Submitted to:
Daikin Industries, Ltd.

REPORT

Developmental Toxicity Test of APFHx with Embryo of Zebrafish (*Danio rerio*)

(Study No. A180072)

Sealed date: May 7, 2018

Testing facility: Environmental Risk Assessment Center,
LSI Medience Corporation
1000 Kamoshida-cho, Aoba-ku, Yokohama,
Kanagawa, Japan

Study Director: _____

Summary

Embryo of zebrafish was exposed to the test substance in referred to " Fish Embryo Acute Toxicity (FET) Test " (OECD Test Guideline No.236) from 4-6 to above 120 hours post fertilization (hpf). At the end of exposure, embryo or larvae were evaluated for the mortality, the hatching rate and the morphology under the microscopy. No observed effect concentration (NOEC) value for the frequency of morphological abnormalities was 500 mg/L.

Purpose

Embryo of zebrafish was exposed to the test substance in order to calculate mortality and hatching rate and to evaluate developmental toxicity of the test substance on embryo to larval stages.

Test Guideline

The study was referred to " Fish Embryo Acute Toxicity (FET) Test " (OECD Test Guideline No.236).

Study Period

Exposure period: From April 12 to 17, 2018

Test Substance

Substance name:	APFHx
Chemical name:	2,2,3,3,4,4,5,5,6,6,6-undecafluorohexanoic acid, ammonium salt
Lot number:	C150E57002
Purity:	99.8%
Impurities:	Water

Positive Control

Substance name:	Sodium Valproate
Supplier:	FUJIFILM Wako Pure Chemical Corporation
Lot number:	PDN2525
Purity:	98.0%

Test Organisms

1) Common name (Scientific name): Zebrafish (*Danio rerio*)

2) Source: National Institute for Environmental Studies, Japan and Tsunashima Fishing Co. (The fish have been maintained at the testing facility.)

3) Collection of fertilized eggs: Freshly fertilized eggs were obtained from natural mating. All eggs ware collected in 0.003% Sodium hypochlorite solution as fungicide and rinsed three times with rearing water. Viable fertilized eggs were selected number of needed eggs by means of microscopy.

Method

Viable fertilized eggs were exposed to the test solutions within 4-6 hpf, respectively. Each egg was individually transferred into a well of a 24-well plate counting each test solution (Total volume was 2 ml/well). After 48-hour exposure, semi-static renewal was performed. The test ended at above 120 hpf. At the end of exposure, embryo or larvae were evaluated for the mortality, the hatching rate and the morphology under the microscopy. The details were determined as follows:

1) Dilution water: Dechlorinated water*1
*1: Dechlorinated water processed by treating Yokohama city tap water with an activated charcoal filter and sodium thiosulfate

2) Preparation of Test Solutions: The test substance and sodium valproate (positive control) were weighed, then dissolved in dilution water and used as the test solution. The test solution of Control was only dilution water.

3) Duration: 4-6 to above 120 hpf (6 days)

4) Exposure procedure: Semi-static (batchwise renewal of the test solutions at 48 hours after the start of exposure)

5) Nominal concentration: Control, Positive control (33.2 mg/L sodium valproate), 100, 500, 1000, 1500, 2000 mg/L

6) Vessel: 24-multiwell plate with cover

7) Volume of test solution: 2 mL/well

8) Number of vessels: 1 vessels/test group (20 well/ test group)

9) Number of loading eggs: 20 eggs/test group (1 eggs/well)

10) Temperature: 28±0.5°C

11) pH: Not adjusted

12) Light: Fluorescent light,
16-hour light (800 lux or less) /8-hour dark

13) Feeding: None

14) Observation of the loaded embryos: Morphology of the embryo or larvae was observed and assessed at 24-, 48-, 96-hour and the end of exposure under a microscope. At the end of exposure, viable larvae were anesthetized with 1000 mg/L 2-Phenoxyethanol (FUJIFILM Wako Pure Chemical Corporation), and then recorded as morphological abnormalities.

