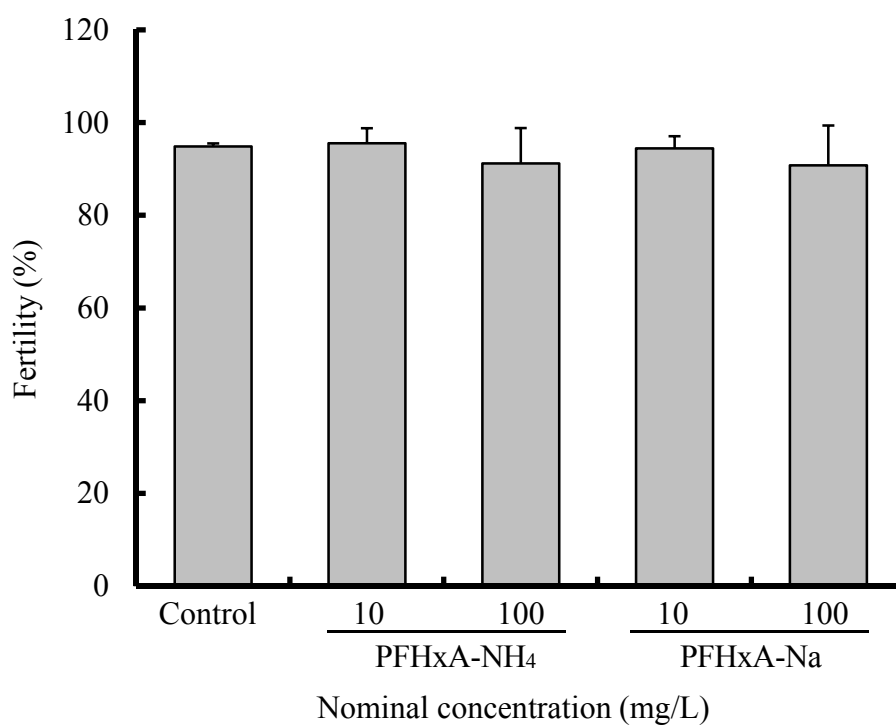


Figure 2 Fertility.

