CERI

Receipt number	662-06-E-4230
Study number	94230

FINAL REPORT

A 48-hour Acute Immobilization Study of 13F-OLE with *Daphnia magna*

September 10, 2007

Kurume Laboratory
Chemicals Evaluation and Research Institute, Japan

STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title A 48-hour Acute Immobilization Study of 13F-OLE with *Daphnia magna*

Study number 94230

I, the undersigned, hereby declare that this report provides a correct English translation of the Final Report (Study No. 94230, issued on September 10, 2007)

Date October 27, 2009

Study Director

GLP STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title A 48-hour Acute Immobilization Study of 13F-OLE with *Daphnia magna*

Study number 94230

The study described in this report was conducted in compliance with the following GLP principles:

(1) "Standard Concerning Testing Facility Relating to New Chemical Substances" (November 21, 2003; No. 1121003, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 17, 2003, No. 3, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121004, Environmental Policy Bureau, Ministry of the Environment)
(2) "OECD Principles of Good Laboratory Practice" (November 26, 1997, ENV/MC/CHEM (98)17)

This final report reflects the raw data accurately and it has been confirmed that the test data are valid.

Date September 10, 2007

Study Director Signed in original

QUALITY ASSURANCE STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title A 48-hour Acute Immobilization Study of 13F-OLE with *Daphnia magna*

Study number 94230

I assure that the final report accurately describes the test methods and procedures, and that the reported results accurately reflect the raw data of the study.

The inspections and audit of this study were carried out and the results were reported to the Study Director and the Test Facility Management by Quality Assurance Unit as follows..

Item of inspection / audit	Date of inspection / audit	Date of report to Study Director and Test Facility Management
Study plan draft	August 10, 2007	August 10, 2007
Study plan	August 13, 2007	August 13, 2007
Amendment of study plan	August 31, 2007	August 31, 2007
Measurement of solubility	August 22, 2007	August 23, 2007
	August 23, 2007	August 23, 2007
Start of the exposure and after the exposure	August 20, 2007	August 24, 2007
	August 22, 2007	August 24, 2007
	August 24, 2007	August 24, 2007
Raw data and final report draft	September 7, 2007	September 7, 2007
Final report	September 11, 2007	September 11, 2007

Date September 11, 2007

Head of Quality Assurance Unit Signed in original

CONTENTS

Page

Title	A 48-hour Acute Immobilization Study of 13F-OLE with *Daphnia magna*
Sponsor	DAIKIN INDUSTRIES, LTD. 1-1, Nishi-Hitotsuya, Settsu, Osaka 566-8585, Japan
Test facility	Kurume Laboratory Chemicals Evaluation and Research Institute, Japan 3-2-7 Miyanojin, Kurume-shi, Fukuoka 839-0801, Japan
Objective	The purpose of this study is to determine the acute toxicity of 13F-OLE to *Daphnia* sp.
Test method	This study was performed according to the following test methods and guidance document. (1) *Daphnia* sp., Acute Immobilization Test stipulated in the "Testing Methods for New Chemical Substances" (November 21, 2003; No. 1121002, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 13, 2003, No. 2, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121002, Environmental Policy Bureau, Ministry of the Environment) (2) OECD Guidelines for Testing of Chemicals, Section 2 : Effects on Biotic Systems, 202 "Daphnia sp., Acute Immobilisation Test (Guideline 202, April 13, 2004)" (3) OECD Guidance Document No. 23 "Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures" (September 2000)
Applied GLP	This study was conducted in compliance with the following GLP principles: (1) "Standard Concerning Testing Facility Relating to New Chemical Substances" (November 21, 2003; No. 1121003, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 17, 2003, No. 3, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121004, Environmental Policy Bureau, Ministry of the Environment) (2) "OECD Principles of Good Laboratory Practice" (November 26, 1997, ENV/MC/CHEM (98)17).

Dates

Study initiation date	August 13, 2007
Experimental starting date	August 22, 2007
Solubility study starting date	August 22, 2007
Bioassay starting date	August 22, 2007
Experimental completion date	August 24, 2007
Solubility study completion date	August 24, 2007
Bioassay completion date	August 24, 2007
Study completion date	September 10, 2007

Storage of test item, raw data, etc.

(1) Test item

The test sample[*1] will be sealed in a storage vessel and stored in archives in this laboratory for ten years after the receipt of notice specified under Clause 1 or Clause 2 in Article 4, Clause 2 or Clause 3 or Clause 8 in Article 4-2, and Clause 2 in Article 5-4 or Clause 2 in Article 24 or Clause 2 in Article 25-3 of "Law Concerning Examination and Regulation of Manufacture, etc. of Chemical Substances". If it is not stable for the storage period, it will be stored as long while it is kept stable. Treatment of the sample after the storage period will be discussed with sponsor.

*1 It will be stored as the common sample for storage of these studies (Study Nos. 94229, 94230 and 94231).

(2) Raw data and materials

Raw data, the study plan, documents concerning the study presented by the sponsor, the final report and necessary materials will be stored in archives in this laboratory for ten years after the receipt of the notice specified under Clause 1 or Clause 2 in Article 4, Clause 2 or Clause 3 or Clause 8 in Article 4-2, and Clause 2 in Article 5-4 or Clause 2 in Article 24 or Clause 2 in Article 25-3 of "Law Concerning Examination and Regulation of Manufacture, etc. of Chemical Substances". Treatment of raw data and materials, etc. after the storage period will be discussed with sponsor.

