Comparison of fish acute toxicity tests using zebrafish reports from China, Japan and Korea
－ 3,4-Dichloroaniline －

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

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Abstract

In this study, the final reports of fish acute toxicity tests of 3,4-dichloroaniline using zebrafish in China, Japan, and Korea were compared and examined to analyze the differences among the three test results.

All tests were conducted in compliance with GLP and followed OECD test guideline 203. One of the important differences in the three countries was test type, i.e., static regime was selected in China and Korea, whereas flow-through regime was used in Japan. However, mean measured concentrations of test solutions were almost same as the nominal concentrations (87-103%) in all three countries. Body weight of the tested fish in China were twice as heavy as those used in Japan, however, the total length were almost same. As for the conditions of exposure, the hardness in China was moderate, and those in Japan and Korea were relatively low.

Although several differences were found in the test conditions of the three tests, the difference of the resultant 96h LC_{50} was within two times the amount of each respective test. 96h LC_{50} of 3,4-dichloroaniline for zebrafish was 7.15 mg/L (measured concentration) in China, 12.9 mg/L (nominal concentration) and 12.6 mg/L (measured concentration) in Japan, and 12.013 mg/L (nominal concentration) and 12.054 mg/L (measured concentration) in Korea, respectively. These values indicated that these toxicity levels were very close to each other. Therefore, it is concluded that the differences in the three countries fall in the allowable level of fluctuations.

1. Introduction

At the 8th expert meeting held in Korea, conducting a comparison study on fish acute toxicity test was agreed upon as a joint research among the three countries, and each country conducted the test using zebrafish in compliance to GLP following OECD test guideline 203. In the 9th meeting held in November 2015 in China, each country showed their results for 3,4-dichloroaniline. The three countries agreed that we need to analyze the differences in the tests in more detail and make a summary report based on the GLP final report in each country as the next step in the joint research.

2. Reports on fish acute toxicity test for joint research

In the 9th expert meeting, the countries agreed that we need the GLP final reports for this joint research. According to the agreement, the following test reports were finalized. These reports were used in this comparative study. In addition, related presentations showed by Chinese and Korean experts and also discussions in the meeting were also included. For comparison and analyses of the differences of the results, this investigation focuses on the items which are considered to affect the result of the acute toxicity test using zebrafish. In addition, the present investigation takes into consideration the "OECD test guideline 203: Fish, Acute Toxicity Test", if necessary.

<China>

- Report for Acute Toxicity to Fish (*Brachydanio rerio*) of 3,4-Dichloroaniline
  Study No.: S2015KH005-01, Report No.: R2015KH005-01, Nanjing Institute of Environmental Sciences, MEP, 2015

<Japan>

- A 96-hour Acute Toxicity Study of 3,4-Dichloroaniline in Zebrafish
  Study No.: 990006, Chemicals Evaluation and Research Institute, Japan, Kurume, 2016

<Korea>

- Acute Toxicity Study of 3,4-Dichloroaniline to Zebrafish (*Danio rerio*)
  Study No: GT15-00309, Korea Conformity Laboratories, 2015

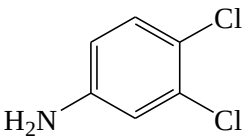
3. Comparison of fish acute toxicity tests conducted in the three countries

3.1 Test substance

3,4-dichloroaniline is a chemical substance indicated in the following structural formula, and has relatively high water solubility. It was speculated that 3,4-dichloroaniline is present in non-dissociated form in water around pH 7 based on the value of dissociation constant (pKa = 2.97).

CAS No:	95-76-1
Other names:	1-Amino-3,4-dichlorobenzene
	3,4-Dichlorobenzenamine
	Benzenamine, 3,4-dichloro-
Molecular formula:	$Cl_2C_6H_3NH_2$
Molecular weight:	162.01[1)]
Water solubility:	92 mg/L (20 ºC)[2)], 580 mg/L (20 ºC)[3)]
Partition coefficient:	log Kow = 2.69 (*n*-octanol/water)[2)]
Dissociation constant:	pKa = 2.97 (25 ºC)[2)]

Structural formula:



1) Calculated value based on atomic weight table (2013)
2) Howard, P.H., and Meylan, W.M. ed. (1997): Handbook of Physical Properties of Organic Chemicals, Boca Raton, New York, London, Tokyo, CRC Lewis Publishers: 126.
3) Verschueren, K. ed. (2009): Handbook of Environmental Data on Organic Chemicals, 5th Edition, New York, Chichester, Weinheim, Brisbane, Singapore, Toronto, John Wiley & Sons, Inc. (CD-ROM).

3.2 Comparison for items affecting test results based on each test report

Results of comparisons for items affecting the test results based on each test report are shown in Table 1.

