Comparison of acute immobilization tests using Daphnia reported from Japan, China and Korea

— *o*-aminophenol —

Expert Meeting of Joint Research for Chemicals among China, Japan and Korea

2015

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1. Introduction

In the 7th Japan expert's meeting of Policy Dialogue on Chemicals and Environment held on November 2013, the results of an acute immobilization test using Daphnia with *o*-aminophenol, conducted in Japan, China and Korea were reported. In this present investigation, reports of the acute immobilization tests using Daphnia from these three countries were acquired through an expert of each government, and the obtained reports were compared and examined in detail to analyze the differences between the results obtained from the tests.

2. Acquisition of the reports for the acute immobilization test using Daphnia

We asked the governmental experts of China and Korea that attended the 7th Japan expert's meeting of Policy Dialogue on Chemicals and Environment to send each test report of the acute immobilization test using Daphnia with *o*-aminophenol, and received each test report from China and Korea. The test report in Japan was acquired from the duty office of the Ministry of the Environment. Because the test report sent from Korea was written in Korean, the Korean test report was translated into Japanese. As the test report sent from China was written in English, it was directly used for comparative investigation. The obtained test reports were as follows.

<Japan>

- Acute immobilization test of *o*-aminophenol using Daphnia (*Daphnia magna*)
  Study No: 1108EDI, 2012

<China>

- Report for Acute Immobilization Test to Daphnids of 2-Aminophenol
  Study No: SQC2013NC001, Report No: RQC2013NC001, Nanjing Institute of Environmental Sciences, 2013

<Korea>

- 물벼룩(Daphnia magna)을 이용한2-Aminophenol의 급성독성시험
  한국건설생활환경시험연구원 바이오융합연구소
  Acute toxicity test of 2-aminophenol using Daphnia (*Daphnia magna*)
  Study No: GT13-00465, Korean Conformity laboratories, 2013

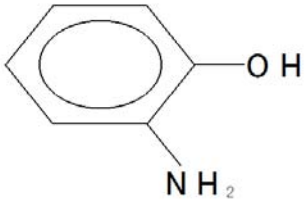
3. Comparative method relating to the acute immobilization test using Daphnia and the like

In addition to the test reports as mentioned, some documents used by Chinese and Korean experts in the 7th Japan expert's meeting of Policy Dialogue on Chemicals and Environment were also referred. For comparison and analyses of the differences of the results, this investigation focuses on the test protocols which are considered to affect the result of the acute immobilization test using Daphnia. In addition, the present investigation makes consideration with the "OECD test guideline 202: Acute Immobilisation Test using Daphnia" and "OECD guidance document No. 23: Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures", if necessary.

4. Comparison and analysis of the protocol and result on the Acute Immobilization Tests using Daphnia

4.1. Comparison of the Acute Immobilization Tests of *o*-aminophenol using Daphnia

o-Aminophenol is a chemical substance indicated in the following structural formula, and has high water solubility. It was speculated that *o*-aminophenol is present in non-dissociated form in water around pH 7 based on the value of dissociation constants (pKa_1 = 4.72, pKa_2 = 9.71). It is known that *o*-aminophenol has low stability in water and induces the polymerization reaction in water. Stability seems to depend on the condition of water (e.g. pH, hardness etc.).

CAS No:	95-55-6
Other name:	2-aminophenol
	2-amino-1-hydroxy aniline
	o-hydroxy aniline
Molecular formula:	C_6H_7NO
Molecular weight:	109.13
Water solubility:	1 g / 50 ml[1)]
Dissociation constant:	pKa_1 = 4.72 at 21 ℃, pKa_2 = 9.71 at 25 ℃[2)]
Structural formula:	

1) Merck Index
2) Kirk-Othmer Encyclopedia of Chemical Technology, fifth edition, volume 2, Wiley-Interscience, 2004

Results of comparison for items affecting the test result based on each test report from each country were shown as follows. EC_{50} values reported in each test report were as 0.574 mg/L (measured concentration) in the test report in Japan, 1.012 mg/L (nominal concentration) and 1.002 mg/L (measured concentration) in the test report in Korea and 1.13 mg/L (nominal concentration) in the test report in China.