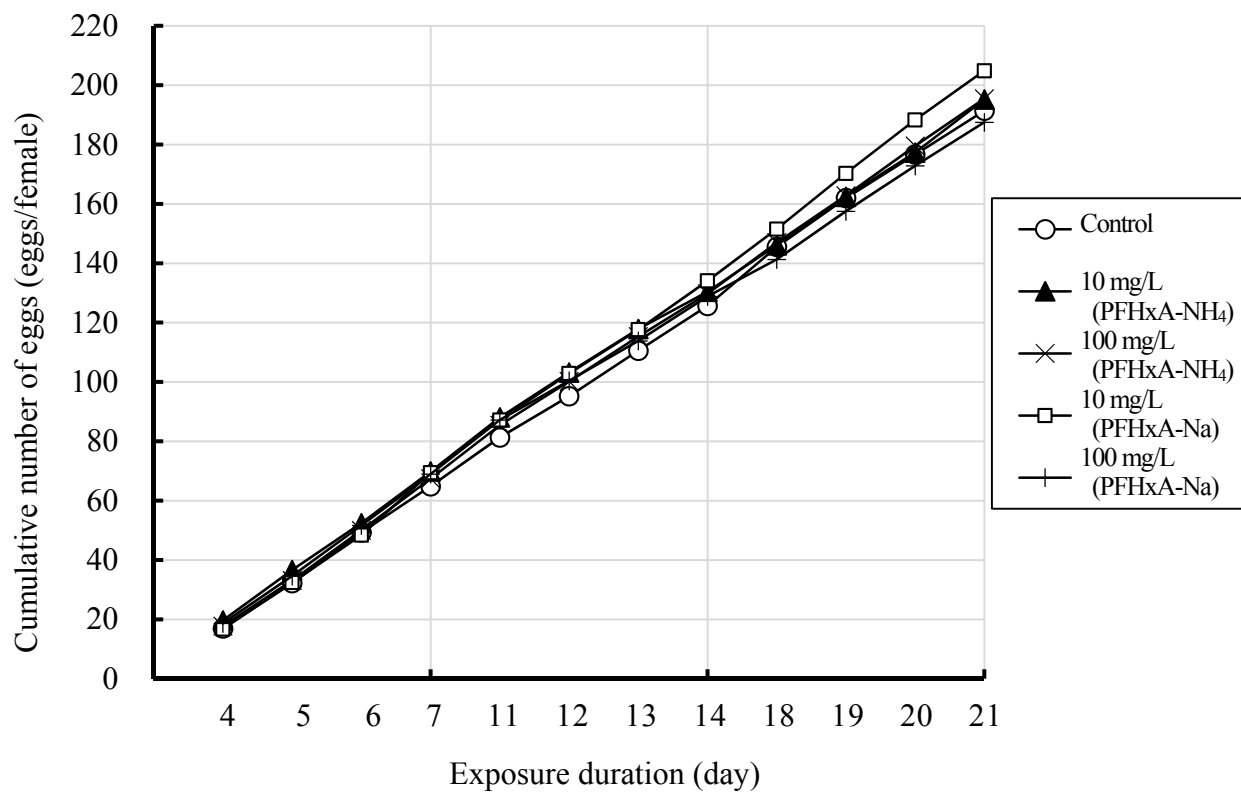


Figure 3 Cumulative number of eggs per female.

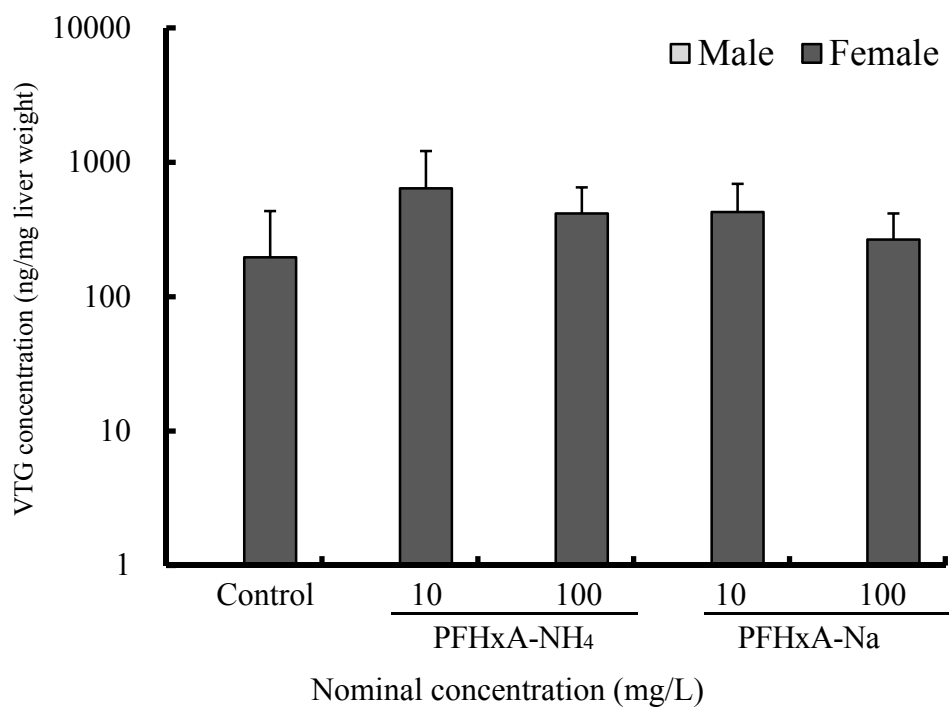


Figure 4 VTG concentration after 21-d exposure.

## Appendix 1

Chemical characteristics of dilution water

## Chemical characteristics of dilution water (Sampling on July 7, 2015)

Parameter	Unit	Results	Determination limit
Total hardness (as CaCO <sub>3</sub> )	mg/L	28	1
Suspended solid	mg/L	< 1	1
pH	-	7.7 (25.0°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	29	1
Electric conductivity	mS/m	11	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	< 0.1	0.1
Sulfate ion	mg/L	9.7	0.5
Chloride ion	mg/L	8.1	0.2
Sodium	mg/L	8.0	0.2
Potassium	mg/L	2.0	0.2
Calcium	mg/L	7.9	0.1
Magnesium	mg/L	2.1	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 solutions.

