CERI

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Study number	94223

FINAL REPORT

Algae Growth Inhibition Study of 13F-SFA with *Pseudokirchneriella subcapitata*

July 25, 2007

Kurume Laboratory
Chemicals Evaluation and Research Institute, Japan

STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title Algae Growth Inhibition Study of 13F-SFA with *Pseudokirchneriella subcapitata*

Study number 94223

I, the undersigned, hereby declare that this report provides a correct English translation of the Final Report (Study No. 94223, issued on July 25, 2007) and reflects the amendments of the Final Report (issued on November 7, 2007) audited by Quality Assurance Unit of Kurume Laboratory, Chemicals Evaluation and Research Institute, Japan.

The Study Director was changed from Masanori Seki to Mika Ono, because Masanori Seki had been reshuffled.

Date October 1, 2009

Study Director _____

GLP STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title Algae Growth Inhibition Study of 13F-SFA with *Pseudokirchneriella subcapitata*

Study number 94223

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 July 25, 2007

Study Director Signed in original

QUALITY ASSURANCE STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title Algae Growth Inhibition Study of 13F-SFA with *Pseudokirchneriella subcapitata*

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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	June 1, 2007	June 1, 2007
Study plan	June 4, 2007	June 4, 2007
Amendment of study plan	June 13, 2007	June 13, 2007
	June 14, 2007	June 14, 2007
	July 23, 2007	July 24, 2007
Measurement of solubility	June 5, 2007	June 6, 2007
	June 6, 2007	June 6, 2007
Start of the exposure and after the exposure	June 4, 2007	June 8, 2007
	June 5, 2007	June 8, 2007
	June 8, 2007	June 8, 2007
Raw data and final report draft	July 23, 2007	July 24, 2007
Final report	July 25, 2007	July 25, 2007

Date July 25, 2007

Quality Assurance Unit, Head Signed in original

CONTENTS

Title	Algae Growth Inhibition Study of 13F-SFA with *Pseudokirchneriella subcapitata*
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 objective of this study is to determine the effect of 13F-SFA on growth of algae.
Test method	The study was performed according to the following test methods and guidance document. (1) Algal Growth Inhibition 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, Partial amendment November 20, 2006) (2) OECD Guidelines for Testing of Chemicals, Section 2: Effects on Biotic Systems, "Freshwater Alga and Cyanobacteria, Growth Inhibition Test (Guideline 201, 23 March 2006)" (3)OECD Guidance Document 23 "Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures" (September 2000)

Applied GLP

The 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	June 4, 2007
Experimental starting date	June 5, 2007
Solubility study starting date	June 5, 2007
Bioassay starting date	June 5, 2007
Experimental completion date	June 8, 2007
Solubility study completion date	June 6, 2007
Bioassay completion date	June 8, 2007
Study completion date	July 25, 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 is 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. 94223, 94224 and 94225).

(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 :

Analysis :

Approval of final report

Study Director Date July 25, 2007

Signature Signed in original

SUMMARY

Title

Algae Growth Inhibition Study of 13F-SFA with *Pseudokirchneriella subcapitata*

Test condition

(1) Test item	13F-SFA
(2) Test organism	*Pseudokirchneriella subcapitata*
(3) Exposure duration	72 hours
(4) Test concentration	Saturated solution of test item (nominal concentration: approximately 100 mg/L) and control
(5) Type of test	Incubation with shaking (approximately 100 rpm)
(6) Preparation of test solution	The test sample and medium were mixed to prepare approximately 100 mg/L (nominal concentration), and they were stirred under closed system for about 24 hours. After settlement for 1 hour, test solution was prepared by taking out from the middle layer.
(7) Replicate	Six replicates / test level
(8) Volume of test solution	600 mL / test level (100 mL / test vessel) (two additional vessels for analytical chemistry of the test item were set.)
(9) Temperature in incubator	21 to 24°C, not varied more than ± 2°C
(10) Light condition	Continuous illumination using a fluorescent light [The measured light intensity was 60 to 120 $\mu E/m^2/s$ (not varied more than 20%) at the level of the test solutions during exposure period.]
(11) Measurement of cell growth	Cell concentration
(12) Analysis of concentration of test item and 13F-EtOH (hydrolyzed product) in test solution	GC analysis (at the start of the exposure, 24 and 48 hours after the start of the exposure, and the end of the exposure)

Results

(1) Solubility of test item in medium (23 ± 1°C)	0.208 mg/L
(2) Concentration of test item in test solution	
(Percent of measured concentration versus that at the start of the exposure)	
At the start of the exposure	0.129 mg/L
24 hours after the start of the exposure	0.0211 mg/L (16.4%)
48 hours after the start of the exposure	0.0162 mg/L (12.6%)
At the end of the exposure	0.00655 mg/L (5.09%)
(3) Concentration of 13F-EtOH in test solution	
At the start of the exposure	0.00827 mg/L
24 hours after the start of the exposure	0.00635 mg/L
48 hours after the start of the exposure	0.00622 mg/L
At the end of the exposure	< 0.00605 mg/L (below the determination limit)
(4) EC_{50} (E_rC_{50})	> 0.0215 mg/L
(5) NOEC (Growth rate 0-3d)	≥ 0.0215 mg/L

[The above-mentioned concentrations (4) and (5) are based on geometric mean of measured concentrations.]

Conclusion

This study was conducted as a limit test in order to confirm the effect of the test item on the test organisms at around the solubility of the test item in medium. The concentration of test item in the test solution at the preparation was a low value (0.129 mg/L) compared with the solubility in medium (0.208 mg/L). However, considering the variation of each test vessel at the solubility measurement (0.144 to 0.266 mg/L), it was judged that the concentration of test item at the preparation was almost around solubility in medium. The concentrations of test item in the test solutions were significantly decreased during the exposure. The reason was estimated to the limit in algae growth inhibition study of the volatile substance (volatilization to headspace in the test vessel) and hydrolysis of test item. It was concluded that the test item did not have an acute effect on the test organisms at around the solubility of the test item in medium because no adverse effect was observed under the conditions of the definitive study. Furthermore, though the hydrolysate of test item was confirmed during exposure, it also had no adverse effect to test organisms. Therefore, it is considered that there was no effect to exposure by hydrolysis of test item.

1. Test item

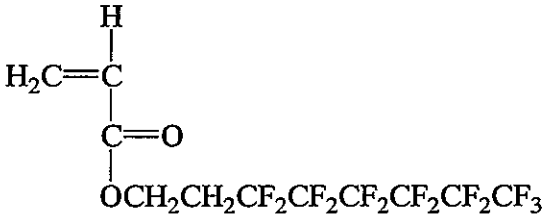
In this report, 13F-SFA has the following name etc.

1.1 Chemical name*2

3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl acrylate

1.2 Chemical structure etc.*2

Structural formula



Molecular formula	$C_{11}H_7F_{13}O_2$
Molecular weight	418.15
CAS Number	17527-29-6

*2 Information supplied by the sponsor

2. Test sample

2.1 Supplier and lot number*2

Supplier	DAIKIN INDUSTRIES, LTD.
Lot number	6X002

2.2 Purity*2

Test item	99.7%
Impurity	Unknown constituent component 0.3%

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	78°C (8 mmHg)	
Density	1.554 g/cm^3(25°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

Pseudokirchneriella subcapitata (ATCC 22662)

(The old scientific name "*Selenastrum capricornutum*")

(2) Reason for selection of species

Species recommended in the test guideline

(3) Source

Pseudokirchneriella subcapitata which originally came from the American Type Culture Collection (12301 Parklawn Drive Rockville, Maryland 20852-1776 U.S.A.) on Jun. 30, 1995 and has been cultured in this laboratory was used. An algae growth inhibition test of potassium dichromate (Reagent grade, Wako Pure Chemical Industries, Ltd.) with the test organisms was conducted to confirm the reproducibility of the test system (on May 21 - May 24, 2007). The E_rC_{50} (0-3d) of potassium dichromate was 0.900 mg/L. This value was within the normal range of the reference substance in this laboratory (mean ± 2S.D.: 0.698 to 1.08 mg/L) [mean ± S.D.: 0.891 ± 0.097 mg/L (n=3)].