Personnel

Study Director :

Section 4 (Eco-toxicity test area)

Study personal

Biology :

Analytical chemistry :

Approval of final report

Study Director Date September 10, 2007

Signature Signed in original

SUMMARY

Title

A 48-hour Acute Immobilization Study of 13F-OLE with *Daphnia magna*

Test conditions

(1) Test item	13F-OLE
(2) Test organism	*Daphnia magna*
(3) Exposure duration	48 hours
(4) Test concentration	Middle layer of suspended solution (nominal concentration: 100 mg/L) and control
(5) Number of organism	Twenty daphnids/test level (five daphnids/test vessel)
(6) Dilution water	Dechlorinated tap water
(7) Type of test	Semi-static regime (renewal at 24 hours after) and closed system
(8) Preparation of test solution	The test sample and dilution water were mixed to prepare 100 mg/L (nominal concentration) and they were stirred under closed system for about 48 hours. After settlement for 1 hour, test solution was prepared by taking out from the middle layer.
(9) Replicate	Four replicates/test level
(10) Volume of test solution	About 1000 mL/test level (about 250 mL/test vessel)
(11) Temperature of test solutions	20±1°C
(12) Irradiation condition	Artificial light of white fluorescent lamp, 16-hour light / 8-hour dark
(13) Feeding	No feeding
(14) Aeration	No aeration
(15) Analysis of concentration of test item in test solution	GC-MS analysis (at the start of the exposure, before and after the renewal and the end of the exposure)

Results

(1) Solubility of test item in dilution water (20±1°C)	0.0606 mg/L
(2) Concentration of test item in test solution (Percentage of concentration at preparation)	
At the start of the exposure and after the renewal	0.0623 and 0.0970 mg/L
Before the renewal and at the end of the exposure	0.0629 and 0.0704 mg/L (101 and 72.6%)
(3) 48-hour EC_{50} (Median Effective Concentration)	> 0.0719 mg/L

[The values of (3) is based on geometric mean of the measured concentrations.]

Conclusion

This study was conducted as a limit test at the concentration around solubility of the test item in dilution water to confirm the effect on the test organisms. It was concluded that the test item has no acute toxicity to the test organisms at the concentration around water solubility, since the measured concentrations of the test solutions were around the solubility in dilution water at the preparation and no effect on the test organisms was observed under the test condition.

1. Test item

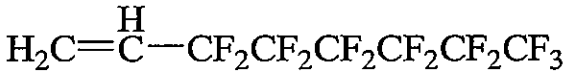
In this report, 13F-OLE has the following name etc.

1.1 Chemical name*2

3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluoro-octa-1-ene

1.2 Chemical structure etc.*2

Structural formula



Molecular formula	$C_8H_3F_{13}$
Molecular weight	346.09
CAS Number	25291-17-2

*2 Information supplied by the sponsor

2. Test sample

2.1 Supplier and lot number*2

Supplier	DAIKIN INDUSTRIES, LTD.
Lot number	061122HM

2.2 Purity*2

Test item	99.8%	
Impurity	Unknown constituent component	0.2%

2.3 Confirmation of test item

It was confirmed that infrared (IR) spectrum of the test item provided by the sponsor coincided with IR spectrum analyzed in this laboratory.

2.4 Physicochemical properties*2

Appearance at normal temperature	Colorless and clear liquid	
Boiling point	106°C (760 mmHg)	
Density	1.560 g/cm^3 (20°C)	
Solubility	Water	Insoluble
	Dimethylsulfoxide	Soluble (fully miscible)
	Acetone	Soluble (fully miscible)

*2 Information supplied by the sponsor

2.5 Storage condition and confirmation of stability at storage condition

Storage condition	Dark storage place at room temperature
Confirmation of stability	The stability of the test item during the test period was confirmed by no alteration in the IR spectra of the test item before the experimental start and after the experimental completion.

3. Test materials and methods

3.1 Test organism

(1) Species

Daphnia magna (Clone A)

(2) Reason for selection of species

Species recommended in the test guidelines

(3) Source

Young daphnids produced by parents which were cultured in the Kurume Laboratory were used. Daphnids [Daphnia magna (Clone A)] originally came from the University of Sheffield (Address: Sheffield S10 2UQ, United Kingdom). The parents to obtain young daphnids were bred in the same quality of water (dechlorinated tap water), water temperature (20±1°C), photoperiod (16-hour light/8-hour dark) as used in the test. Parents used for the test were same lot and bred for more than 14 days, and their age and survival rate were 21-days old and 100%, respectively. Chlorella vulgaris of 0.1 to 0.2 mgC/day per daphnia was fed to the parents once a day. A 48-hour acute immobilization test of K2Cr2O7 (Reagent grade, Wako Pure Chemical Industries, Ltd.) with the test organisms was conducted (on June 26 to June 28, 2007) to confirm the reproducibility of the test conditions. The 48-hour EC50 of K2Cr2O7 was 0.296 mg/L. This value was within the normal range in this laboratory (mean ± 2S.D.: 0.124 - 0.350 mg/L) [mean ± S.D.: 0.237± 0.057 mg/L (n=59)].

(4) Selection of young daphnids

Less than 24-hour old daphnids were used for the test.

(5) Allocation to the test groups

Test organisms were placed at random to each test vessel.

3.2 Dilution water

Dechlorinated tap water, aerated sufficiently and controlled temperature, was used. Some chemical characteristics of the dilution water measured regularly are listed in Appendix 1.

3.3 Test apparatus and equipment

(1) Test apparatus

Test vessel : Petri dish (diameter: 8.0 cm, depth: 5.0 cm)

The test vessels were covered and closed with glass lid in order to prevent dust, and volatilization of the test solution.

(2) Test equipment

Water bath : Plastic tank

Warming / cooling unit (Type HCA 250, Sato craft)

3.4 Test conditions

(1) Conditions of exposure

(a) Type of test

The test organisms were exposed to the test solution containing the test item.

The test was conducted using semi-static regime of whole test solution replacement after 24 hours and closed system.

(b) Exposure duration

48 hours

(c) Test concentration

Based on the results of the preliminary studies, it was expected that the test solution at around the solubility in dilution water would have no immobility of the test organism. Therefore, the definitive study was conducted as the limit test with suspended solution which was prepared by taking out from the middle layer of 48-hour mixed solution (nominal concentration: 100 mg/L). The results of the preliminary studies are shown in Additional data.

(d) Control

The dilution water without the test item, which was stirred in the same manner as the test solution, was used as the control.

(e) Replicates

Four replicates/test level

(f) Number of organism

Twenty daphnids/test level (five daphnids/test vessel)

(g) Volume of test solution

About 1000 mL/test level (about 250 mL/test vessel)

(2) Conditions of test environment

(a) Temperature

20±1°C

(b) Dissolved oxygen concentration

The study was performed in the condition where dissolved oxygen concentration was more than 3 mg/L. No aeration was used for the test during the exposure.

(c) pH

The test was performed without adjusting pH.

(d) Irradiation condition

Artificial light of white fluorescent lamp, 16-hour light/8-hour dark

(e) Feeding

Test organisms were not fed during the exposure.