Table 1-1 Comparison of Acute immobilization test of *o*-aminophenol using Daphnia

	Acute Immobilization test using Daphnia conducted in Japan	**Acute Immobilization test using Daphnia conducted in China**	**Acute Immobilization test using Daphnia conducted in Korea**
Test summary			
Year Report published	2012	2013	2013
GLP adapted	GLP adapted	GLP adapted	GLP adapted
Purity of substance	99.4 %	99 %	99 %
Impurity	Unknown	Unknown	Unknown
Test organism			
Test organism	Daphnia *Daphnia magna*	Daphnia *Daphnia magna*	Daphnia *Daphnia magna*
Provider	National Institute for Environmental Studies	Unknown	National Institute of Environmental Research
Conditioning of parent generation	Breed in M4 medium. During the conditioning, the breeding is maintained as good. The feed contains raw chlorella concentrate (*Chlorella vulgaris*) and others.	Breed in M4 medium with the same condition during the test. The breeding was maintained as healthy.	Generally, breed in M4 medium. The feed contains raw chlorella concentrate (*Chlorella vulgaris*) and others.
Confirmation of sensitivity	Confirm the sensitivity using potassium dichromate every six months. Current EC_{50} value (48-h): 1.05 mg/L	EC_{50} value (24-h) for potassium dichromate: 1.19 mg/L	EC_{50} values (24-h) for potassium dichromate: 1.592 mg/L (nominal concentration) 1.581 mg/L (measured concentration)
Age	Less than 24 hours after birth	Less than 6 to 24 hours after birth	Less than 24 hours after birth

	Acute Immobilization test using Daphnia conducted in Japan	**Acute Immobilization test using Daphnia conducted in China**	**Acute Immobilization test using Daphnia conducted in Korea**
Medium			
Medium	M4 medium	M4 medium	ISO medium for the main test
Test condition			
Exposure method	Semi-static (replace the test solution after 24 hours)	Semi-static (replace the test solution after 24 hours)	Semi-static (replace the test solution after 24 hours)
Exposure time	48 hours	48 hours	48 hours
Temperature	19.5 – 19.8 ℃ (20.0±1 ℃)	21.5 ℃	19.9 – 20.3 ℃
Ventilation	None	None	None
Dissolved oxygen	8.3 – 8.8 mg/L (> 3 mg/L)	88 – 99 % (> 3 mg/L)	7.84 – 8.23 mg/L
pH adjustment	No pH adjustment	No pH adjustment	pH adjusted (adjusted at 7.5) * Generally no pH adjustment
pH	7.8 – 7.9	7.5 – 7.9	7.22 – 7.87
Hardness	230 – 240 mg/L as $CaCO_3$ (measure M4 medium)	Unknown	232 – 236 mg/L as $CaCO_3$
Alkalinity	Unknown	Unknown	44 – 48 mg/L
Lighting	16 hours light/8 hours dark room light (6.33 – 9.08 $\mu E/m^2/s$)	16 hours light/8 hours dark	16 hours light/8 hours dark (lighting from 8:00 to 24:00)
Feeding during testing	No feed	No feed	No feed
Amount of test liquid and the like			
Amount of test media	100 mL	50 mL	50 mL
Test vessel	100 mL Glass beaker	100 mL Glass beaker	Glass beaker
Replicate	4 replicates/test concentration	4 replicates/test concentration	4 replicates/test concentration
Number of organism	20/test section (5/vessel×4)	20/test section (5/vessel×4)	20/test section (5/vessel×4)

	Acute Immobilization test using Daphnia conducted in Japan	**Acute Immobilization test using Daphnia conducted in China**	**Acute Immobilization test using Daphnia conducted in Korea**
Preliminary test			
Nominal concentration	0.0, 0.40, 0.71, 2.2, 4.0 mg/L	0.0, 0.10, 0.50, 1.00, 5.00 mg/L	0.196, 0.391, 0.782, 1.563, 3.125, 6.250, 12.500, 25.000 mg/L
Result	Nominal concentration / immobilization rate after each exposure time 24-h 48-h 0.0 mg/L 0 0 0.40 mg/L 0 0 0.71 mg/L 0 10 2.2 mg/L 5 95 4.0 mg/L 80 100 EC_{50} value: 0.71 – 2.2 mg/L	Nominal concentration / Immobilization rate after each exposure time 24-h 48-h 0.00 mg/L 0 0 0.10 mg/L 0 0 0.50 mg/L 20 20 1.00 mg/L 40 40 5.00 mg/L 100 100	See Annex 1
Replicate	4 replicates/test concentration	1 replicate/test concentration	1 replicate/test concentration
Organism numbers	20/test section	5/test section	5/test section
Others	Upon conducting a preliminary test with static condition, higher toxicity was observed compared to the toxicity with semi-static condition. Therefore, it is suggested that produced compound may have a toxicity of higher than *o*-aminophenol.	—	Upon conducting preliminary tests using M4 medium, M7 medium and ISO medium, ISO medium was determined as the most appropriate medium.
Main test			
Nominal concentration	0.0, 0.32, 0.56, 1.0, 1.8, 3.2 mg/L	0.0, 0.56, 1.0, 1.8, 3.2 , 5.6 mg/L	0.000, 0.7000, 0.840, 1008, 1.210, 1.452 mg/L
Geometric factor	1.8	1.8	1.2
Solubilizing agent	Not used	Not used	Not used
Concentration of solubilizing agent	—	—	—