For each of PFHxA-NH<sub>4</sub> and PFHxA-Na were performed as follows.

The calibration curve was drawn by using four standard solutions of 1.00, 5.00, 10.0 and 20.0 mg/L 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 (1.00 mg/L) within the range of the calibration confirmed.

### b) Analytical condition

Instrument	High-performance liquid chromatograph LC-2010A <sub>HT</sub> (Equipped with UV-VIS detector) (Shimadzu) (Equipped with UV-VIS detector)
Column	L-column2 ODS (150 mm × 2.1 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- <i>n</i> -butylammonium phosphate solution (100/1 v/v)
Flow rate	0.2 mL/min
Wave length	215 nm
Injection volume	20 μL

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

Preparation of standard solutions as follows were performed on each for the PFHxA-NH<sub>4</sub> and PFHxA-Na.

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 10.0 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-NH<sub>4</sub>) (supplied by the sponsor)

Name	PFHxA-NH <sub>4</sub>
Purity	99.78 mass%
Lot number	C15FD57002
Storage condition	PFHxA-NH <sub>4</sub> 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.

The standard sample for analysis of the test item (PFHxA-Na) (supplied by the sponsor)

Name	PFHxA-Na
Purity	99.83 mass%
Lot number	C15SD57001
Storage condition	PFHxA-Na 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	7days	14days	At the end	Arithmetic mean
Control	n.d.	n.d.	n.d.	n.d.	
10.0 (PFHxA-NH <sub>4</sub> )	9.99 (99.9)	10.1 (101)	10.2 (102)	10.3 (103)	10.2 (102)
100 (PFHxA-NH <sub>4</sub> )	104 (104)	105 (105)	104 (104)	102 (102)	104 (104)
10.0 (PFHxA-Na)	10.4 (104)	10.5 (105)	10.4 (104)	10.2 (102)	10.4 (104)
100 (PFHxA-Na)	97.1 (97.1)	101 (101)	102 (102)	99.9 (99.9)	10.0 (100)

n.d. : <1.00 mg/L

The arithmetic mean is calculated by the following expression:

$$(C_0 + C_7 + C_{14} + C_{21}) / 4$$

where

C<sub>0</sub> : the measured concentration at the start

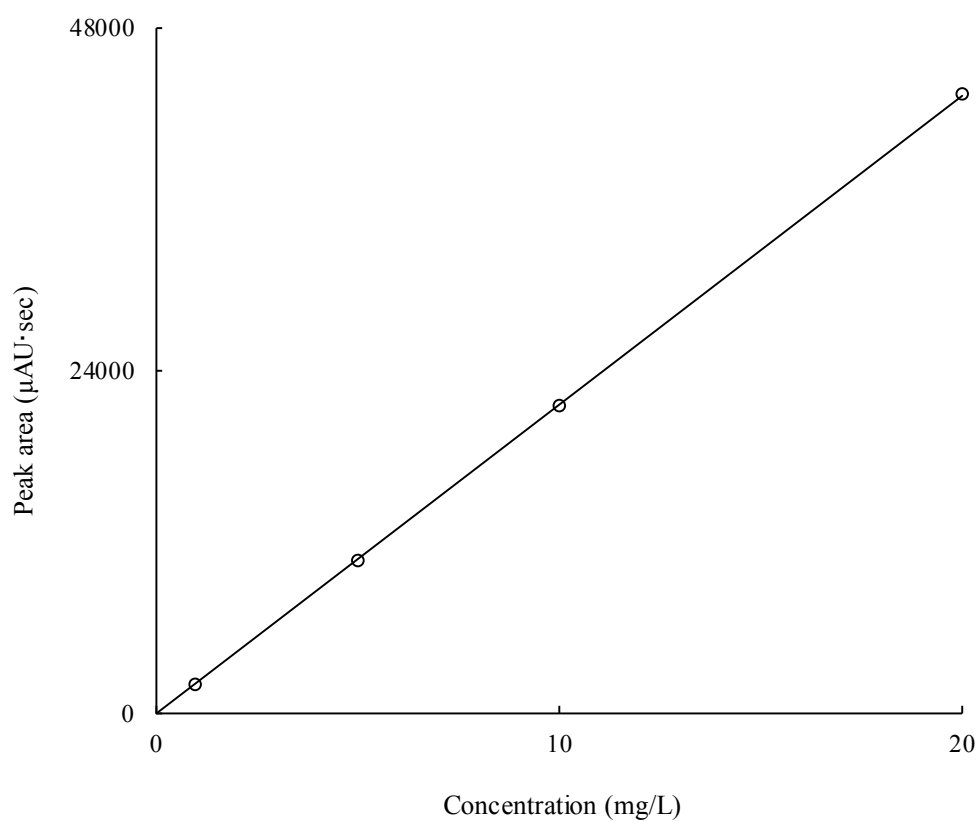
C<sub>7</sub> : the measured concentration at 7 days