Table 1 Comparison of fish acute toxicity test of 3,4-dichloroaniline using zebrafish

Country	**China**	**Japan**	**Korea**
Test summary			
Date of exposure	September 9, 2015- September 13, 2015	September 28, 2015- October 2, 2015	September 14, 2015- September 18, 2015
GLP	Yes	Yes	Yes
Test method	-OECD Guidelines for Testing of Chemicals, No.203 -The guidelines for the testing of chemicals (HJ/T 153-2004)	-OECD Guidelines for Testing of Chemicals, No.203 -Fish, Acute Toxicity Test stipulated in the "Testing Methods for New Chemical Substances" of Japan -OECD Guidance Document, No.23, September 2000	-OECD Guidelines for Testing of Chemicals, No.203 -Guideline for testing of chemical, National Institute of Environment Research [Notice No. 2015-8 (revised 30th Apr., 2015)]
Test substance			
Purity of substance	99.9%	100.0%	99.3%
Impurity	Not specified	Not specified	Not specified
Test organism			
Species	Zebrafish (*Brachydanio rerio*)	Zebrafish (*Danio rerio*)	Zebrafish (*Danio rerio*)
Strain	Not specified	NIES-R	Not specified
Provider	Institute of Hydrobiology, Chinese Academy of Sciences (fish supplier)	CERI Kurume (in-laboratory production)	Guppy bank (supplier)

Country	China	Japan	Korea
Acclimation	-Duration of acclimation: 7 days -Duration of no feeding: approximately 24 hours before the start of exposure	-Hatching date: June 18, 2015 (age at the start of exposure; three-month-old) -Duration of acclimation: 27 days (September 1, 2015-September 28, 2015) -Feeding amount and frequency: Amount corresponding to 3% of body weight was fed every day -Duration of no feeding: 24 hours before the start of exposure	-Duration of acclimation: 7 days -Duration of no feeding: the day before the start of exposure. -Feeding amount and frequency: approximately 2-4% of body weight per day
Size of test organisms	(Subsample) -Total length: 2.24 cm (RSD: 5.63%, 2.02-2.41 cm) -Body weight: 0.128 g (RSD: 7.15%, 0.114-0.147 g) Values show the mean, RSD, and range. Number of fish was 10.	(Control fish at the end of exposure) -Total length: 2.1±0.1 cm -Body weight: 0.051±0.010 g Values show the mean ± SD. Number of fish was 7.	(Minimum - Maximum) -Total length: 1.906-2.383 cm -Body weight: 0.051-0.115 g Values show the range. (Before the beginning of exposure) -Total length: 2.138±0.033 cm -Body weight: 0.073±0.008 g Values show the mean ± SD. (After the end of exposure) -Total length: 2.144±0.112 cm -Body weight: 0.080±0.016 g Values show the mean ± SD. Number of fish was 10.
Test condition			
Exposure duration	96 hours	96 hours	96 hours
Dilution water	Good quality tap water which had been dechlorinated for at least 24 hours	Dechlorinated tap water	Dechlorinated water
Vehicle	Not used	Not used	Not used

Country	**China**	**Japan**	**Korea**
Temperature	23.2-23.6 ℃	23.9-24.2 ℃	22.3-22.5 ℃
Aeration	None	None	Not specified
Dissolved oxygen	71-95% of the air saturation	8.1-8.2 mg/L (98-99% of the air saturation)	91.1-98.8% of the air saturation
pH adjustment	Not specified	No	Not specified
pH (treatment groups)	7.11-7.32	7.8-7.9	7.53-7.88
pH (control group)	7.19-7.29	7.8	7.83-7.87
Hardness	146-159 mg/L as $CaCO_3$ (test solution)	28 mg/L as $CaCO_3$ (dilution water)	38 mg/L as $CaCO_3$ (test solution)
Photoperiod	16 hours light/8 hours dark (1,000 lux-1,500 lux)	16 hours light/8 hours dark (Room light)	12 hours light/12 hours dark (lighting on at 08:00 a.m. and off at 08:00 p.m.)
Feeding during testing	No feeding	No feeding	No feeding
Range finding study			
Test type	Static	(1st) Semi-static(renewal at 48 hours) (2nd) Flow-through (renewal rate: approx. 24 times/day)	Static
Nominal concentration	0, 1.00, 10.0, 100 mg/L	(1st) Control, 0.300, 0.949, 3.00, 9.49, 30.0 mg/L (2nd) Control, 2.50, 5.00, 10.0, 20.0 mg/L	0, 5.821, 7.451, 9.537, 12.207, 15.625, 20.000 mg/L
Replicate	1	1	1
Number of fish (volume of test solution) and density	5 (3 L)	(1st) 2 (1 L) (2nd) 4 (approx. 1.8 L)	5 (more than 1.0 L/1.0 g fish)