15) Test Results: Mortality[*2], hatching rate[*3], morphological assessment [*4] at the end of exposure (Parameters of morphological assessment are listed in Table 1)

*2: The ratio of dead embryos or larvae at the end of exposure relative to the loading eggs (Mortality: %)

*3: The ratio of the eggs hatched at the end of exposure relative to the loading eggs (Hatching rate: %)

*4: The ratio of morphological abnormalities on the viable larvae at the end of exposure (frequency of morphological abnormalities: %) and paramerters observed for morphological abnormalities

Results

The mortality, the hatching rate and the frequency of morphological abnormalities are given in Table 2, 3 and 4.

In the control and positive control group, the mortality were both 0% and hatching rate were both 100%. In the conc.1-5, the mortality were 0, 0, 20, 80 and 100% and hatching rate were 100, 100, 90, 90 and 90%, respectively.

In the control and positive control group, the frequency of morphological abnormalities were 10 and 100%. In the conc.1-4, the frequency of morphological abnormalities were 5, 20, 100 and 100%, respectively. The details of morphological assessment are given in Table 5 and figure 1.

The median lethal concentration (LC50) values of each period are shown in Table 6, and that at the end of exposure is given below.

120 hpf-LC50: 1220 mg/L (95% confidence limits: 1080 - 1340 mg/L, Probit method)

No observed effect concentration (NOEC) for the frequency of morphological abnormalities was determined by χ^2 test (Yukms Statlight #5 software, Yukms Corp., Tokyo). The NOEC value is given below. The details are described in Appendix 1.

NOEC for the frequency of morphological abnormalities: 500 mg/L

Table 1 Parameters of morphological assessment*1

Organs	Parameters
Body	Axis, Somite, Notochord, Fin (pectoral fin and whole fin expect pectoral fin)
Face/head	Eye, Head, Low jaw, Otolith
Thoracic region	Edema, Heart
Abdominal region	Edema, Intestinal tissue (including liver)
Circulation	Blood Circulation, Heartbeat

*1 Reference: Yamashita A. et al., Improvement of the evaluation method for teratogenicity using zebrafish embryos. J Toxicol Sci. 2014, vol.39, no.3, 453-464.

Table 2 Mortality

Test group	Nominal concentraion (mg/L)	Cumulative number of dead (Mortality, %)			
		24hpf	48hpf	96hpf	120hpf
Control	-	0 (0)	0 (0)	0 (0)	0 (0)
Positive control	33.2	0 (0)	0 (0)	0 (0)	0 (0)
Conc.1	100	0 (0)	0 (0)	0 (0)	0 (0)
Conc.2	500	0 (0)	0 (0)	0 (0)	0 (0)
Conc.3	1000	0 (0)	0 (0)	2 (10)	4 (20)
Conc.4	1500	0 (0)	0 (0)	7 (35)	16 (80)
Conc.5	2000	0 (0)	0 (0)	17 (85)	20 (100)

Table 3 Hatching rate

Test group	Nominal concentraion (mg/L)	Number of hatching	Hatching rate (%)
Control	-	20	100
Positive control	33.2	20	100
Conc.1	100	20	100
Conc.2	500	20	100
Conc.3	1000	18	90
Conc.4	1500	18	90
Conc.5	2000	18	90

Table 4 Frequency of morphological abnormalities

Test group	Nominal concentraion (mg/L)	Number of morphological assessment *1	Number of morphological abnormality *2	Frequency of morphological abnormality (%)
Control	-	20	2	10
Positive control	33.2	20	20	100
Conc.1	100	20	1	5
Conc.2	500	20	4	20
Conc.3	1000	15	15	100
Conc.4	1500	4	4	100
Conc.5	2000	-	-	-

*1: Assessed at the survival larvae

*2: Non inflated swim bladder was not considered as morphological abnormality

Table 5 The results of morphological assessment

Organs	Major parameters observed as abnormality	
	Positive control	Test substance
Body	Somite anomalies Notochord anomalies Fin anomalies	Somite anomalies Notochord anomalies Fin anomalies
Face/head	Lower jaw anomalies	-
Thoracic region	Pericardial edema Heart anomalies	Pericardial edema
Abdominal region	Abdominal edema	Abdominal organ anomalies Opaque intestinal tissue
Circulation	Blood circular anomalies Heartbeat anomalies	Blood circular anomalies Heartbeat anomalies