C<sub>14</sub> : the measured concentration at 14 days

C<sub>21</sub> : the measured concentration at the end

## Appendix 3

Calibration curve and chromatogram

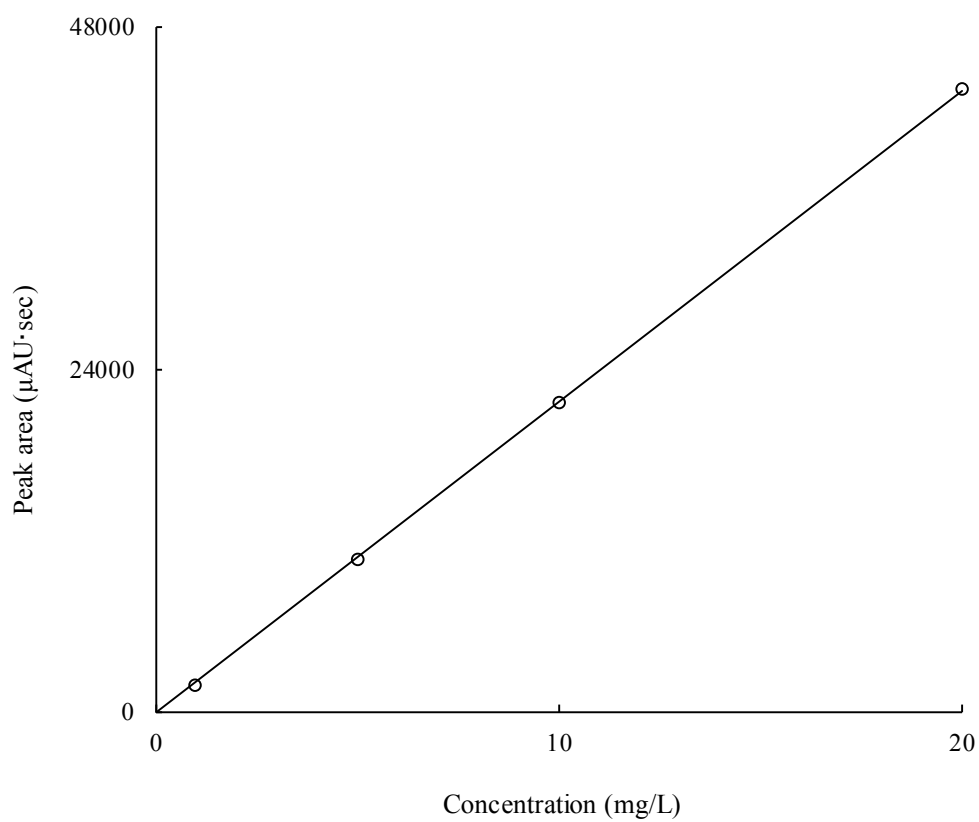


$$y = 2161x$$

$$r = 1.00$$

Concentration (mg/L)	Peak area (μAU·sec)
1.00	1962
5.00	10660
10.0	21531
20.0	43305

