American Chemistry Council

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May 15, 2008

TSCA Confidential Business Information Center (7407M)
EPA East - Room 6428 Attn: Section 8(e)
U.S. Environmental Protection Agency
1200 Pennsylvania Avenue, NW
Washington, DC 20460-0001
Via Hand Delivery

8EHQ-08-17149

88080000192

Attention: TSCA 8(e) Coordinator

RE: Information Acquired on the Toxicity of 4,4'-methylenedianiline (MDA) (CASRN 101-77-9) to Aquatic Invertebrates

Dear TSCA 8(e) Coordinator:

The American Chemistry Council's Diisocyanates Panel (Panel), on behalf of its members,[1] is submitting two reports to EPA pursuant to Section 8(e) of the Toxic Substances Control Act (TSCA). These reports were prepared by the Yokohama Laboratories, Mitsubishi Chemical Safety Institute Ltd, under the direction of the Japan Ministry of Environment. Translations of the reports were recently distributed to the Panel. The two reports describe studies of:

- Chronic Toxicity of 4,4'-MDA to Daphnia magna
- Acute Toxicity of 4,4'-MDA to Daphnia magna

A brief summary of the reports is provided below:

The attached aquatic toxicity studies for 4,4'-MDA (CASRN 101-77-9) describe potential new findings on acute and chronic toxicity to aquatic invertebrates. The potential acute toxicity of this substance to aquatic invertebrates (Cladocera) has been assessed previously using a 24 hr static exposure with *Moina macropopa* (EC_{50} = 2.3 mg/L), and the potential effect of chronic exposure on reproduction was evaluated with the same species over 15 days (NOEC = 0.15 mg/L). The new Japanese studies report both 24 and 48 hr acute EC_{50} values for *Daphnia magna* (according to OECD TG 202), and chronic reproduction inhibition with this same species over 21 days (OECD TG 211). Thus, the submitted studies provide information on aquatic toxicity endpoints and species which have not been investigated previously.

Contains No CBI CONTAINS NO CBI

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[1] The members of the Panel are BASF Corporation, Bayer MaterialScience, The Dow Chemical Company, and Huntsman Corporation.

While being submitted in accordance with TSCA 8(e), the Panel has made no determination as to whether a substantial risk of injury to health or the environment is actually presented by these findings.

If you have any questions, please contact me at 703-741-5609, or at chris_bryant@americanchemistry.com.

Sincerely,

Chris Bryant
Managing Director,
Chemical Products & Technology Division

Attachments:
Chronic Toxicity of 4,4'-MDA to *Daphnia magna* (Test No. A010459-3)
Acute Toxicity of 4,4'-MDA to *Daphnia magna* (Test No. A010459-2)

[illegible]

III Report 11546

Acute toxicity of 4,4'-MDA to *Daphnia magna*

Yokohama Laboratories
Mitsubishi Chemical Safety Institute Ltd
Japan

English translation by I Matsumura, for the International Isocyanate Institute, of the Japanese report "Test No: A010459-2, September 30th 2002, Acute Immobility Test against Daphnia magna by 4,4'-methylene bis benzene amine"

Issued: March 2008

Number of pages: 32

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III Report

International Isocyanate Institute Inc.

The Scientific Office, Bridgewater House, Whitworth Street, Manchester M1 6LT, UK.

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Report
III

Disclaimer

The translation herein is presented in good faith, is believed to be accurate and reliable, but may well be incomplete and/or not applicable to all conditions or situations that may be encountered.

No representation, guarantee or warranty is made as to the accuracy, reliability or completeness of this translation, or that the application or use of any of the information, analysis, methods and recommendations herein will avoid, reduce or ameliorate hazards, accidents, losses, damages or injury of any kind to persons or property. Readers are therefore cautioned to satisfy themselves as to the applicability and suitability of said information, analysis, methods and recommendations for the purposes intended prior to use.

REPORT TO MINISTRY OF THE ENVIRONMENT

FINAL REPORT

OF

ACUTE IMMOBLITY TEST AGAINST

DAPHNIA MAGNA BY

4, 4'- METHYLENE BIS BENZENE AMINE

(TEST NUMBER: A010459-2)

SEPTEMBER 30, 2002

MITSUBISHI CHEMICAL SAFETY INSTITUTE LTD.

September 30, 2002

Yokohama Laboratories

Mitsubishi Chemical Safety Institute Ltd.

Declaration

Consignor : Ministry of the Environment

Title: Acute Immobility Test against *Daphnia magna* by 4,4'- methylene bis benzene amine

Test Number: A010459-2

The experiments were enforced in accordance with the experimental program and the results were exactly described in the report.
The experiments were enforced under the GLP stated below.

Standard on the Implementation of Eco-toxic Tests (Kan-Ho-An No.242, 2001)
Notice on the Amendment of Standard on the Implementation of Eco-toxic Tests by Office of Environmental Impact Assessment Review, Environmental Impact Assessment Division, Environmental Policy Bureau, Ministry of the Environment, Japan

Signed by The Supervisor for the experiments

September 30, 2002

Yokohama Laboratories

Mitsubishi Chemical Safety Institute Ltd.

Certification of the Reliability

Consignor: Ministry of the Environment

Title: Acute immobility Test against *Daphnia magna* by 4,4'- methylene bis benzene amine

Test Number: A010459-2

The below inspection verified that the tests were enforced in accordance with the test program and the standard operation manuals, that the methods and the operation applied to the tests were exactly described in the report, and that the test results reflected the original data precisely.

Items for the Inspection	Date of the Inspection	Date of the report to the Administrator and the Manger
Test program	January 28, 2002	January 28, 2002
Test operation		
Preparation of test solution	June 4, 2002	June 4, 2002
Pouring daphnia	June 4, 2002	June 4, 2002
Observation of daphnia	June 6, 2002	June 6, 2002
Final report	September 30, 2002	September 30, 2002

Signed and sealed by two persons in charge in Reliability Certification Division

General Outline of the Test

1. Title: Acute immobility test against *Daphnia magna* by 4,4'- methylene bis benzene amine (Test Number: A010459-2)

2. Purpose of the test: The acute immobility test is to be enforced on *Daphnia magna* for 72 hours. The concentration for the 50% acute immobility (EiC50) and the maximum concentration for no effect (NOECi) are to be determined.

3. Guideline applied: OECD Guideline for chemical substances tests No. 202
Test for acute immobility and reproduction of the daphnia (1984)

4. GLP applied: Standard on the Implementation of Eco-toxic Tests (Kan-Ho-An No.242, 2001)
Notice on the Amendment of Standard on the Implementation of Eco-toxic Tests by Office of Environmental Impact Assessment Review, Environmental Impact Assessment Division, Environmental Policy Bureau, Ministry of the Environment, Japan

5. Consignor : Ministry of the Environment
2-2, Kasumigaseki1 Chome, Chiyoda-ku, Tokyo 100-8975, Japan
Person in Charge; Office of Environmental Impact Assessment Review, Environmental Impact Assessment Division, Environmental Policy Bureau

6. Consignee: Mitsubishi Chemical Safety Institute Ltd.
1-20, Shiba 2 Chome, Minato-ku, Tokyo 105-0014, Japan

7. Test facilities: Yokohama Laboratories, Mitsubishi Chemical Safety Institute Ltd.
1000, Kamoshida, Aoba-ku, Yokohama, Kanagawa 227-0033, Japan

8. Administrator: Named

9. Supervisor for the experiments: Ecological Chemistry Group XXXX (September 30, 2002)

10. Person in charge for the experiments:
Experiments: Named and sealed by four persons (September 30, 2002)
Analysis: Named and sealed (September 30, 2002)

11. Work schedule:

Start of the test	January 28,2002
Start of the experimentation	June 4, 2002
Completion of the experimentation	June 6, 2002
Completion of the test	September 30, 2002

12. Custody: The test program, the original data, the test articles, records and the final report are to be kept in the archive of Yokohama Laboratories. They are kept for 10 years after the preparation of the final report, and the further custody was to be discussed between the consignor and the consignee. However the test articles are kept for the shorter term either 10 years after the preparation of the final report or the term for stable custody without deterioration of the quality.

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Summary

Consignor: Ministry of the Environment

Title: Acute immobility test against *Daphnia magna* by 4,4'- methylene bis benzene amine

Test Number: A010459-2

Test method:

1) Guideline applied: OECD Guideline for Chemical Substance Test No. 202
Test for acute immobility and reproduction of the daphnia (1984)

2) Exposure method: Semi-static method (Exchange the whole quantity of the test solution in 24 hr)
The surface was covered with Teflon sheet

3) Living organism under test: *Daphnia magna*

4) Exposure term: 48 hours

5) Test concentration: Control, 0.200, 0.630, 2.00, 6.30, 20.0, 63.0, and 200 mg/Lit
(Set value) Common ratio: 3.2

6) Test liquid quantity: 100 ml / vessel

7) Sequence number: 4 vessels / experimental section

8) Living organism number under test: 20 pieces / experimental section (5 pieces / vessel)

9) Test temperature: 20±1 deg C

10) Lighting: Room light, lighting for 16 hrs on (lower than 800 lux) and 8 hrs off

11) Analysis: High Performance Liquid Chromatography (HPLC)

Test results:

1) Test substance concentration in the test solution
The set value was applied to the calculation of the inhibition concentration because the ratio of the observed values at the initiation of the exposure to the set value was within ±20% under the analytical result of the test solution.

2) Result after 24 hrs exposure

	(mg/L)	95% confidence limits (mg/L)
50% acute immobility ratio (EiC50)	8.08	5.23 ~ 12.8
Maximum No-Observed Effect Concentration (NOECi)	0.630	--
100% inhibition minimum concentration	200	--

2) Result after 48 hrs exposure

	(mg/L)	95% confidence limits (mg/L)
50% acute immobility ratio (EiC50)	2.47	1.27 ~ 4.40
Maximum No-Observed Effect Concentration (NOECi)	0.200	--
100% inhibition minimum concentration	200	--

1. Test Substance

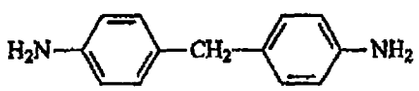
1.1 Name, Chemical structure and Physico-chemical properties

Name: 4,4' methylene bis benzene amine (Abbreviation MBBA)

Alternatives: *p,p'* methylene dianiline, 4,4' diamino diphenylmethane

CAS No.: 101-77-9

Chemical structure



Molecular formula: $C_{13}H_{14}N_2$

Molecular weight: 198.26

Boiling point*1: 232 deg C / 9 mmHg

Melting point*2: 91.5 deg C

Solubility*2: Easily soluble in alcohols, ethers, and benzene

Water solubility*2,*3: 0.1% (25 deg C) *2: 840 mg/L *3:

Specific gravity*2: 1.1 (20 deg C)

log Pow*2: 1.6 -2.5

Other*2: Discolored to dark brown by oxygen in air and light

Degradability 0% (by BOD)

*1: The Merck Index, Thirteenth Ed., 2001

*2: References by the supplier

*3: Measured by the consignee (purified water, 20 deg C, stirring for 48 hours, analyzed by HPLC)

1.2 Sample for the test

Purity*[1]: 99.6%

Lot Number*[1]: 102D2146

Supplier: Kanto Chemical Co., Inc.

Quantity supplied*[1]: 25g

Date of Supply: November 6, 2001

Appearance*[1]: Pale yellow flakes, Peculiar odor

*1: References by the supplier

1.3 Identification and the storage stability of the test substance

Before initiation of the test the test substance supplied was identified with the characteristic band by infrared absorption spectrometry.

During the test, the test substance was stored in a refrigerator for the test materials in the Laboratories (storage condition: chilled, dark under nitrogen filled). The infrared spectrum after the test was confirmed no difference compared to one at the initial. The test substance was concluded to be stable during the test term.

2. Living organism under test

1) Name: Water flea

2) Scientific Name: *Daphnia magna*

3) Supplier: National Institute for Environmental Studies

4) Date of supply: July 18, 1995

5) Sensitivity: 50% acute immobility concentration (EiC50) after 48 hrs against the standard substance (potassium bichromate, special grade chemical) was 0.88 mg/L (95% confidence limits: 0.73 ~1.07 mg/L). The value was nearly consistent with the EiC50 value at the Laboratories (since June 1998, n=7) as follows:

mean value ± standard deviation = 0.73 ± 0.19 mg/L

minimum value~ maximum value = 0.57 ~ 1.02 mg/L

Pre cultivation: Pre cultivated from March 29, 2002 to September 2, 2002

The algae grew logarithmically during the term (under similar condition at the test).

6) Developing stage: Female infant (Younger than 24 hours)

7) Condition for raising parent water flea

Water for raising: Dilution water (refer to 3.2)

Raising density: Under One piece/80 mL (25 pieces/2L)

Container for raising: 2 L glass container

Water temperature: 20±1 deg C

Dissolved oxygen concentration: Over 60% of saturated concentration

pH: 6.7 ~ 8.5

Lighting: Room light, lighting for 16 hrs on (lower than 800 lux) and 8 hrs off

Generation of dormant spawn and male: None

Feed: *Chlorella vulgaris* (mono-cellular green alga, Algae cultivation solution was centrifuged and the water layer was exchanged with the dilution water)

Feed quantity: 0.2 mg C (organin carbon content) / piece/ day

Exchange of raising water: Regularly (3 times/week)

Infants were daily eliminated as much as possible.