Country	**China**	**Japan**	**Korea**
Result	Nominal concentration Cumulative mortality (No. of fish) 48h 96h 0 mg/L 0 0 1.00 mg/L 0 0 10.0 mg/L 2 5 100 mg/L 5 5	(1st) Nominal concentration Cumulative mortality (%) 48h 96h Control 0 0 0.300 mg/L 0 0 0.949 mg/L 0 0 3.00 mg/L 0 0 9.49 mg/L 0 0 30.0 mg/L 100 100 (2nd) Nominal concentration Cumulative mortality (%) 48h 96h Control 0 0 2.50 mg/L 0 0 5.00 mg/L 0 0 10.0 mg/L 0 0 20.0 mg/L 75 100	Nominal concentration Cumulative mortality (%) 96h Control 0 5.821 mg/L 0 7.451 mg/L 0 9.537 mg/L 20 12.207 mg/L 60 15.625 mg/L 100 20.000 mg/L 100
Others	-	In the range finding study conducted by semi-static regime, degradation product was confirmed in the test solution. Therefore, the flow-through regime was adopted in the definitive study to reduce the degradation product concentration in the test solution.	-
Definitive study			
Test type	Static	Flow-through (renewal rate: approx. 24 times per day)	Static
Nominal concentration	0, 1.00, 2.00, 4.00, 6.00, 8.00, 10.0 mg/L	Control, 4.94, 7.41, 11.1, 16.7, 25.0 mg/L Concentration was arranged as geometrically with a ratio of 1.5.	0, 5.821, 7.451, 9.537, 12.207, 15.625 mg/L Concentration was arranged as geometrically with a ratio of 1.28.
Replicate	1	1	Not specified
No. of fish	10 fish/test group	7 fish/test group	10 fish/test group

Country	**China**	**Japan**	**Korea**
Test vessel	Approximately 5 L Glass tank, with a sealable inert lid	3 L Glass tank, transparent plastic lid	Chemically inert 11 L glass tank, cover for reducing the loss of water and entry of dust
Volume of test solution	3 L/test group	Approx. 1.8 L/test group	10 L/test group
Frequency of chemical analysis	At the start of exposure, 24, 48, 72 and 96 h	At the start and end of exposure or at the time that mortality of all test organisms were confirmed to be dead	At the beginning (0 h), 48 h and 96 h
Analytical method	UPLC	HPLC	HPLC
Statistical method (software)	Trimmed Spearman-Karber Method (Version 1.5, USEPA)	Binomial test (Microsoft Excel)	Probit method (SPSS Ver. 12.0)
Results			
Measured concentration	Nominal concentration Measured concentration as mean 1.00 mg/L → 0.888 mg/L (88.8%) 2.00 mg/L → 1.78 mg/L (89.0%) 4.00 mg/L → 3.48 mg/L (87.0%) 6.00 mg/L → 6.18 mg/L (103%) 8.00 mg/L → 7.45 mg/L (93.1%) 10.0 mg/L → 9.48 mg/L (94.8%)	Nominal concentration Measured concentration as arithmetic mean 4.94 mg/L → 4.94 mg/L (100%) 7.41 mg/L → 7.42 mg/L (100%) 11.1 mg/L → 11.0 mg/L (98.8%) 16.7 mg/L → 15.9 mg/L (95.1%) 25.0 mg/L → 24.5 mg/L (98.1%)	Nominal concentration Measured concentration as geometric mean 5.821 mg/L → 5.855 mg/L (100.6%) 7.451 mg/L → 7.445 mg/L (99.9%) 9.537 mg/L → 9.507 mg/L (99.7%) 12.207 mg/L→12.303 mg/L (100.8%) 15.625 mg/L→15.583 mg/L (99.7%)
Cumulative mortality	Nominal concentration Cumulative mortality (%) 48h 96h 0 mg/L 0 0 1.00 mg/L 0 0 2.00 mg/L 0 0 4.00 mg/L 0 0 6.00 mg/L 0 20 8.00 mg/L 10 40 10.0 mg/L 80 100	Nominal concentration Cumulative mortality (%) 48h 96h Control 0 0 4.94 mg/L 0 0 7.41 mg/L 0 0 11.1 mg/L 0 14 16.7 mg/L 14 100 25.0 mg/L 100 100	Nominal concentration Cumulative mortality (%) 48h 96h 0.000 mg/L 0 0 5.821 mg/L 0 0 7.451 mg/L 0 0 9.537 mg/L 0 20 12.207 mg/L 10 40 15.625 mg/L 40 100