3.5 Preparation of test solution

No correction with purity was done for the preparation of the test concentration. The test sample was employed in terms of volume using the density [1.560 g/cm^3 (20°C)] for the preparation of test solution.

After the test sample was added into the dilution water filled in Erlenmeyer flask with micro volumeter (Eppendorf Co., Ltd) to prepare test solution of 100 mg/L as nominal concentration, the flask was immediately sealed with a plug not to produce head space. The solution was gently stirred by magnetic stirrer for about 48 hours under 20±1°C to produce dispersed solution with suspended test item. After cease of stirring, the solution was settled for 1 hour and then test solution was prepared by taking out from the middle layer of the settled solution. The prepared test solution was immediately divided into each test vessel and covered with glass lid not to produce head space.

3.6 Observation and measurements

(1) Observation of test organisms

Immobility and symptom were observed at 24 and 48 hours after the exposure. Daphnids were considered immobile if they were not able to swim within 15 seconds after gentle agitation of the test vessel.

(2) Appearance of test solution

Appearance of the test solutions were observed at the start of the exposure and before the renewal (after 24 hours).

(3) Condition of test solutions

Dissolved oxygen concentration, pH and temperature of the test solutions were measured at the start of the exposure, before and after the renewal and the end of the exposure(twice of a set of preparation and 24 hours after). At the preparation, another solution sampled from the container for preparation was used for the measurement. At 24 hours after the preparation, the measurement was carried out for one test vessel in each level. The dissolved oxygen concentration measurements were carried out with an oxygen meter (YSI Incorporated., YSI Model 58). The pH measurements were carried out with a portable pH meter (DKK-TOA, Model HM-21P). The temperature measurements were carried out with a calibrated red alcohol thermometer of glass stick type.

(4) Concentration of test item in test solution

The concentration of the test item in the test solutions was measured at the start of the exposure, before and after the renewal and the end of the exposure (twice of a set of preparation and 24 hours after). At the start of the exposure, another solution sampled from the container for preparation was used for analysis. At 24 hours after the preparation, the test solution for analysis was taken out with equal volume from the middle layer of the test solution in test vessels in each test level, and mixed. The concentration of the test item was analyzed by gas chromatography - mass spectrometry (GC-MS). Analytical method and measured concentration of test item are shown in Appendix 2, and analytical calibration curve and chromatograms are shown in Appendix 3.

(5) Solubility of test item in dilution water

Since the solubility of the test item was expected less than 100 mg/L, it was measured concurrently with the definitive study. The detail and the result of the measurement of the solubility are shown in Appendix 4.

3.7 Calculating method of EC_{50} *3

The EC_{50} value was estimated as "> test concentration" since no less than 50% of immobility was not observed in the present exposure level.

The results of the study were estimated based on a geometric mean of the measured concentrations as the test concentration.

*3 EC_{50} (Median Effective Concentration) is the concentration at which causes 50% immobility of tested population during exposure.

3.8 Validity of the test

(1) The immobilization rate should not exceed 10% in control group during exposure.

(2) Not more than 10% of the control daphnids should show the signs of disease or stress, for example, discoloration or unusual behavior such as trapping at surface of water.

(3) Dissolved oxygen concentration should be more than 3mg/L at the end of the exposure.

3.9 Treatment of numerical values

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

4. Results and discussion

4.1 Immobility

No immobility of the test organism was observed in the exposure level during exposure. Immobility at 24 and 48 hours are shown in Table 1. Immobility in the control during exposure was 0% and no abnormal response (discolor of body, trapping at the surface of the water and so on) was observed, which meets the criterion for the validity of the test (i.e. not more than 10%).

4.2 Observed abnormal response

There was no abnormal response in the control.

The following results of observation were based on the comparison with the control organisms. No abnormal responses were obtained in the test level during exposure. The result of the observation during exposure is shown in Table 2.

4.3 Observation and measurement of test solution

(1) Appearance of test solution

The test solutions were clear and colorless at the start of the exposure and before the renewal.

(2) Condition of test solutions

The measured values of dissolved oxygen concentration, pH and temperature during the exposure ranged from 8.2 to 8.3 mg/L, 7.8 to 7.9 and 19.9 to 20.0°C, respectively. Conditions of the test solutions are shown in Tables 3-1, 3-2 and 3-3. The measured values of dissolved oxygen concentration met the criterion for the validity of the test (more than 3 mg/L at the end of exposure).

(3) Concentration of test item in test solution

The measured concentrations of the test item in the test solution were 0.0623 and 0.0970 mg/L at the start of the exposure and after the renewal. Before the renewal and at the end of the exposure, they were 0.0629 and 0.0704 mg/L which were 101 and 72.6% of the concentration at the preparation. The results of the measured concentrations of the test item are shown in Appendix 2.

4.4 EC_{50}

Both the 24-hour and 48-hour EC_{50}s to *Daphnia magna* were >0.0719 mg/L (based on a geometric mean of the measured concentrations). The EC_{50}s at each observation time are shown in Table 4.

4.5 Discussion

This study was conducted as a limit test in order to confirm the effect of the test item on the test organisms at the concentration around the solubility of the test item in dilution water. As a result, the measured concentration of the test solution at the preparation was almost the same concentration as the solubility in the dilution water. Although it decreased at the end of the exposure, it is thought that the definitive study was appropriate for the test at the concentration around the solubility since the test was performed using semi-static replacement regime (renewal at 24 hours after) to maintain the test concentration. No adverse effect was found under the condition in the definitive study, therefore, it was concluded that the test item had no adverse acute effect on the test organisms at around the solubility in dilution water.

5. Factors that affected reliability of test results

There were no factors which might have affected the reliability of the test.