	Acute Immobilization test using Daphnia conducted in Japan	**Acute Immobilization test using Daphnia conducted in China**	**Acute Immobilization test using Daphnia conducted in Korea**
Preparation of test solution	0.01 g of the substance to be tested was dissolved in 200 mL of diluting water (M4 medium) to produce an original test solution. The original test solution was diluted with the diluting water to produce the test solution having a targeted concentration.	0.0100 g of the substance to be tested was dissolved in 100 mL of diluting water (M4 medium) to product an original test solution. The original test solution was diluted with the diluting water to prepare the test solution having a targeted concentration.	0.25 g of the substance to be tested was dissolved in diluting water (ISO medium) to product 247.5 mg/L of an original test solution. The original test solution was diluted with the diluting water to prepare the test solution having a targeted concentration. The ISO medium is preliminary adjusted as pH 7.5.
Analysis of the test solution	Conducted (immediately afterwards, before replacement of water at 24 hours, after replacement of water at 24 hours, after 48 hours)	Conducted (immediately afterwards, before replacement of water at 24 hours, after replacement of water at 24 hours, after 48 hours)	Conducted (immediately afterwards, before replacement of water at 24 hours, after replacement of water at 24 hours, after 48 hours)
Analyzed concentration	Nominal concentration → Measured concentration (Time-weight average) 0.32 mg/L → 0.115 mg/L (35.9 %) 0.56 mg/L → 0.228 mg/L (40.7 %) 1.0 mg/L → 0.442 mg/L (44.2 %) 1.8 mg/L → 0.837 mg/L (46.5 %) 3.2 mg/L → 1.48 mg/L (46.3 %)	Nominal concentration → Measured concentration (Arithmetical average) 0.56 mg/L → 0.53 mg/L 1.00 mg/L → 0.91 mg/L 1.80 mg/L → 1.67 mg/L 3.20 mg/L → 2.64 mg/L 5.60 mg/L → 4.61 mg/L Averages of the measured concentrations were within 20% against the each nominal concentration.	Nominal concentration → Measured concentration (Geometric average) 0.700 mg/L → 0.691 mg/L (98.7 %) 0.840 mg/L → 0.836 mg/L (99.5 %) 1.008 mg/L → 1.006 mg/L (99.8 %) 1.210 mg/L → 1.216 mg/L (100.5 %) 1.452 mg/L → 1.373 mg/L (94.6 %)
Color of the test solution	Yellow coloring was observed in all test solutions with concentration-dependent manner after 24 hours.	No related description	Slightly yellow coloring was observed in all test solutions after 24 hours.

	Acute Immobilization test using Daphnia conducted in Japan	**Acute Immobilization test using Daphnia conducted in China**	**Acute Immobilization test using Daphnia conducted in Korea**
Immobilization ratio	Nominal concentration / Immobilization ratio after each exposure time 24-h / 48-h 0.0 mg/L: 0 / 0 0.32 mg/L: 0 / 0 0.56 mg/L: 0 / 0 1.0 mg/L: 0 / 35 1.8 mg/L: 0 / 80 3.2 mg/L: 40 / 95	Nominal concentration / Immobilization ratio after each exposure time 24-h / 48-h 0.0 mg/L: 0 / 0 0.56 mg/L: 0 / 20 1.00 mg/L: 5 / 30 1.80 mg/L: 40 / 100 3.20 mg/L: 45 / 100 5.60 mg/L: 100 / 100	Nominal concentration / Immobilization ratio after each exposure time 24-h / 48-h 0.0 mg/L: 0 / 0 0.700 mg/L: 5 / 10 0.840 mg/L: 5 / 30 1.008 mg/L: 35 / 50 1.210 mg/L: 60 / 75 1.452 mg/L: 75 / 95
Result			
EC_{50} (48-h)	0.574 mg/L (based on the measured concentration)	1.13 mg/L (based on the nominal concentration) As the average measured concentration was within 20 % against the nominal concentration.	1.012 mg/L (based on the nominal concentration) 1.002 mg/L (based on the measured concentration)
95 % confidence interval	0.473 – 0.693 mg/L	1.03 – 1.25 mg/L	0.930 – 1.096 mg/L (Nominal concentration) 0.923 – 1.080 mg/L (Measured concentration)
Concentration used for estimating EC_{50} value	Using the measured concentration	Using the nominal concentration	Using both measured and nominal concentrations
Average of the measured concentration	Time-weight average	Measured concentration is not used for the estimation of LC_{50} value.	Geometric average
Statistical method	Probit method	Trimmed Spearman-Karber method	Probit method

4.2. Changes of *o*-aminophenol in water

It is known that aminophenol is unstable in water and is polymerized therein. Kirk-Othmer Encyclopedia of Chemical Technology[3] describes its nature as follows.

> "3-Aminophenol is fairly stable in air unlike 2-aminophenol and 4-aminophenol that easily undergo oxidation to colored products. …. The aminophenols are chemically reactive. Oxidation leads to the formation of highly colored polymeric quinoid structures."

