CERI

Receipt number	662-16-E-7725
Study number	97725

FINAL REPORT

Algae Growth Inhibition Study of APFHx (C-1500N) in *Pseudokirchneriella subcapitata*

This is a correct copy of the original.
Chemicals Evaluation and Research Institute, Japan, Kurume (CERI Kurume)

Date July 26, 2017

Study Director

July, 2017

Chemicals Evaluation and Research Institute, Japan, Kurume

GLP STATEMENT

Chemicals Evaluation and
Research Institute, Japan, Kurume

Sponsor	DAIKIN INDUSTRIES, LTD.
Title	Algae Growth Inhibition Study of APFHx (C-1500N) in *Pseudokirchneriella subcapitata*
Study number	97725

The study described in this report was conducted in compliance with the following GLP principle:
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 11, 2017

Study Director

QUALITY ASSURANCE STATEMENT

Chemicals Evaluation and Research Institute, Japan, Kurume

Sponsor: DAIKIN INDUSTRIES, LTD.

Title: Algae Growth Inhibition Study of APFHX (C-1500N) in *Pseudokirchneriella subcapitata*

Study number: 97725

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 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	Date of inspection	Date of report
Study plan	June 1, 2017	June 1, 2017
Start of exposure	June 5, 2017	June 5, 2017
Completion of exposure	June 8, 2017	June 8, 2017
Raw data and draft final report	July 3, 2017	July 3, 2017
Final report	July 11, 2017	July 11, 2017

Date July 11, 2017

Personnel of Quality Assurance Unit:

QUALITY ASSURANCE STATEMENT

Chemicals Evaluation and Research Institute, Japan, Kurume

Sponsor: DAIKIN INDUSTRIES, LTD.

Title: Algae Growth Inhibition Study of $APFH_X$ (C-1500N) in *Pseudokirchneriella subcapitata*

Study number: 97725

Study inspection of the corrected parts in the final report was carried out and it was confirmed that the correction has no problem.

The inspections 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	Date of inspection	Date of report
Draft final report amendment No.1	July 25, 2017	July 25, 2017
Final report amendment No.1	July 26, 2017	July 26, 2017

This statement was issued as a supplement to the quality assurance statement issued on July 11, 2017.

Date July 26, 2017

Personnel of Quality Assurance Unit:

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1. Title

Algae Growth Inhibition Study of APFHx (C-1500N) in *Pseudokirchneriella subcapitata*

2. Sponsor

Name DAIKIN INDUSTRIES, LTD.

Address 1-1, Nishi Hitotsuya, Settsu-shi, Osaka 566-8585, Japan

3. Test facility

Name Chemicals Evaluation and Research Institute, Japan, Kurume (CERI Kurume)

Address 3-2-7 Miyanojin, Kurume-shi, Fukuoka 839-0801, Japan

4. Objective

The objective of this study is to determine the 0-72-hour median effective concentration (EC_{50}) and no observed effect concentration (NOEC) by conducting an algae growth inhibition test of APFHx (C-1500N) in *Pseudokirchneriella subcapitata.*

5. Test method

OECD Guidelines for Testing of Chemicals, No.201, March 23, 2006, Annex 5 corrected: July 28, 2011, "Freshwater Alga and Cyanobacteria, Growth Inhibition Test"

6. GLP principle

"OECD Principles of Good Laboratory Practice" November 26, 1997, ENV/MC/CHEM (98)17

7. Dates

Study initiation date	May 30, 2017
Experimental starting date	June 5, 2017
Experimental completion date	June 8 2017
Study completion date	July 11, 2017

8. Storage of test item, raw data, etc.

The study plan (original), the final report (original), the raw data, documents concerning the study presented by the sponsor, the test sample survey sheets and other reports are stored in the archives of this laboratory. The test item is returned to the sponsor.

The storage period is 10 years after submission of the final report.

Treatment of the raw data, etc. after the storage period (continue, reject, or return) is discussed with the sponsor.

9. Personnel

Study Director

Study personnel (Biological study)

Study personnel (Analytical chemistry)

10. Approval of final report

Date July 11, 2017

Study Director

11. Summary

Test item

APFHx (C-1500N)

Objective

The objective of this study is to determine the 0-72-hour median effective concentration (EC_{50}) and no observed effect concentration (NOEC) by conducting an algae growth inhibition test of APFHx (C-1500N) in *Pseudokirchneriella subcapitata*.

Test method

OECD Guidelines for Testing of Chemicals, No.201, March 23, 2006, Annex 5 corrected: July 28, 2011, "Freshwater Alga and Cyanobacteria, Growth Inhibition Test"

Test conditions

Test organism	*Pseudokirchneriella subcapitata*
	100 mg/L (upper limit concentration of test method as limit test) and a control
Preparation of test solution	Test sample and medium were mixed and stirred for one minute to prepare the nominal concentration of 100 mg/L as the test solution.
Type of test	Incubation with rotary shaking (approximately 100 rpm)
Exposure duration	72 hours
Replicate	6 replicates/test level
Volume of test solution	600 mL/test level (100 mL/test vessel)
Temperature in incubator	22.3-22.5°C
Light condition	90-91 $\mu mol \cdot m^{-2} \cdot s^{-1}$
Measurement of cell growth	Cell concentration
Analysis of concentration of test item in test solution	
	HPLC analysis (at the start and end of exposure)

Results

EC_{50} (E_rC_{50})	>100 mg/L
NOEC (Growth rate 0-3d)	≥100 mg/L

(Concentrations described above were based on the nominal concentration)

12. Test materials

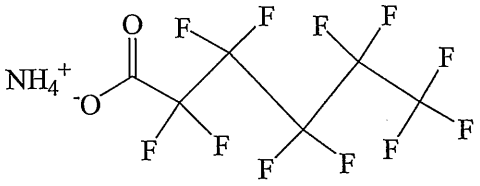
12.1 Test item

a) Chemical name etc.