Table 1 Immobility

Measured Concentration*4 (mg/L)		Immobility (%)			
		24 hours		48 hours	
		Replicate	Test level	Replicate	Test level
Control	A	0	0	0	0
	B	0		0	
	C	0		0	
	D	0		0	
0.0719	A	0	0	0	0
	B	0		0	
	C	0		0	
	D	0		0	

*4: geometric mean of measured concentration

(The followings are expressed as measured concentration)

Table 2 Observed abnormal response

Measured concentration (mg/L)	Observed abnormal response	
	24 hours	48 hours
Control	-	-
0.0719	-	-

- : No abnormal response

Table 3-1 Dissolved oxygen concentration of test solutions

Measured concentration (mg/L)	At the start	24 hours		At the end
		Before the renewal	After the renewal	
Control	8.3	8.2	8.2	8.2
0.0719	8.3	8.2	8.2	8.2

Unit : mg/L

Table 3-2 pH of test solutions

Measured concentration (mg/L)	At the start	24 hours		At the end
		Before the renewal	After the renewal	
Control	7.9	7.9	7.9	7.8
0.0719	7.9	7.9	7.9	7.8

Table 3-3 Temperature of test solutions

Measured concentration (mg/L)	At the start	24 hours		At the end
		Before the renewal	After the renewal	
Control	19.9	19.9	19.9	20.0
0.0719	20.0	19.9	19.9	20.0

Unit: °C

Table 4 EC_{50} to *Daphnia magna*

Exposure duration	EC_{50} (mg/L)	95% confidence interval (mg/L) (Slope of the dose-response curve)	Statistical procedure used for determination of EC_{50}
24-hour	>0.0719	- (-)	-
48-hour	>0.0719	- (-)	-

-: Not obtained

Appendix 1

Chemical characteristics of dilution water

Chemical charasteristics of dilution water (Sampling on July 2, 2007))

Parameter	Unit	Results	Lower limit of determination
Total hardness (as $CaCO_3$)	mg/L	37.0	0.1
Suspended solid	mg/L	< 1	1
pH	—	7.7 (24℃)	—
Total organic carbon	mg/L	< 0.1	0.1
Chemical oxygen demand	mg/L	< 0.5	0.5
Residual chlorine	mg/L	< 0.02	0.02
Ammonium ion	mg/L	0.02	0.01
Total cyan	mg/L	< 0.01	0.01
Alkalinity	mg/L	29	1
Electric conductivity	mS/m	15.5	—
Organic phosphorous	mg/L	< 0.1	0.1
Alkylmercury	mg/L	< 0.0005	0.0005
Mercury	mg/L	< 0.0005	0.0005
Cadmium	mg/L	< 0.001	0.001
Chromium	mg/L	< 0.02	0.02
Lead	mg/L	< 0.005	0.005
Arsenic	mg/L	< 0.001	0.001
Boron	mg/L	0.04	0.02
Fluorine	mg/L	0.1	0.1
Iron	mg/L	< 0.01	0.01
Copper	mg/L	< 0.005	0.005
Cobalt	mg/L	< 0.001	0.001
Manganese	mg/L	< 0.01	0.01
Zinc	mg/L	< 0.005	0.005
Aluminum	mg/L	0.051	0.001
Nickel	mg/L	< 0.001	0.001
Silver	mg/L	< 0.0001	0.0001
Sulfate ion	mg/L	12.9	0.1
Chloride ion	mg/L	15	1
Sodium	mg/L	13.1	0.01
Potassium	mg/L	3.6	0.01
Calcium	mg/L	10.3	0.01
Magnesium	mg/L	2.8	0.01
1,2-dichloropropane	mg/L	< 0.0001	0.0001
Chlorothalonil	mg/L	< 0.0001	0.0001
Propyzamide	mg/L	< 0.0001	0.0001
Chlornitrofen	mg/L	< 0.0001	0.0001
Simazine	mg/L	< 0.001	0.001
Thiobencarb	mg/L	< 0.0001	0.0001
Diazinon	mg/L	< 0.0001	0.0001
Isoxathion	mg/L	< 0.0001	0.0001
Fenitrothion	mg/L	< 0.0001	0.0001
EPN	mg/L	< 0.0001	0.0001
Dichlorvos	mg/L	< 0.0001	0.0001
Iprobenfos	mg/L	< 0.0001	0.0001
PCB	mg/L	< 0.0005	0.0005

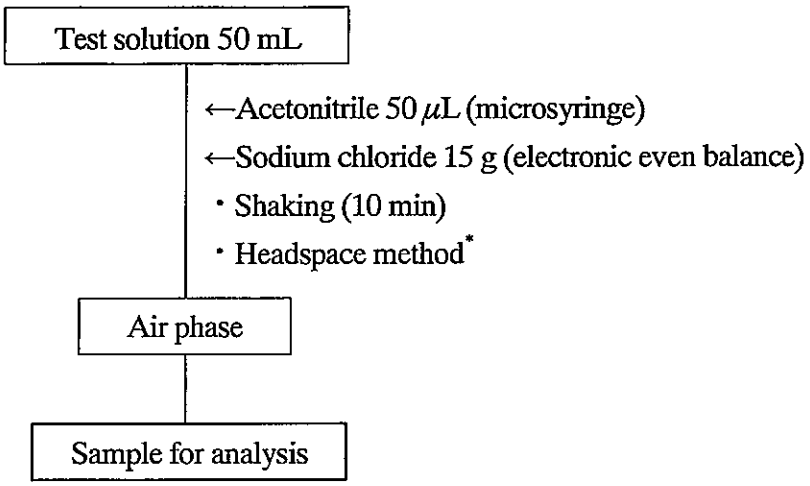
Appendix 2

Analytical method and measured concentration of test item

1. Pretreatment of test solution

The test solution sampled was pretreated according to the flow scheme to prepare the sample for analysis.

Flow scheme



* Headspace method condition
Vessel: 125 mL vial container
Warming: 70°C, more than 20 min

2. Method of analysis

The pretreated samples for analysis were quantitatively analyzed by gas chromatograph-mass spectrometry (GC-MS) under the following conditions to determine the concentration of the test item. The concentration of the test item in each sample for analysis was determined on the basis of a comparison of the peak area on the chromatogram of the sample with that of a standard sample. Some chromatograms obtained are shown in Appendix 3.