3. Test method

3.1 Test condition

1) Exposure method: Semi-static method (Exchange the whole quantity of the test solution in 24 hr) The surface was covered with Teflon sheet

2) Exposure term: 48 hours

3) Test liquid quantity: 100 ml / vessel

4) Sequence number: 4 vessels / experimental section

5) Living organism number under test: 20 pieces / experimental section (5 pieces / vessel)

6) Test temperature: 20±1 deg C

7) Dissolved oxygen concentration: Over 60% of saturated concentration

8) pH: No pH adjustment for the test solution

9) Lighting: Room light, lighting for 16 hrs on (lower than 800 lux) and 8 hrs off

10) Feed: None

3.2 Dilution water

Modified water Elendt M4 recommended in OECD Test Guideline No.211 "Daphnia magna Reproduction Test" The composition is shown in Addendum 1.

3.3 Test vessel, constant temperature bath etc.

1) Test vessel: 100 mL glass beaker (The surface was covered with Teflon sheet.)

2) Thermostat bath: Water bath made of PVC (TAITEC Corp, Coolnit Model CL-80F)

3) Thermometer: Yokogawa Electric Corp. Model 2455 02 No.1

4) Dissolved Oxygen meter: Electric Chemical Gauge Co., Ltd, Model DOL-10 No.2

5) pH tester: Toa Radio-wave Industries, Model HM-40V No.1

3.4 Establishment of the test concentration

The below preliminary experiments (each 2 series, 10 pieces/test section) suggested that the test substance should influence in the wide range of the concentration. The concentration for the tests was set as follows;

Test concentration: Control, 0.200, 0.630, 2.00, 6.30, 20.0, 63.0, and 200 mg/Lit

(Common ratio: 3.2)

The results of the preliminary tests

First Trial

Concentration (mg/L)	Immobility Ratio (%) after 24 hrs	after 48 hrs
Control	0	0
1.00	10	30
2.80	0	20
7.70	30	50
21.5	50	80
60.0	50	60

Static water method

Second Trial

Concentration (mg/L)	Immobility Ratio (%) after 24 hrs	after 48 hrs
Control	0	0
0.200	0	0
2.00	0	0
20.0	30	40
200	100	100

Static water method

Third Trial

Concentration (mg/L)	Immobility Ratio (%) after 24 hrs	after 48 hrs
Control	0	0
0.200	0	0
2.00	40	50
20.0	40	50

Static water method

3.5 Preparation of the test solution

The dilution water was aerated and adjusted to 20 ±1 deg C in the thermostat bath before preparation of the test solution. The concentration solution of the test substance was prepared as shown in the below table. The preparation was performed under mechanical dissolution with ultrasonic for 45 min.

	Concentration solution of the test substance
Test substance quantity	200 mg
Additive for dissolution	None
Filled up volume (by dilution water)	1000 mL
Test substance concentration	200 mg/L
Additive concentration	--

The concentration test solution of each quantity as shown in 3.4 was filled to 500 mL with the dilution water. Each 100 mL of the solution was taken in 4 test vessel per a concentration.

The control was the dilution water without the test substance.

The appearance of the test solution at the preparation was clear and colorless for the control and the concentration section of 0.200 ~ 20.0 mg/L, and clear and light brown for the concentration section of 63.0 ~ 200 mg/L,

3.6 Analysis of the test solution

Each 0.75 mL of the analysis sample was taken from each one test vessel at the initiation of the exposure and before the exchange of the water (24 hrs after starting the exposure), and added with equal quantity of acetonitrile, mixed, then analyzed by using of HPLC. The concentration of the test substance in each test solution was determined from the peak area compared to that of the standard solution. The details are described in Addendum 2.

3.7 Test operation

Water temperature, dissolved oxygen concentration and pH value of the test solution were determined, then test daphnia was added to the solution with glass pipette. The time of the addition was the initiation of the test. The raising water amount in the pipette was controlled under 1 % to the test solution amount.

The immobility number of daphnia was counted at 24 hrs and 48 hrs after exposure. After the test vessel was slowly moved, a daphnia which could not move for 15 sec. was considered immobile. (Mobility is defined as moving in the water but not the bottom. One moving on the surface often moved in the water when it was force to sink by the drop of water. It was counted as immobile when it came to float again.)

Water temperature, dissolved oxygen content and pH value were measured for each one test vessel of the whole test section at the initiation of the exposure, and before the exchange of water (at 24 hrs after initiation of the exposure).

4. Calculation of the test results

4.1 Determination of the test substance concentration for the calculation of the inhibition concentration

The test substance concentration for the calculation of immobility concentration was determined by the ratio of the observer values to the set value as the below table under the analytical results of the test solution (refer to 3.6).

Ratio of the observed value against the set value	All values are within ±20 %	Any value is over ±20 %
Concentration for the calculation (all experimental section)	Set value	Geometric mean of the observer values

4.2 Calculation of 50% acute immobility ratio (EiC50)

The 50 % immobility concentration (EiC50) was computed as the below scheme with the immobility ratio by the numbers immobile daphnia and the test number (20 pieces) at 24 and 48 hrs.

Inhibition ratio at the highest concentration section	>and= 50 %	< 50 %
Appropriateness for the calculation of EiC50	Possible	Impossible
Determination of EiC50	Accept appropriate one from the calculation results by Binomial method, Moving average method and Probit method. The 95% confidence limits was determined as far as possible.	> Highest concentration section
Record of concentration - immobility curve	Record	Record

4.3 Maximum No Observed Effect Concentration (NOEC) and 100% inhibition minimum concentration

The highest concentration section (maximum no-observed effect concentration (NOECi)) where daphnia did not have the influence of immobility was recorded at 24 and 48 hrs as far as possible. Similarly the lowest concentration section (100% inhibition minimum

concentration), which all daphnia had the influence of immobility, was recorded.

5. Results and discussion

5.1 Factors influencing to the reliability of the test results

No correspondent phenomenon was observed.

5.2 Test substance concentration in the test solution

The test substance concentration in the test solution was measured at the exposure initiation and before water exchange (24 hrs after exposure initiation). The result is shown in Table 1.

Because the ratio of the initial concentration to the set value was within ±20 % under the analysis of the test solution as 81 ~99% at the exposure initiation and 90~98% before water exchange, the set value was used for below result (50% immobility concentration, maximum no observed effect concentration and 100% inhibition minimum concentration)

5.3 50% immobility ratio (EiC50)

Table 2 and 3 shows immobility ratio and 50% immobility ratio (EiC50) at each time interval. Figure 1 shows concentration - immobility curve.

The immobility ratio of the control during the exposure term was 5% and the number of daphnia floating on surface was 0% and met the required test condition.

The below is concluded under the above results.

24 hrs EiC50: 8.08 mg/L (95% confidence limits: 5.23 ~ 12.8 mg/L)

48 hrs EiC50: 2.47 mg/L (95% confidence limits: 1.27 ~ 4.40 mg/L)

5.4 Maximum No-Observed Effect Concentration (NOECi) and 100% inhibition minimum concentration

Maximum no-observed effect concentration (NOECi) and 100% inhibition minimum concentration are shown in Table 4 and below.

24 hrs NOECi: 0.630 mg/L

24 hrs 100% inhibition minimum concentration: 200 mg/L

48 hrs NOECi: 0.200 mg/L

48 hrs 100% inhibition minimum concentration: 200 mg/L

5.5 Water temperature, dissolved oxygen concentration and pH of the test solution

Table 5 shows the test solution temperature, Table 6 does dissolved oxygen content and Table 7 does pH values.

The water temperature was maintained at 20 ±1 deg C at the whole test sections. The

dissolved oxygen content was over 60% of the saturated dissolved oxygen content (8.8 mg/L at 20.0 deg C) and met the test requirement. The pH values were in the appropriate range (6.0 ~ 8.5) for daphnia raising.

<End of the report>

Table 1 Measured Concentration of the Test Substance in Test Water
(Semi-Static Condition)

Nominal Concentration (mg/L)	Measured concentration (mg/L)				Geometric Mean During 24 Hours (mg/L)
	0 Hour New	Percent of Nominal	24 Hours Old	Percent of Nominal	
Control	< 0.005	—	< 0.005	—	—
0.200	0.162	81	0.179	90	0.170
0.680	0.572	91	0.574	91	0.573
2.00	1.85	93	1.86	93	1.85
6.30	5.93	94	5.95	94	5.94
20.0	19.3	97	19.2	96	19.2
63.0	62.1	99	61.9	98	62.0
200	193	97	193	97	193

New: freshly prepared test solutions
Old: test solutions after 24 hours exposure

Table 2 The Number of Immobilized *Daphnia magna* (Percent Immobility)

Nominal Concentration (mg/L)	Cumulative Number of Immobilized *Daphnia* (Percent Immobility)	
	24 Hours	48 Hours
Control	0 (0)	1 (5)
0.200	0 (0)	0 (0)
0.630	0 (0)	5 (25)
2.00	12 (60)	16 (80)
6.30	10 (50)	13 (65)
20.0	13 (65)	16 (80)
63.0	13 (65)	15 (75)
200	20 (100)	20 (100)

Table 3 Calculated EiC50 Values

Exposure Period (Hours)	EiC50 (mg/L)	95-Percent Confidence Limits (mg/L)	Statistical Method
24	8.08	5.23 - 12.8	Moving average
48	2.47	1.27 - 4.40	Moving average

Table 4 No Observed Effect Concentration (NOECi) and Lowest Concentration in 100% Immobility

Exposure Period (Hours)	No Observed Effect Concentration (NOECi) (mg/L)	Lowest Concentration in 100% Immobility (mg/L)
24	0.630	200
48	0.200	200

Table 5 Temperature

Nominal Concentration (mg/L)	Temperature (℃)	
	0 Hour New	24 Hours Old
Control	20.4	20.0
0.200	20.0	19.9
0.630	20.0	20.0
2.00	20.0	19.9
6.30	20.2	19.9
20.0	20.2	19.9
63.0	20.2	20.0
200	20.4	20.1

New: freshly prepared test solutions
Old: test solutions after 24 hours exposure

Table 6 Dissolved Oxygen Concentrations

Nominal Concentration (mg/L)	Dissolved Oxygen Concentration (mg/L)	
	0 Hour New	24 Hours Old
Control	8.8	8.6
0.200	8.8	8.7
0.630	8.9	8.7
2.00	8.9	8.6
6.30	9.0	8.8
20.0	8.8	8.6
63.0	8.7	8.6
200	8.4	8.5

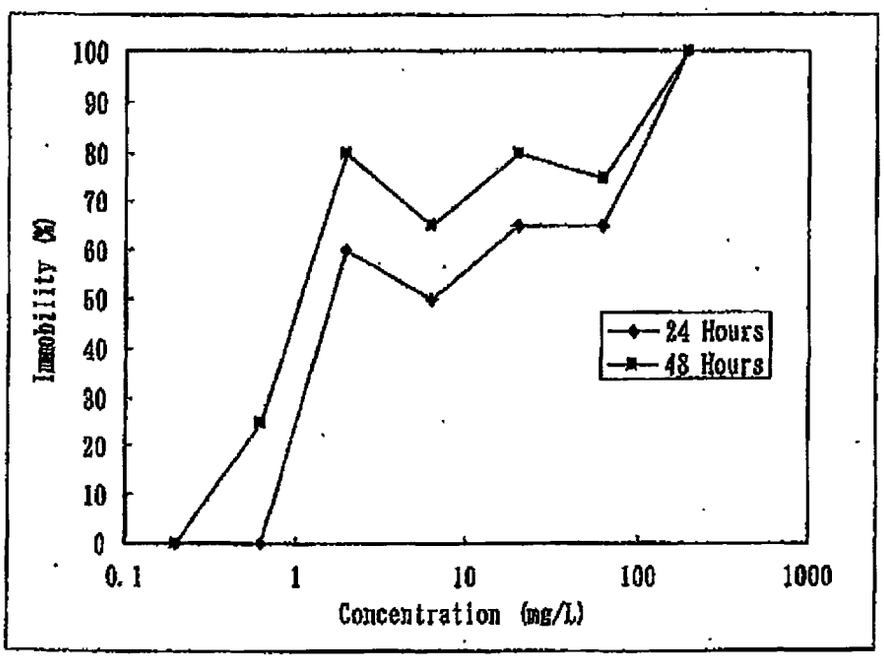
New: freshly prepared test solutions
Old: test solutions after 24 hours exposure

Table 7 pH Values

Nominal Concentration (mg/L)	pH	
	0 Hour New	24 Hours Old
Control	8.1	7.9
0.200	8.1	7.9
0.630	8.1	7.9
2.00	8.1	7.9
6.30	8.1	7.9
20.0	8.1	7.9
63.0	8.1	7.9
200	8.2	8.0

New: freshly prepared test solutions
Old: test solutions after 24 hours exposure

Figure 1 Concentration-Immobility Curve



Addendum - 1

Composition of Dilution Water

Table A-1 Blendt M4 medium recommended by OECD Guideline No. 211 used as dilution water

Macro-nutrients	Concentration (mg/L)
$CaCl_2 \cdot 2H_2O$	293.8
$MgSO_4 \cdot 7H_2O$	123.3
KCl	5.80
$NaHCO_3$	64.8
$Na_2SiO_3 \cdot 9H_2O$	10.0
$NaNO_3$	0.274
KH_2PO_4	0.143
K_2HPO_4	0.184

Trace elements	Concentration (μg/L)
H_3BO_3	2859.5
$MnCl_2 \cdot 4H_2O$	360.5
LiCl	306.0
RbCl	71.0
$SrCl_2 \cdot 6H_2O$	152.0
NaBr	16.0
$Na_2MoO_4 \cdot 2H_2O$	63.0
$CuCl_2 \cdot 2H_2O$	16.8
$ZnCl_2$	13.0
$CoCl_2 \cdot 6H_2O$	10.0
KI	3.25
Na_2SeO_3	2.19
NH_4VO_3	0.575
$Na_2EDTA \cdot 2H_2O$	2500
$FeSO_4 \cdot 7H_2O$	995.5

Vitamines	Concentration (μg/L)
Thiamine hydrochloride	75.0
Cyanocobalamine (B12)	1.00
Biotine	0.750

Addendum - 2

Analysis of Test Solution

1 Analytical method of the test solution

1) Each sample solution of 0.75 mL was taken in a vial container for the measurement and added with equal amount of acetonitrile, then mixed. The analysis was performed by HPLC. The typical chromatograms are shown in Figures A-2-2 (2), (3), (4), (5), (7), (8), (9), and (10).