Chemical name	2,2,3,3,4,4,5,5,6,6,6-undecafluorohexanoic acid, ammonium salt
Another name	APFHx (C-1500N)
CAS number	21615-47-4

b) Chemical structure etc.

Structural formula



Molecular formula	$C_6H_4F_{11}NO_2$
Molecular weight	331.08

c) Test sample

Purity of test item	50%
Impurity	Water 50%
Supplier	DAIKIN INDUSTRIES, LTD.
Lot number	C150E62004

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

d) Physicochemical property

Appearance	Colorless and clear liquid

e) Storage condition

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

f) Identification and stability of test item under the storage condition

The infrared (IR) spectrum of the test item measured at this laboratory was confirmed to be identical to that provided by the sponsor.

The stability of the test item was confirmed by comparing the IR spectrum of the test item after the completion of the experiment under the storage condition with that before the start of the experiment.

g) Safety and handling

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

12.2 Test organisms

Species	*Pseudokirchneriella subcapitata*
Reason for selection of species	Species recommended in the test guideline
Source	American Type Culture Collection
Strain number	ATCC 22662
Supplied date	June 30, 1995
Subculture	Passage cultured under sterile conditions in this laboratory

Confirmation of reproducibility of test system

Algae growth inhibition test with a reference substance was periodically conducted. The latest data is shown below.

Reference substance; Potassium dichromate
(JIS special grade, Wako Pure Chemical Industries, Ltd. Lot No. JPJ7565)

Test period; May 22 to May 25, 2017

E_rC_{50} (0-3d); 1.3 mg/L

This value was within the normal range of the reference substance in this laboratory (mean ± 2S.D.) [mean ± S.D.: 1.00 ± 0.20 mg/L (n=31)].

13. Test methods

13.1 Culture medium

At the pre-culture and algae growth inhibition test, the OECD medium (OECD TG 201; March 23, 2006) prepared with purified water was used.

Component	mg/L	Component	mg/L
H_3BO_3	0.185	$CuCl_2 \cdot 2H_2O$	0.00001
$MnCl_2 \cdot 4H_2O$	0.415	$CaCl_2 \cdot 2H_2O$	18.0
$ZnCl_2$	0.00300	NH_4Cl	15.0
$FeCl_3 \cdot 6H_2O$	0.0640	KH_2PO_4	1.60
$Na_2EDTA \cdot 2H_2O$	0.100	$NaHCO_3$	50.0
$CoCl_2 \cdot 6H_2O$	0.00150	$MgCl_2 \cdot 6H_2O$	12.0
$Na_2MoO_4 \cdot 2H_2O$	0.00700	$MgSO_4 \cdot 7H_2O$	15.0

13.2 Test apparatus and equipment

Test vessel: 300 mL Erlenmeyer flask (with gas-permeable Silicosen®)

Incubator: Incubator with temperature and illumination control, continuous shaking [Incubator with rotary shaker and artificial illumination, U.S.I Corp. (Instrument No.SIN-002)]

13.3 Preparation of test solution

The test sample of 0.160 g and medium of 800 mL were mixed and it was stirred for one minute to prepare the dissolved test solution of 200 mg/L as test sample (corresponding to 100 mg/L as the test item). The test solution was divided into each test vessel.

13.4 Test conditions

Type of test: Incubation with rotary shaking (approximately 100 rpm)

Duration: 72 hours

Test concentration: 100 mg/L (upper limit concentration of test method as a limit test)
The test concentration was decided based on the results of preliminary study.
The results of preliminary study are shown in Additional data.

Control: The medium without the test item

Replicate: 6 replicates/test level

Volume of test solution:
600 mL/test level (100 mL/test vessel)

Initial cell concentration

The algae were counted in pre-culture incubated under the same conditions as the test for 3 days (from June 2 to June 5, 2017) as inoculums, and were added to test vessels to bring the initial cell concentration of 0.75×10^4 cells/mL.

Operation: All operations were carried out under sterile conditions.

Temperature: 21-24°C (not varied more than ±2°C)

Light intensity: Nominal 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$ ±20% (within ±15% from the average light intensity)
Continuous illumination provided by fluorescent lights with wavelength range of 400-700 nm

13.5 Observation and measurement

a) Cell growth, etc.

Item of measurement: Biomass (cell concentration)

Frequency: Every 24 hours after the start of exposure
(The blank correction was conducted by measuring the value of blank solution of test vessel prepared for background in each test level.)

Observation of cell condition: One vessel in each test level at the end of exposure

Instrument: Particle counter; Model COULTER Z2
(Beckman Coulter, Inc., Instrument No.CC-004)
System biological microscope; Model BX41 (Olympus Corporation)

b) Appearance of test solution

Observation at the start and end of exposure

c) Condition of test solutions and exposure environment

pH: Another solution sampled from the preparation container was measured (at the start of exposure).
One test vessel in each test level was measured (at the end of exposure).

Culture temperature

It was measured at the start, 1-day, 2-day after the start, and the end of exposure in the incubator.

Light intensity

It was measured at the start, 1-day, 2-day after the start, and the end of exposure in the incubator.