Analytical conditions

Instrument	Gas chromatograph-mass spectrometer
Gas chromatograph	Agilent 6890 Series Plus^{+}
Mass spectrometer	Agilent 5973N MSD

Gas chromatograph conditions

Column	HP-PONA film thickness 0.5 μm (Agilent Technologies) 50 m × 0.2 mmI.D. Fused silica
Column temperature	40°C (2 min) $\xrightarrow{①}$ 70°C (0 min) $\xrightarrow{②}$ 150°C (0.1 min)
Temp. rate	①15°C/min ②30°C/min
Carrier gas	Helium
Column flow	24.1 mL/min
Injection temp.	150°C
Injection volume	0.1 mL
Inlet mode	Split
Split ratio	13:1
Pressure	40 kPa

Mass spectrometer conditions

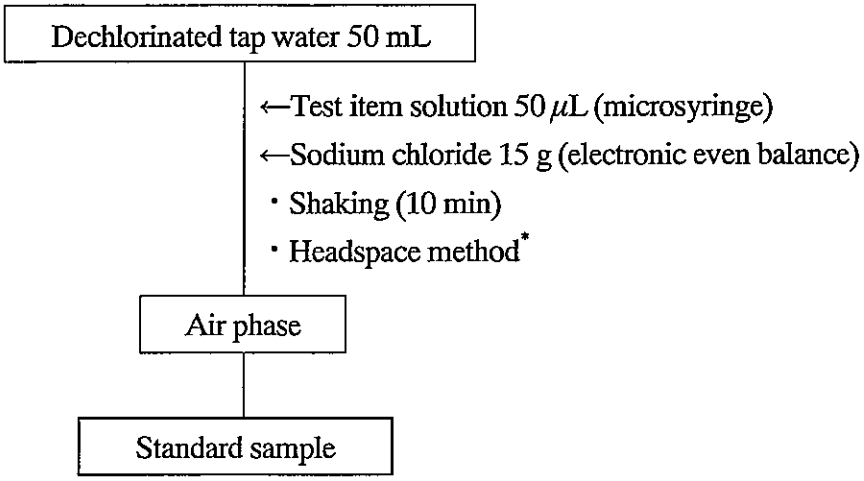
Ionization method	Electron ionization (EI)
Detecting method	Selected ion monitoring (SIM)
Measurement (m/z)	77
Ion source temp.	230°C
MS quadrupole temp.	150°C
Ionization voltage	69.9 eV
Interface temp.	200°C

3. Preparation of standard sample

The standard sample to determine the concentration of the test item in the sample for analysis was prepared as follows. The standard sample was prepared with correcting by the purity (99.8%) of the test item.

The test sample of 100.2 mg was precisely weighed with an electronic balance and dissolved in acetonitrile to obtain 1000 mg/L solution of the test item. The solution was diluted with acetonitrile to prepare 200 mg/L solution of the test item. And the solution was pretreated according to the flow scheme to prepare the 0.200 mg/L standard sample.

Flow scheme



4. Calibration curve

The test item solution of 20.0, 100, 200 and 400 mg/L were prepared by the same procedure as described in section 3. And they were pretreated according to the flow scheme of section 3 to prepare the standard sample of 0.0200, 0.100, 0.200 and 0.400 mg/L respectively. These samples were analyzed according to the quantitative analytical conditions described in section 2. A calibration curve was drawn from the relationship between the concentrations of standard sample and the peak area on the chromatogram, and the determination was confirmed. The calibration curve is shown in Appendix 3. The determination limit of the test item was the lowest concentration of the standard sample (0.0200 mg/L) within the range of the calibration confirmed. Therefore, the determination limit of the test item in the test solution was 0.0200 mg/L in consideration of pretreatment.

5. Results of the measurement

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

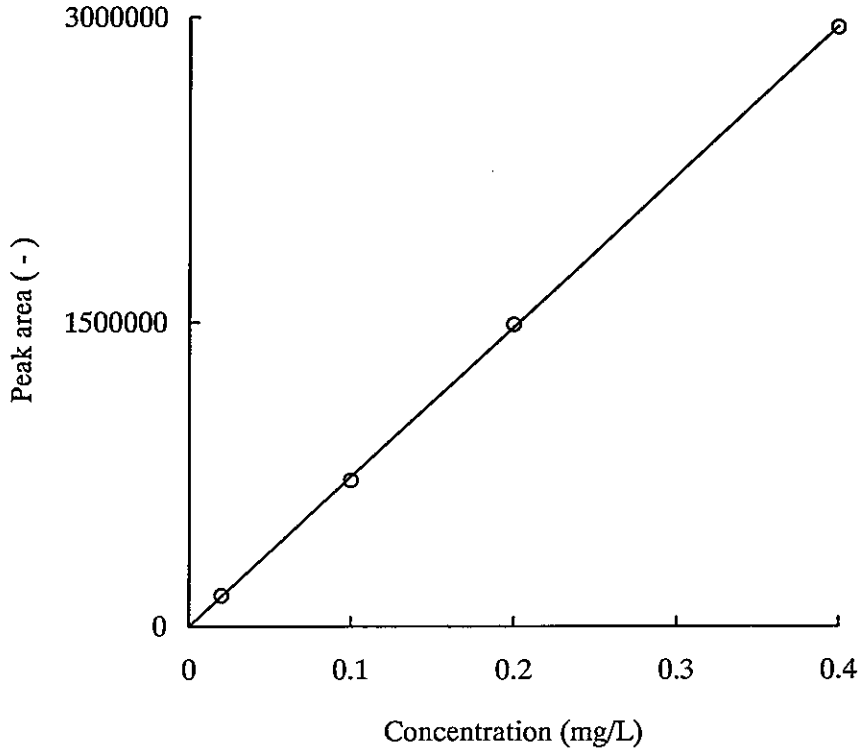
Appendix table 2-1 Measured concentrations of test item in test solutions

Nominal concentration (mg/L)	Measured concentration (mg/L) (Percentage of measured concentration versus that at each reparation%)				
	At the start	24 hours		At the end	Geometric mean
		Before the renewal	After the renewal		
Control	n.d.	n.d.	n.d.	n.d.	
100	0.0623	0.0629 (101)	0.0970	0.0704 (72.6)	0.0719

n.d. : < 0.0200 mg/L

Appendix 3

Calibration curve and chromatogram



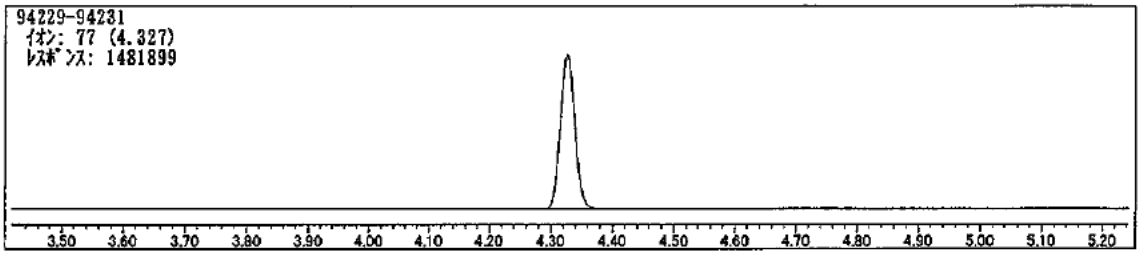
y = 7379807x
r = 1.00

Concentration (mg/L)	Peak area (-)
0.0200	151365
0.100	719864
0.200	1489138
0.400	2949675

Appendix figure 3-1 Calibration curve of 13F-OLE for analysis by GC-MS.