2) The standard solution of 0.75 mL, prepared with acetonitrile, was taken in a vial container and added with equal amount of purified water, then mixed. The analysis was performed by HPLC. The typical chromatograms are shown in Figures A-2-2 (1) and (6).

3) The test substance concentration of each test solution was determined under the one-point calibration with the peak area of the standard solution observed in each analysis.
The linearity was confirmed with the calibration curve covering the whole test concentration range before the exposure initiation. (Refer to Chapter 3 Calibration curve)

2 Measurement condition of high performance liquid chromatography (HPLC)

(Equipment)

High performance liquid chromatograph: Hewlett Packard Model HP-1100 (No. 1)

Work station:	HP Chemistation (Windows 95)
Degasser:	Model G1322A
Pump for solution sending:	Model G1312A
Autosampler:	Model G1313A
Column oven:	Model G1316A
UV-visual spectroscopic sensor:	Model G1314A

(Condition)

Column:	Inertsil ODS-3V, 5μm, 4.6 x 150 mm (GL Sciences Inc.)
Eluent:	50 mM $NH_4H_2PO_4$ - $(NH_4)_2HPO_4$ (pH 6.7): methanol = 40 : 60
Flow rate:	1.0 mL/min.
Wave length:	250 nm
Injection volume:	10 μL
Temperature of column oven:	37 deg C

3 Calibration curve

The standard solutions of 0, 0.050 ~ 200 mg/L were prepared with acetonitrile. Specific

amount of the standard solution was diluted with purified water and measured by HPLC. The calibration curve was prepared of the concentration (mg/L) as the horizontal axis and the peak area (count) as the vertical axis. The correlative relationship of the linear regression formula was well as 1.00 for the calibration curve with least square method. The calibration curve is shown in Figure A-2-1.

4 Detection limit

The least detection peak area was set 0.1 count and the correspondent test material concentration in the test solution, 0.005 mg/L was regarded the detection limit.

5 Addition recovery test

The pre-analysis operation was the sampling of the test solution as stated in "Chapter 1 Analytical method of the test solution", the addition recovery test was not necessary. So the compensation of the recovery rate was not enforced.

Figure A-2-1 Calibration curve

No.	Concentration (mg/L)	Peak Area (count)
1	0	0
2	0.050	1.2
3	0.100	2.1
4	0.200	4.2
5	0.500	10.6
6	1.00	21.8
7	2.00	48.3
8	5.00	110.1
9	10.0	220.1
10	20.0	434.8
11	50.0	1098.8
12	100	2182.4
13	200	4262.7

Y= 21.4X
r= 1.00

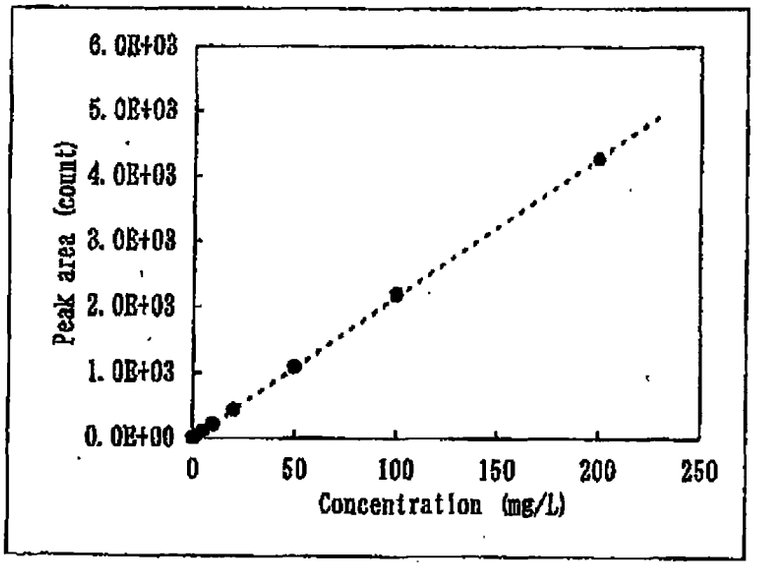
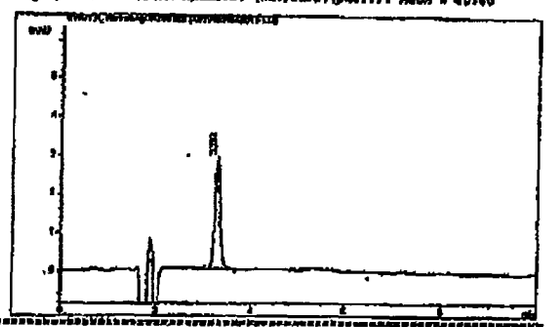


Figure A-2-2 Representative chromatograms

(1) Standard 1.00 mg/L ; 0 Hour

Injection Date :02/05/01
Test No. :A010459-2
Test Substance :MBBA
Sample Name :[illegible]
Acq Operator :[redacted]
Acq. Method :A010459.M
Analysis Method :C:\HPCHEM\1\METHODS\2001\A010459.M
Sample Info :50mM NH4H2PO4-(NH4)2HPO4(pH6.7): MeOH = 40:60

Seq Line : 11
Vial No. : 1
Inj. Vol. : 10 µl



Area Percent Report:

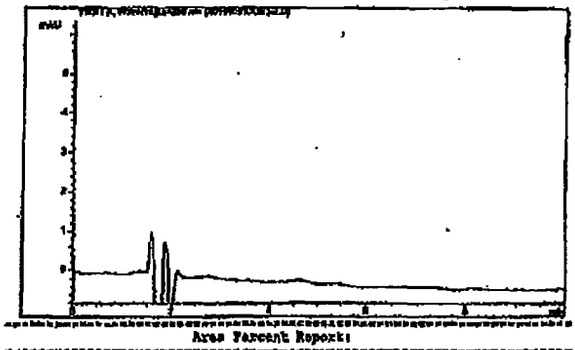
Peak #	RetTime [min]	Type	Width [min]	Area [count]	Height [count]	Area % [%]
1	3.262	MM	0.131	22.9	5.91	100
				22.9		

*** End of Report ***

(2) Control ; 0 Hour

Injection Date :02/06/04
Test No. :A010459-2
Test Substance :MBBA
Sample Name :CONT-2
Acq Operator :[redacted]
Acq. Method :A010459.M
Analysis Method :C:\HPCHEM\1\METHODS\2001\A010459.M
Sample Info :50mM NH4H2PO4-(NH4)2HPO4(pH6.7): MeOH = 40:60

Seq Line : 3
Vial No. : 3
Inj. Vol. : 10 µl



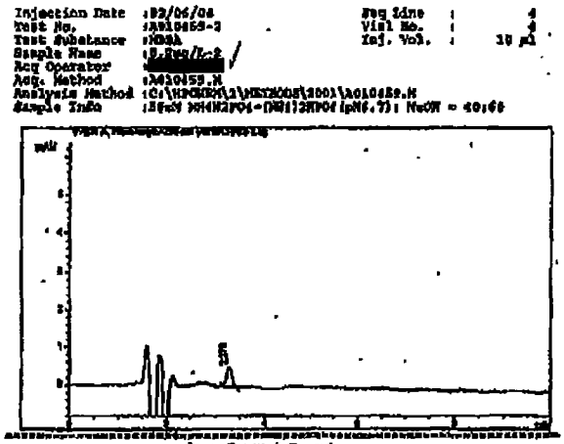
Area Percent Report:

Peak #	RetTime [min]	Type	Width [min]	Area [count]	Height [count]	Area % [%]

*** End of Report ***

Figure A-2-2 Continued

(3) 0.200 mg/L nominal ; 0 Hour

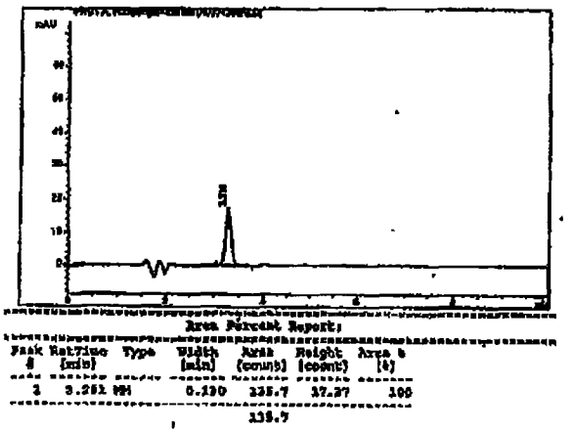


Area Percent Report:

Peak #	RetTime [min]	Type	Width [min]	Area (count)	Height (count)	Area % (%)
1	3.278	MM	0.111	3.7	0.51	100
				3.7		

*** End of Report ***

(4) 6.30 mg/L nominal ; 0 Hour



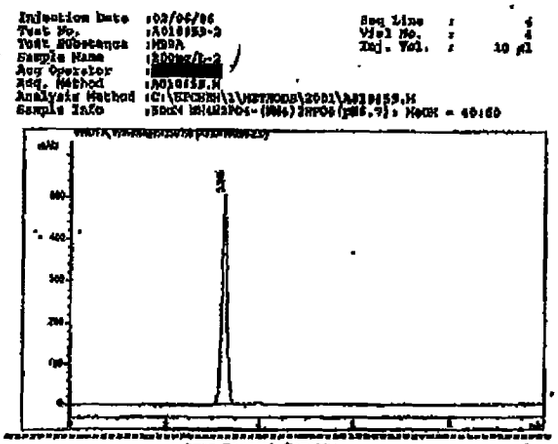
Area Percent Report:

Peak #	RetTime [min]	Type	Width [min]	Area (count)	Height (count)	Area % (%)
1	3.281	MM	0.190	335.7	37.37	100
				335.7		

*** End of Report ***

Figure A-2-2 Continued

(5) 200 mg/L nominal ; 0 Hour



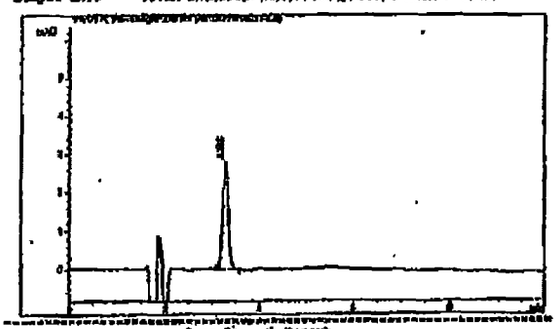
Area Percent Report:

Peak #	RetTime [min]	Type	Width [min]	Area [count]	Height [count]	Area % [%]
1	3.268	MM	0.156	4219.5	584.68	100

4219.5

*** End of Report ***

(6) Standard 1.00 mg/L ; 24 Hours



Area Percent Report:

Peak #	RetTime [min]	Type	Width [min]	Area [count]	Height [count]	Area % [%]
1	3.245	MM	0.133	22.2	2.80	100

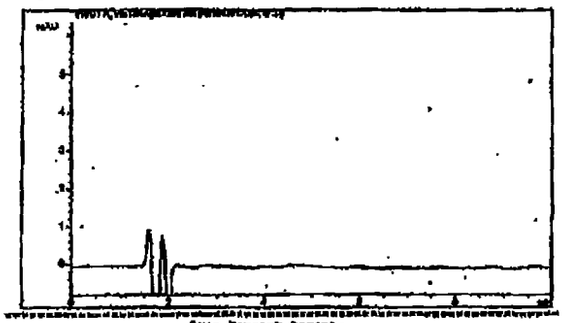
22.2

*** End of Report ***

Figure A-2-2 Continued

(7) Control ; 24 Hours

Injection Date	:02/05/85	Seq Line :	3
Test No.	:A010459-2	Vial No. :	3
Test Substance	:MBBA	Inj. Vol. :	10 µl
Sample Name	:cont-2old		
Acq Operator	:[redacted]		
Acq. Method	:A010459.M		
Analysis Method	:C:\HPCHEM\1\METHODS\2001\A010459.M		
Sample Info	:50mM NH4H2PO4-(NH4)2HPO4(pH6.7): MeOH = 40:60		



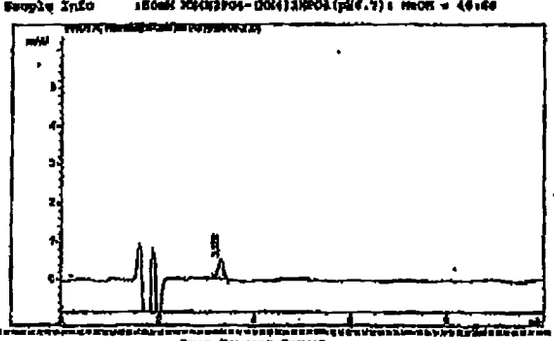
Area Percent Report:

Peak #	RetTime [min]	Type	Width [min]	Area [count]	Height [count]	Area % [%]

*** End of Report ***

(8) 0.200 mg/L nominal ; 24 Hours

Injection Date	:02/05/85	Seq Line :	4
Test No.	:A010459-2	Vial No. :	4
Test Substance	:MBBA	Inj. Vol. :	10 µl
Sample Name	:0.2mg/L-2old		
Acq Operator	:[redacted]		
Acq. Method	:A010459.M		
Analysis Method	:C:\HPCHEM\1\METHODS\2001\A010459.M		
Sample Info	:50mM NH4H2PO4-(NH4)2HPO4(pH6.7): MeOH = 40:60		



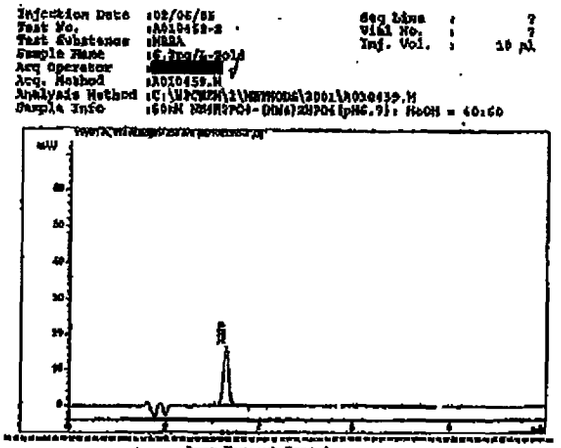
Area Percent Report:

Peak #	RetTime [min]	Type	Width [min]	Area [count]	Height [count]	Area % [%]
1	3.278	MM	0.330	4.0	0.52	100
				4.0		

*** End of Report ***

Figure A-2-2 Continued

(9) 6.30 mg/L nominal ; 24 Hours



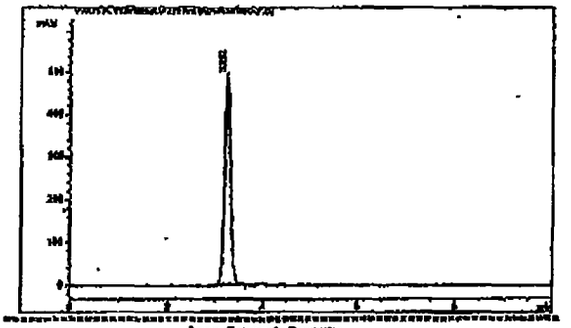
Area Percent Report:

Peak #	RetTime [min]	Type	Width [min]	Area [count]	Height [count]	Area % [%]
1	3.879	MM	0.133	331.7	16.60	100

331.7

*** End of Report ***

(10) 200 mg/L nominal ; 24 Hours



Area Percent Report:

Peak #	RetTime [min]	Type	Width [min]	Area [count]	Height [count]	Area % [%]
1	3.882	MM	0.138	4128.9	496.12	100

4128.9

*** End of Report ***

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III Report 11547

Chronic toxicity of 4,4'-MDA to *Daphnia magna*

Yokohama Laboratories
Mitsubishi Chemical Safety Institute Ltd
Japan

English translation by I Matsumura, for the International Isocyanate Institute, of the Japanese report "Test No: A010459-3, November 29th 2002, Reproduction Inhibition Test against Daphnia magna by 4,4'-methylene bis benzene amine"

Issued: March 2008

Number of pages: 41

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III Report

International Isocyanate Institute Inc.

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REPORT TO MINISTRY OF THE ENVIRONMENT

FINAL REPORT

OF

REPRODUCTION INHIBITION TEST AGAINST

DAPHNIA MAGNA BY

4, 4'- METHYLENE BIS BENZENE AMINE

(TEST NUMBER: A010459-3)

NOVEMBER 29, 2002

MITSUBISHI CHEMICAL SAFETY INSTITUTE LTD.

November 29, 2002

Yokohama Laboratories

Mitsubishi Chemical Safety Institute Ltd.

Declaration

Consignor : Ministry of the Environment

Title: Reproduction Inhibition Test against *Daphnia magna* by 4, 4'- methylene bis benzene amine

Test Number: A010459-3

The experiments were enforced in accordance with the experimental program and the results were exactly described in the report.
The experiments were enforced under the GLP stated below.

Standard on the Implementation of Eco-toxic Tests (Kan-Ho-An No.242, 2001)
Notice on the Amendment of Standard on the Implementation of Eco-toxic Tests by Office of Environmental Impact Assessment Review, Environmental Impact Assessment Division, Environmental Policy Bureau, Ministry of the Environment, Japan

Signed by The Supervisor for the experiments

November 29, 2002

Yokohama Laboratories

Mitsubishi Chemical Safety Institute Ltd.

Certification of the Reliability

Consignor: Ministry of the Environment

Title: Reproduction Inhibition Test against *Daphnia magna* by 4,4'- methylene bis benzene amine

Test Number: A010459-3

The below inspection verified that the tests were enforced in accordance with the test program and the standard operation manuals, that the methods and the operation applied to the tests were exactly described in the report, and that the test results reflected the original data precisely.

Items for the Inspection	Date of the Inspection	Date of the report to the Administrator and the Manger
Test program	August 13, 2002	August 13, 2002
Test operation		
Preparation of test solution	August 20, 2002	August 20, 2002
Pouring daphnia	August 20, 2002	August 20, 2002
Observation of daphnia	September 10, 2002	September 10, 2002
Final report	November 29, 2002	November 29, 2002

Signed and sealed by two persons in charge in Reliability Certification Division

General Outline of the Test

1. Title: Reproduction inhibition test against *Daphnia magna* by 4,4'- methylene bis benzene amine (Test Number: A010459-3)

2. Purpose of the test: The reproduction inhibition test is to be enforced on *Daphnia magna* for 21 days. The lowest no effect concentration (LOEC) and highest no effect concentration (NOEC) are to be determined. The 50% reproduction inhibition concentration (EC50) is to be determined as far as possible.

3. Guideline applied: OECD Guideline for chemical substances tests No. 211
Test for reproduction of daphnia (1998)

4. GLP applied: Standard on the Implementation of Eco-toxic Tests (Kan-Ho-An No.242, 2001)
Notice on the Amendment of Standard on the Implementation of Eco-toxic Tests by Office of Environmental Impact Assessment Review, Environmental Impact Assessment Division, Environmental Policy Bureau, Ministry of the Environment, Japan

5. Consignor : Ministry of the Environment
2-2, Kasumigaseki1 Chome, Chiyoda-ku, Tokyo 100-8975, Japan
Person in Charge; Office of Environmental Impact Assessment Review, Environmental Impact Assessment Division, Environmental Policy Bureau

6. Consignee: Mitsubishi Chemical Safety Institute Ltd.
1-20, Shiba 2 Chome, Minato-ku, Tokyo 105-0014, Japan

7. Test facilities: Yokohama Laboratories, Mitsubishi Chemical Safety Institute Ltd.
1000, Kamoshida, Aoba-ku, Yokohama, Kanagawa 227-0033, Japan

8. Administrator: Named

9. Supervisor for the experiments: Ecological Chemistry Group XXXX (November 29, 2002)

10. Person in charge for the experiments:
Experiments: Named and sealed by five persons (November 29, 2002)
Analysis: Named and sealed (November 29, 2002)

11. Work schedule:

Start of the test	August 13,2002
Start of the experimentation	August 20, 2002
Completion of the experimentation	September 10, 2002
Completion of the test	November 29, 2002

12. Custody: The test program, the original data, the test articles, records and the final report are to be kept in the archive of Yokohama Laboratories. They are kept for 10 years after the preparation of

the final report, and the further custody was to be discussed between the consignor and the consignee. However the test articles are kept for the shorter term either 10 years after the preparation of the final report or the term for stable custody without deterioration of the quality.

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Addendum 2 Analysis of the test solution

Addendum 3 Observation result of daphnia

Summary

Consignor: Ministry of the Environment

Title: Reproduction inhibition test against *Daphnia magna* by 4,4'- methylene bis benzene amine

Test Number: A010459-3

Test method:

1) Guideline applied: OECD Guideline for Chemical Substance Test No. 211
Test for reproduction of daphnia (1998)

2) Exposure method: Semi-static method (Exchange the whole quantity of the test solution daily)
The surface was covered with Teflon sheet

3) Living organism under test: *Daphnia magna*

4) Exposure term: 21 days

5) Test concentration: Control, 0.00600, 0.0190, 0.0600, 0.190, and 0.600 mg/Lit
(Set value) Common ratio: 3.2

6) Test liquid quantity: 100 ml / vessel

7) Sequence number: 10 vessels / experimental section

8) Living organism number under test: 10 pieces / experimental section (1 piece / vessel)

9) Test temperature: 20±1 deg C

10) Lighting: Room light, lighting for 16 hrs on (lower than 800 lux) and 8 hrs off

11) Analysis: High Performance Liquid Chromatography (HPLC)

Test results:

1) Test substance concentration in the test solution
The time-weighted average of the measured values was applied to the calculation of the result because the ratio of the observed values at the initiation of the exposure to the set value included values over ±20% under the analytical result of the test solution.

2) Result after 21 days exposure

	(mg/L)	95% confidence limits (mg/L)
Median lethal concentration (LC50) of parental daphnia	0.0291	0.0182 ~ 0.0599
50% reproduction inhibition ratio (EC50)	0.0149	0.00963 ~ 0.0176
50% no-observed effect concentration (NOEC)	0.00525	---
Lowest observed effect concentration (LOEC)	0.0182	---

2) Result after 48 hrs exposure

	(mg/L)	95% confidence limits (mg/L)
50% reproduction inhibition ratio (EiC50)	2.47	1.27 ~ 4.40
Highest No-Observed Effect Concentration (NOECi)	0.200	--
100% inhibition minimum concentration	200	--

1. Test Substance

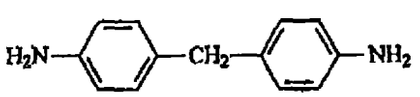
1.1 Name, Chemical structure and Physico-chemical properties

Name: 4,4' methylene bis benzene amine (Abbreviation MBBA)

Alternatives: *p,p'* methylene dianiline, 4,4' diamino diphenylmethane

CAS No.: 101-77-9

Chemical structure



Molecular formula: $C_{13}H_{14}N_2$

Molecular weight: 198.26

Boiling point*1: 232 deg C / 9 mmHg

Melting point*2: 91.5 deg C

Solubility*2: Easily soluble in alcohols, ethers, and benzene

Water solubility*2,*3: 0.1% (25 deg C) *2: 840 mg/L *3:

Specific gravity*2: 1.1 (20 deg C)

log Pow*2: 1.6 -2.5

Other*2: Discolored to dark brown by oxygen in air and light

Degradability 0% (by BOD)

*1: The Merck Index, Thirteenth Ed., 2001

*2: References by the supplier

*3: Measured by the consignee (purified water, 20 deg C, stirring for 48 hours, analyzed by HPLC)

1.2 Sample for the test

Purity*[1]: 99.6%

Lot Number*[1]: 102D2146

Supplier: Kanto Chemical Co., Inc.

Quantity supplied*[1]: 25g

Date of Supply: November 6, 2001

Appearance*[1]: Pale yellow flakes, Peculiar odor

*1: References by the supplier

1.3 Identification and the storage stability of the test substance

Before initiation of the test the test substance supplied was identified with the characteristic band by infrared absorption spectrometry.

During the test, the test substance was stored in a refrigerator for the test materials in the Laboratories (storage condition: chilled, dark under nitrogen filled). The infrared spectrum after the test was confirmed no difference compared to one at the initial. The test substance was concluded to be stable during the test term.

2. Living organism under test

1) Name: Water flea

2) Scientific Name: *Daphnia magna*

3) Supplier: National Institute for Environmental Studies

4) Date of supply: July 18, 1995

5) Sensitivity: 50% reproduction inhibition concentration (EiC50) after 48 hrs against the standard substance (potassium bichromate, special grade chemical) was 0.89 mg/L (95% confidence limits: 0.75 ~1.06 mg/L). The value was nearly consistent with the EiC50 value at the Laboratories (since June 1998, n=8) as follows:

mean value ± standard deviation = 0.75 ± 0.18 mg/L

minimum value~ maximum value = 0.57 ~ 1.02 mg/L

6) Developing stage: Female infant (younger than 24 hours)

7) Condition for raising parental daphnia

Water for raising: Dilution water (refer to 3.2)

Raising density: Under One piece/80 mL (25 pieces/2L)

Container for raising: 2 L glass container

Water temperature: 20±1 deg C

Dissolved oxygen concentration: Over 60% of saturated concentration

pH: 6.7 ~ 8.5

Lighting: Room light, lighting for 16 hrs on (lower than 800 lux) and 8 hrs off

Raising term: July 30, 2002 ~ August 20, 2002

Mortality of parental at 2 weeks before the exposure initiation: 0%

Generation of dormant spawn and male: None

Feed: *Chlorella vulgaris* (mono-cellular green alga, Algae cultivation solution was centrifuged and the water layer was exchanged with the dilution water)

Feed quantity: 0.2 mg C (organic carbon content) / piece/ day

Exchange of raising water: Regularly (3 times/week)

Infants were daily removed as much as possible.