Table 6 Median Lethal Concentrations (LC50)

Fertilization period (hpf)	LC50 (mg/L)	95-Percent confidence limits (mg/L)	Statistical method
24	**>2000**	-	-
48	**>2000**	-	-
96	**1560**	1380-1770	Probit
120	**1220**	1080-1340	Probit

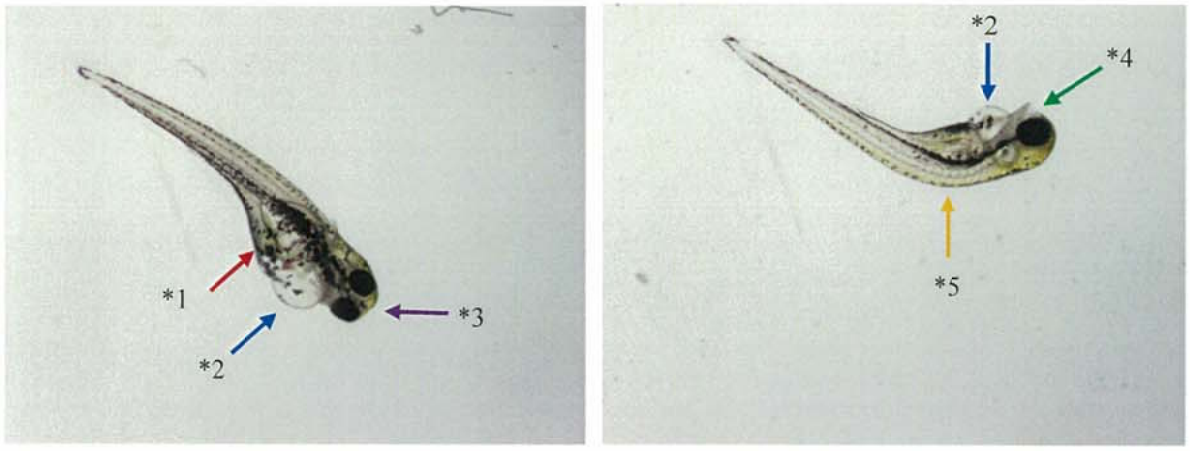
Data-processing program: TOXDAT3_02*1

*1: EPA/600/4-85/013 March 1985

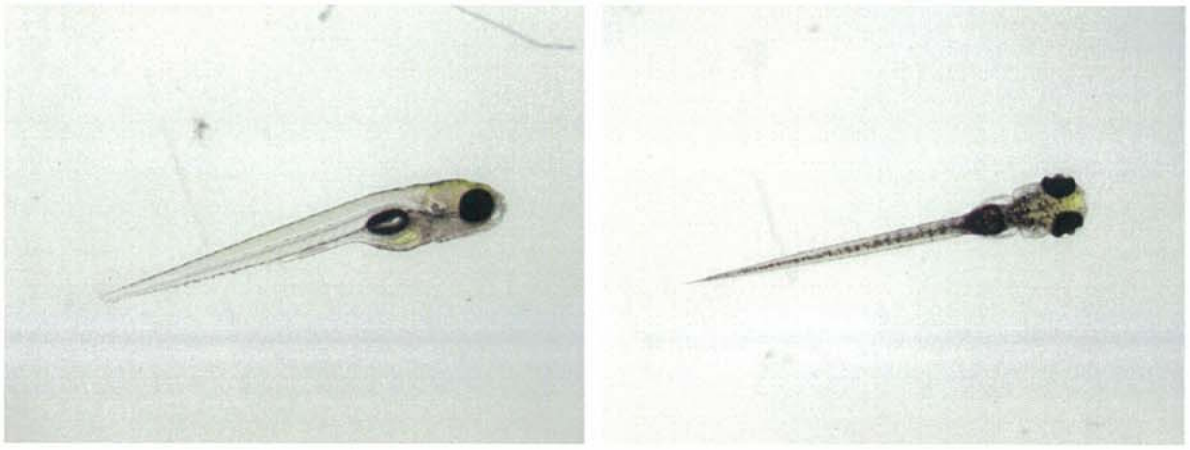
Figure 1　The results of morphological assessment