Instrument: Portable pH meter Model HM-21P (DKK-TOA CORPORATION)
Thermometer of glass stick type
Quantum scalar laboratory irradiance meter Model LI-250A (LI-COR, Inc.)

d) Concentration of test item in test solution

Frequency of measurement: At the start and end of exposure

Sample for measurement: Another solution sampled from the preparation container (at the start of exposure)
The mixed solution taken out with equal volume of the test solution from the test vessels in each test level (at the end of exposure)

Removal of algae: Centrifugation (3000 rpm, 10 minutes) (at the end of exposure)

Volume of sample Approximately 10 mL (at the start of exposure, all test levels)
9 mL (at the end of exposure, all test levels)

Analytical condition Referred in Appendix 1

13.6 Treatment of results

The results of the study were estimated by the nominal concentration, because the measured concentration in the test solution during the exposure was within the range of ±20% of the nominal concentration.

a) 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:

$$\mu_{i\text{-}j} = \frac{lnX_j - lnX_i}{t_j - t_i}$$

where

$\mu_{i\text{-}j}$ = Specific growth rate from time i to j (normally d^{-1})

X_i = Value of biomass at t_i : Set value of biomass was used at the start of the exposure (t_0).

X_j = Value of biomass at t_j

t_i = Time (d) of i^{th} measurement after beginning of exposure

t_j = Time (d) of j^{th} measurement after beginning of exposure

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

The percentage inhibition for each exposure level was mean value of the percent inhibition in average specific growth rate for a replicate (I_μ) in test level. The percent inhibition (I_μ) was calculated from mean value for average specific growth rate in control (μ_c), average specific growth rate for each replicate in exposure level (μ_T), and following formula:

$$I\mu = \frac{\mu_c - \mu_T}{\mu_c} \times 100$$

b) Estimation of EC_{50}

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

c) Estimation of NOEC

Regarding the growth rate, after F test was done to determine the homogeneity of variance for the data, Student's t-test was used to estimate the significant difference in comparison with the control. The statistical analysis was conducted using computer program (running on Microsoft software "Excel") constructed by our laboratory. NOEC was determined by the results of statistical analysis and whole test results.

Corrected page

13.7 Validity of test

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

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

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

13.8 Treatment of numerical values

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

14. Results and discussion

14.1 Observation and measurement of test solution

a) Appearance of test solution

At the start of exposure, test solutions of the exposure level and the control were colorless and clear. At the end of exposure, they were green due to the algal growth.

b) Water quality and environmental conditions

The measured values of pH of the test solution are shown in Table 1, and culture temperature and light intensity in the incubator are shown in Table 2.

The measured values of pH were 7.8-8.3. Culture temperatures in incubator were 22.3-22.5°C and light intensities were 90-91 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

c) Concentration of test item in test solution

The results of measured concentration of test item are shown in Appendix 1. The calibration curve and the chromatograms are shown in Appendix 2.

The measured concentration of test item in the test solution at the start of exposure was 98.8 mg/L and that at the end of exposure was 98.7 mg/L, which were 98.8% and 98.7% of the nominal concentration, respectively. The measured concentrations of test item were kept within ± 20% of the nominal concentration.

14.2 E_rC_{50}

Values of biomass at each time, growth rate and growth inhibition rate, and the E_rC_{50} are shown in Table 3, Table 4 and Table 5, respectively.

E_rC_{50} of the test item based on the growth rate was >100 mg/L.

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

NOEC, the result of statistical analysis of significant difference, and growth curve are shown in Table 5, Table 6 and Figure 1, respectively.

The algal growth in exposure level was same as the control.

The following results of cell observation were based on the comparison with the control. The condition of cells in exposure level was same as the control. In the control, the condition of cells was not abnormal.

On the growth rate, the significant difference was found in the exposure level. In this level, mean of the inhibition rate was low (1.3%), and there was no effect on the test organism in the preliminary study. It was considered that the effect in the exposure level was within the variation range of the test operation and the statistic significant difference was not caused by the effect of the test item. Therefore, it was decided that the test item had no adverse effect on the test organisms in the exposure level and NOEC based on the growth rate was estimated at ≥100 mg/L.

Corrected page

14.4 Validity of test

a) Growth of control

The cell in the control grew exponentially during the exposure (ref. Figure 1). At the end of exposure, it increased to 187 or more times of the number of initial cells in the control (ref. Table 3). This meets the validity of test: the cell growth in the control should have increased by a factor of at least 16 at 72 hours after the start of exposure.

b) Specific growth rates of section-by-section in controls

The mean coefficient of variation for section-by-section specific growth rates in the controls was 8.3% (ref. Table 7). It meets the validity of test: the mean coefficient of variation in the control must not exceed 35%.

c) Specific growth rates in replicate controls

The coefficient of variation of specific growth rates in replicate controls was 0.49% (ref. Table 7). It meets the validity of test: the coefficient of variation in controls must not exceed 7%.

14.5 Discussion

This study was conducted as a limit test in order to confirm the effect of the test item on the test organisms at upper limit concentration of test method (100 mg/L).

On the growth rate, although the significant difference was found in the exposure level, mean of the inhibition rate was low and also there was no effect on the test organism in the preliminary study. It was considered that the effect in the exposure level was not inhibition caused by the test item but was acceptable variation range of the test operation. Therefore, it was decided that the test item had no adverse effect on the test organisms at upper limit concentration of test method. The measured concentrations of the test item in the test solution were within the range of ±20% of the nominal concentration. The environmental conditions were within the suitable range; therefore, it is concluded that this study complied with the applied test method.