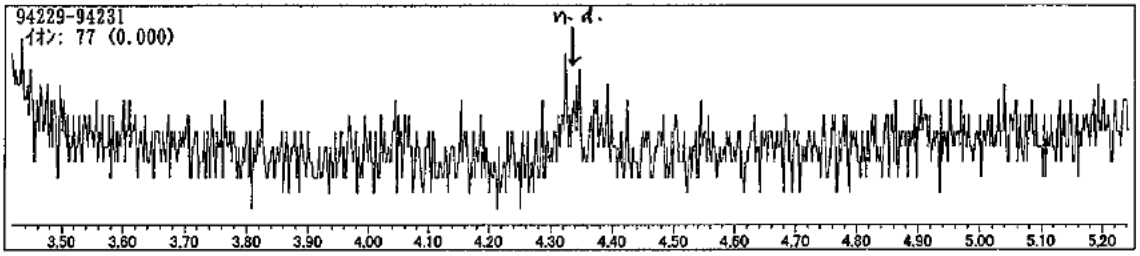
Standard sample 0.200 mg/L

データ ファイル : C:¥MSDCHEM¥1¥DATA¥94229¥070822¥0822S01.D バイアル : 1
測定日 : 22 Aug 2007 13:33 オペレータ :
サンプル : Standard sample 0.200mg/L 装置 : Instrumen
一般情報 : 希釈率 : 1.00
サンプル アマウント : 0.00
MS 積分パラメータ : autointl.e
定量 メソッド : C:¥MSDCHEM¥1¥METHODS¥94231(B).M (ケミステーション インテグレータ) 94230



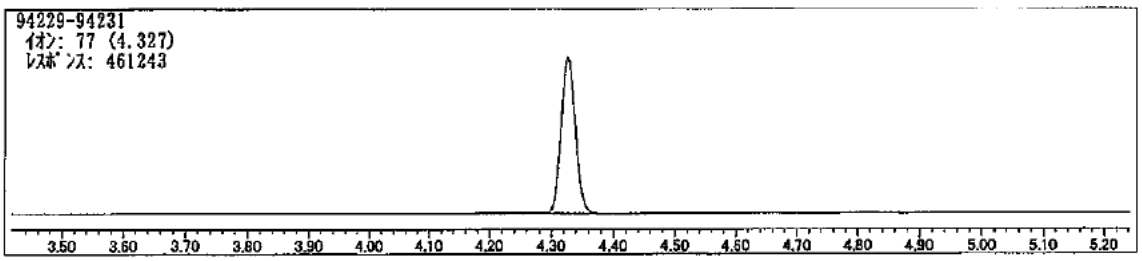
Control

データ ファイル : C:¥MSDCHEM¥1¥DATA¥94229¥070822¥30H0HZ.D バイアル : 1
測定日 : 22 Aug 2007 13:45 オペレータ :
サンプル : 94230 本試験 0h Control 装置 : Instrumen
一般情報 : 希釈率 : 1.00
サンプル アマウント : 0.00
MS 積分パラメータ : autointl.e
定量 メソッド : C:¥MSDCHEM¥1¥METHODS¥94231(B).M (ケミステーション インテグレータ) 94230



100 mg/L (Nominal concentration)

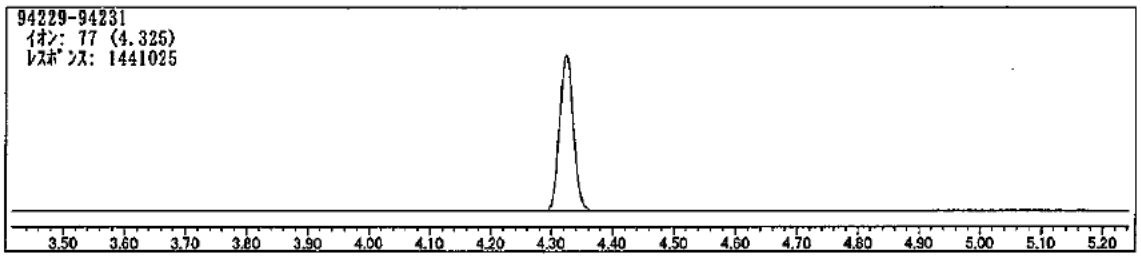
データ ファイル : C:¥MSDCHEM¥1¥DATA¥94229¥070822¥30H0HA.D バイアル : 1
測定日 : 22 Aug 2007 13:56 オペレータ :
サンプル : 94230 本試験 0h 100mg/L 装置 : Instrumen
一般情報 : 希釈率 : 1.00
サンプル アマウント : 0.00
MS 積分パラメータ : autointl.e
定量 メソッド : C:¥MSDCHEM¥1¥METHODS¥94231(B).M (ケミステーション インテグレータ) 94230



Appendix figure 3-2 GC-MS chromatograms at start of exposure.