Appendix figure 3-1 Calibration curve of test item (PFHxA-NH<sub>4</sub>) for analysis by HPLC.



$$y = 2175x$$

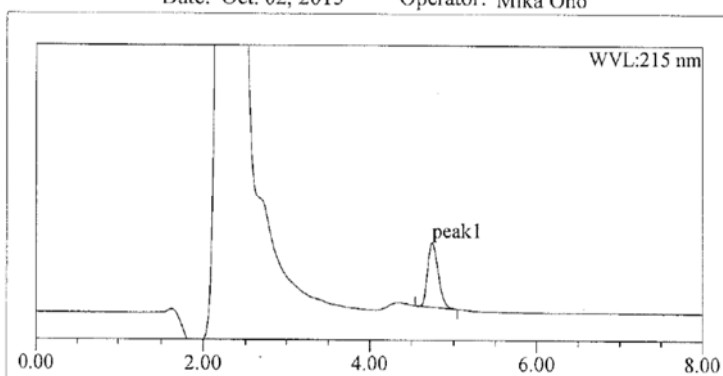
$$r = 1.00$$

Concentration (mg/L)	Peak area ( $\mu\text{AU}\cdot\text{sec}$ )
1.00	1841
5.00	10633
10.0	21631
20.0	43638

Appendix figure 3-2 Calibration curve of test item (PFHxA-Na) for analysis by HPLC.

## PFHA-NH4 Standard solution 10.0 mg/L

Date: Oct. 02, 2015 Operator: Mika Ono

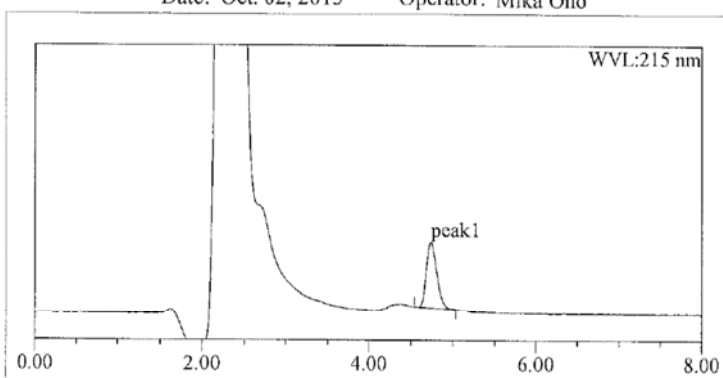


97026 151002 PFHA-NH4 S2

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
peak1	4.74	2402	21402	100.00
Total	-	-	21402	100.00

## PFHA-NH4 10.0 mg/L exposure level

Date: Oct. 02, 2015 Operator: Mika Ono

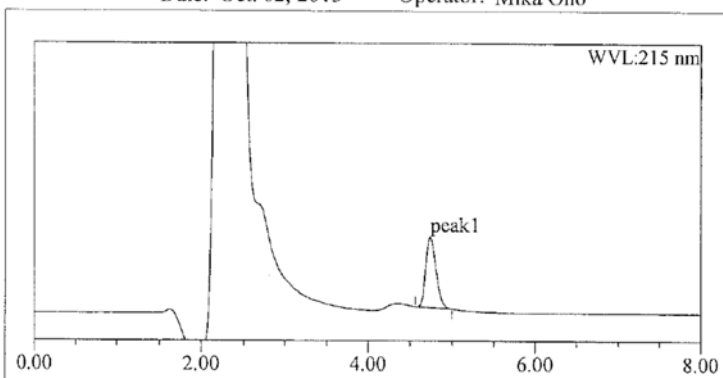


97026 151002 PFHA-NH4 H0dB

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
peak1	4.74	2465	21390	100.00
Total	-	-	21390	100.00

## PFHA-NH4 100 mg/L exposure level

Date: Oct. 02, 2015 Operator: Mika Ono



97026 151002 PFHA-NH4 H0dA

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
peak1	4.74	2606	22252	100.00
Total	-	-	22252	100.00