3. Test method

3.1 Test condition

1) Exposure method: Semi-static method (Exchange the whole quantity of the test solution daily) The surface was covered with Teflon sheet

2) Exposure term: 21 days

3) Test liquid quantity: 80 ml / vessel

4) Sequence number: 10 vessels / experimental section

5) Living organism number under test: 10 pieces / experimental section (one piece / vessel)

6) Test temperature: 20 ±1 deg C

7) Dissolved oxygen concentration: Under 3 mg/L, preferably over 60% of saturated concentration

8) pH: 6 ~ 9 (however except the case due to the test substance)

9) Hardness: about 250 mg/L (correspondent to $CaCO_3$)

9) Lighting: Room light, lighting for 16 hrs on (lower than 800 lux) and 8 hrs off

10) Feed: *Chlorella vulgaris* (mono-cellular green alga, Algae cultivation solution was centrifuged and the water layer was exchanged with the dilution water)
Feed quantity: 0.15 mg C (organic carbon content) / piece/ day

3.2 Dilution water

Modified water Elendt M4 recommended in OECD Test Guideline No.211 "*Daphnia magna* Reproduction Test " The composition is shown in Addendum 1.

3.3 Test vessel, constant temperature bath etc.

1) Test vessel: 100 mL glass beaker (The surface was covered with Teflon sheet.)

2) Thermostat bath: Water bath made of PVC (TAITEC Corp, Coolnit Model CL-80F)

3) Thermometer: Yokogawa Electric Corp. Model 2455 02 No.1

4) Dissolved Oxygen meter: Electric Chemical Gauge Co., Ltd, Model DOL-10 No.2

5) pH tester: Toa Radio-Wave Industries, Model HM-40V No.1

6) Hardness measurement kit: Kyoritsu Chemical-Check Lab. Corp. Model WAD-TH

3.4 Establishment of the test concentration

Usually the test concentration is determined with the highest concentration section at about 48 hrs EiC50 value (set concentration: 2.47 mg/L) in acute immobility test against *daphnia magna*, but in this experiments the test concentration was determined as below because the inhibition of 25% was observed at the acute immobility test and the influence of aniline derivatives on the reproduction is considered acute than expected under our former experience.

Control, 0.00600, 0.0190, 0.0600, 0.190 and 0.0600 mg/Lit (Common ratio: 3.2)

3.5 Preparation of the test solution

The dilution water was aerated and adjusted to 20 ±1 deg C in the thermostat bath before preparation of the test solution. The stock solution of the test substance was prepared as shown in the below table. The preparation was performed under mechanical dissolution with ultrasonic for 30 min.

	Stock solution of the test substance
Test substance quantity	50 mg
Additive for dissolution	None
Filled up volume (by dilution water)	500 mL
Test substance concentration	100 mg/L
Additive concentration	--

The stock solution of each quantity as shown in 3.4 was filled to 1.0 L with dilution water. Each 80 mL of the solution was taken in 10 test vessel per a concentration.

The control was the dilution water without the test substance.

The appearance of the test solution at the preparation was clear and colorless for the whole experiment sections and the control

3.6 Analysis of the test solution

The analysis sample was taken from each one test vessel for the whole experiment section three times during the exposure and before/after water exchange and analyzed as the below procedure. In case of the control and 0.00600 mg/L concentration section the test solution of 50 mL was run through Sep-pack C18, which was conditioned with acetonitrile and purified water, then eluted by acetonitrile and filled up to 5 mL (10 times concentrate). The equal quantity of purified water was added to the sample solution and served to HPLC. In case of 0.0190 ~ 0.600 mg/L section, the sample solution was added with equal quantity of acetonitrile, mixed and analyzed by HPLC. The test substance concentration was determined

with the ratio the peak area to that of the standard solution. The details were described in the Addendum 2.

3.7 Test operation

Water temperature, dissolved oxygen concentration, pH value and hardness of the test solution were determined, then test daphnia was added to the solution with glass pipette. The time of the addition was the initiation of the test. The raising water amount in the pipette was controlled less than 1 % to the test solution amount. Daphnia was transferred to the new test solution at every water exchange and raised for 21 days. It was daily fed of constant amount. (refer to 3.1) Daphnia was observed with water quality measurement as follows;

1) Observation of daphnia

Parental daphnia: Daily observation and record on live/death, mobility, abnormal appearance. Any dead piece was removed.

Born infant daphnia: Born infant was daily counted of numbers and removed. Dead infant, abortion spawn, and quiescence spawn were observed and recorded. The date of the first brood production was recorded (Day to first brood production)

2) Water quality

Water temperature, dissolved oxygen concentration, pH value and hardness were measured four times during the exposure at before/after water exchange for each test vessel of the whole experimental section

4. Calculation of the test results

4.1 Determination of the test substance concentration for the calculation of the inhibition concentration

The test substance concentration for the calculation of immobility concentration was determined by the ratio of the observed values to the set value as the below table under the analytical results of the test solution (refer to 3.6).

Ratio of the observed value against the set value	All values are within ±20 %	Any value is over ±20 %
Concentration for the calculation (all experimental sections)	Set value	Time-weighted geometric mean of the observed values

4.2 Calculation of median lethal inhibition ratio (LC50)

The median lethal inhibition ratio (LC50) was computed as the below scheme with mortality by the death numbers of parental daphnia and the test number (10 pieces).

Mortality at the highest concentration section	>and= 50 %	< 50 %
Appropriateness for the calculation of LC50	Possible	Impossible
Determination of LC50	Accept appropriate one from the calculation results by Binomial method, Moving average method and Probit method. The 95% confidence limits was determined as far as possible.	> Highest concentration section
Record of death number variation with time curve	Record	Record

4.3 Calculation of 50% reproduction inhibition concentration (EC50)

The 50% reproduction inhibition concentration (EC50) for 21 days and 95% confidence limits was computed as far as possible by mean cumulative brood production number (survival infant) per one survival parent at the control and each concentration section and made regression analysis (Logi method) with Logistic curve. The variance with time of mean cumulative brood production number for each experimental section was recorded as a graph.

4.4 Highest no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC)*

Cumulative brood production number per one survival parent for each test vessel after 21 days was counted and the significant difference between each concentration section and the control by statistic method,** then maximum no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) were determined.

* Maximum no observed effect concentration (NOEC): Highest concentration with no significant reproduction inhibition observed in comparison to the control

Lowest observed effect concentration (LOEC): Lowest concentration with significant reproduction inhibition observed in comparison to the control

** Statistic method: Bartlett's test for equality variance, One-way analysis of variance (ANOVA) and Dunnett's multiple comparison test

Statistics analysis was performed with Yukms Software Starlight "#4 Multigroup comparison", Yukms Corp. Japan.

5. Results and discussion

5.1 Factors influencing to the reliability of the test results

No correspondent phenomenon was observed.

5.2 Test substance concentration in the test solution

The test substance concentration in the test solution was measured three times during the exposure before/after water exchange. The result is shown in Table 1.

Because the ratio of the initial concentration to the set value includes values over ±20 % under the analysis of the test solution as 81 ~99%, the time -weighted average value is used for below result (median lethal concentration, 50% reproduction inhibition concentration, highest no observed effect concentration and lowest observed effect concentration)

5.3 Result of observation on daphnia

Mortal number and mortality of parental daphnia

Cumulative number of dead parental daphnia and mortality in each experimental section during the exposure are shown in Tables 2-1, 2-2 and Figure 1

The mortality of parental daphnia in the control was 0% at the end of the exposure and met the test requirement, which is under 20%. The mortality at the highest concentration section was 100% at the end of the exposure.

First brood production day

Table 3 shows the first brood production day in each experimental section.

The first brood production day for the control was 8th day after exposure initiation and was considered in the normal range. In the highest concentration section all parental daphnia died before the first brood production.

Quiescence spawn etc

No quiescence spawn was observed during the exposure for all experimental section.

5.4 Median lethal concentration (LC50) of parental daphnia

Median lethal concentration (LC50) of parental daphnia at 21days exposure is shown in Table 5 and below.

LC50 at 21days: 0.0291 mg/L (95% confidence limits: 0.0182 ~ 0.0599 mg/L)

5.5 50% reproduction inhibition ratio (EC50)

The 50% reproduction inhibition ratio (EC50) at 21 days exposure is shown in Table 6 and below.

EC50 at 21days: 0.0149 mg/L (95% confidence limits: 0.00963 ~ 0.0176 mg/L)

5.6 Highest no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) influencing on cumulative reproduction number

Highest no observed effect concentration (NOEC) and lowest observed effect

concentration (LOEC) influencing on cumulative reproduction number per a parental daphnia at 21 days exposure are shown in Table 7 and below.

NOEC at 21 days: 0.00525 mg/L

LOEC at 21 days: 0.0182 mg/L

5.7 Water temperature, dissolved oxygen concentration, and pH value of test solution

Table 8 shows the test solution temperature during the exposure, Table 9 does dissolved oxygen concentration, Table 10 does pH values and Table 11 does hardness.

The water temperature was maintained at 20 ±1 deg C at the whole experimental sections. The dissolved oxygen content was over 60% of the saturated dissolved oxygen content (8.8 mg/L at 20.0 deg C) and met the test requirement. The pH values were in the appropriate range (6.0 ~ 9.0 in the variance of 1.5) for daphnia raising. The hardness is considered in the appropriate range (around 250 mg/L)

<End of the report>

Table 1-1 Measured Concentration of the Test Substance in Test Water during a 21-day Exposure Period
(*Daphnia* Reproduction Inhibition Test under the Semi-Static Test Condition)

Nominal Concentration (mg/L)	Date→	Measured Concentration (mg/L) 0 New	1 Old	7 New	8 Old	14 New	15 Old	TWM*1 (mg/L)	% of Nominal
Control		<0.00005	<0.00005	<0.00005	<0.00005	<0.00005	<0.00005	–	–
0.00600		0.00548	0.00476	0.00598	0.00534	0.00566	0.00436	0.00525	88
0.0190		0.0186	0.0186	0.0195	0.0176	0.0181	0.0171	0.0182	96
0.0600		0.0614	0.0571	0.0620	0.0595	*	*	0.0599	100
0.190		0.188	0.177	*	*	*	*	0.182	96
0.600		0.596	0.578	*	*	*	*	0.587	98

Table 1-2 Measured Concentration as a Percentage of Nominal

Nominal Concentration (mg/L)	Date→	Measured Concentration as a Percentage of Nominal 0 New	1 Old	7 New	8 Old	14 New	15 Old
0.00600		91	79	100	89	94	73
0.0190		98	98	103	93	95	90
0.0600		102	95	103	99	*	*
0.190		99	93	*	*	*	*
0.600		99	96	*	*	*	*

New: Freshly prepared test solution
Old: Old test solution before renewal
*1: Time-weighted mean measured concentration during 21 days.
*: No measurement was made because all parental *Daphnia* were dead.

	Concentration (mg/L) Min.		Max.	% of Nominal Min.		Max.
New	0.00548	~	0.596	91	~	103
Old	0.00436	~	0.578	73	~	99

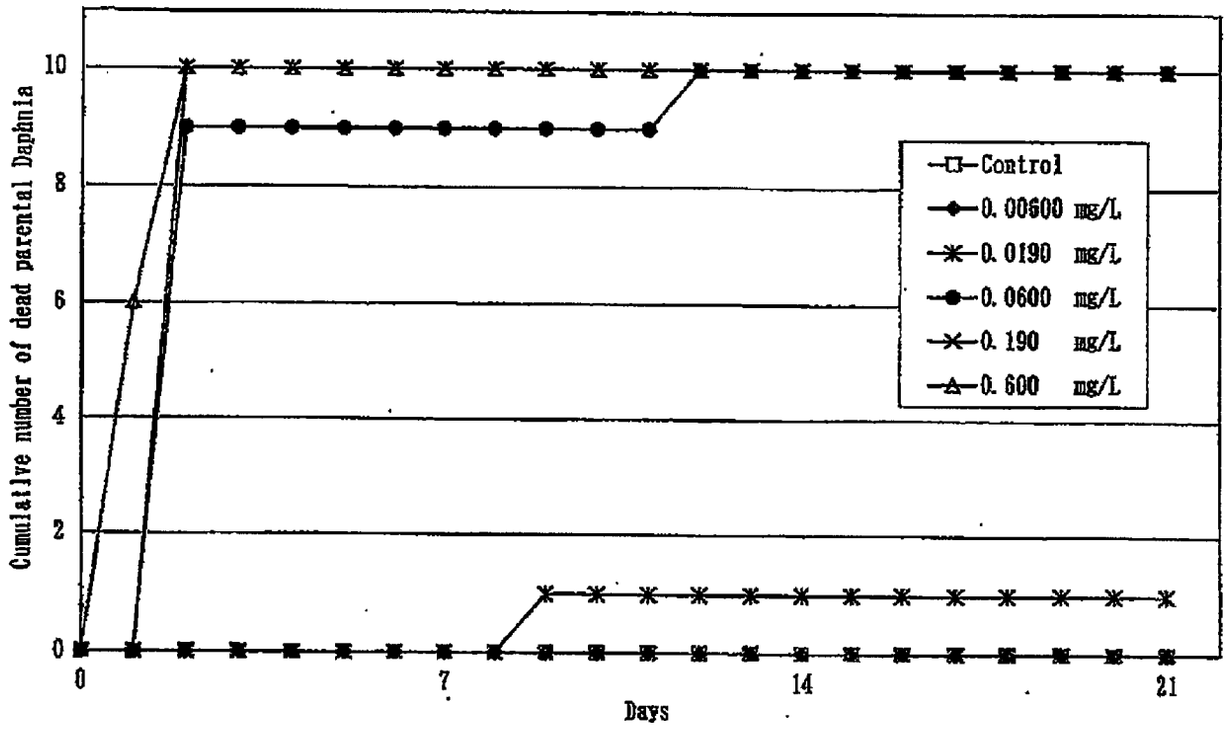
Table 2-1 Cumulative Number of Dead Parental *Daphnia*

Nominal conc.	Days																				
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.00600 mg/L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.0190 mg/L	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
0.0600 mg/L	0	0	9	9	9	9	9	9	9	9	9	9	10	10	10	10	10	10	10	10	10	10
0.190 mg/L	0	0	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
0.600 mg/L	0	6	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10

Table 2-2 Mortality (%) of Parental *Daphnia*

Nominal conc.	Days					
	1	2	4	7	14	21
Control	0	0	0	0	0	0
0.00600 mg/L	0	0	0	0	0	0
0.0190 mg/L	0	0	0	0	10	10
0.0600 mg/L	0	90	90	90	100	100
0.190 mg/L	0	100	100	100	100	100
0.600 mg/L	60	100	100	100	100	100

Figure 1 Cumulative Number of Dead Parental *Daphnia*



Values in legend are given in the nominal concentration.