3.2 Culture medium

The medium recommended in OECD test guideline (Guideline 201, 23 March 2006). The composition of medium is shown in Appendix 1. Medium was used under sterile condition.

3.3 Test apparatus and equipment

(1) Test apparatus

Test vessel : Sterilized 500 mL Erlenmeyer flask (closed vessel)

(2) Test equipment

Incubator : Incubator with temperature control, continuous shaking and continuous illumination, maintained the uniform light intensity (Incubator with rotary shaker and artificial illumination, TB-C-50RL, Takasaki Scientific Instruments Corp.)

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 added to medium. The test vessels that contain test solution and test organisms were shaken (approximately 100 rpm) during the exposure.

(b) Exposure duration

72 hours

(c) Test concentration

Based on the results of the preliminary studies, it was expected that the test solution at around the solubility in medium had no impact on the growth of algae. Therefore, the definitive study was conducted as the limit test with the saturated test solution which was stirred for 24 hours of test item (nominal concentration : approximately 100 mg/L). The results of preliminary studies are shown in Additional data.

(d) Control

The medium without the test item, which was treated in the same manner as the test solution (except the collection of the solution from the middle layer) was used as the control.

(e) Replicates

Six replicates / test level

(f) Initial cell concentration

The pre-culture, incubated under the same conditions as the test for 3 days and exponentially growing was used as inoculum to prepare the initial cell concentration of approximately 5×10^3 cells/mL.

(g) Operation

All operations were carried out under sterile conditions.

(h) Volume of test solution

600 mL / test level (100 mL / test vessel: two additional vessels for analytical chemistry of the test item were set.)

(2) Conditions of test environment

(a) Temperature in the incubator

21 to 24°C, not varied more than ± 2°C

(b) Light

Continuous illumination provided with 60 to 120 $\mu E/m^2/s$ (fluctuation range: mean ± 20%) at the level of the test solutions, using a fluorescent light with wavelength range of 400 to 700 nm.

*3 $120\ \mu E/m^2/s = 0.72 \times 10^{20}$ photons/m^2/s

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 [1.554 g/cm^3(25°C)] for the preparation of test solution.

The test sample and medium were mixed to prepare approximately 100 mg/L (nominal concentration). Then, they were stirred for about 24 hours under the closed system with little headspace. After the mixture was left for about 1 hour at rest, the saturated solution of test item was collected from the middle layer for use as the test solution. The test solution was divided into each test vessel.

3.6 Observation and measurements

(1) Cell growth, etc.

Biomass was shown as cell concentration.

Cell concentration was counted with particle counter (Model COULTER Z1, Beckman Coulter) at 24, 48, and 72 hours after the start of the exposure. The blank value correction was conducted by measuring simultaneously the blank values of the blank solutions without algae which were separately prepared in each test level when the test solutions were prepared. Furthermore, the cell condition for one vessel in each test level was observed under microscope (Model BX41, Olympus Co., Ltd.) at the end of the exposure.

(2) Appearance of test solution

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

(3) Water quality and environmental conditions

The pH of the test solution was measured at the start and end of the exposure. For the measurement of pH, another solution sampled from the vessel for preparation was used at the start of the exposure and one test vessel in each test level was used for the measurement at the end of exposure. The culture temperature and light intensity in the incubator were measured once a day during the exposure. The pH measurements were carried out on a portable pH meter (Model HM-21P, DKK-TOA Co.). The temperature was measured on a calibrated thermometer of glass stick type. Light intensity was measured on quantum scalar laboratory irradiance meter (Model LI-250A, LI-COR).

(4) Concentration of test item in the test solution

It was considered that the measurement was necessary with time from the results of the preliminary study, therefore, the concentration of test item in the test solution was measured at the start of the exposure, 24 and 48 hours after the start of the exposure, and the end of the exposure. Since the test item was hydrolyzed and produced 2-(perfluorohexyl)ethanol (abbreviation: 13F-EtOH which is the test item of study number 94232 to 94234), the concentration of 13F-EtOH was also measured. At the start, the solution sampled from the container for preparation in each test level was used for the measurement. At 24 and 48 hours after the start of the exposure, total volume of solution (100 mL) sampled from the test vessel for analytical chemistry. At the end of the exposure, equal volume of the test solution was taken out from the test vessels in each test level and mixed. The concentration of the test item and 13F-EtOH were analyzed by gas chromatography (GC). Analytical method and measured concentration of test item and 13F-EtOH are shown in Appendix 2, and analytical calibration curve and chromatograms are shown in Appendix 3.

(5) Solubility of test item in medium

Solubility of the test item in medium was measured in this study because the solubility was estimated to be below 100 mg/L. Detail of the measurement and results are shown in Appendix 4.

3.7 Treatment of results

The results of the study were estimated by geometric mean of measured concentration.

(1) Calculation of concentration-inhibition rates

The mean value of biomass for each test level was plotted against time to produce growth curves. Using this curve, inhibition rates were calculated comparing with control values on growth rate.

Comparison of growth rates

The specific growth rate for a specific period was calculated as the logarithmic increase in biomass according to the following formula:

$$\mu_{i-j}=\frac{lnN_j - lnN_i}{t_j - t_i}$$

where

μ_{i-j} : specific growth rate from time i to j (normally day^{-1})
N_i : measured number of cells/mL at t_i, nominal number at start t_0
N_j : measured number of cells/mL at t_j
t_i : time of i^{th} measurement after beginning of test (day)
t_j : time of j^{th} measurement after beginning of test (day)

Specific growth rate over the exposure duration (0-72 h) was calculated for determination of EC_{50}. In control, specific growth rates for section-by-section were calculated for check of validity of the test.

The percentage inhibition of the cell growth at each exposure level (I_μ) was calculated as the difference between the average specific growth rate at control level (μ_c) and that at exposure level (μ_t) as:

$$I_\mu=\frac{\mu_c - \mu_t}{\mu_c}\times 100$$

(2) Estimation of EC_{50} *3

The EC_{50} was estimated as "> the concentration of the test item" since no less than 50% of inhibition rate was not obtained within the exposure levels. The EC_{50} was denoted as E_rC_{50} based on growth rate.

*3 EC_{50} (Median Effective Concentration) is the concentration of the test item that results in 50% reduction in growth of the test organisms during the exposure.

(3) Estimation of No Observed Effect Concentration (NOEC*4)

Regarding the growth rate, *F* test was done to determine the homogeneity of variance for the data. Then Aspin-Welch *t*-test was used to determine the significant difference between the control level and exposure level. NOEC was determined by the results of statistical analysis and cell condition.

*4 NOEC (No Observed Effect Concentration) is the highest concentration of the test item that does not cause any observed adverse effects on growth of the test organisms during the exposure.

3.8 Validity of the test

(1) The cell growth in the control cultures should have increased by a factor of at least 16 within the 72-hour exposure period.

(2) The mean coefficient of variation for section-by-section specific growth rates in the control cultures must not exceed 35%.

(3) The coefficient of variation of average specific growth rates in replicate control cultures must not exceed 7%.