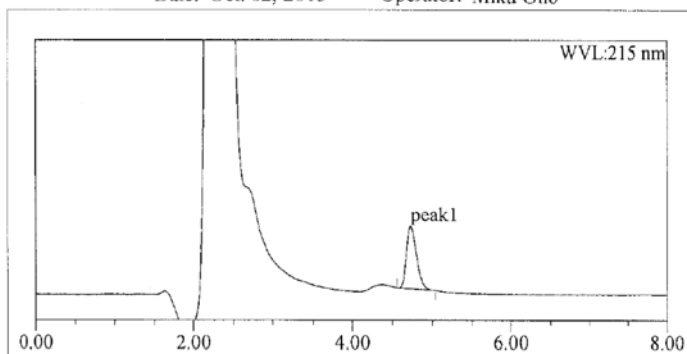
Appendix figure 3-3 HPLC chromatograms at start of exposure (PFHxA-NH<sub>4</sub>).



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## PFHA-Na Standard solution 10.0 mg/L

Date: Oct. 02, 2015 Operator: Mika Ono

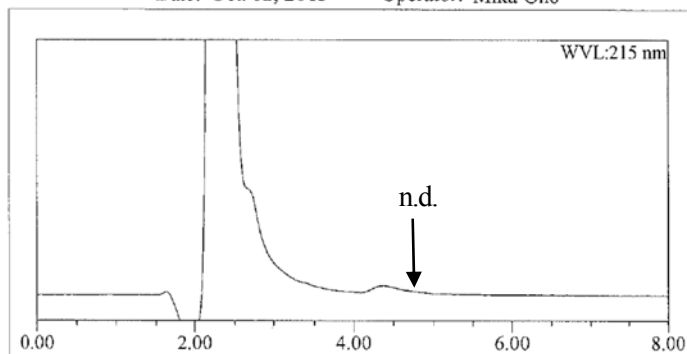


97026 151002 PFHA-Na S2

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
peak1	4.73	2467	21420	100.00
Total	-	-	21420	100.00

## Control

Date: Oct. 02, 2015 Operator: Mika Ono

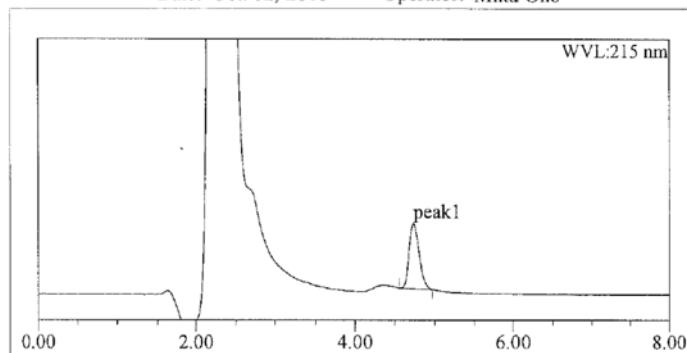


97026 151002 PFHA-Na H0dZ

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
peak1	-	-	-	-
Total	-	-	0	0.00

## PFHA-Na 10.0 mg/L exposure level

Date: Oct. 02, 2015 Operator: Mika Ono

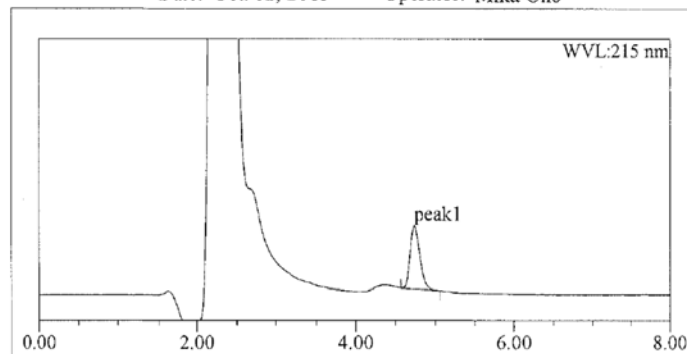


97026 151002 PFHA-Na H0dB

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
peak1	4.74	2600	22268	100.00
Total	-	-	22268	100.00