Table 3 Time (Days) to First Brood Production

Vessel No.-	Nominal Concentration, mg/L (Measured Concentration[*1], mg/L)					
	Control	0.00800 (0.00525)	0.0190 (0.0182)	0.0800 (0.0599)	0.190 (0.182)	0.600 (0.587)
1	8	8	8	-	-	-
2	8	8	15	-	-	-
3	8	8	8	-	-	-
4	8	8	9	-	-	-
5	8	8	8	-	-	-
6	8	8	15	-	-	-
7	8	8	8	-	-	-
8	8	8	8	-	-	-
9	8	8	8	-	-	-
10	8	8	8	-	-	-
Min	8	8	8	-	-	-
Max	8	8	15	-	-	-

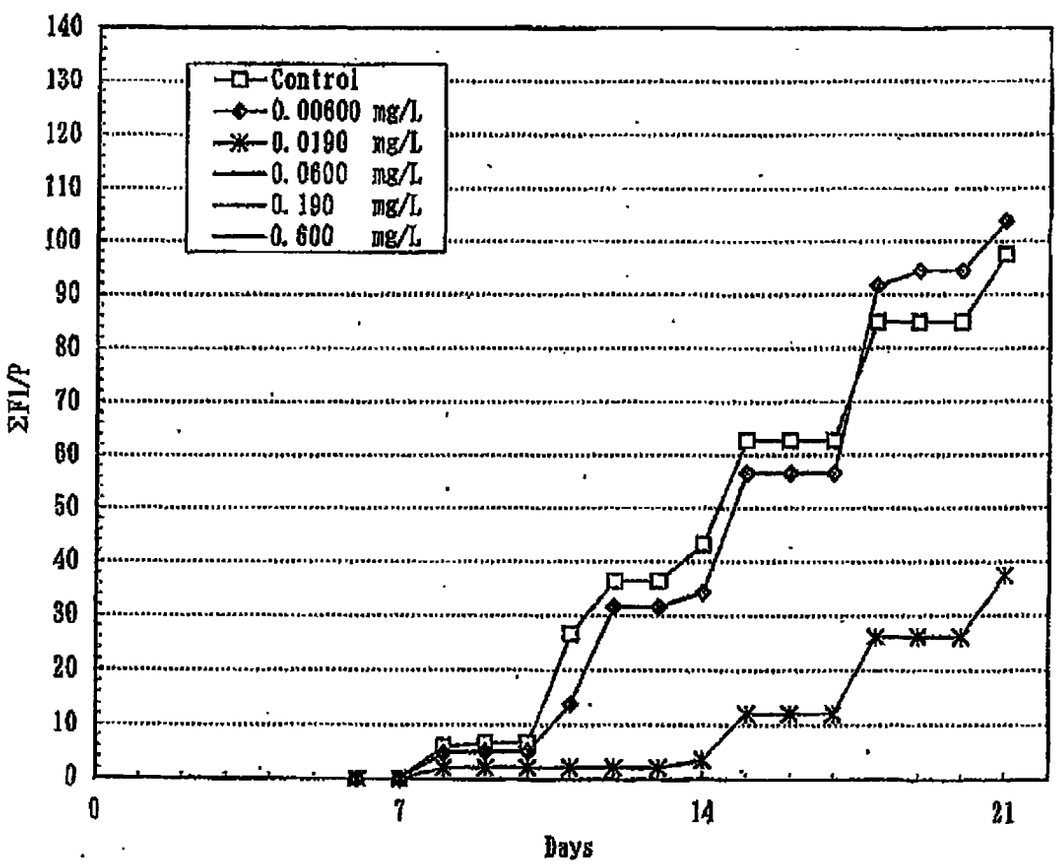
*1: Time-weighted mean measured concentration.
-: The parental *Daphnia* was dead before first brood production.

Table 4 Mean Cumulative Number of Juveniles Produced per Adult Alive for 21 Days (ΣF1/P)

Nominal Conc.	Days															
	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Control	0.0	0.0	5.9	6.6	6.6	26.4	36.4	36.4	43.2	62.7	62.7	62.7	84.9	84.9	84.9	97.5
0.00600 mg/L	0.0	0.0	4.7	5.0	5.0	13.8	31.6	31.6	34.3	56.6	56.6	56.6	91.7	94.4	94.4	103.8
0.0190 mg/L	0.0	0.0	2.0	2.1	2.1	2.1	2.1	2.1	3.6	12.1	12.1	12.1	26.0	26.0	26.0	37.4
0.0600 mg/L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.190 mg/L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.600 mg/L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

-: All parental *Daphnia* were dead during a 21-day testing period.

Figure 2 Time Course of ΣF1/P for Each Concentration Level



Values in legend are given in the nominal concentration.

Table 5 Calculated LC50 Values for Parental *Daphnia*

Exposure Period (day)	LC50*1 (mg/L)	95% Confidence Limits (mg/L)	Statistical method
21	0.0291	0.0182 — 0.0599	Binomial

*1: Based on the Time-weighted mean measured concentration

Table 6 Calculated EC50 Values for Inhibition of Reproduction

Exposure Period (day)	EC50*1 (mg/L)	95% Confidence limits (mg/L)	Statistical method
21	0.0149	0.00963 — 0.0176	Logit

*1: Based on the Time-weighted mean measured concentration

Table 7 Cumulative Number of Juveniles Produced per Adult Alive for 21 Days in Each Test Vessel and Results of Statistical Comparison of the Mean Values (by Dunnett's Multicomparison Test)

Vessel No.	Nominal Concentration, ng/L (Measured Concentration*1, ng/L)					
	Control	0.00600 (0.00525)	0.0190 (0.0182)	0.0600 (0.0599)	0.190 (0.182)	0.600 (0.587)
1	111	96	D	D	D	D
2	109	114	50	D	D	D
3	81	136	27	D	D	D
4	86	93	21	D	D	D
5	86	95	37	D	D	D
6	97	105	18	D	D	D
7	81	111	43	D	D	D
8	106	128	33	D	D	D
9	108	82	49	D	D	D
10	110	78	59	D	D	D
Mean	97.5	103.8	37.4	0.0	0.0	0.0
S.D.	12.7	18.8	14.0			
Inhibition rate (%)		-6.5	61.6	100.0	100.0	100.0
Significant difference		-	**	++	++	++

*1: Time-weighted mean measured concentration.
D: Were not included for calculation because the parental *Daphnia* was dead during a 21-day testing period.
-: Indicates no significant difference.
*: Indicates a significant difference (α=0.05) from the control. (There was no sign in this test.)
**: Indicates a significant difference (α=0.01) from the control.
++: Statistical comparison test could not be performed for this concentration because adult alive after 21 days was none. However, we concluded that this concentration level showed adverse effect on *Daphnia* reproduction.

No Observed Effect Concentration (NOEC): 0.00525 ng/L
Lowest Observed Effect Concentration (LOEC): 0.0182 ng/L

Table 8 Temperature during a 21-day Period under the Semi-Static Condition

Nominal Concentration (mg/L)	Date→	Temperature (℃) 0 new	1 old	7 new	8 old	14 new	15 old	20 new	21 old	Min.	Max.
Control		20.3	20.2	20.2	20.2	20.4	20.2	20.2	20.3	20.2	20.4
0.00600		20.1	20.2	20.2	20.3	20.4	20.3	20.2	20.3	20.1	20.4
0.0190		20.4	20.2	20.2	20.3	20.4	20.3	20.3	20.3	20.2	20.4
0.0600		20.1	20.2	20.2	20.3	–	–	–	–	20.1	20.3
0.190		20.1	20.3	–	–	–	–	–	–	20.1	20.3
0.600		20.2	20.3	–	–	–	–	–	–	20.2	20.3
									Total	20.1	20.4

new: freshly prepared test-solution. old: old test solution before renewal
–: No measurement was made because all parental *Daphnia* were dead.

Table 9 Dissolved Oxygen Concentration (D.O.) during a 21-day Period under the Semi-Static Condition

Nominal Concentration (mg/L)	Date→	D.O. (mg/L) 0 new	1 old	7 new	8 old	14 new	15 old	20 new	21 old	Min.	Max.
Control		8.8	8.6	8.8	7.9	8.8	8.5	8.8	7.2	7.2	8.8
0.00600		8.8	8.6	8.7	7.9	8.7	8.1	8.7	7.2	7.2	8.8
0.0190		8.8	8.7	8.7	8.1	8.7	8.2	8.7	7.2	7.2	8.8
0.0600		8.8	8.7	8.7	8.2	–	–	–	–	8.2	8.8
0.190		8.8	8.6	–	–	–	–	–	–	8.6	8.8
0.600		8.8	8.6	–	–	–	–	–	–	8.6	8.8
									Total	7.2	8.8

new: freshly prepared test solution. old: old test solution before renewal
–: No measurement was made because all parental *Daphnia* were dead.

Table 10 pH during a 21-day Period under the Semi-Static Condition

Nominal Concentration (mg/L)	Date→	pH 0 new	1 old	7 new	8 old	14 new	15 old	20 new	21 old		Min.	Max.
Control		8.2	8.1	8.2	7.7	8.3	7.7	8.1	7.3		7.3	8.3
0.00600		8.2	8.1	8.3	7.7	8.2	7.6	8.1	7.3		7.3	8.3
0.0190		8.2	8.1	8.2	7.8	8.3	7.7	8.1	7.3		7.3	8.3
0.0600		8.2	8.1	8.2	7.9	–	–	–	–		7.9	8.2
0.190		8.2	8.1	–	–	–	–	–	–		8.1	8.2
0.600		8.3	8.1	–	–	–	–	–	–		8.1	8.3
										Total	7.3	8.3

new: freshly prepared test solution, old: old test solution before renewal
–: No measurement was made because all parental *Daphnia* were dead.

Table 11 Total Hardness (as $CaCO_3$) during a 21-day Period under the Semi-Static Condition

Nominal Concentration (mg/L)	Date→	Total hardness (as CaCO3, mg/L) 0 new	1 old	7 new	8 old	14 new	15 old	20 new	21 old		Min.	Max.
Control		255	260	255	255	255	260	250	250		250	260
0.00600		250	260	260	260	260	260	250	255		250	260
0.0190		250	250	255	250	250	260	250	255		250	260
0.0600		255	250	255	250	–	–	–	–		250	255
0.190		250	250	–	–	–	–	–	–		250	250
0.600		250	255	–	–	–	–	–	–		250	255
										Total	250	260

new: freshly prepared test solution, old: old test solution before renewal
–: No measurement was made because all parental *Daphnia* were dead.

Addendum - 1

Composition of Dilution Water

Table A-1 Elendt M4 medium recommended by OECD Guideline No. 211 used as dilution water

Macro nutrients	Concentration (mg/L)
$CaCl_2 \cdot 2H_2O$	293.8
$MgSO_4 \cdot 7H_2O$	123.3
KCl	5.80
$NaHCO_3$	64.8
$Na_2SiO_3 \cdot 9H_2O$	10.0
$NaNO_3$	0.274
KH_2PO_4	0.143
K_2HPO_4	0.184

Trace elements	Concentration (μg/L)
H_3BO_3	2859.5
$MnCl_2 \cdot 4H_2O$	360.6
LiCl	306.0
RbCl	71.0
$SrCl_2 \cdot 6H_2O$	152.0
NaBr	16.0
$Na_2MoO_4 \cdot 2H_2O$	63.0
$CuCl_2 \cdot 2H_2O$	16.8
$ZnCl_2$	13.0
$CoCl_2 \cdot 6H_2O$	10.0
KI	3.25
Na_2SeO_3	2.19
NH_4VO_3	0.575
$Na_2EDTA \cdot 2H_2O$	2500
$FeSO_4 \cdot 7H_2O$	995.5

Vitamines	Concentration (μg/L)
Thiamine hydrochloride	75.0
Cyanocobalamine (B12)	1.00
Biotine	0.750

Addendum - 2

Analysis of Test Solution

1 Analytical method of the test solution

1) The analysis sample was taken from each one test vessel for the whole experiment section three times during the exposure and before/after water exchange and analyzed as the below procedure. In case of the control and 0.00600 mg/L concentration section the test solution of 50 mL was run through SEP-PAK C18, which was conditioned with acetonitrile and purified water, then eluted by acetonitrile and filled up to 5 mL (10 times concentrate). The equal quantity of purified water was added to the sample solution and served to HPLC.
In case of 0.0190 ~ 0.600 mg/L section, the sample solution was added with equal quantity of acetonitrile, mixed and analyzed by HPLC. The test substance concentration was determined with the ratio the peak area to that of the standard solution. The details were described in the Addendum 2. Chromatograms are shown in Figures A 2-2 (2), (3), (5) and (6).