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 Observation of test solution and measurement of water quality variables

The contrast table of nominal concentration and geometric mean of the measured concentrations is shown below.

Nominal concentration (mg/L)	Geometric mean of measured concentration (mg/L)
100	0.0215

The value of geometric mean of the measured concentration is used in this report.

(1) Appearance of test solution

In the 0.0215 mg/L exposure level and control, test solutions were colorless and clear at the start of the exposure and the appearance of the test solutions at the end of the exposure were green due to the algae growth.

(2) Water quality and environmental conditions

The measured values of pH in the test solutions were 7.9 and 8.0 at the start, 9.6 and 9.8 at the end of the exposure. The fluctuation of pH in control was out of the range of the regulation of the test method (not increase by more than 1.5 units in ordinary practice). Temperature in incubator ranged from 22.9 to 23.1°C and light intensity was 94 to 99 $\mu E/m^2/s$. The measured values of pH of test solution are shown in Table 1, and temperature and light intensity in the incubator are shown in Table 2.

(3) Concentration of test item in test solution

The measured concentrations of the test item in the test solution were 0.129, 0.0211, 0.0162 and 0.00655 mg/L at the start of the exposure, 24 and 48 hours after the start of the exposure, and the end of the exposure, and those were 16.4, 12.6 and 5.09% of measured concentrations at the start of the exposure, respectively. The measured concentrations of 13F-EtOH in the test solution were 0.00827, 0.00635, 0.00622 and < 0.00605 mg/L (below the determination limit) at the start of the exposure, 24 and 48 hours after the start of the exposure, and the end of the exposure, respectively. The results of the measured concentrations of the test item are shown in Appendix 2.

4.2 EC_{50}

EC_{50} (E_rC_{50}) of the 13F-SFA based on the growth rate was > 0.0215 mg/L. Values of biomass at each time, growth rate and growth inhibition rates, and the EC_{50} are shown in Table 3, Table 4, and Table 5, respectively.

4.3 Growth curves in each test level, cell observations and NOEC

In the 0.0215 mg/L exposure level, the algae growth was close to the control. The following results of cell observation were based on the comparison with the control. No abnormality was observed in control. In the 0.0215 mg/L exposure level, the condition of cells was the same as the control.

By the results in statistical analysis and cell observation showed above, NOEC based on growth rate was $\geq$ 0.0215 mg/L. NOEC, the result of statistical analysis of significant difference, and growth curve are shown in Table 5, Table 6 and Figure 1, respectively.

4.4 Validity of test

Detailed result on validity of the test is shown in Table 7.

(1) Growth of control

The cell in the control grew exponentially during the exposure. At the end of exposure, it increased to 59.2 or more times of the number of initial cells in the control. This meets the validity of the test: the cell growth in control should have increased by a factor of at least 16 times at 72 hours after the start of the exposure.

(2) The mean coefficient of variation for section-by-section specific growth rates in the controls

The mean coefficient of variation in the control was 17.0%. It meets the validity of the test: the mean coefficient of variation in the control must not exceed 35%.

(3) The coefficient of variation of average specific growth rates in replicate controls

The coefficients of variations were 1.12% in the control. They meet the validity of the test: the mean coefficient of variation in controls must not exceed 7%.

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 around the solubility of the test item in medium. The concentration of test item in the test solution at the preparation was a low value (0.129 mg/L) compared with the solubility in medium (0.208 mg/L). However, considering the variation of each test vessel at the solubility measurement (0.144 to 0.266 mg/L), it was judged that the concentration of test item at the preparation was almost around solubility in medium. The concentrations of test item in the test solutions were significantly decreased during the exposure. The reason was estimated to the limit in algae growth inhibition study of the volatile substance (volatilization to headspace in the test vessel) and hydrolysis of test item. It was concluded that the test item did not have an acute effect on the test organisms at around the solubility of the test item in medium because no adverse effect was observed under the conditions of the definitive study. Furthermore, though the hydrolysate of test item was confirmed during exposure, it also had no adverse effect to test organisms. Therefore, it is considered that there was no effect to exposure by hydrolysis of test item.

With regard to the environmental conditions of the test, increase in the unit of pH in control, which is out of the range of the regulation of the test method, was observed. It was decided that the increase of the unit of pH in control was due to the limitation of the algae growth inhibition study with volatile substance (gas exchange between outside and inside of the test vessel is impossible because of the test vessel of closed system). The environmental conditions except pH were within the suitable range. Therefore, it is concluded that this study complied with the applied test guidelines.

5. Factors that affected reliability of test results

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

6. Content of deviation from protocol

None.

Table 1 pH of test solutions at start and end of exposure

Measured concentration*5 (mg/L)	pH	
	At the start	At the end
Control	8.0	9.8
0.0215	7.9	9.6

*5 Geometric mean of measured concentrations (also expressed as measured concentration in the following table)

Table 2 Culture temperature and light intensity in incubator

Time	At the start	1-day	2-day	At the end
Culture temperature (°C)	23.1	23.1	23.0	22.9
Light intensity ($\mu E/m^2/s$)	99	94	95	99

Table 3 Value of cell concentration at each time

Measured concentration (mg/L)	No.	Cell concentration ($\times 10^4$ cells/mL)			
		0 hour*6	24 hours	48 hours	72 hours
Control	1	0.500	1.99	9.36	32.2
	2	0.500	1.93	10.0	30.1
	3	0.500	1.78	9.66	29.6
	4	0.500	1.90	8.86	33.2
	5	0.500	1.97	10.1	30.8
	6	0.500	1.75	9.54	29.8
	Mean	0.500	1.89	9.58	30.9
	S.D.	0	0.0999	0.445	1.45
0.0215	1	0.500	2.06	8.44	25.9
	2	0.500	2.03	10.3	24.5
	3	0.500	1.71	8.27	26.9
	4	0.500	2.30	11.6	31.9
	5	0.500	2.11	11.2	32.2
	6	0.500	2.07	10.0	33.1
	Mean	0.500	2.04	9.97	29.1
	S.D.	0	0.191	1.37	3.74

*6 The value based on the measured value of pre-culture

Table 4 Growth inhibition rates at exposure level

Measured concentration (mg/L)	No.	Growth rate (0-3d)	Inhibition rate (%)
Control	1	1.39	-
	2	1.37	-
	3	1.36	-
	4	1.40	-
	5	1.37	-
	6	1.36	-
	Mean	1.37	-
0.0215	1	1.32	4.26
	2	1.30	5.62
	3	1.33	3.36
	4	1.39	-0.778
	5	1.39	-0.986
	6	1.40	-1.69
	Mean	1.35	1.63

Table 5 EC_{50} and NOEC on growth rate

Endpoint	EC_{50} (mg/L)	NOEC (mg/L)
Growth rate	> 0.0215	$\geq$ 0.0215

Table 6 Result of statistical analysis

Measured concentration (mg/L)	Endpoint Growth rate
0.0215	-
Statistical procedure	*F*-test Aspin Welch *t*-test

-: no significant difference

Table 7 Validity of test

< Variation for section-by-section specific growth rates in the controls >

Control No.	Mean	Standard deviation	Coefficient of variation (%)	
1	1.39	0.16	11.2	
2	1.37	0.28	20.1	
3	1.36	0.30	21.9	17.0
4	1.40	0.12	8.63	(Mean)
5	1.37	0.26	18.7	
6	1.36	0.29	21.6	