1) Control

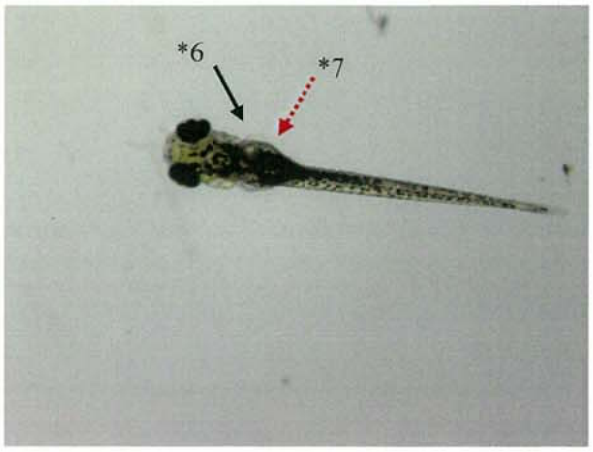
2) Positive Control

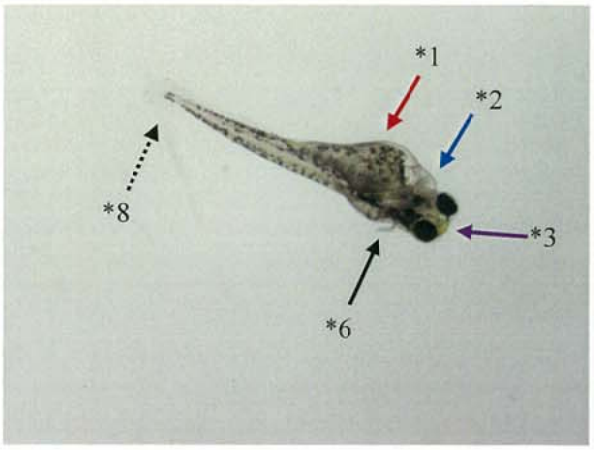


3) Conc.1

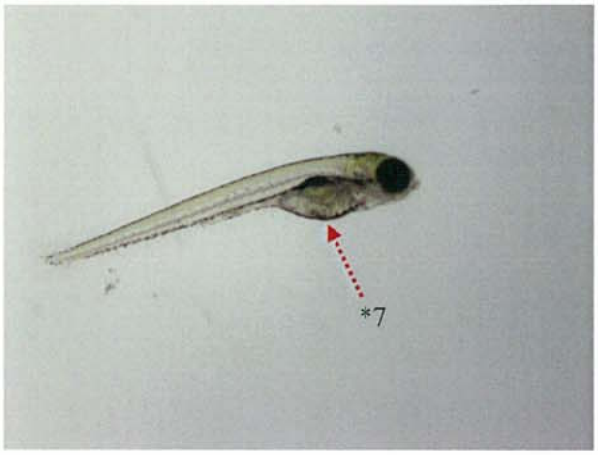


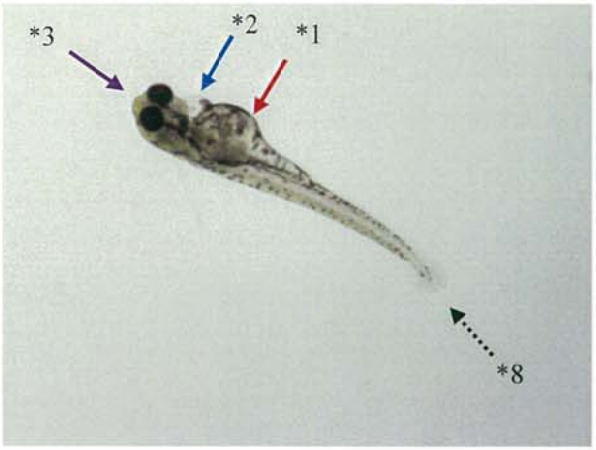
4) Conc.2



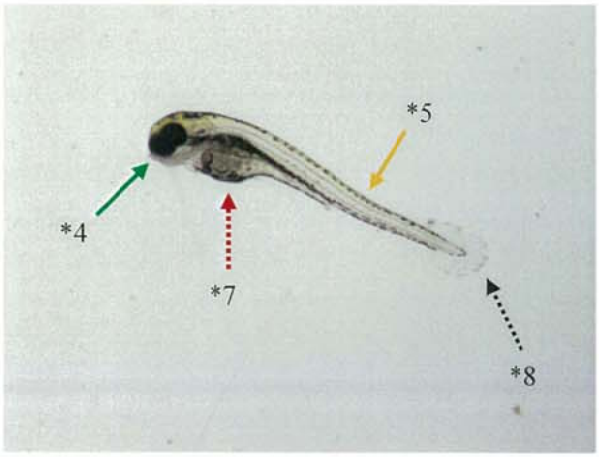


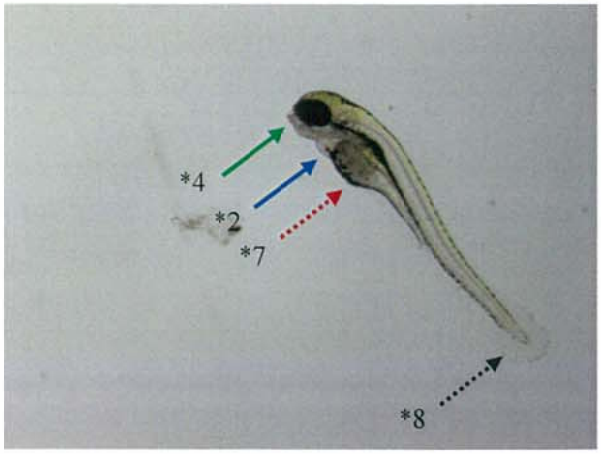
5) Conc.3





6) Conc.4





*1 (→): Abdominal edema

*2 (→): Pericardial edema, Heart anomaly (Unfold)

*3 (→): Abnormal Eye and Head shape (Edema and small size)

*4 (→): Abnormal Low jaw shape (Opening)

*5 (→): Abnormal Notochord shape (Bent)

*6 (→): Abnormal Fin shape

*7 (-->): Abnormal Fin shape (Necrosis)

*8 (-->): Abnormal Abdominal Organ shape

Appendix 1 Results of the χ^2 test for the frequency of morphological abnormalities

A significant difference from the control was detected by step-down $\chi 2$ test using the data of the control and Conc. 1-4.