Bio-degradation test on *p*-aminophenol[4] although which is not ortho-body, according to inspection of existing chemical substances program, conducted in Ministry of Economy, Trade and Industry was conducted and its test report mentioned as follows.

> "In both the (test substance + water) system and the (test substance + activated sludge) system, test solutions showed the coloring and insoluble matter in brown appeared. According to the LC analysis at the end of the test, it was confirmed that the test substance was completely disappeared and it was transformed to the compound of 1,000 to 4,000 in molecular weight. This compound did not show the absorption around 3,400 cm^{-1} in the infra-red spectrum, which corresponding to the amino group. Therefore, it is supposed that the test substance was oxidized in water and polymerized through the forming of the quinone structure."

In addition, there is a scientific paper which mentions that *o*-aminophenol has a tendency to make dimerization through the reacting pathway as follows[5].

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3) Kirk-Othmer Encyclopedia of Chemical Technology, fifth edition, volume 2, Wiley-Interscience, 2004

4) Test report of degradation for p-aminophenol (K-191), Ministry of Economy, Trade and Industry, 1982

5) K. Jackowska, J. Bukowska, and A. Kudelski, Electro-oxidation of *o*-aminophenol studied by cyclic voltammetry and surface enhanced Raman scattering (SERS) J. Electroanal. Chem., 350, 177-187, 1993

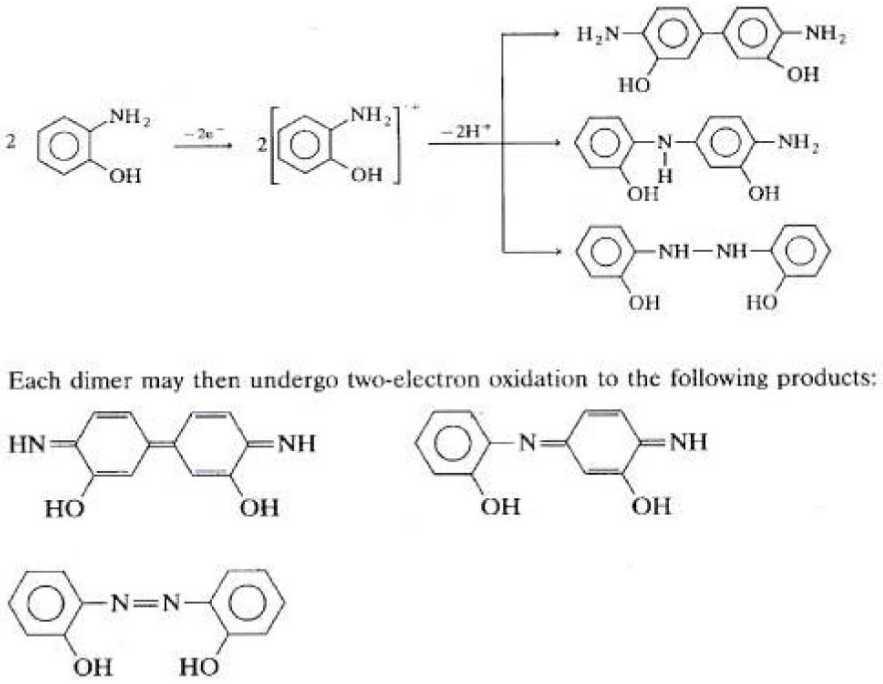

Figure 1-1 Polymerization reacting pathway for *o*-aminophenol

As mentioned above, it is anticipated that *o*-aminophenol is dimerized through forming of the quinone structure and the like in water, and is finally polymerized. It is thought that these reactions lead to the coloring, which was observed in the tests conducted in Japan and Korea.

4.3. Discussion

The most important difference between the tests conducted in the three countries is the selection of the medium. Whereas M4 medium was used as the medium to conduct the tests in Japan and China, ISO medium was used as the medium to test in Korea.

In Korea, prior to conducting the test, which medium should be selected to use was examined. Using M4 medium, M7 medium and ISO medium which are recommended for use in the OECD test-guideline 202, solutions of 0.196 mg/L and 25.000 mg/L of *o*-aminophenol in each medium were prepared and the concentrations of *o*-aminophenol were measured after 48 hours. The following table shows the results of this preliminary examination.