< Variation of average specific growth rates in replicate controls >

	0-3day
Mean	1.37
Standard deviation	0.02
Coefficient of variation (%)	1.12

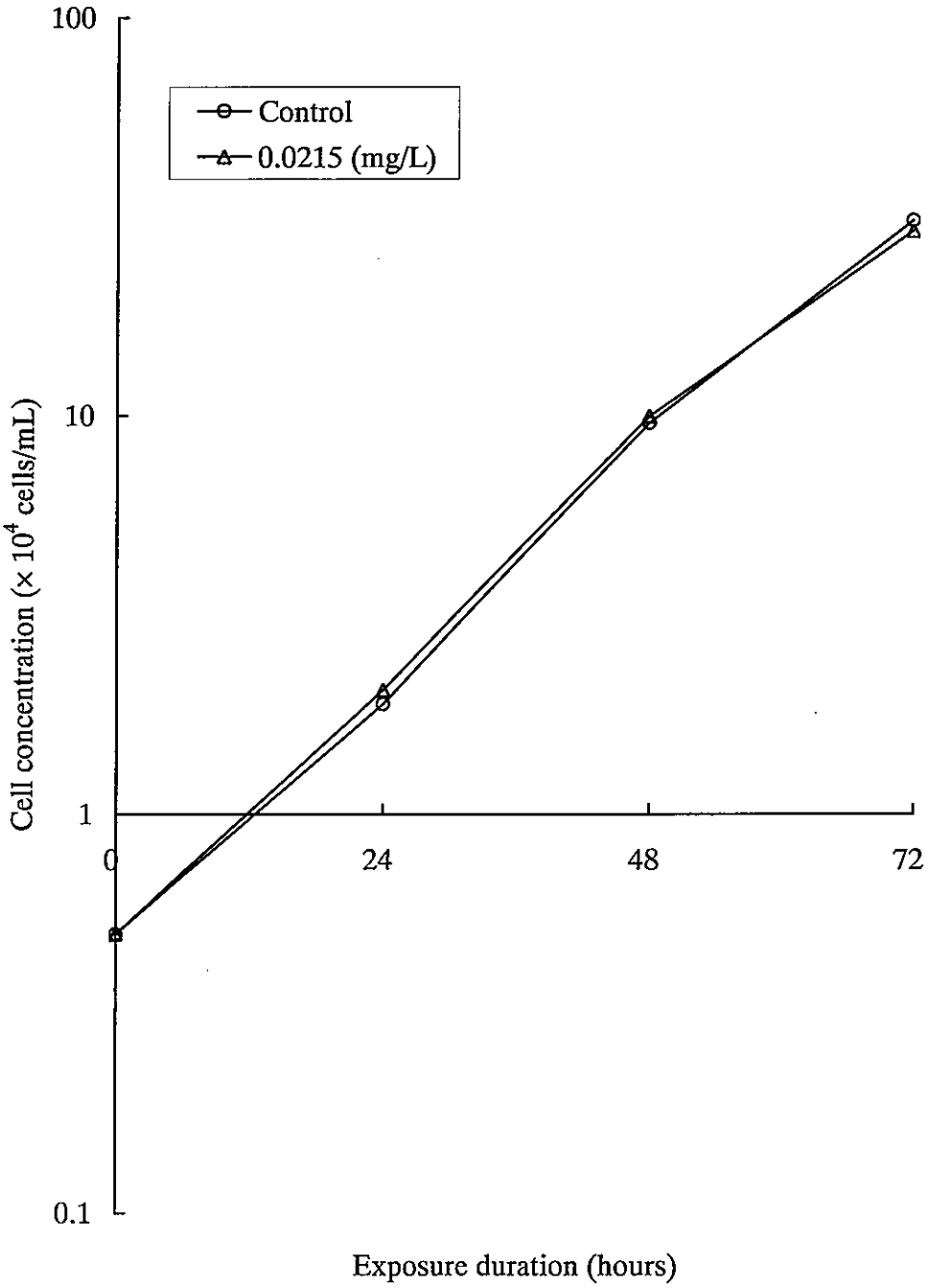


Figure 1 Growth curve in each test level.

Appendix 1

Chemical characteristics of dilution water

Composition of OECD medium
[Guideline 201 (March 23, 2006)]

Nutrient salts	Amount	
H_3BO_3	0.185	mg
$MnCl_2 \cdot 4H_2O$	0.415	mg
$ZnCl_2$	0.003	mg
$FeCl_3 \cdot 6H_2O$	0.064	mg
$Na_2EDTA \cdot 2H_2O$	0.1	mg
$CoCl_2 \cdot 6H_2O$	0.0015	mg
$Na_2MoO_4 \cdot 2H_2O$	0.007	mg
$CuCl_2 \cdot 2H_2O$	0.00001	mg
$CaCl_2 \cdot 2H_2O$	18	mg
NH_4Cl	15	mg
KH_2PO_4	1.6	mg
$NaHCO_3$	50	mg
$MgCl_2 \cdot 6H_2O$	12	mg
$MgSO_4 \cdot 7H_2O$	15	mg

The constituents mentioned above were filled up to 1L with purified water.

Appendix 2

Analytical method and measured concentration of test item and13F-EtOH (hydrolyzed product)

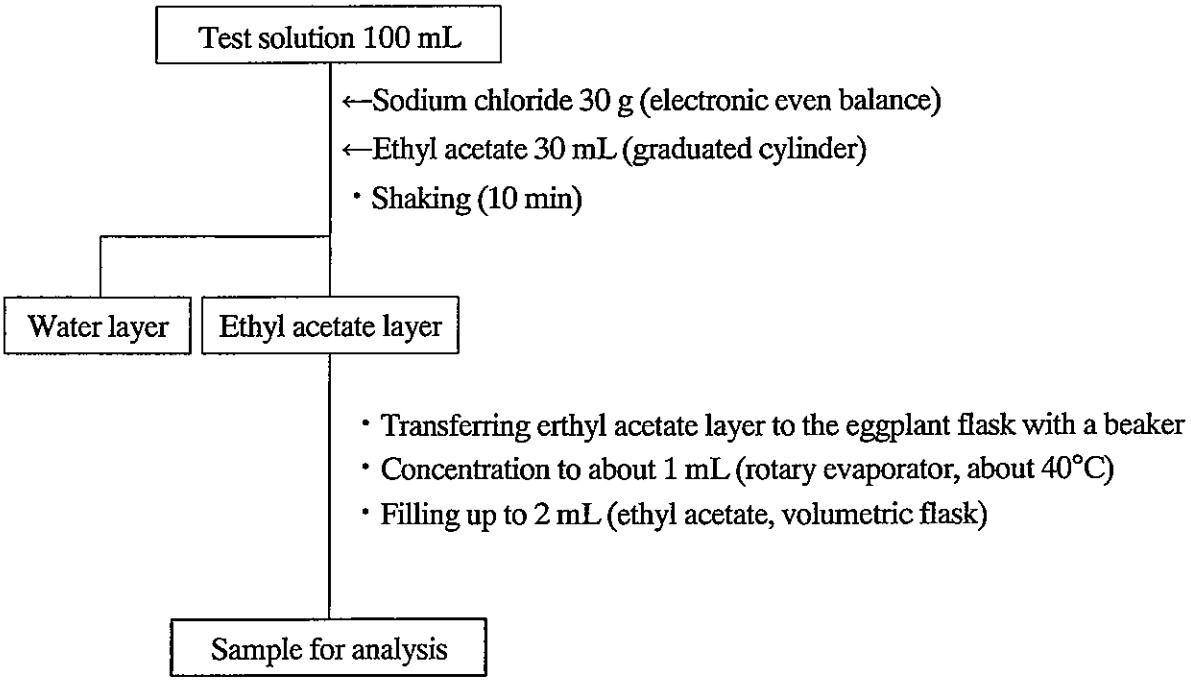
1. Analysis of test solution

By the result of preliminary study, it was expected that the test item was hydrolyzed into 13F-EtOH during the exposure. Therefore, the concentration of the test item and 13F-EtOH were measured.