15. Factors that affected the reliability of test results

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

Corrected page

Table 1 pH of test solutions

Nominal concentration (mg/L)	pH	
	At the start	At the end
Control	7.8	8.3
100	7.8	8.1

Table 2 Culture temperature and light intensity in incubator

Time	At the start	1-day	2-day	At the end
Culture temperature (°C)	22.5	22.4	22.3	22.5
Light intensity ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	90	91	91	91

Table 3 Value of biomass at each time

Nominal concentration (mg/L)	No.	Cell concentration ($\times 10^4$ cells/mL)			
		0 hour[a]	24 hours	48 hours	72 hours
Control	A	0.75	5.1	25	150
	B	0.75	5.0	27	150
	C	0.75	5.3	26	150
	D	0.75	4.9	27	140[b]
	E	0.75	5.1	27	150
	F	0.75	5.3	26	140[b]
	Mean	0.75	5.1	26	150
	S.D.	0	0.15	0.48	3.8
100	A	0.75	5.1	26	130
	B	0.75	5.0	27	130
	C	0.75	5.0	27	140
	D	0.75	5.1	26	120
	E	0.75	5.0	28	150
	F	0.75	5.1	26	140
	Mean	0.75	5.0	27	140
	S.D.	0	0.064	0.84	7.3

a The value is based on the measured cell concentration of pre-culture.

b The minimum cell growth in control (biomass at the end of exposure/biomass at the start of exposure)

140/0.75 = 187

Table 4 Growth rate and growth inhibition rate

Nominal concentration (mg/L)	No.	Growth rate (0-3d)	Growth inhibition rate (%)
Control	A	1.76	-
	B	1.76	-
	C	1.76	-
	D	1.75	-
	E	1.77	-
	F	1.75	-
	Mean	1.76	-
	S.D.	0.00869	-
100	A	1.73	1.5
	B	1.73	1.6
	C	1.74	1.1
	D	1.70	3.0
	E	1.76	0.026
	F	1.75	0.64
	Mean	1.73	1.3
	S.D.	0.0181	1.0

Table 5 E_rC_{50} and NOEC

E_rC_{50} (mg/L)	NOEC (mg/L)
>100	≥100

Table 6 Result of statistical analysis

Nominal concentration (mg/L)	Statistical analysis	Statistical procedure
100	(*)	*F* test Student's *t*-test

(*) : Although there was significant difference (p< 0.05), it was judged that the test item caused no adverse effect on the test organism.

Table 7 Variation of growth rates in control

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

Control No.	Mean	Standard deviation	Coefficient of variation (%)	
A	1.76	0.162	9.2	8.3 (Mean)
B	1.76	0.127	7.2	
C	1.76	0.173	9.8	
D	1.75	0.106	6.1	
E	1.77	0.126	7.1	
F	1.75	0.178	10	

<Variation of average specific growth rates in replicate controls>

	0-3day
Mean	1.76
Standard deviation	0.00869
Coefficient of variation (%)	0.49

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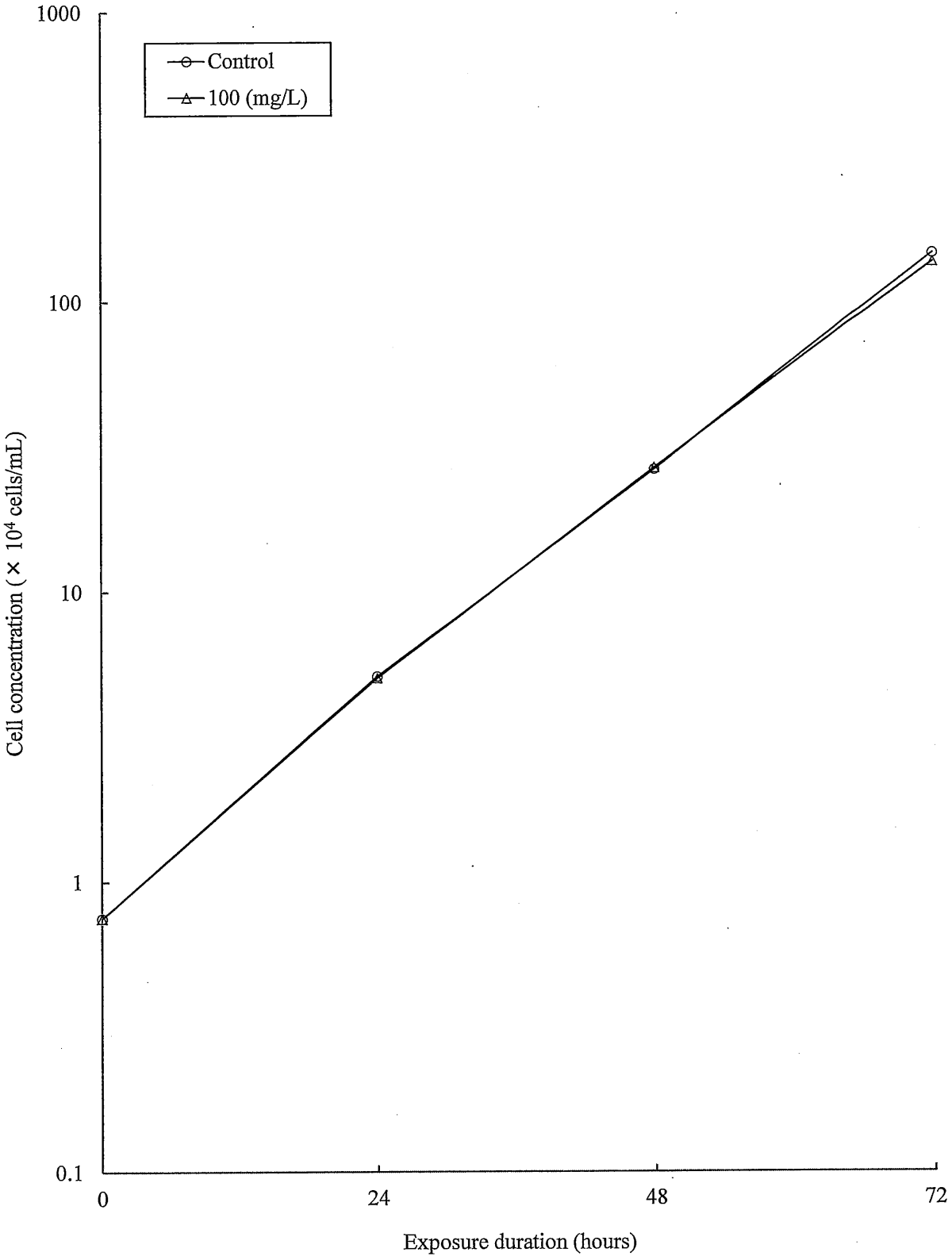


Figure 1 Growth curve.