Table 1-2 Stability of *o*-aminophenol in medium

Medium	Nominal concentration	Time as measured	Measured concentration	Geometric average concentration (percent against to the nominal concentration)
M4 medium	0.196 mg/L	Immediately afterwards	n.d.	n.d.
		48 hours later	n.d.	
	25.000 mg/L	Immediately afterwards	20.190 mg/L	9.436 mg/L (37.7 %)
		48 hours later	4.410 mg/L	
M7 medium	0.196 mg/L	Immediately afterwards	n.d.	n.d.
		48 hours later	n.d.	
	25.000 mg/L	Immediately afterwards	20.660 mg/L	10.474 mg/L (41.9 %)
		48 hours later	5.310 mg/L	
ISO medium	0.196 mg/L	Immediately afterwards	n.d.	n.d.
		48 hours later	n.d.	
	25.000 mg/L	Immediately afterwards	20.530 mg/L	18.399 mg/L (73.6 %)
		48 hours later	16.490 mg/L	

It was confirmed from these results that *o*-aminophenol is more stable in ISO medium, rather than in M4 medium and M7 medium. As ISO medium does not contain $CuCl_2$, $ZnCl_2$ and $FeSO_4$ which are contained in M4 medium and M7 medium, it is supposed that these constituents are responsible for the stability of *o*-aminophenol.

Next, the effect on pH to the stability of *o*-aminophenol in medium was examined in Korea using ISO medium. *o*-Aminophenol was set to have its concentration as 0.196 to 25.000 mg/L in ISO medium adjusted with pH 6.5, 7.5 and 8.5, and the concentrations were measured afterwards, after 24 hours later and after 48 hours.

Table 1-3 Stability of *o*-aminophenol in ISO medium

Nominal concentration	Average arithmetic concentration afterwards (percent against the nominal concentration)		
	pH 6.5	pH 7.5	pH 8.5
0.196 mg/L	n.d. (0%)	n.d. (0 %)	n.d. (0 %)
0.391 mg/L	0.244 mg/L (62.4 %)	0.251 mg/L (64.1 %)	0.119 mg/L (30.4 %)
0.782 mg/L	0.606 mg/L (77.5 %)	0.600 mg/L (76.7 %)	0.378 mg/L (48.3 %)
1.563 mg/L	1.245 mg/L (79.6 %)	1.256 mg/L (80.4 %)	1.034 mg/L (66.2 %)
3.125 mg/L	2.580 mg/L (82.6 %)	2.624 mg/L (84.0 %)	2.109 mg/L (67.5 %)
6.250 mg/L	5.872 mg/L (94.0 %)	5.558 mg/L (88.9 %)	4.604 mg/L (73.7 %)
12.500 mg/L	13.287 mg/L (106.3 %)	12.545 mg/L (100.4 %)	10.412 mg/L (83.3 %)
25.000 mg/L	23.607 mg/L (94.4 %)	23.263 mg/L (93.0 %)	20.873 mg/L (83.5 %)

It was found from these results that *o*-aminophenol is most stable at pH 7.5 in ISO medium. From the results of the preliminary investigations above mentioned, ISO medium is decided to be used with the adjustment of its pH as 7.5 for the acute immobilization test using Daphnia. Such an examination for the medium was not conducted in Japan and China, and M4 medium was used for the testing in two countries. The effect on pH in M4 medium was also not confirmed in Japan and China.

M4 medium was used for the tests both in Japan and China, and the set concentrations in the tests were very close, which were 0 to 3.2 mg/L in the test in Japan and 0 to 5.60 mg/L in the test in China. The tests were conducted as semi-static manner in the tests conduced in three countries. The test solution was replaced every 24 hours, and the concentration of *o*-aminophenol was analyzed before and after the replacement of the test solution at 24 hours and 48 hours later in the tests in three countries.

In the test conducted in Japan, the time-weight average concentrations in each test solution were 35.9 to 46.5% against the nominal concentration, showing relatively high level of decrease in the concentration. However, in the test conducted in China, the average concentrations of *o*-

aminophenol in the test solutions were maintained within 80 % against the nominal concentration. From these results, EC_{50} value was estimated in the test conducted in China, using the nominal concentration. In the test conducted in Japan, as the concentration of *o*-aminophenol remarkably decreased in the test solutions, measured concentrations were used for the determination of EC_{50} value.

The reason why the concentration of *o*-aminophenol decreased in the test in Japan and the concentration of *o*-aminophenol did not decrease in the test in China is not clear. One of the reasons is the difference of the chemical analysis method, including the column, detecting wavelength and mobile phase etc. Stability of *o*-aminophenol in each test solution during the test period in the test conduced in China is shown in the following table.

Table 1-4 Stability of *o*-aminophenol in the test conduced in China (Percent against the nominal concentration)

Nominal concentration	Immediately afterwards	After 24 hours		After 48 hours
		Before the replacement	After the replacement	
0.56 mg/L	107 %	88 %	96 %	86 %
1.00 mg/L	99 %	86 %	94 %	86 %
1.80 mg/L	99 %	89 %	95 %	89 %
3.20 mg/L	95 %	72 %	85 %	78 %
5.60 mg/L	87 %	69 %	90 %	84 %

As shown here, the concentrations of *o*-aminophenol in the test solution have a tendency to decrease. Specially the percentage of *o*-aminophenol against the nominal concentration is 69 % after the 24 hours at the highest test solution, although the average concentration was within 80 % against the nominal concentration.