2. Pretreatment of test solution

The test solutions sampled were pretreated according to the following flow scheme to prepare the sample for analysis.

Flow scheme



3. Method of analysis

The pretreated samples for analysis were quantitatively analyzed by gas chromatography (GC) under the following conditions to determine the concentrations of the test item and 13F-EtOH (hydrolyzed product). The concentrations of the test item and 13F-EtOH in each sample for GC analysis were 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 samples that the concentration of the test item and 13F-EtOH exceeded the range of the calibration curve were diluted to fall within the range and analyzed. Some chromatograms obtained are shown in Appendix 3.

Analytical conditions

Instrument	Gas chromatograph
	Hewlett Packard HP 6890 Series GC System
Auto injector	Hewlett Packard HP6890 Series
Detector	Flame ionization detector (FID)
Column	DB-WAX film thickness 0.50 μm
	(Agilent Technologies)
	30 m × 0.32 mm I.D. Fused silica
Column temp.	40°C(5 min) $\xrightarrow{①}$ 150°C(0 min) $\xrightarrow{②}$ 240°C(2 min)
Temp. rate	①15°C /min ②50°C /min
Injection temp.	200°C
Carrier gas	Helium
Column flow	1.8 mL/min
Hydrogen	40.0 mL/min
Air	400 mL/min
Injection volume	2 μL
Inlet mode	Splitless
Purge flow	20.0 mL/min
Purge time	0.50 min
Detector	
Temp.	240°C
Sensitivity	Range 2^0

4. Preparation of standard solution

The standard solutions to determine the concentrations of the test item and 13F-EtOH in the sample for analysis were prepared as follows. The standard solution was each prepared with correcting by the purity (99.7%) of the test item and the purity (99.8%) of 13F-EtOH.

The test sample of 100.3 mg was precisely weighed with an electronic balance and dissolved in ethyl acetate to obtain 1000 mg/L solution of the test item. The reference standard for 13F-EtOH component analysis(the test sample of study number 94232-94234) of 100.2 mg was precisely weighed with an electronic balance and dissolved in ethyl acetate to obtain 1000 mg/L solution of 13F-EtOH. The test item solution was diluted with ethyl acetate to prepare 25.0 mg/L (as the concentrations of the test item and 13F-EtOH) of test item and 13F-EtOH solution, after 13F-EtOH solution was added. The solution was diluted with ethyl acetate to prepare 2.50 mg/L (as each concentration of the test item and 13F-EtOH) of standard solution.

5. Calibration curve

The standard solutions of 0.250、1.25、2.50 and 5.00 mg/L (as the concentrations of the test item and 13F-EtOH) were prepared by the same procedure as described in section 4. These solutions were analyzed according to the quantitative analytical conditions described in section 3. The calibration curves were drawn from the relationship between the concentrations of standard solution (the test item and 13F-EtOH) 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 solution (0.250 mg/L) within the range of the calibration confirmed. Therefore, the determination limit of the test item in the test solution was 0.00613 mg/L in consideration of pretreatment. The determination limit of 13F-EtOH was the lowest concentration of the standard solution (0.250 mg/L) within the range of the calibration confirmed. Therefore, the determination limit of 13F-EtOH in the test solution was 0.00605 mg/L in consideration of pretreatment.

6. Recovery test and blank test

6.1 Method

The recovery test was conducted by adding the test item solution (prepared with acetone) to medium according to pretreatment of test solution described in section 2. Similarly, the recovery test was conducted by adding 13F-EtOH solution (prepared with acetone) to medium. The blank test was also conducted using medium (added acetone) without the test item and 13F-EtOH in the same way as the recovery test. The recovery test and the blank test were performed in duplicate.

Amount of the test item added	5.00 μg
Amount of 13F-EtOH added	5.00 μg

6.2 Result

As a result of analysis by the method of section 6.1, no peak of the test item and 13F-EtOH appeared in the chromatogram of the blank test. Two individual recovery rates and their average on the analytical procedure are shown below. The averages of recovery rate were used as correction factor, for the determination of the test item and 13F-EtOH concentrations in the test solutions.

Recovery rate of the test item for pretreatment

81.0%, 82.1% average 81.6%

Recovery rate of 13F-EtOH for pretreatment

83.3%, 81.8% average 82.6%

7. Results of measurement

The results of the measured concentrations of the test item and 13F-EtOH 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 of test item (mg/L) (Percentage of measured concentration versus that at the start %)				
	At the start	24 hours	48 hours	At the end	Geometric mean
Control	n.d.	n.d.	n.d.	n.d.	
100	0.129	0.0211 (16.4)	0.0162 (12.6)	0.00655 (5.09)	0.0215

n.d. : <0.00613mg/L

Appendix table 2-2 Measured concentrations of 13F-EtOH in test solutions

Nominal concentration (mg/L)	Measured concentration of 13F-EtOH (mg/L)			
	At the start	24 hours	48 hours	At the end
Control	n.d.	n.d.	n.d.	n.d.
100	0.00827	0.00635	0.00622	n.d.

n.d. : <0.00605mg/L

The geometric mean is calculated by the following expression:

$$\text{antilog}\left(\frac{1}{2(t_n - t_1)}\sum_{i=1}^{n-1}\left[\left(\log(conc_i) + \log(conc_{i+1})\right)\cdot(t_{i+1} - t_i)\right]\right)$$

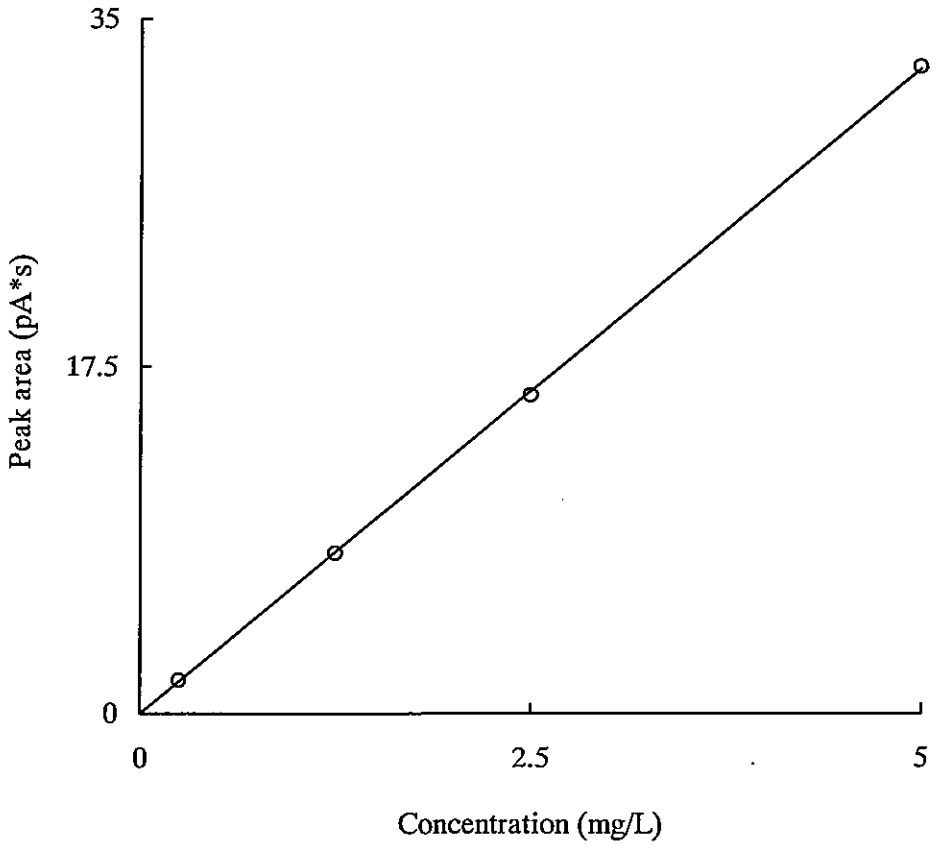
where

t_1 = at the start < t_2 < $\cdots$ t_n = at the end

$conc_1$ = concentration at the start, $conc_2$, $\cdots$, $conc_n$ = concentration at the end