Appendix 1

Analytical method and measured concentration of test item

1. Pretreatment of test solution

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

2. Determination of test item

a) Method of determination

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

The calibration curve was drawn by using four standard solutions of 1.00, 5.00, 10.0 and 20.0 mg/L which were prepared in the same way described in c) to confirm the effectiveness of this quantity method. As a result, the regression line of the calibration curve was a straight line from the origin. Therefore, the determination method was valid. The drawn calibration curve and chromatograms which obtained by analysis of samples for HPLC are shown in Appendix 2.

The determination limit of the test item in the test solution was the lowest concentration of the standard solution (1.00 mg/L) within the range of the calibration confirmed.

b) Analytical condition

Instrument	High-performance liquid chromatograph (Instrument No. LC-166)
Pump	LC-20AD (Shimadzu)
UV-VIS detector	SPD-20AV (Shimadzu)
Column oven	CTO-20A (Shimadzu)
Auto injector	SIL-20AC (Shimadzu)
System controller	SCL-10A$_{VP}$ (Shimadzu)
Degasser	DGU-20A$_3$ (Shimadzu)
Column	L-column2 ODS (150 mm × 2.1 mm I.D., particle size 5 μm, Chemicals Evaluation and Research Institute, Japan)
Column temp.	40°C
Eluent	A (50%) : Acetonitrile B(50%):Ultra pure water/0.5 mol/L tetra-*n*-butylammonium phosphate solution (100/1 v/v)
Flow rate	0.2 mL/min
Wave length	215 nm
Injection volume	20 μL

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

The standard sample for analysis of the test item (50.1 mg) was precisely weighed by an electronic analytical balance, dissolved in ultra pure water and filled up to 50 mL to obtain 1000 mg/L solution of the standard sample. The solution was diluted with medium to prepare 10.0 mg/L standard solution

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

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

Name	APFHx (C-1500N)
Purity	99.8%
Lot number	C150E57002
Storage condition	The standard sample was stored in a dark storage place at room temperature in a desiccator.
Appearance	White powder

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

3. Results of measurement

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

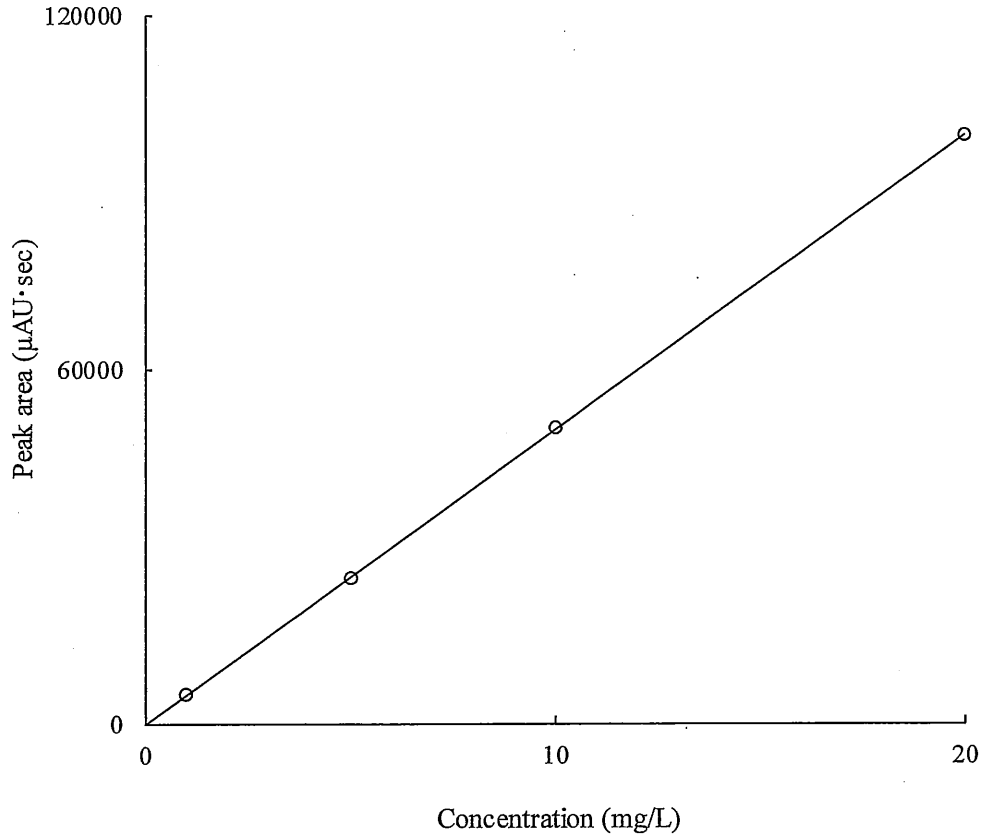
Appendix table 1-1 Measured concentrations of test item in test solutions