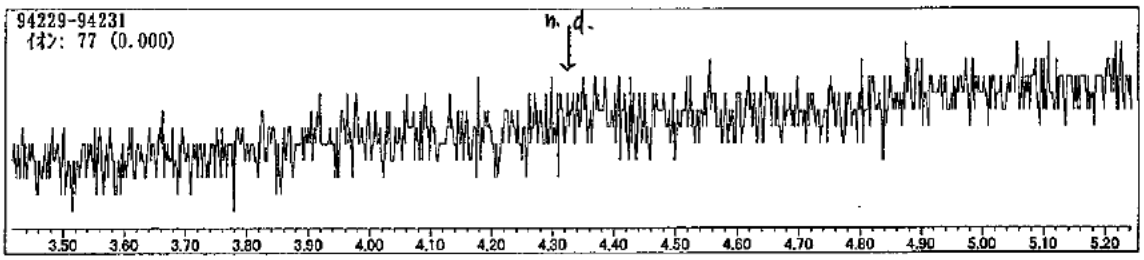
Standard sample 0.200 mg/L

データ ファイル : C:¥MSDCHEM¥1¥DATA¥94229¥070823¥0823S01.D バイアル : 1
測定日 : 23 Aug 2007 12:17 オペレータ :
サンプル : Standard sample 0.200mg/L 装置 : Instrumen
一般情報 : 希釈率 : 1.00
サンプル アマウント : 0.00
MS 積分パラメータ : autoint1.e
定量 メソッド : C:¥MSDCHEM¥1¥METHODS¥94231(B).M (ケミステーション インテグレータ) 94230



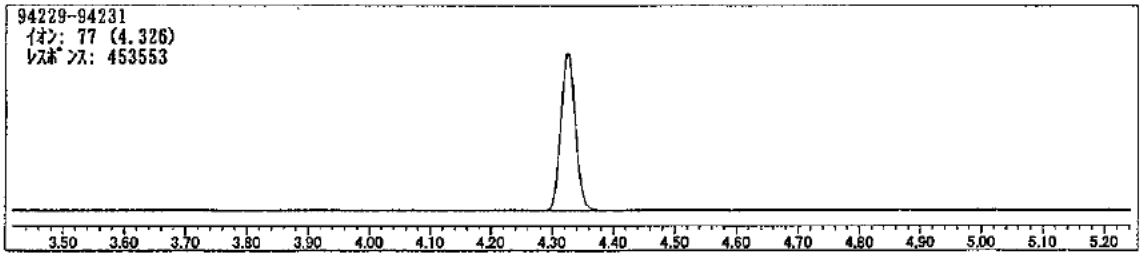
Control

データ ファイル : C:¥MSDCHEM¥1¥DATA¥94229¥070823¥30H24HZO.D バイアル : 1
測定日 : 23 Aug 2007 12:28 オペレータ :
サンプル : 94230 本試験 24h Control 換水前 装置 : Instrumen
一般情報 : 希釈率 : 1.00
サンプル アマウント : 0.00
MS 積分パラメータ : autoint1.e
定量 メソッド : C:¥MSDCHEM¥1¥METHODS¥94231(B).M (ケミステーション インテグレータ) 94230



100 mg/L (Nominal concentration)

データ ファイル : C:¥MSDCHEM¥1¥DATA¥94229¥070823¥30H24HAO.D バイアル : 1
測定日 : 23 Aug 2007 12:39 オペレータ :
サンプル : 94230 本試験 24h 100mg/L 換水前 装置 : Instrumen
一般情報 : 希釈率 : 1.00
サンプル アマウント : 0.00
MS 積分パラメータ : autoint1.e
定量 メソッド : C:¥MSDCHEM¥1¥METHODS¥94231(B).M (ケミステーション インテグレータ) 94230



Appendix figure 3-3 GC-MS chromatograms at 24 hours after exposure (before renewal).

Appendix 4

Solubility of test item in dilution water

1. Title

Solubility of test item in dilution water

2. Objective

The objective of this study is to estimate the solubility of the test item to dilution water.

3. Outline

Test item mixed with dilution water was stirred for 24 or 48 hours under the test temperature. After leaving at rest, the middle layer was sampled and analyzed.

4. Performance of test

4.1 Test equipments and instruments

Water bath :	Plastic tank
	Warming/cooling unit (Type HCA250, Sato craft)
Mixing apparatus :	Magnetic stirrer
Vessel :	Devised glass container (Interior volume : About 600 mL)

4.2 Test conditions

(1) Test temperature :	20±1°C
(2) Measurement :	Twice (24 and 48 hours after the mixture was stirred)
(3) Dilution water :	Dechlorinated tap water
(4) Repitition :	24 hours n=3 (Sample-1, Sample-2 and Sample-3)
	48 hours n=3 (Sample-4, Sample-5 and Sample-6)

4.3 Test procedures

(1) Test sample and dilution water were mixed in a devised glass container to prepare approximately 100 mg/L* solution and sealed without headspace.

* The additive amount (38.5 μL) was calculated from the density of the test item (1.560 g/cm^3).

(2) The test solution was stirred slowly with a magnetic stirrer under the test temperature in a water bath.

(3) After the solution was stirred for 24 or 48 hours, the flask was settled in a water bath for approximately 1 hour.

(4) After settling, the samples were quantitatively analyzed to measure the concentration of the test item

4.4 Analysis of test solution

(1) Pretreatment of test solution

The middle layer of the test solutions were collected carefully from sampling spout of the devised glass container by syringe. The collected solution was pretreated according to the flow scheme described in Appendix 2 1. Pretreatment of test solution.

(2) Method for analysis

See Appendix 2 2. Method of analysis.

4.5 Preparation of standard solution

See Appendix 2 3. Preparation of standard sample.

4.6 Calibration curve

See Appendix 2 4. Calibration curve.

5. Results

Measured solubility of the test item after 48 hours was higher than that of after 24 hours. Therefore, value of after 48 was adopted to the solubility in dilution water. The solubility of the test item to dilution water was 0.0606 mg/L. The results of analyses are shown below.

Appendix table 4-1 Value measured after stirring for 24 hours

Sample name	Measured value (mg/L)	Arithmetic mean (mg/L)
Sample-1	0.0540	0.0456
Sample-2	0.0232	
Sample-3	0.0597	

Appendix table 4-2 Value measured after stirring for 48 hours

Sample name	Measured value (mg/L)	Arithmetic mean (mg/L)
Sample-4	0.0414	0.0606
Sample-5	0.0606	
Sample-6	0.0799	

Additional data

Results of preliminary studies

1. Solubility of test item in dilution water

It was expected that the solubility of the test item in dilution water was below 100 mg/L, therefore, the measurement of the solubility of the test item in dilution water was conducted. The following preliminary study 1 was performed in fish acute toxicity test (Study number: 94231).