Appendix 3

Calibration curve and chromatogram



y = 6.49x
r = 1.00

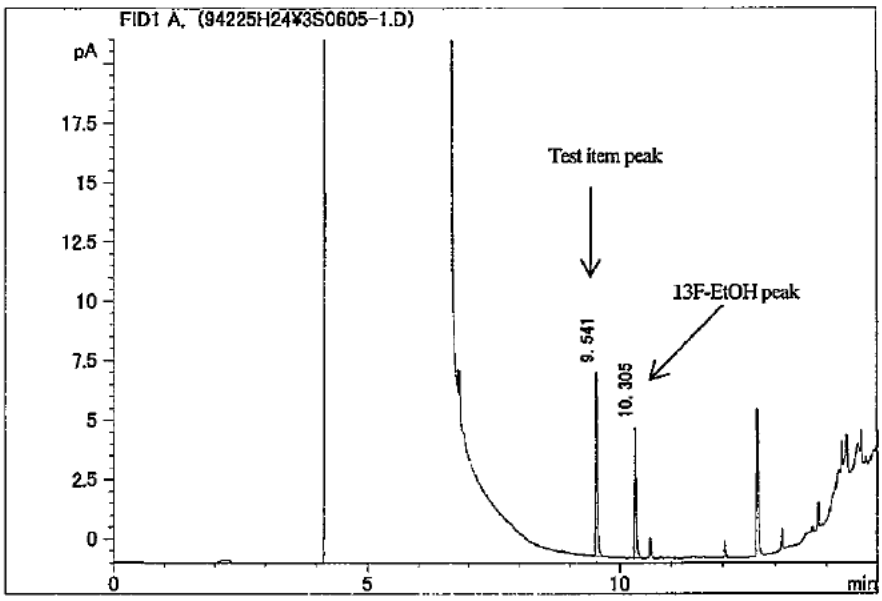
Concentration (mg/L)	Peak area (pA*s)
0.250	1.667
1.25	8.062
2.50	16.015
5.00	32.546

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

Standard solution 2.50 mg/L

Study No. 94223

測定オペレータ　　:
メソッド名　　: C:¥HPCHEM¥1¥METHODS¥94223.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥94225H24¥3S0605-1.D
サンプル名　　: std 2.50mg/L



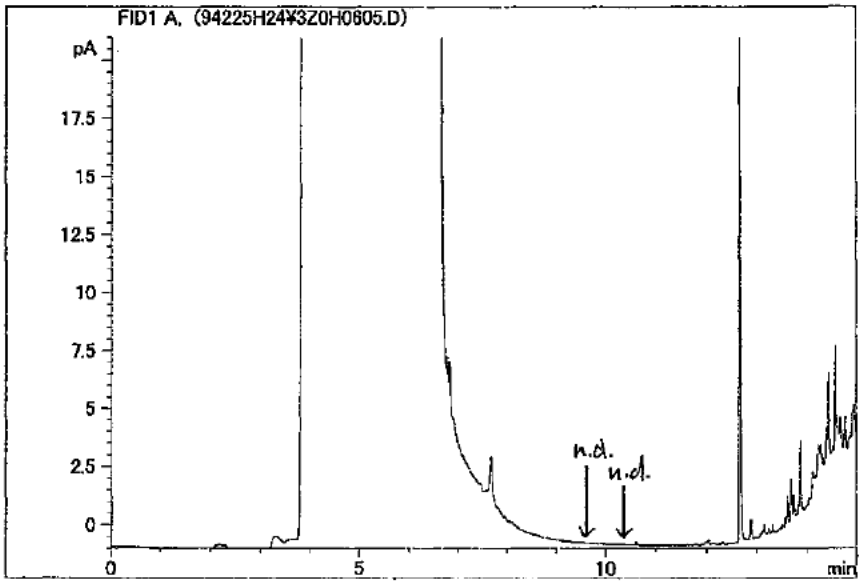
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	16.620	7.740	9.541
2	12.379	5.492	10.305

Appendix figure 3-2-1　GC chromatogram at start of exposure.

Control

Study No. 94223

測定オペレータ　　:
メソッド名　　: C:¥HPCHEM¥1¥METHODS¥94223.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥94225H24¥3ZOH0605.D
サンプル名　　: Cont. (*50) H0h



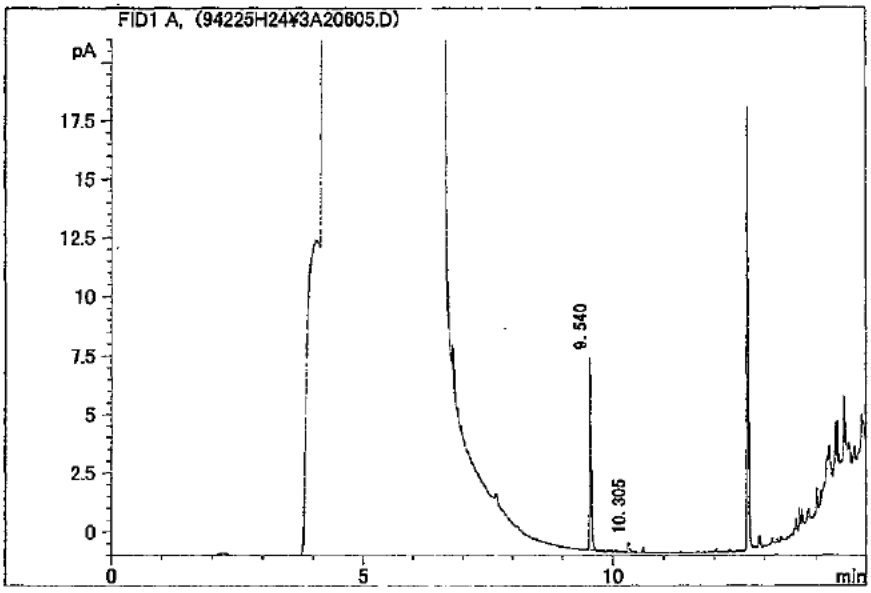
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT

Appendix figure 3-2-2　GC chromatogram at start of exposure.