50 mL of test solution
|
|
Eluted through SEP-PAK C18 (Conditioned with each 5 mL of acetonitrile and purified water in advance)
| Sucked by an aspirator and flew with water
|
|
Flew with 2 mL of purified water and wasted
|
|
Eluted with 5 mL of acetonitrile and filled up to 5 mL
|
|
750 µL of test solution + 750 µL of purified water
|
|
Measurement of HPLC (100 µL)

Flew
|
|
Wasted

2) The standard solution of 0.75 mL, prepared with acetonitrile was taken in a vial container and added with equal amount of purified water, then mixed. The analysis was made with HPLC. The chromatograms are shown in Figures A-2-2 (1) and (4).

3) The test substance concentration of each test solution was determined under the one-point calibration with the peak area of the standard solution observed in each analysis.

The linearity was confirmed with the calibration curve covering the whole test concentration range before the exposure initiation. (Refer to Chapter 3 Calibration curve)

2 Measurement condition of high performance liquid chromatography (HPLC)

(Equipment)

High performance liquid chromatograph: Hewlett Packard Model HP-1100 (No. 1)

Work station:	HP Chemistation (Windows 95)
Degasser:	Type G1322A
Pump for solution sending:	Model G1312A
Autosampler:	Model G1313A
Column oven:	Model G1316A
UV-visual spectroscopic sensor:	Model G1314A

(Condition)

Column:	Inertsil ODS-3V, 5μm, 4.6 x 150 mm (GL Science Inc.)
Eluent:	50 mM $NH_4H_2PO_4$ - $(NH_4)_2HPO_4$ (pH 6.7): methanol = 40 : 60
Flow rate:	1.0 mL/min.
Wave length:	250 nm
Injection volume:	10 μL
Temperature of column oven:	37 deg C

3 Calibration curve

The standard solutions of 0, 0.050 ~ 200 mg/L were prepared with acetonitrile. Specific amount of the standard solution was diluted with purified water and measured by HPLC. The calibration curve was prepared of the concentration (mg/L) as the horizontal axis and peak are (count) as the vertical axis. The correlative relationship of the linear regression formula was well as 1.00 for the calibration curve with least square method. The calibration curve was shown in Figure A-2-1.

4 Detection limit

The least detection peak area was set 0.1 count and the correspondent test material concentration in the test solution, 0.00005 mg/L was regarded the detection limit.

5 Addition recovery test

The test solution of 0.006 mg/L of test substance concentration was prepared and analyzed under description in "1 Analytical method of the test solution". The recovery ratio of two times addition recovery test was 110% and 102%, therefore the mean value was 106%. The analytical results of the control and the 0.00600 concentration section were corrected with the mean value.

Figure A-2-1 Calibration curve

No.	Concentration (mg/L)	Peak Area (count)
1	0	0
2	0.0500	1.2
3	0.100	2.1
4	0.200	4.2
5	0.500	10.6
6	1.00	21.8
7	2.00	48.3
8	5.00	110.1
9	10.0	220.1
10	20.0	434.8
11	50.0	1093.8
12	100	2182.4
13	200	4262.7

Y= 21.4X
r= 1.00

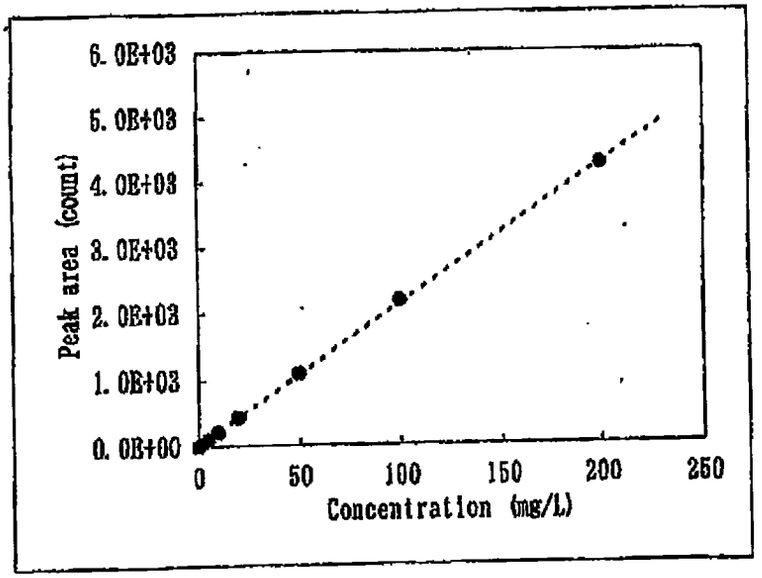
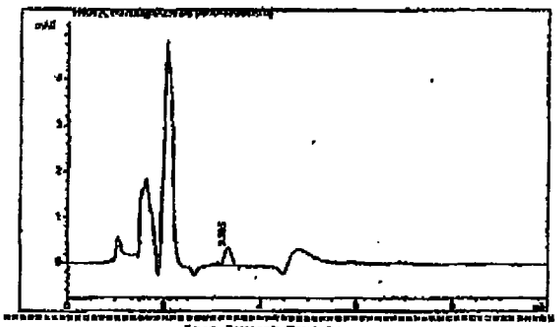


Figure A-2-2 Representative chromatograms

(1) Standard 0.0200 mg/L ; Day 0

Injection Date :02/08/20 Seq Line : 1
Test No. :A010459-3 Vial No. : 1
Test Substance :HSBA Inj. Vol. : 100 µl
Sample Name :[illegible]
Acq Operator :[redacted]
Acq. Method :A010459.M
Analysis Method :C:\[illegible]\METHODS\2001\A010459.M
Sample Info :50mM NH4H2PO4-(NH4)2HPO4(pH6.7): MeOH = 40:60



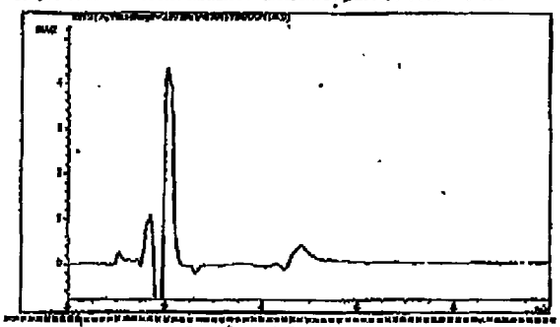
Area Percent Report:

Peak #	RetTime [min]	Type	Width [min]	Area [count]	Height [count]	Area % [%]
1	3.305	MM	0.175	4.2	0.40	100
				4.2		

*** End of Report ***

(2) Control ; Day 0

Injection Date :02/08/20 Seq Line : 3
Test No. :A010459-3 Vial No. : 1
Test Substance :HSBA Inj. Vol. : 100 µl
Sample Name :[illegible]
Acq Operator :[redacted]
Acq. Method :A010459.M
Analysis Method :C:\[illegible]\METHODS\2001\A010459.M
Sample Info :50mM NH4H2PO4-(NH4)2HPO4(pH6.7): MeOH = 40:60



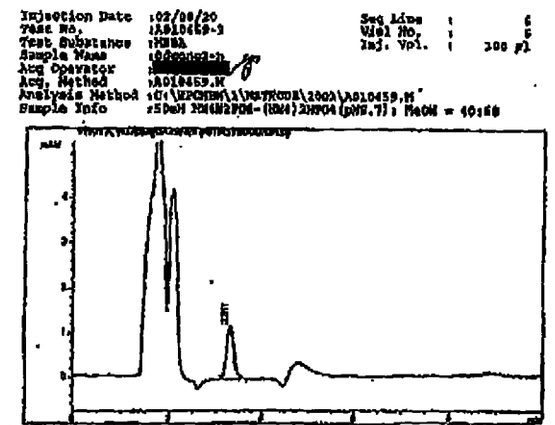
Area Percent Report:

Peak #	RetTime [min]	Type	Width [min]	Area [count]	Height [count]	Area % [%]

*** End of Report ***

Figure A-2-2 Continued

(3) 0.0600 mg/L nominal ; Day 0



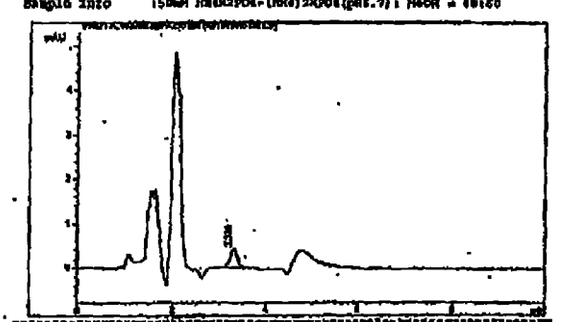
Injection Date :02/08/20 Seq Line : 6
Test No. :A010459-3 Vial No. : 6
Test Substance :NEBA Inj. Vol. : 100 µl
Sample Name :[redacted]
Acq Operator :[redacted]
Acq. Method :A010459.M
Analysis Method :C:\EPCHEM\1\METHODS\2002\A010459.M
Sample Info :50mM KH2PO4-(NH4)2HPO4(pH5.7) : MeOH = 40:60

Area Percent Report:

Peak #	RetTime [min]	Type	Width [min]	Area [count]	Height [count]	Area % [%]
1	2.311	MM	0.186	12.8	2.19	100
				12.8		

*** End of Report ***

(4) Standard 0.0200 mg/L ; Day 1



Injection Date :02/08/21 Seq Line : 1
Test No. :A010459-3 Vial No. : 1
Test Substance :NEBA Inj. Vol. : 100 µl
Sample Name :[redacted]
Acq Operator :[redacted]
Acq. Method :A010459.M
Analysis Method :C:\EPCHEM\1\METHODS\2002\A010459.M
Sample Info :50mM KH2PO4-(NH4)2HPO4(pH5.7) : MeOH = 40:60

Area Percent Report:

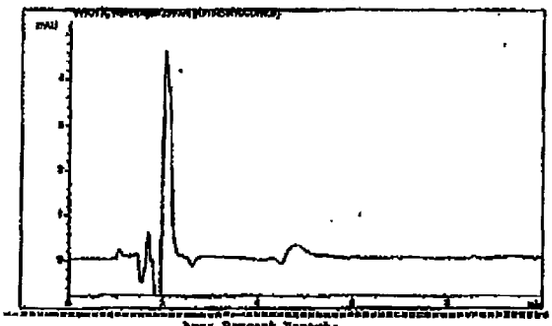
Peak #	RetTime [min]	Type	Width [min]	Area [count]	Height [count]	Area % [%]
1	3.388	MM	0.172	4.2	0.41	100
				4.2		

*** End of Report ***

Figure A-2-2 Continued

(5) Control ; Day 1

Injection Date :02/08/21 Seq Line : 3
Test No. :A010459-3 Vial No. : 3
Test Substance :NBBA Inj. Vol. : 100 µl
Sample Name :1dcont
Acq Operator :[redacted]
Acq. Method :A010459.M
Analysis Method :C:\HPCHEM\1\METHODS\2001\A010459.M
Sample Info :50mM NH4H2PO4-(NH4)2HPO4(pH6.7): MeOH = 40:60



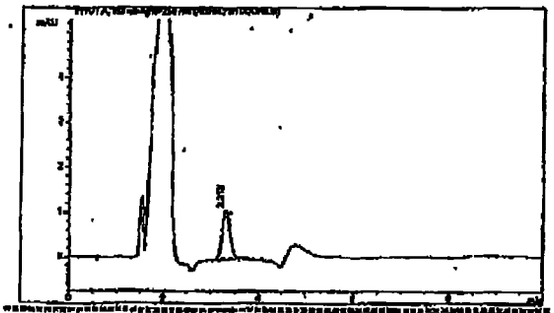
Area Percent Report:

Peak #	RetTime [min]	Type	Width [min]	Area [count]	Height [count]	Area % [%]

*** End of Report ***

(6) 0.0600 mg/L nominal ; Day 1.