1) Preliminary study 1 for measurement of solubility

(1) Method

Since the test item was expected to volatile due to the chemical structure, the test item and the dilution water (dechlorinated tap water) were mixed and gently stirred in a devised glass container under closed system with no head space and test temperature (24±1°C) for 24 and 48 hours. For removal of insoluble substance, the procedure of gentle stirring and taking out from the middle layer of the settled solution for 1 hour was employed since centrifugation made the concentration of the test item decreased. The concentration of the test item in the collected sample was analyzed with pretreatment by gas chromatography-mass spectrometry (GC-MS). For 48 hours stirring, a sample of approximately 10 mg/L as nominal concentration was additionally measured.

(2) Result

Nominal concentration (mg/L)	Measured concentration (mg/L)	
	24-hour stirring	48-hour stirring
Approx. 100 (Sample-1)	0.0934	-
Approx. 100 (Sample-2)	0.129	-
Approx. 100 (Sample-3)	-	0.0949
Approx. 100 (Sample-4)	-	0.135
Approx. 10 (Sample-5)	-	0.0791

The measured value was around 0.1 mg/L with a little variance.

2) Preliminary study 2 for measurement of solubility

(1) Method

Similarly in the preliminary study 1, the test item and the dilution water (dechlorinated tap water) were mixed and gently stirred in a devised glass container under closed system with no head space and daphnids test temperature (20±1°C) for 24 and 48 hours. For removal of insoluble substance, the procedure of gentle stirring and taking out from the middle layer of the settled solution for 1 hour was employed. The concentration of the test item in the collected sample was analyzed with pretreatment by GC-MS.

(2) Result

Nominal concentration (mg/L)	Measured concentration of test item (mg/L)	
	24-hour stirring	48-hour stirring
Approx. 100 (Sample-1)	0.0666	-
Approx. 100 (Sample-2)	0.0737	-
Approx. 100 (Sample-3)	-	0.105
Approx. 100 (Sample-4)	-	0.0603

The solubility of the test item in dilution water was around 0.1 mg/L.

3) Summary of preliminary study for measurement of solubility

From the results of the preliminary studies, the solubility of the test item in the dilution water was estimated at around 0.1 mg/L. Since centrifugation made the concentration of the test item decreased, the procedure of gentle stirring and taking out from the middle layer of the settled solution for 1 hour was employed for removal of insoluble substance. Since the measured concentrations were almost same even in the different nominal concentrations of ten times (approximately 10 and 100 mg/L), it was thought that insoluble substance could be removed.

From the results mentioned above, in definitive study, the devised glass container would be used for the preparation, and the procedure of gentle stirring and taking out from the middle layer of the settled solution for 1 hour was employed in order to remove insoluble substance.

2. Effect on test organism

1) Preliminary study

(1) Method

After the test sample was added into the dilution water filled in Erlenmeyer flask with micro volumeter (Eppendorf Co., Ltd) to prepare test solution of 100 mg/L as nominal concentration, the flask was immediately sealed with a plug not to produce head space. The solution was gently stirred by magnetic stirrer for about 48 hours under about 20±1°C to produce dispersed solution with suspended test item. After cease of stirring, the solution was settled for 1 hour and then test solution was prepared by taking out from the middle layer of the settled solution. The preliminary study to investigate the effect of the test item on the test organisms was performed under closed system, and static and semi-static regime (renewal after 24 hours). The measurement of the test item in the test solution was also carried out. The test sample was employed in terms of volume using the density [1.560 g/cm^3 (20°C)] for the preparation of test solution.

(2) Result

Nominal concentration (mg/L)	24 hours		48 hours	
	Immobility (%)	Others	Immobility (%)	Others
100 (Static)	0	-	0	-
100 (Semi-static)	0	-	0	-

The number of organisms: ten daphnids/test level (five daphnids/replicate), Closed system
- shows that no other abnormal response was observed.

Nominal concentration (mg/L)	Measured concentration (mg/L) (percentage of measured concentration at start)		
	At the start	after 24 hours	At the end (after 48 hours)
100	0.108	0.102 (94.3)	0.0926 (85.4)

No effect of the test item on the test organisms was observed in the test using the static and semi-static regime. The measured test concentration at the start of the exposure was around the solubility of the test item in the dilution water, but it gradually decreased slightly during the exposure.

2) Summary of effect on test organisms (preliminary study)

The test item had no effect on the test organisms under the dispersed solution, which was prepared by mixing the test sample and the dilution water for 48 hours to produce 100 mg/L of an upper limit concentration on the test method for New Chemical Substances and by taking out from the middle layer of the solution. Since the test item was expected to volatile, the preliminary study was carried out under closed system. Since the test concentration in the test solution decreased in some degree, the definitive study was planed to be conducted under semi-static replacement regime.

3. Operation of definitive study

1) Measurement of solubility of test item in dilution water

From the result of the preliminary study, the measurement of the solubility was performed using the solution taken out from the middle layer of the solution which was prepared by mixing the test sample and the dilution water to produce about 100 mg/L and stirred gently for 48 hours under the condition of 20±1°C and closed system. For removal of insoluble substance, the procedure of centrifugation or filtration was not used, but it of settling for about 1 hour after cease of stirring and then taking out from the middle layer of the settled solution was used as a method to remove as much as possible. The measurement of the test item concentration was employed for this test solution.

2) Definitive study

Since no effect of the test item on the test organisms was expected from the result of the preliminary studies, the definitive study was conducted at 100 mg/L of an upper limit concentration as nominal, and under closed system and using the test solution of middle layer from the dispersed solution prepared by stirring for about 48 hours and a control. The preparation of test solution was done as follows; The test sample was added in terms of volume using the density into the dilution water filled in Erlenmeyer flask with micro volumeter to prepare test solution of 100 mg/L as nominal concentration. After the flask was immediately sealed with a plug not to produce head space, the solution was gently stirred by magnetic stirrer for about 48 hours under about 20±1°C to produce dispersed solution with suspended test item. After cease of stirring, the solution was settled for 1 hour and then test solution was prepared by taking out from the middle layer of the settled solution. No correction with purity was done for the preparation of the test concentration. The measurement of the test concentration in the test solution was carried out at the start of the exposure, before and after the renewal, and at the end of the exposure.