100 mg/L (Nominal concentration)

Study No. 94223

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94223.M
生データファイル名 : C:¥HPCHEM¥1¥DATA¥94225H24¥3A20605.D
サンプル名 : 100(*50D2)H0h



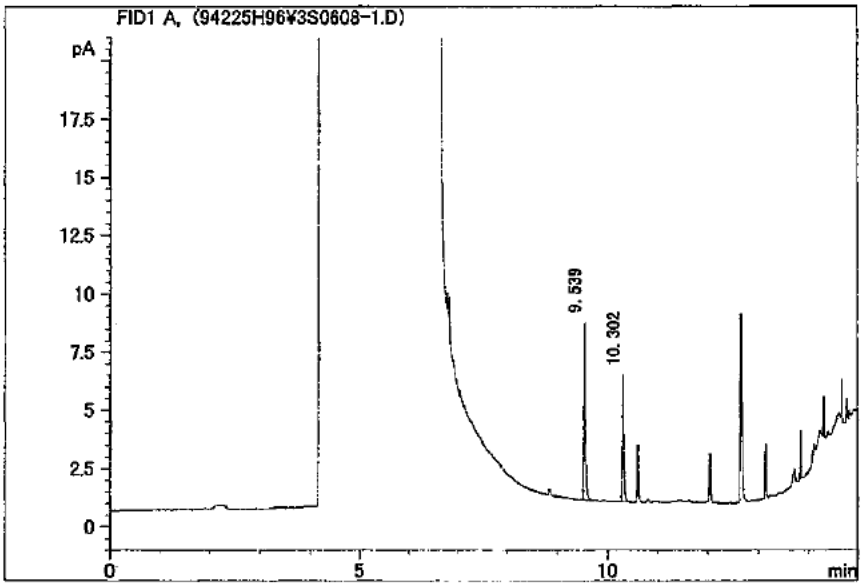
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	17.459	8.147	9.540
2	0.879	0.393	10.305

Appendix figure 3-2-3 GC chromatogram at start of exposure.

Standard solution 2.50 mg/L

Study No. 94223

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94223.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥94225H96¥3S0608-1.D
サンプル名 : std 2.50mg/L



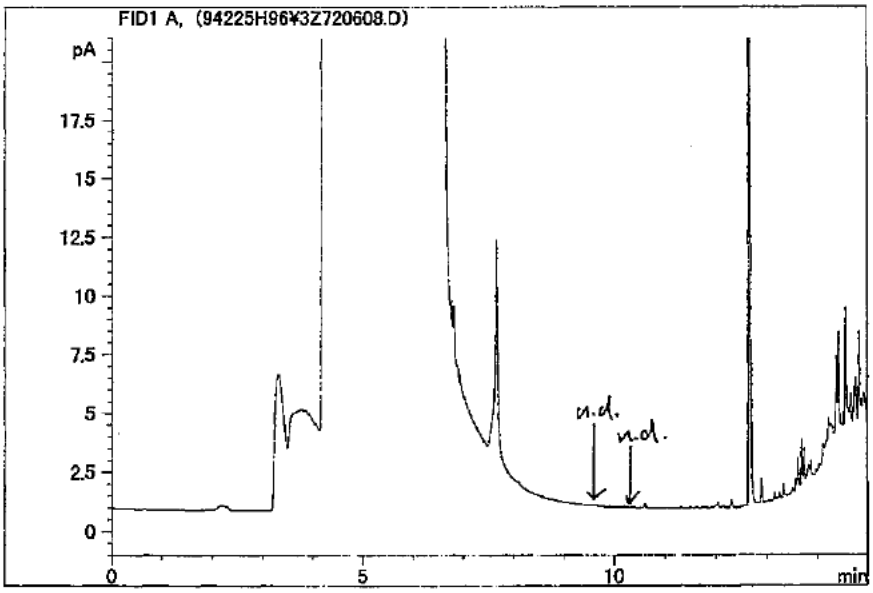
#	メインピーク面積	メインピーク高さ	実測 RT
1	16.380	7.631	9.539
2	12.268	5.450	10.302

Appendix figure 3-3-1 GC chromatogram at end of exposure.

Control

Study No. 94223

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94223.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥94225H96¥3Z720608.D
サンプル名 : Cont. (*50)H72h



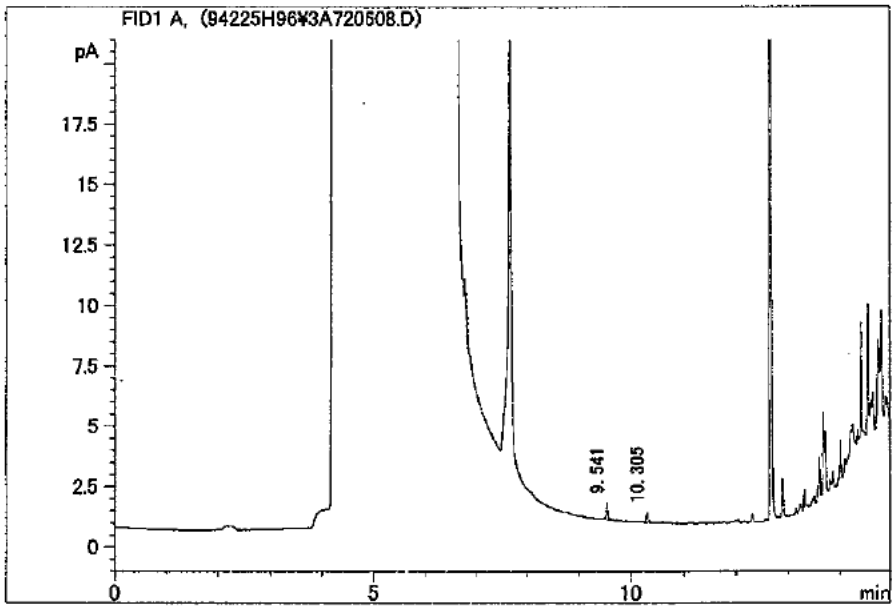
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT

Appendix figure 3-3-2 GC chromatogram at end of exposure.

100 mg/L (Nominal concentration)

Study No. 94223

測定オペレータ　　:
メソッド名　　: C:¥HPCHEM¥1¥METHODS¥94223.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥94225H96¥3A720608.D
サンプル名　　: 100(*50)H72h



#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	1.750	0.691	9.541
2	1.094	0.414	10.305

Appendix figure 3-3-3　GC chromatogram at end of exposure.

Appendix 4

Solubility of test item in medium

1. Title

Solubility of test item in medium

2. Objective

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

3. Outline

Test item mixed with medium was stirred for 24 hours under the test temperature. After clearing insoluble matter, solubility was 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 : 23±1°C

(2) The number of measurement: Once (after the mixture was stirred for 24 hours)

(3) Repetition : n=3 (Sample-1, Sample-2 and Sample-3)

4.3 Test procedures

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

* The additive amount (38.6 μL) was caluculated from the density of the test item (1.554 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 hours, the flask was settled in a water bath for about 1 hour.

(4) After settling, the samples were quantitatively analyzed to measure the concentration of the test item and 13F-EtOH (hydrolyzed product).

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 (2. Pretreatment of test solution).

(2) Method for analysis

See Appendix 2 3. Method of analysis.

4.5 Preparation of standard solution

See Appendix 2 4. Preparation of standard solution.

4.6 Calibration curve

See Appendix 2 5. Calibration curve.

4.7 Recovery test and blank test

See Appendix 2 6. Recovery test and blank test.

5. Results

Measured solubility of the test item in medium was 0.208 mg/L. In addition, the measured concentration of 13F-EtOH was 0.0169 mg/L. The results of analyses are shown below.

Appendix table 4-1 Value measured after stirring for 24 hours (test item)

Sample name	Measured value (mg/L)	Arithmetic mean (mg/L)
Sample-1	0.214	0.208
Sample-2	0.144	
Sample-3	0.266	

Appendix table 4-2 Value measured after stirring for 24 hours (13F-EtOH)

Sample name	Measured value (mg/L)	Arithmetic mean (mg/L)
Sample-1	0.0204	0.0169
Sample-2	0.0112	
Sample-3	0.0191	

Additional data

Results of preliminary studies

1. Solubility of test item in medium

It was expected that the solubility of the test item in medium was below 100 mg/L, therefore, the measurement of the solubility of the test item in medium was conducted.