Nominal concentration (mg/L)	Measured concentration (mg/L) (Percentage of measured concentration versus nominal concentration %)		
	At the start	At the end	Geometric mean
Control	n.d.	n.d.	
100	98.8 (98.8)	98.7 (98.7)	98.8 (98.8)

n.d. : <1.00 mg/L

Appendix 2

Calibration curve and chromatogram



y = 4980x

r = 1.00

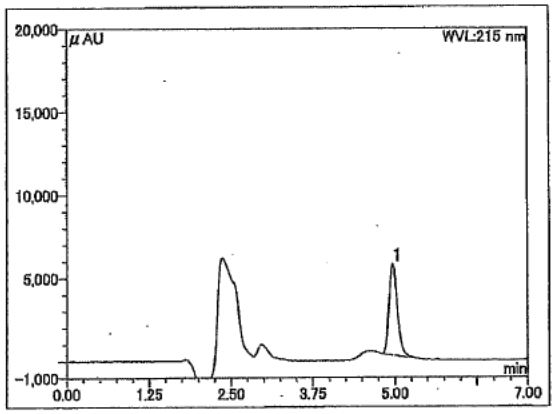
Concentration (mg/L)	Peak area (μAU·sec)
1.00	4847
5.00	24616
10.0	49930
20.0	99610

Appendix figure 2-1 Calibration curve of test item for analysis by HPLC.

Study No. 97725

Standard solution 10.0 mg/L

Operator:	
Operating date:	05/Jun/2017
Sample ID:	97725_170605_S2
Program:	97725_97726_iso
Vial No.:	1_1
Channel:	UV_VIS_1

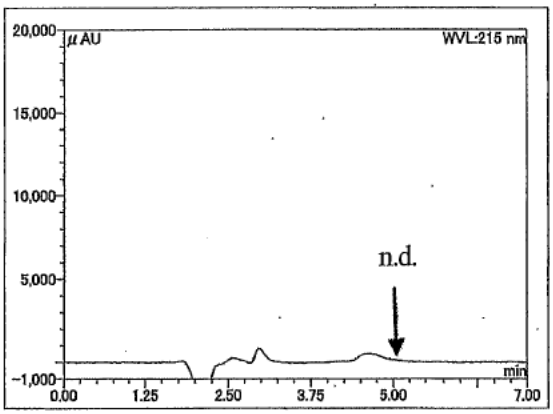


Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	4.96	5487	48785	100.00
Total	–	–	48785	100.00

Study No. 97725

Control

Operator:	
Operating date:	05/Jun/2017
Sample ID:	97725_170605_H0hZ
Program:	97725_97726_iso
Vial No.:	1_2
Channel:	UV_VIS_1

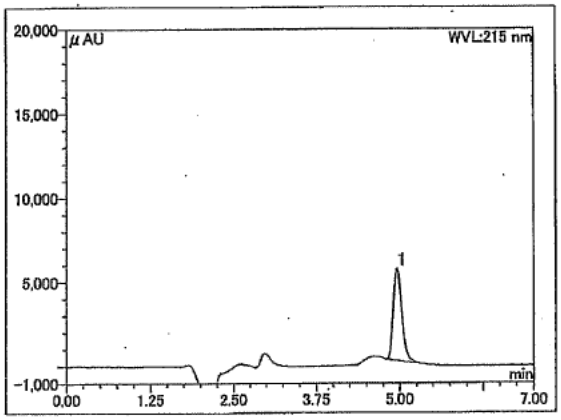


Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
Total	–	–	0	0.00

Study No. 97725

100 mg/L exposure level

Operator:	
Operating date:	05/Jun/2017
Sample ID:	97725_170605_H0hA
Program:	97725_97726_iso
Vial No.:	1_3
Channel:	UV_VIS_1



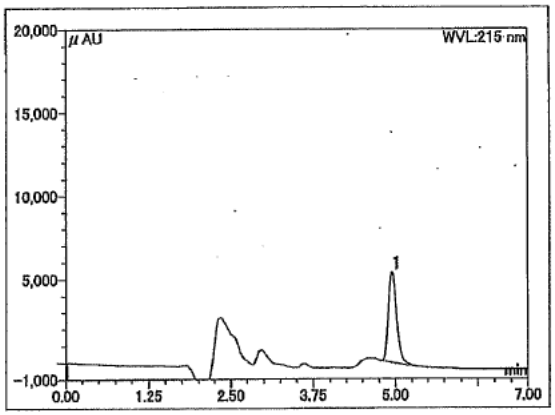
Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	4.96	5448	48202	100.00
Total	–	–	48202	100.00

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

Study No. 97725

Standard solution 10.0 mg/L

Operator:	
Operating date:	08/Jun/2017
Sample ID:	97725_170608_S2
Program:	97725_97726_iso
Vial No.:	1_1
Channel:	UV_VIS_1

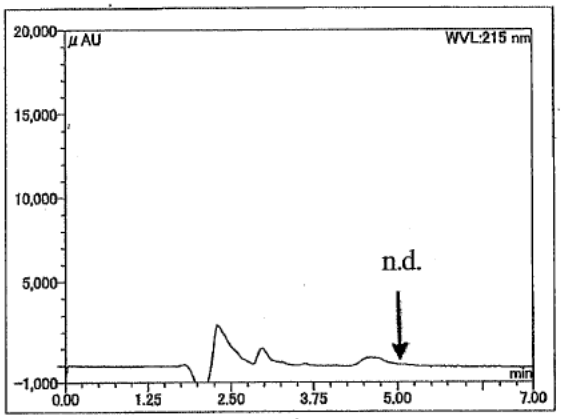


Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	4.95	5434	48615	100.00
Total	–	–	48615	100.00

Study No. 97725

Control

Operator:	
Operating date:	08/Jun/2017
Sample ID:	97725_170608_H72hZ
Program:	97725_97726_iso
Vial No.:	1_2
Channel:	UV_VIS_1