## PFHA-Na 100 mg/L exposure level

Date: Oct. 02, 2015 Operator: Mika Ono



97026 151002 PFHA-Na H0dA

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
peak1	4.74	2470	20800	100.00
Total	-	-	20800	100.00

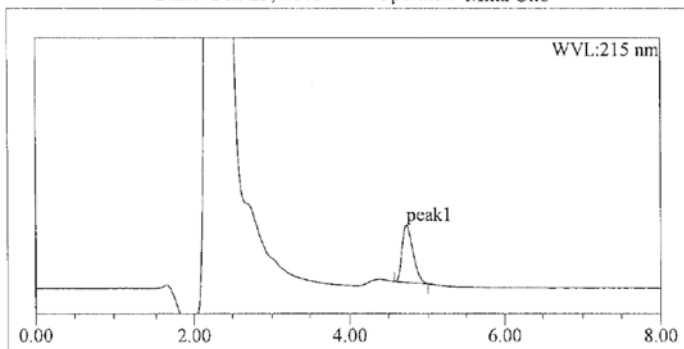
Appendix figure 3-4 HPLC chromatograms at start of exposure (PFHxA-Na).

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PFHA-NH4 Standard solution 10.0 mg/L

Date: Oct. 23, 2015 Operator: Mika Ono

97026 151023 PFHA-NH4 S2

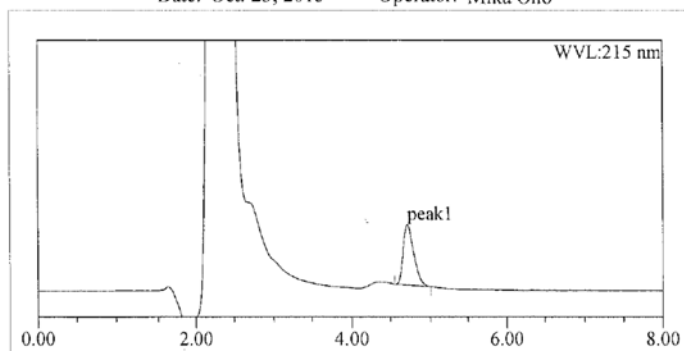


No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
peak1	4.73	2291	21406	100.00
Total	-	-	21406	100.00

PFHA-NH4 10.0 mg/L exposure level

Date: Oct. 23, 2015 Operator: Mika Ono

97026 151023 PFHA-NH4 H21dB

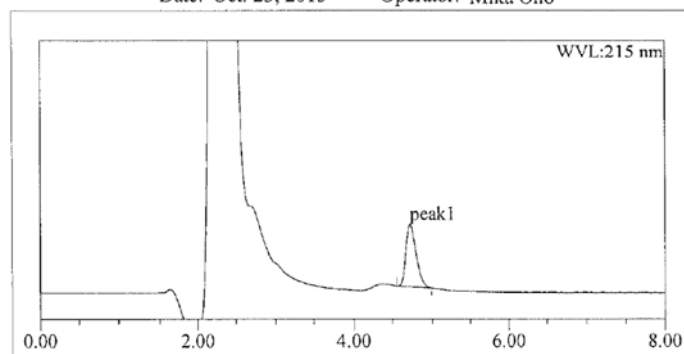


No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
peak1	4.71	2398	22068	100.00
Total	-	-	22068	100.00

PFHA-NH4 100 mg/L exposure level

Date: Oct. 23, 2015 Operator: Mika Ono

97026 151023 PFHA-NH4 H21dA

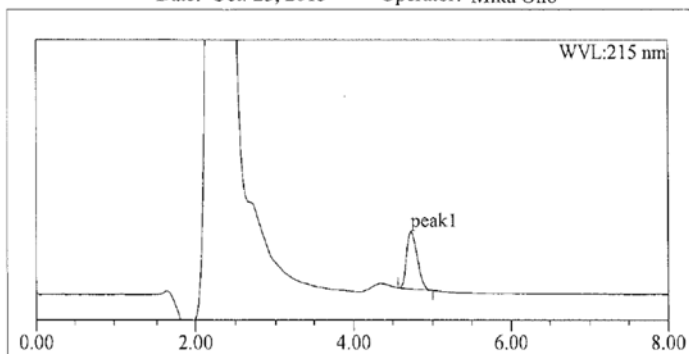


No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
peak1	4.72	2463	21866	100.00
Total	-	-	21866	100.00

Appendix figure 3-5 HPLC chromatograms at end of exposure (PFHxA-NH<sub>4</sub>).