No other major difference was observed in the other experimental conditions. While the test reports in Japan and Korea describe the coloring of the test solution after 24 hours, there is no corresponding description in the test report in China. Accordingly, it is unknown whether the test solution was colored at 24 hours later in the test in China.

As shown in Table 1-5, the cumulative immobilization ratios of Daphnia in the tests in Japan and China are very close based on the nominal concentrations.

Table 1-5 Cumulative immobilization ratios after each exposure time in the tests in Japan and China

Nominal concentration	Cumulative immobilization ratio at 24 hours later		Cumulative immobilization ratio at 48 hours later	
	Test in Japan	Test in China	Test in Japan	Test in China
0 mg/L	0 %	0 %	0 %	0 %
0.32 mg/L	0 %	—	0 %	—
0.56 mg/L	0 %	0 %	0 %	20 %
1.00 mg/L	0 %	5 %	35 %	30 %
1.80 mg/L	0 %	40 %	80 %	100 %
3.20 mg/L	40 %	45 %	95 %	100 %
5.60 mgL	—	100 %	—	100 %

In the test conducted in Japan and the test conducted in China, the following dose-response curves (concentration - cumulative immobilization ratios) are shown. According to the result for both tests, it is supposed that EC_{50} values were much closer if the nominal concentration was used in the test conducted in Japan. However, as the measured concentration (time-weight average concentration) was used in the test conducted in Japan, the difference between EC_{50} values was observed.

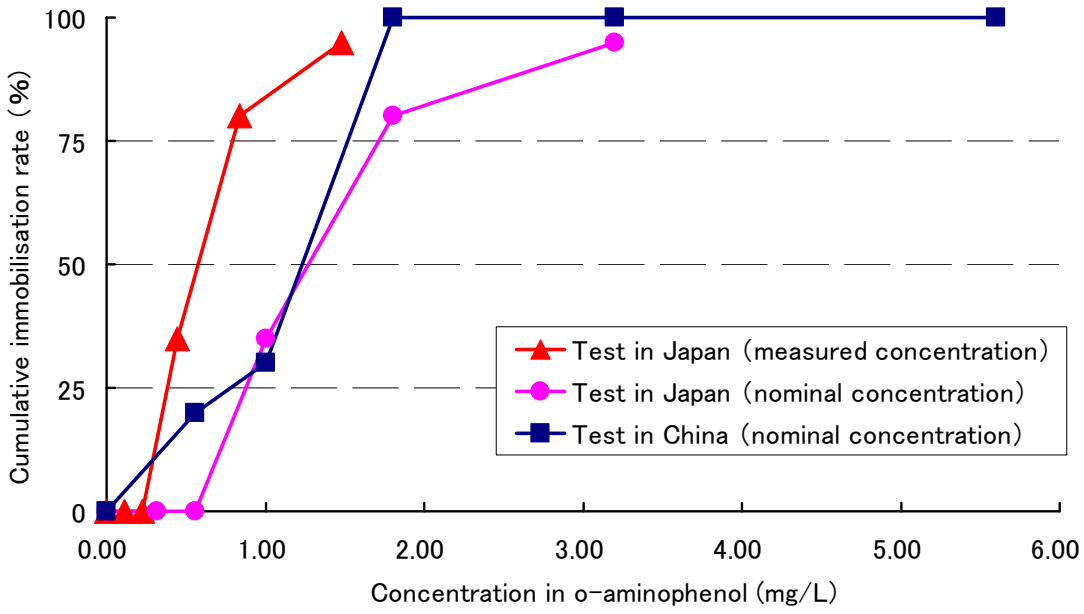

Figure 1-2 Concentration of *o*-aminophenol vs cumulative immobilization ratio of Daphnia

From these comparisons, the reason of the difference of EC_{50} values between the tests conducted in Japan and China is not clear, although the difference may be originated from whether the nominal concentration is used or the measured concentration is used. As shown in Table 1-4, the measured concentrations of *o*-aminophenol have a tendency to be decreased in the test conducted in China although the average concentration is within 80 % against the nominal concentration. Therefore, if the actual concentrations (measured concentration) are used in the test conducted in China, the EC_{50} value of Chinese test would be closer to the EC_{50} value of the Japanese test which used the measured concentration.

The following Figure shows the dose-response curves (concentrations of *o*-aminophenol vs the cumulative immobilization ratios) of the tests conducted in Japan, China and Korea based on the measured concentration.