Group	Test Group	Nominal Cconcentration (mg/L)	Morphological Assessment	
			Abnormal	Normal
1	Control	–	2	18
2	Conc.1	100	1	19
3	Conc.2	500	4	16
4	Conc.3	1000	15	0
5	Conc.4	1500	4	0
6	Conc.5	2000	-	-

Group	Samples	Mean	S.E.	S.D.	Variance
1	*	*	*	*	*
2	*	*	*	*	*
3	*	*	*	*	*
4	*	*	*	*	*
5	*	*	*	*	*

Method	vs	Side	Stat.	0.05	0.01	0.001	Prob.	
m*n Chi-Square test			0 52.0523	>9.4877	>13.2767	18.4668	1.345E-10	**

Group	Samples	Mean	S.E.	S.D.	Variance
1	*	*	*	*	*
2	*	*	*	*	*
3	*	*	*	*	*
4	*	*	*	*	*

Method	vs	Side	Stat.	0.05	0.01	0.001	Prob.	
m*n Chi-Square test			0 46.2961	>7.8147	>11.3449	16.2662	4.906E-10	**

Group	Samples	Mean	S.E.	S.D.	Variance
1	*	*	*	*	*
2	*	*	*	*	*
3	*	*	*	*	*

Method	vs	Side	Stat.	0.05	0.01	0.001	Prob.
m*n Chi-Square test			0 2.2642	<5.9915	<9.2103	13.8155	0.3224

**: Indicates a significant difference (α=0.01) from the control.