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 medium were mixed and gently stirred in a devised glass container under closed system with no head space and test temperature (23±1°C) for 24 and 48 hours. And then the middle layer was sampled after settling for about 1 hour. Centrifugation and filtration with a membrane filter for removal of insoluble substance were not demonstrated, because these methods caused the decrease of test item concentration. The concentration of the test item in the collected sample was analyzed after the pretreatment by gas chromatography (GC) (n=2).

(2) Result

Nominal concentration (mg/L)	Measured concentration (mg/L)	
	24-hour stirring	48-hour stirring
Approx. 100 (Sample-1)	0.178	—
Approx. 100 (Sample-2)	0.238	—
Approx. 100 (Sample-3)	—	0.125
Approx. 100 (Sample-4)	—	0.122

Solubility of test item in medium was around 0.1 to 0.3 mg/L.

2) Preliminary study 2 for measurement of solubility

(1) Method

Since the test item was forecasted to be hydrolyzed into 13F-EtOH, the solubility of the test item in dilution water and 13F-EtOH (the test item of study number 94232-94234) were measured at the same time. Firstly, 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) as condition of fish acute toxicity test for 24 and 48 hours. And then the middle layer was sampled after settling for about 1 hour. The concentration of the test item and 13F-EtOH in the collected sample were analyzed after the pretreatment by gas chromatography (GC) (n=2).

(2) Result

Measured concentration of the test item

Nominal concentration (mg/L)	Measured concentration of test item (mg/L)	
	Stirring for 24 hours	Stirring for 48 hours
Approx. 100 (Sample-1)	0.392	-
Approx. 100 (Sample-2)	0.609	-
Approx. 100 (Sample-3)	-	0.0923
Approx. 100 (Sample-4)	-	0.0856

Measured concentration of 13F-EtOH

Nominal concentration (mg/L)	Measured concentration of 13F-EtOH (mg/L)	
	Stirring for 24 hours	Stirring for 48 hours
Approx. 100 (Sample-1)	0.0843	-
Approx. 100 (Sample-2)	0.0721	-
Approx. 100 (Sample-3)	-	0.552
Approx. 100 (Sample-4)	-	0.291

The solubility of the test item in dilution water at 24-hour stirring was higher than the value of preliminary study 1 for measurement of solubility, because devised glass container was not used but Erlenmeyer flask, so the test solution was contaminated with the suspended test sample at the sampling. The measured concentration of 13F-EtOH (hydrolyzed product) was generated 0.07 to 0.1 mg/L at 24-hour stirring, and 0.2 to 0.6 mg/L 48-hour stirring.

3) Summary of preliminary study for measurement of solubility of test item in medium

Since the test item was expected to volatile due to the chemical structure, the test solution was stirred gently in a devised glass container under closed system with no head space. By the results of preliminary study 1, the solubility of the test item in medium was around 0.1 to 0.3 mg/L. And then, the solubility of the test item in medium was within twice to the difference of the analytical results at 24 and 48-hour stirring. It considered that the solubilities of the test item in medium at 24 and 48-hour stirring were about the same value. On the other hand, by the result of preliminary study 2, the measured concentration of 13F-EtOH (hydrolyzed product) at 24-hour stirring was around 0.08 mg/L, but it was greatly increased to 0.2 to 0.6 mg/L at 48-hour stirring.

From the results mentioned above, in definitive study the devised glass container would be used for the preparation. It was decided that the test solution was stirred for 24 hours, because the solubilities of the test item at 24 and 48-hour stirring were about the same value, and the production amount of hydrolyzed product was low.

2. Study for effect on test organism

1) Preliminary study

(1) Method

The test sample and medium were mixed to prepare approximately 100 mg/L (nominal concentration) as upper limit of test concentration. Then, they were stirred for about 48 hours in the closed bottle with little headspace. After the mixture was left for about 1 hour at rest, the saturated solution of test item was collected from the middle layer for use as the test solution. And then the test organisms were exposed to the test solution to confirm the effect. It was expected that the test item was volatile. Therefore, the test solution was prepared under the closed condition without headspace, and the closed test vessel for volatile substance was used. At the same time, the concentration of the test item in the test solution was measured at the start and end of the exposure. The confirmation whether the algae took in the test item was not conducted because the separate operation such as centrifugation was impossible for volatile substance.

(2) Result

Nominal concentration (mg/L)	Growth inhibition rate (%)
	Growth rate (0-3d)
Approx. 100	-2.83

Replicates: two replicates / test level

Measurement method: cell counting method

No effect was found in the saturated solution of the test item.

Nominal concentration (mg/L)	Measured concentration (mg/L) (Percent of the measured concentration to that at the start %)	
	At the start of the exposure	At the end of the exposure (After 72 hours)
Approx. 100	0.289	0.0175 (6.07)

The concentration of the test item at the start of the exposure was close to solubility in medium. The measured concentration of the test item significantly decreased at the end of the exposure, resulting in about 6% of that at the start of the exposure.

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

The test sample and medium were mixed to prepare the maximum concentration on the applied test guidelines (approximately 100 mg/L), and then they were stirred for 48 hours under closed condition. The mixture was left at rest for 1 hour, and then the solution was collected from the middle layer. The test organisms were exposed to the saturated solution (nominal concentration: 100 mg/L). As a result, no effect was found in the saturated solution. The measured concentration of the test item significantly decreased during the exposure because of the volatilization of the test item. The exchange of the test solution was impossible in the algae growth inhibition study, and appropriate headspace in the test vessel was necessary for the growth of algae. Therefore, it was difficult to maintain the concentration of the saturated solution as a test design.

3. Results of preliminary study (Summary)

As the result of the preliminary study for solubility, it was considered that concentration of the test item was saturated by 24 hours after stirring. Furthermore, it was confirmed that the hydrolyzed product (13F-EtOH) was produced with time.

In the biological preliminary study, the saturated test solution of test item prepared by stirred 48 hours which is usual stirring time had no effect on test organism. However, it was expected that the hydrolyzed product in the saturated solution prepared by 48-hour stirring is more produced than 24-hour stirring, though the dissolved the test item is almost the same concentration between 24- and 48-hour stirring. Therefore, the definitive study was conducted with the saturated solution prepared by 24-hour stirring because it was better that the hydrolyzed product is as little as possible. It was expected that the saturated test solution prepared by 24-hour stirring also has no effect on test organisms.

4. Operation of the definitive study

1) Measurement of solubility of test item in medium

Based on the results of the preliminary study for the solubility, measurement of the solubility of the test item in medium was conducted as follows. The test sample was added to medium to prepare the nominal concentration of 100 mg/L. Then, they were stirred for 24 hours at 23 ± 1°C (temperature of algae growth inhibition study) in the closed vessel. Removal of the insoluble matter such as the centrifugation and filtration was not conducted. After stirring, the test solution was left at rest for 1 hour, and then the solution collected from the middle layer. As the test solution, the concentration of the test item and hydrolyzed product was measured.

2) Definitive study

Based on the results of the preliminary study, it was expected that the test item did not affect the test organisms at around the solubility of the test item in medium. Therefore, the definitive study was conducted with the saturated solution (nominal concentration: approximately 100 mg/L) which was prepared by stirring for about 24 hours and the control. The preparation method of the test solution was as follows. The test sample and medium were added to the closed vessel without headspace to prepare approximately 100 mg/L, and they were stirred for about 24 hours by magnetic stirrer. The mixture was left at rest for 1 hour in order to conform to the measurement of the solubility. Then, the solution was collected from the middle layer, and this was used for the test solution (saturated solution of the test item). The concentrations of the test item and 13F-EtOH in the test solution were measured at the start of the exposure, 24 and 48 hours after the exposure, and the end of the exposure.