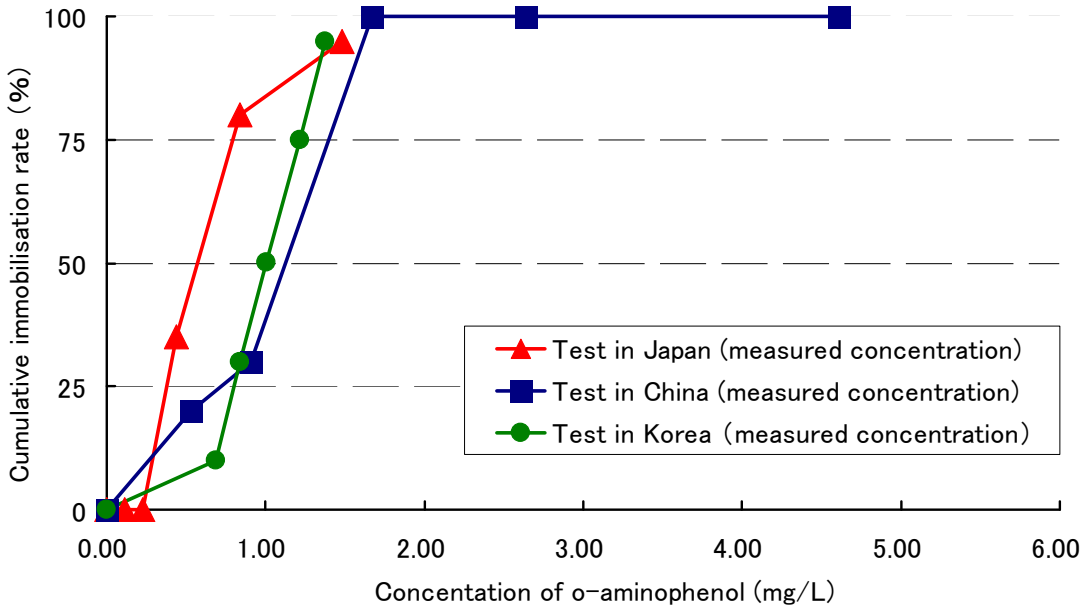

Figure 1-3 Concentrations of *o*-aminophenol vs cumulative immobilization ratios of Daphnia based on the measured concentration base

The following Figures shows the dose-response curves (concentrations of *o*-aminophenol vs the cumulative immobilization ratios) of the test conducted in Japan, China and Korea based on the nominal concentration base.

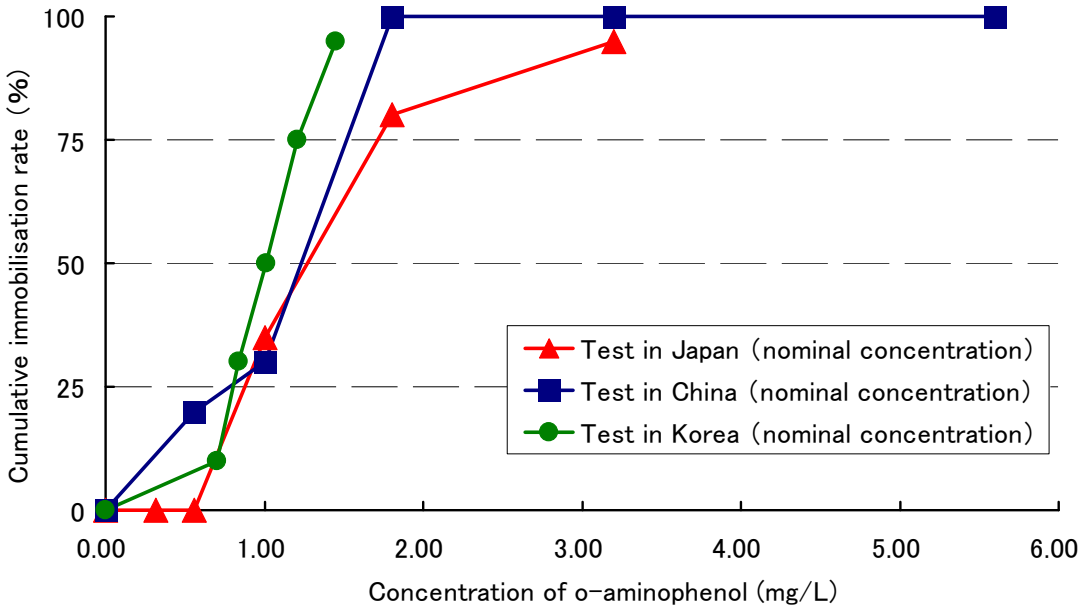

Figure 1-4 Concentrations of *o*-aminophenol vs cumulative immobilization ratios of Daphnia based on the nominal concentration base

As shown in Figure 1-4, the curves of the concentrations vs the cumulative immobilization ratios are generally the same in the tests conducted in three countries. Therefore, the difference of the EC_{50} values observed in the tests conducted in the three countries may be originated whether using the measured concentration or using the nominal concentration for its determination. In the test conduced in China, the decrease in the concentration was not observed, although the decrease in the concentration of *o*-aminophenol was observed in the test conducted in Japan. This difference reflects the difference of EC_{50} values.