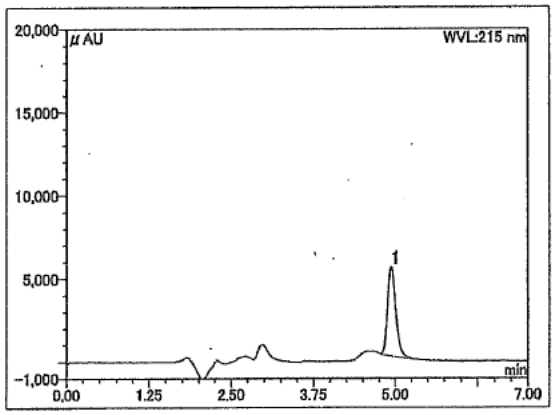


Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
Total	–	–	0	0.00

Study No. 97725

100 mg/L exposure level

Operator:	
Operating date:	08/Jun/2017
Sample ID:	97725_170608_H72hA
Program:	97725_97726_iso
Vial No.:	1_3
Channel:	UV_VIS_1



Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	4.94	5360	47982	100.00
Total	–	–	47982	100.00

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

Additional data

Results of preliminary study

1. Solubility of test item in medium

It was confirmed that the solubility of the test item in medium was more than 100 mg/L for visual observation.

2. Preliminary study of effect on test organism

Replicate: Two replicates/test level

Measurement method: Cell counting method

Preparation of test solution: The test sample and medium were mixed and stirred to prepare the stock solution. The test solution was prepared by diluting the stock solution with medium as appropriate.

Analysis: The concentration of the test item in the test solutions were measured. In addition, the concentration of the test item in the test solution without algae was also measured in order to confirm whether the test item was absorbed to algae or not.

<Result of effect on test organisms>

Nominal concentration (mg/L)	Growth inhibition rate based on growth rate (0-3d) (%)
0.100	-0.55
1.00	0.054
10.0	-0.50
100	0.70

<Result of measured concentration of test item in test solutions>

Nominal concentration (mg/L)	Measured concentration (mg/L) (Percent of the measured concentration versus nominal concentration, %)	
	At the start of exposure	At the end of exposure
1.00	0.969 (96.9)	0.995 (99.5)
1.00 (no algae)		0.963 (96.3)
10.0	10.1 (101)	10.2 (102)
10.0 (no algae)		10.3 (103)
100	102 (102)	104 (104)
100 (no algae)		105 (105)

Absorption to algae: None

3. Condition of definitive study

Test concentration: 100 mg/L and a control (limit test)

CERI

Final Report Amendment No. 1

Chemicals Evaluation and
Research Institute, Japan, Kurume

1. Title (Study number)
Algae Growth Inhibition Study of APFHx (C-1500N) in *Pseudokirchneriella subcapitata* (97725)

2. Content

i) 13.6 c) Estimation of NOEC (Page 12) (See the attached sheet 1 for reason and detail.)
ii) 14.3 Growth curves in each test level, cell observations and NOEC
(Page 13) (See the attached sheet 1 for reason and detail.)
iii) 14.5 Discussion (Page 14) (See the attached sheet 2 for reason and detail.)
iv) Table 6 (Page 18) (See the attached sheet 2 for reason and detail.)

3. Approval
Study Director Date July 26, 2017

Name

Attached sheet 1

Content i) 13.6 c) Estimation of NOEC (Page 12)

Reason

Alteration of the contents in final report based on recalculation of the test results by revised data processing program

Content of correction

Before correction	Regarding the growth rate, after *F* test was done to determine the homogeneity of variance for the data, Student's *t*-test was used to estimate the significant difference in comparison with the control. The statistical analysis was conducted using computer program (running on Microsoft software "Excel") constructed by our laboratory. NOEC was determined by the results of statistical analysis and cell condition. NOEC value was estimated as "> the test concentration" since the growth inhabitation was not observed in the exposure level.
After correction	Regarding the growth rate, after *F* test was done to determine the homogeneity of variance for the data, Student's *t*-test was used to estimate the significant difference in comparison with the control. The statistical analysis was conducted using computer program (running on Microsoft software "Excel") constructed by our laboratory. NOEC was determined by the results of statistical analysis and whole test results.

Content ii) 14.3 Growth curves in each test level, cell observations and NOEC (Page 13)

Reason

Alteration of the contents in final report based on recalculation of the test results by revised data processing program

Content of correction

Before correction	On the growth rate, there was no statistical difference in exposure level. By the results in statistical analysis and cell observation showed above, NOEC based on the growth rate was estimated at >100 mg/L.
After correction	On the growth rate, the significant difference was found in the exposure level. In this level, mean of the inhibition rate was low (1.3%), and there was no effect on the test organism in the preliminary study. It was considered that the effect in the exposure level was within the variation range of the test operation and the statistic significant difference was not caused by the effect of the test item. Therefore, it was decided that the test item had no adverse effect on the test organisms in the exposure level and NOEC based on the growth rate was estimated at >100 mg/L.

Attached sheet 2

Content iii) 14.5 Discussion (Page 14)

Reason

Alteration of the contents in final report based on recalculation of the test results by revised data processing program

Content of correction

Before correction	As a result, no adverse effect was found in the definitive study. Therefore, it was decided that the test item had no adverse effect on the test organisms at upper limit concentration of test method.
After correction	On the growth rate, although the significant difference was found in the exposure level, mean of the inhibition rate was low and also there was no effect on the test organism in the preliminary study. It was considered that the effect in the exposure level was not inhibition caused by the test item but was acceptable variation range of the test operation. Therefore, it was decided that the test item had no adverse effect on the test organisms at upper limit concentration of test method.

Content iv) Table 6 (Page 18)

Reason

Alteration of the contents in final report based on recalculation of the test results by revised data processing program

Content of correction

Before correction

Nominal concentration (mg/L)	Statistical analysis	Statistical procedure
100	n.s.	*F* test Student's *t*-test

n.s. : No significant difference

After correction

Nominal concentration (mg/L)	Statistical analysis	Statistical procedure
100	(*)	*F* test Student's *t*-test

(*) : Although there was significant difference ($p < 0.05$), it was judged that the test item caused no adverse effect on the test organism.