Injection Date :02/08/21 Seq Line : 6
Test No. :A010459-3 Vial No. : 6
Test Substance :NBBA Inj. Vol. : 100 µl
Sample Name :1dconc1
Acq Operator :[redacted]
Acq. Method :A010459.M
Analysis Method :C:\HPCHEM\1\METHODS\2001\A010459.M
Sample Info :50mM NH4H2PO4-(NH4)2HPO4(pH6.7): MeOH = 40:60



Area Percent Report:

Peak #	RetTime [min]	Type	Width [min]	Area [count]	Height [count]	Area % [%]
1	3.313	MM	0.161	72.0	1.11	100
				12.0		

*** End of Report ***

(A010459-3)

Addendum - 3

Observation Result of Daphnia Magna

Appendix 3-1 Result of reproduction test　　Test chemical: MBBA　　(Untreated control)

Rep. No.	Counts		Time 8/21 1 d	8/22 2 d	8/23 3 d	8/24 4 d	8/25 5 d	8/26 6 d	8/27 7 d	8/28 8 d	8/29 9 d	8/30 10 d	8/31 11 d	9/1 12 d	9/2 13 d	9/3 14 d	9/4 15 d	9/5 16 d	9/6 17 d	9/7 18 d	9/8 19 d	9/9 20 d	9/10 21 d	Total
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
1	F1 generation	Live	0	0	0	0	0	0	0	2	0	0	36	0	0	27	0	0	0	19	0	0	27	
	Cumulative reproductivity		0	0	0	0	0	0	0	2	2	2	38	38	38	65	65	65	65	84	84	84	111	111
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
2	F1 generation	Live	0	0	0	0	0	0	0	4	0	0	31	0	0	28	0	0	0	19	0	0	27	
	Cumulative reproductivity		0	0	0	0	0	0	0	4	4	4	35	35	35	63	63	63	63	82	82	82	109	109
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
3	F1 generation	Live	0	0	0	0	0	0	0	5	0	0	0	27	0	0	23	0	0	26	0	0	0	
	Cumulative reproductivity		0	0	0	0	0	0	0	5	5	5	5	32	32	32	55	55	55	81	81	81	81	81
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
4	F1 generation	Live	0	0	0	0	0	0	0	7	0	0	0	29	0	0	28	0	0	22	0	0	0	
	Cumulative reproductivity		0	0	0	0	0	0	0	7	7	7	7	36	36	36	64	64	64	86	86	86	86	86
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
5	F1 generation	Live	0	0	0	0	0	0	0	6	6	0	1	27	0	0	24	0	0	22	0	0	0	
	Cumulative reproductivity		0	0	0	0	0	0	0	6	12	12	13	40	40	40	64	64	64	86	86	86	86	86
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
6	F1 generation	Live	0	0	0	0	0	0	0	13	0	0	31	0	0	0	25	0	0	23	0	0	0	
	Cumulative reproductivity		0	0	0	0	0	0	0	13	13	13	44	44	44	44	69	69	69	97	97	97	97	97
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
7	F1 generation	Live	0	0	0	0	0	0	0	5	1	0	0	17	0	0	34	0	0	24	0	0	0	
	Cumulative reproductivity		0	0	0	0	0	0	0	5	6	6	6	23	23	23	57	57	57	81	81	81	81	81
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
8	F1 generation	Live	0	0	0	0	0	0	0	4	0	0	36	0	0	12	9	0	0	23	0	0	22	
	Cumulative reproductivity		0	0	0	0	0	0	0	4	4	4	40	40	40	52	61	61	61	84	84	84	106	106
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
9	F1 generation	Live	0	0	0	0	0	0	0	8	0	0	30	0	0	0	27	0	0	18	0	0	25	
	Cumulative reproductivity		0	0	0	0	0	0	0	8	8	8	38	38	38	38	65	65	65	83	83	83	108	108
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
10	F1 generation	Live	0	0	0	0	0	0	0	5	0	0	33	0	0	1	25	0	0	21	0	0	25	
	Cumulative reproductivity		0	0	0	0	0	0	0	5	5	5	38	38	38	39	64	64	64	85	85	85	110	110

Appendix 3-2 Result of reproduction test　　Test chemical: MBBA　　(Concentration 1)

Rep. No.	Counts		Time 8/21 1 d	8/22 2 d	8/23 3 d	8/24 4 d	8/25 5 d	8/26 6 d	8/27 7 d	8/28 8 d	8/29 9 d	8/30 10 d	8/31 11 d	9/1 12 d	9/2 13 d	9/3 14 d	9/4 15 d	9/5 16 d	9/6 17 d	9/7 18 d	9/8 19 d	9/9 20 d	9/10 21 d	Total
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
1	F1 generation	Live	0	0	0	0	0	0	0	5	0	0	0	24	0	0	23	0	0	44	0	0	0	
	Cumulative reproductivity		0	0	0	0	0	0	0	5	5	5	5	29	29	29	52	52	52	96	96	96	96	96
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
2	F1 generation	Live	0	0	0	0	0	0	0	5	0	0	23	0	0	0	24	0	0	38	0	0	24	
	Cumulative reproductivity		0	0	0	0	0	0	0	5	5	5	28	28	28	28	52	52	52	90	90	90	114	114
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
3	F1 generation	Live	0	0	0	0	0	0	0	4	0	0	36	0	0	27	1	0	0	44	0	0	24	
	Cumulative reproductivity		0	0	0	0	0	0	0	4	4	4	40	40	40	67	68	68	68	112	112	112	136	136
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
4	F1 generation	Live	0	0	0	0	0	0	0	3	0	0	0	26	0	0	27	0	0	37	0	0	0	
	Cumulative reproductivity		0	0	0	0	0	0	0	3	3	3	3	29	29	29	56	56	56	93	93	93	93	93
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
5	F1 generation	Live	0	0	0	0	0	0	0	6	0	0	0	28	0	0	27	0	0	34	0	0	0	
	Cumulative reproductivity		0	0	0	0	0	0	0	6	6	6	6	34	34	34	61	61	61	95	95	95	95	95
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
6	F1 generation	Live	0	0	0	0	0	0	0	9	0	0	0	28	0	0	26	0	0	42	0	0	0	
	Cumulative reproductivity		0	0	0	0	0	0	0	9	9	9	9	37	37	37	63	63	63	105	105	105	105	105
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
7	F1 generation	Live	0	0	0	0	0	0	0	5	0	0	29	0	0	0	32	0	0	33	0	0	22	
	Cumulative reproductivity		0	0	0	0	0	0	0	5	5	5	34	34	34	34	56	56	56	89	89	89	111	111
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
8	F1 generation	Live	0	0	0	0	0	0	0	3	0	0	0	30	0	0	28	0	0	43	0	0	24	
	Cumulative reproductivity		0	0	0	0	0	0	0	3	3	3	3	33	33	33	61	61	61	104	104	104	128	128
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
9	F1 generation	Live	0	0	0	0	0	0	0	5	0	0	0	26	0	0	24	0	0	27	0	0	0	
	Cumulative reproductivity		0	0	0	0	0	0	0	5	5	5	5	31	31	31	55	55	55	82	82	82	82	82
	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
10	F1 generation	Live	0	0	0	0	0	0	0	2	3	0	0	16	0	0	21	0	0	9	27	0	0	
	Cumulative reproductivity		0	0	0	0	0	0	0	2	5	5	5	21	21	21	42	42	42	51	78	78	78	78

Appendix 3-3 Result of reproduction test Test chemical: MBBA (Concentration 2)

Rep. No.	Counts		Time 8/21 1 d	8/22 2 d	8/23 3 d	8/24 4 d	8/25 5 d	8/26 6 d	8/27 7 d	8/28 8 d	8/29 9 d	8/30 10 d	8/31 11 d	9/1 12 d	9/2 13 d	9/3 14 d	9/4 15 d	9/5 16 d	9/6 17 d	9/7 18 d	9/8 19 d	9/9 20 d	9/10 21 d	Total
1	P generation	Live	1	1	1	1	1	1	1	1	0													
	F1 generation	Live	0	0	0	0	0	0	0	1	0													
	Cumulative reproductivity		0	0	0	0	0	0	0	1	1													—
2	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	F1 generation	Live	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	21	0	0	28	
	Cumulative reproductivity		0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	22	22	22	50	50
3	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	F1 generation	Live	0	0	0	0	0	0	0	3	0	0	0	0	0	0	2	0	0	5	0	0	17	
	Cumulative reproductivity		0	0	0	0	0	0	0	3	3	3	3	3	3	3	5	5	5	10	10	10	27	27
4	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	F1 generation	Live	0	0	0	0	0	0	0	0	1	0	0	0	0	0	6	0	0	14	0	0	0	
	Cumulative reproductivity		0	0	0	0	0	0	0	0	1	1	1	1	1	1	7	7	7	21	21	21	21	21
5	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	F1 generation	Live	0	0	0	0	0	0	0	3	0	0	0	0	0	0	15	0	0	19	0	0	0	
	Cumulative reproductivity		0	0	0	0	0	0	0	3	3	3	3	3	3	3	18	18	18	37	37	37	37	37
6	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	F1 generation	Live	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	0	0	0	0	0	0	
	Cumulative reproductivity		0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	18	18	18	18	18	18	18
7	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	F1 generation	Live	0	0	0	0	0	0	0	5	0	0	0	0	0	8	0	0	0	9	0	0	21	
	Cumulative reproductivity		0	0	0	0	0	0	0	5	5	5	5	5	5	13	13	13	13	22	22	22	43	43
8	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	F1 generation	Live	0	0	0	0	0	0	0	1	0	0	0	0	0	0	10	0	0	22	0	0	0	
	Cumulative reproductivity		0	0	0	0	0	0	0	1	1	1	1	1	1	1	11	11	11	33	33	33	33	33
9	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	F1 generation	Live	0	0	0	0	0	0	0	3	0	0	0	0	0	4	9	0	0	17	0	0	16	
	Cumulative reproductivity		0	0	0	0	0	0	0	3	3	3	3	3	3	7	16	16	16	33	33	33	49	49
10	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	F1 generation	Live	0	0	0	0	0	0	0	3	0	0	0	0	0	1	16	0	0	18	0	0	21	
	Cumulative reproductivity		0	0	0	0	0	0	0	3	3	3	3	3	3	4	20	20	20	38	38	38	59	59

— : Were not included for calculation because the parental *Daphnia* was dead during a 21-day testing period.

Appendix 3-4 Result of reproduction test Test chemical: MBBA (Concentration 3)

Rep. No.	Counts		Time: 8/21 1 d	8/22 2 d	8/23 3 d	8/24 4 d	8/25 5 d	8/26 6 d	8/27 7 d	8/28 8 d	8/29 9 d	8/30 10 d	8/31 11 d	9/1 12 d	9/2 13 d	9/3 14 d	9/4 15 d	9/5 16 d	9/6 17 d	9/7 18 d	9/8 19 d	9/9 20 d	9/10 21 d	Total
1	P generation	Live	1	0																				
	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
2	P generation	Live	1	0																				
	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
3	P generation	Live	1	0																				
	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
4	P generation	Live	1	0																				
	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
5	P generation	Live	1	0																				
	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
6	P generation	Live	1	0																				
	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
7	P generation	Live	1	0																				
	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
8	P generation	Live	1	1	1	1	1	1	1	1	1	1	1	0										
	F1 generation	Live	0	0	0	0	0	0	0	0	0	0	0	0										
	Cumulative reproductivity		0	0	0	0	0	0	0	0	0	0	0	0										—
9	P generation	Live	1	0																				
	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
10	P generation	Live	1	0																				
	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—

— : Were not included for calculation because the parental *Daphnia* was dead during a 21-day testing period.

Appendix 3-5 Result of reproduction test Test chemical: MBBA (Concentration 4)

Rep. No.	Counts		Time 8/21 1 d	8/22 2 d	8/23 3 d	8/24 4 d	8/25 5 d	8/26 6 d	8/27 7 d	8/28 8 d	8/29 9 d	8/30 10 d	8/31 11 d	9/1 12 d	9/2 13 d	9/3 14 d	9/4 15 d	9/5 16 d	9/6 17 d	9/7 18 d	9/8 19 d	9/9 20 d	9/10 21 d	Total
	P generation	Live	1	0																				
1	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
	P generation	Live	1	0																				
2	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
	P generation	Live	1	0																				
3	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
	P generation	Live	1	0																				
4	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
	P generation	Live	1	0																				
5	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
	P generation	Live	1	0																				
6	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
	P generation	Live	1	0																				
7	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
	P generation	Live	1	0																				
8	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
	P generation	Live	1	0																				
9	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
	P generation	Live	1	0																				
10	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—

— : Were not included for calculation because the parental *Daphnia* was dead during a 21-day testing period.

Appendix 3-6 Result of reproduction test Test chemical: MBBA (Concentration 5)

Rep. No.	Counts		Time 8/21 1 d	8/22 2 d	8/23 3 d	8/24 4 d	8/25 5 d	8/26 6 d	8/27 7 d	8/28 8 d	8/29 9 d	8/30 10 d	8/31 11 d	9/1 12 d	9/2 13 d	9/3 14 d	9/4 15 d	9/5 16 d	9/6 17 d	9/7 18 d	9/8 19 d	9/9 20 d	9/10 21 d	Total
1	P generation	Live	0																					
	F1 generation	Live	0																					
	Cumulative reproductivity		0																					—
2	P generation	Live	0																					
	F1 generation	Live	0																					
	Cumulative reproductivity		0																					—
3	P generation	Live	0																					
	F1 generation	Live	0																					
	Cumulative reproductivity		0																					—
4	P generation	Live	0																					
	F1 generation	Live	0																					
	Cumulative reproductivity		0																					—
5	P generation	Live	1	0																				
	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
6	P generation	Live	1	0																				
	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
7	P generation	Live	1	0																				
	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
8	P generation	Live	1	0																				
	F1 generation	Live	0	0																				
	Cumulative reproductivity		0	0																				—
9	P generation	Live	0																					
	F1 generation	Live	0																					
	Cumulative reproductivity		0																					—
10	P generation	Live	0																					
	F1 generation	Live	0																					
	Cumulative reproductivity		0																					—

— : Were not included for calculation because the parental *Daphnia* was dead during a 21-day testing period.