The geometric factors were set as 1.8 in the tests conducted in Japan and China, the geometric factor was set as 1.2 in the test conducted in Korea. Otherwise, the experimental conditions were generally the same in the tests conducted in the three countries, and there were no differences which are suspected to affect the results.

In addition to the above, a preliminary test with static manner was conducted in Japan, although the preliminary test with semi-static manner was conducted. The set concentration with static manner was set to be as 1.0 mg/L, and the immobilization rate was measured for ten Daphnias in one test section (5/vessel with two sets). As for the result, the toxicity was strongly detected in the preliminary test with the static manner compared to the results of the preliminary with the semi-static manner. It is suggested from the result that transformed compounds may have stronger toxicity than those of *o*-aminophenol.

Table 1-6 Cumulative immobilization ratios in the semi-static manner and the static manner

Concentration	Cumulative immobilization number and immobilization rate (%)	
	24 hours later	48 hours later
Control section	0 (0 %)	0 (0 %)
1.0 mg/L (Static manner)	0 (0 %)	7 (70 %)
1.0 mg/L (Semi-static manner)	0 (0 %)	1 (10 %)

In the semi-static manner, test solution was replaced at 24 hours later.

5. Summary

While the test methods utilized in the three countries were generally the same, M4 medium was used for the tests conducted in Japan and in China, and ISO medium, in which *o*-aminophenol can be more stable, was used for the test conducted in Korea. In addition, although the experimental conditions for the tests conducted in Japan and China were generally the same, whereas the decrease in the concentration of *o*-aminophenol was observed in the test conducted in Japan, the decrease of the *o*-aminophenol concentration was not observed in the test conducted in China. Upon these observations, EC_{50} values were determined in the test conducted in Japan, using the time-weight average for the measured concentrations after the exposure, before and after the replacement of the water and at 48 hours later, while the nominal concentrations were used for calculating EC_{50} value in the test conducted in China. Because ISO medium, which is more stable than those in the other medium, was used for the test conducted in Korea, the measured concentration was close to the nominal concentration. Concerning the method for calculating the average, the time-weight average was used for the measured concentrations in the test conducted in Japan and the geometric average was used for the measured concentrations in the test conducted in Korea. In the test conducted in China, arithmetical average was used for the measured concentrations in order to check the stability of *o*-aminophenol although measured concentrations were not used for the determination of EC_{50} value.

The EC_{50} values reported in the test reports in each countries were to be as 0.574 mg/L (based on the measured concentration) in the test conducted in Japan, 1.012 mg/L (based on the nominal concentration) and 1.002 mg/L (based on the measured concentration) in the test conducted in Korea, and 1.13 mg/L (based on the nominal concentration) in the test conducted in China. The difference in the values observed in the tests conducted in the three countries may be originated because the medium as used were different and that the determination of the EC_{50} values was made using the measured concentration, or using the nominal concentration. The reason is unknown as for why that the analyzed result for the concentration of *o*-aminophenol in the test solution in the test conducted in Japan was different from those in China. However, the concentration of *o*-aminophenol in the test solution in the test conducted in China has a tendency to decrease and this tendency is same to the Japanese results.

Because the EC_{50} values obtained in the tests conducted in the three countries were very close to each other, it is thought that the difference of the results of the tests in three testing laboratories is in the level which does not cause any problems. The EC_{50} value for *o*-aminophenol relating to the acute immobilization test using Daphnia was speculated as around 0.5 to 1.2 mg/L.

Annex 1

Comparison of Daphnia magna acute toxicity according to pH condition in the preliminary test (static system)

(a) pH 6.5

Nominal Concentration (mg/L)	Measured Concentration (mg/L)	Immobilised *Daphnia* (%)	
		24 h	48 h
Control	-	0	0
0.196	ND*	0	0
0.391	0.244	0	0
0.782	0.606	20	20
1.563	1.245	60	100
3.125	2.580	100	100
6.250	5.872	100	100
12.500	13.287	100	100
25.000	23.607	100	100

* Not detected, ** LOD: 0.022 mg/L, **** LOQ: 0.066 mg/L

(b) pH 7.5

Nominal Concentration (mg/L)	Measured Concentration (mg/L)	Immobilised *Daphnia* (%)	
		24 h	48 h
Control	-	0	0
0.196	ND*	0	0
0.391	0.251	0	0
0.782	0.600	0	0
1.563	1.256	20	80
3.125	2.624	100	100
6.250	5.558	100	100
12.500	12.545	100	100
25.000	23.263	100	100

(c) pH 8.5

Nominal Concentration (mg/L)	Measured Concentration (mg/L)	Immobilised *Daphnia* (%)	
		24 h	48 h
Control	-	0	0
0.196	ND*	0	0
0.391	0.119	0	0
0.782	0.378	0	0
1.563	1.034	60	100
3.125	2.109	100	100
6.250	4.604	100	100
12.500	10.412	100	100
25.000	20.873	100	100