Volume of sample Approximately 10 mL (at the start of exposure, all test levels)
9 mL (at the end of exposure, all test levels)

Analytical condition Referred in Appendix 1

13.6 Treatment of results

The results of the study were estimated by the nominal concentration, because the measured concentration in the test solution during the exposure was within the range of ±20% of the nominal concentration.

a) 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:

$$\mu_{i\text{-}j} = \frac{lnX_j - lnX_i}{t_j - t_i}$$

where

$\mu_{i\text{-}j}$ = Specific growth rate from time i to j (normally d^{-1})

X_i = Value of biomass at t_i : Set value of biomass was used at the start of the exposure (t_0).

X_j = Value of biomass at t_j

t_i = Time (d) of i^{th} measurement after beginning of exposure

t_j = Time (d) of j^{th} measurement after beginning of exposure

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

The percentage inhibition for each exposure level was mean value of the percent inhibition in average specific growth rate for a replicate (I_μ) in test level. The percent inhibition (I_μ) was calculated from mean value for average specific growth rate in control (μ_c), average specific growth rate for each replicate in exposure level (μ_T), and following formula:

$$I\mu = \frac{\mu_c - \mu_T}{\mu_c} \times 100$$

b) Estimation of EC_{50}

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

c) Estimation of NOEC

Regarding the growth rate, after F test was done to determine the homogeneity of variance for the data, Student's t-test was used to estimate the significant difference in comparison with the control. The statistical analysis was conducted using computer program (running on Microsoft software "Excel") constructed by our laboratory. NOEC was determined by the results of statistical analysis and cell condition. NOEC value was estimated as "≥ the test concentration" since the growth inhabitation was not observed in the exposure level.

13.7 Validity of test

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

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

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

13.8 Treatment of numerical values

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

14. Results and discussion

14.1 Observation and measurement of test solution

a) Appearance of test solution

At the start of exposure, test solutions of the exposure level and the control were colorless and clear. At the end of exposure, they were green due to the algal growth.

b) Water quality and environmental conditions

The measured values of pH of the test solution are shown in Table 1, and culture temperature and light intensity in the incubator are shown in Table 2.

The measured values of pH were 7.8-8.3. Culture temperatures in incubator were 22.3-22.5°C and light intensities were 90-91 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

c) Concentration of test item in test solution

The results of measured concentration of test item are shown in Appendix 1. The calibration curve and the chromatograms are shown in Appendix 2.

The measured concentration of test item in the test solution at the start of exposure was 98.8 mg/L and that at the end of exposure was 98.7 mg/L, which were 98.8% and 98.7% of the nominal concentration, respectively. The measured concentrations of test item were kept within ± 20% of the nominal concentration.

14.2 E_rC_{50}

Values of biomass at each time, growth rate and growth inhibition rate, and the E_rC_{50} are shown in Table 3, Table 4 and Table 5, respectively.

E_rC_{50} of the test item based on the growth rate was >100 mg/L.

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

NOEC, the result of statistical analysis of significant difference, and growth curve are shown in Table 5, Table 6 and Figure 1, respectively.

The algal growth in exposure level was same as the control.

The following results of cell observation were based on the comparison with the control. The condition of cells in exposure level was same as the control. In the control, the condition of cells was not abnormal.

On the growth rate, there was no statistical difference in exposure level. By the results in statistical analysis and cell observation showed above, NOEC based on the growth rate was estimated at ≥100 mg/L.

14.4 Validity of test

a) Growth of control

The cell in the control grew exponentially during the exposure (ref. Figure 1). At the end of exposure, it increased to 187 or more times of the number of initial cells in the control (ref. Table 3). This meets the validity of test: the cell growth in the control should have increased by a factor of at least 16 at 72 hours after the start of exposure.

b) Specific growth rates of section-by-section in controls

The mean coefficient of variation for section-by-section specific growth rates in the controls was 8.3% (ref. Table 7). It meets the validity of test: the mean coefficient of variation in the control must not exceed 35%.

c) Specific growth rates in replicate controls

The coefficient of variation of specific growth rates in replicate controls was 0.49% (ref. Table 7). It meets the validity of test: the coefficient of variation in controls must not exceed 7%.

14.5 Discussion

This study was conducted as a limit test in order to confirm the effect of the test item on the test organisms at upper limit concentration of test method (100 mg/L).

As a result, no adverse effect was found in the definitive study. Therefore, it was decided that the test item had no adverse effect on the test organisms at upper limit concentration of test method. The measured concentrations of the test item in the test solution were within the range of ±20% of the nominal concentration. The environmental conditions were within the suitable range; therefore, it is concluded that this study complied with the applied test method.

15. Factors that affected the reliability of test results

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

Table 5 E_rC_{50} and NOEC

E_rC_{50} (mg/L)	NOEC (mg/L)
>100	≥100

Table 6 Result of statistical analysis

Nominal concentration (mg/L)	Statistical analysis	Statistical procedure
100	n.s.	*F* test Student's *t*-test

n.s. : No significant difference

Table 7 Variation of growth rates in control

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

Control No.	Mean	Standard deviation	Coefficient of variation (%)	
A	1.76	0.162	9.2	
B	1.76	0.127	7.2	
C	1.76	0.173	9.8	8.3
D	1.75	0.106	6.1	(Mean)
E	1.77	0.126	7.1	
F	1.75	0.178	10	

< Variation of average specific growth rates in replicate controls >

	0-3day
Mean	1.76
Standard deviation	0.00869
Coefficient of variation (%)	0.49