## PFHA-Na Standard solution 10.0 mg/L

Date: Oct. 23, 2015 Operator: Mika Ono

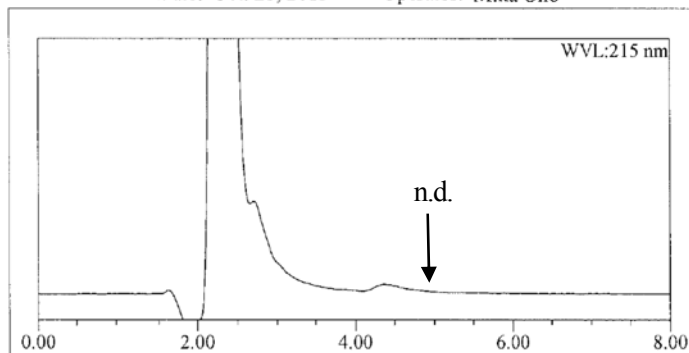


97026 151023 PFHA-Na S2

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
peak1	4.73	2244	21047	100.00
Total	-	-	21047	100.00

## Control

Date: Oct. 23, 2015 Operator: Mika Ono

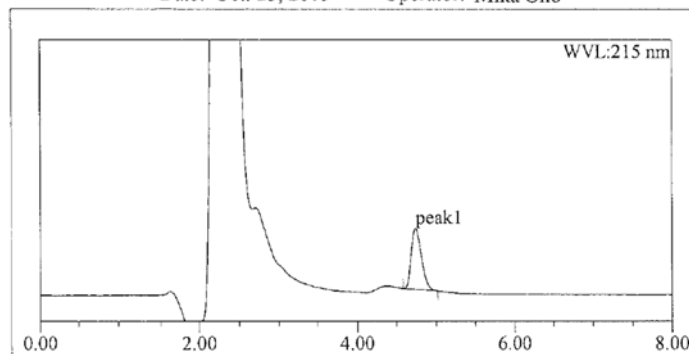


97026 151023 PFHA-Na H21dZ

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
peak1	-	-	-	-
Total	-	-	0	0.00

## PFHA-Na 10.0 mg/L exposure level

Date: Oct. 23, 2015 Operator: Mika Ono

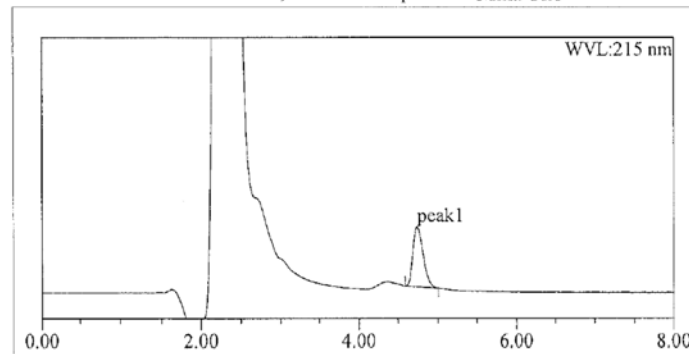


97026 151023 PFHA-Na H21dB

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
peak1	4.74	2403	21462	100.00
Total	-	-	21462	100.00

## PFHA-Na 100 mg/L exposure level

Date: Oct. 23, 2015 Operator: Mika Ono



97026 151023 PFHA-Na H21dA

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
peak1	4.74	2340	21028	100.00
Total	-	-	21028	100.00

Appendix figure 3-6 HPLC chromatograms at end of exposure (PFHxA-Na).