Country	**China**	**Japan**	**Korea**
LC_{50} (95% confidence limits: mg/L)	-24h: 9.11 mg/L -48h: 8.55 mg/L (8.08-9.05) -72h: 7.59 mg/L (6.90-8.34) **-96h: 7.15 mg/L** (6.36-8.04) (measured concentration)	-24h: 21.6 mg/L (nominal concentration) -48h: 19.3 mg/L (nominal concentration) -72h: 14.4 mg/L (nominal concentration) **-96h: 12.9 mg/L** (nominal concentration) **-96h: 12.6 mg/L** (measured concentration)	-48h: > 15.625 mg/L (nominal concentration) -48h: > 15.583 mg/L (measured concentration) -**96h: 12.013 mg/L** (10.871-13.541) (nominal concentration) **-96h: 12.054 mg/L** (10.896-13.559) (measured concentration)

4. Discussion

4.1 Test type

One of the important differences in the three countries is test type. Static regime was selected in China and Korea, whereas flow-through regime was used for the 2nd range finding and definitive tests in Japan.

In the 1st range-finding experiment conducted by semi-static regime in Japan, degradation product was detected in the test solution of 0.300 mg/L. Zok et al.[4)] exposed zebrafish to 3,4-dichloroaniline for 96 hours in a semi-static regime and the biotransformation product was analyzed. As a result, they found that the aniline was transformed into the corresponding acetanilide. Thus, there is a possibility that the degradation product was a metabolite of the test item produced by zebrafish. In addition, there is another possibility of the degradation product being caused by oxidation because aniline is known to be easily oxidized. Therefore, the flow-through regime was adopted in the definitive study to reduce the degradation product concentration in the test solution in Japan. As a result, degradation product was not detected in the definitive study in Japan.

China and Korea conducted the test in static regime, and no degradation products were detected in the chromatogram in the definitive study for either country. It is not clear why the degradation product was detected only in Japan. One of the reasons is the difference of the chemical analysis method, including the column, detecting wavelength and mobile phase etc. Other reasons might be the difference in fish strain or environmental factors, which might affect the generation of degradation product.

In any case no degradation product was detected in the definitive study in the three countries. In addition, the mean concentrations of 3,4-dichloroaniline in the test solution were maintained within 87-103% of the nominal concentrations in the definitive study in the three countries. Therefore, the difference in test type does not essentially come to an issue in comparison of fish acute toxicity tests in the three countries.

4.2 Test fish

Acclimation period of zebrafish was at least 7 days in the three countries, which is appropriate to the OECD test guideline 203.

Whereas the number of organisms in Japan was 7 fish/test level, those in China and Korea were

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4) Zok S, Görge G, Kalsch W, Nagel R. 1991. Bioconcentration, metabolism and toxicity of substituted anilines in the zebrafish (*Brachydanio rerio*). *Sci Total Environ* 109/110: 411-421.

10 fish/test level. However, these are appropriate to OECD test guideline 203, i.e., at least 7 fish/test level.

The mortality in the control was 0% at the end of the test in the three countries, which met the criterion for the validity of the OECD test guideline 203 (not exceed 10% or one fish if less than ten are used).

Total length was 2.24 cm (average) in China, 2.1 cm (average) in Japan and 19.06-23.83 mm (minimum - maximum) in Korea, respectively. These values were within the range of 2.0±1.0 cm, which is the recommended value in the OECD test guideline 203.

But the body weight was significantly different among these countries. The largest body weight was 0.128 g (average) in China, 0.051-0.115 g (minimum - maximum) in Korea, and 0.051 g (average) in Japan, indicating that the difference was more than double between China and Japan. The reason for the difference in body weight was not clarified. We may need to conduct another comparison study on rearing and acclimation conditions, including feeding, origin of test fish (strain), age, and density. In any case, no standard values concerning body weight were indicated in OECD test guideline 203. Therefore, there is no problem regarding the difference in body weight in terms of adhering to the test guideline.

4.3 Conditions of exposure

During the exposure, the water temperatures of the test solution of the three countries were 23.2-23.6 °C in China, 23.9-24.2 °C in Japan, and 22.3-22.5 °C in Korea, respectively. These showed that each test was conducted in a constant temperature within the range of 2 °C and also appropriate to the recommended value for zebrafish (21-25 °C) in OECD test guideline 203.

The pH values in the test solution during the exposure were 7.11-7.32 in China, 7.8-7.9 in Japan, and 7.53-7.88 in Korea, respectively. The pH values in the three countries were near neutral and the pKa of 3,4-dichloroaniline was 2.97, indicating that 3,4-dichloroaniline was present in non-dissociated form in the test solutions and pH values were not related to the toxicity in the three countries.

The dissolved oxygen in the test solution during the exposure was 71-95% of air saturation in China, 8.1-8.2 mg/L (98-99% of air saturation) in Japan, and 91.1-98.8% of air saturation in Korea, respectively. These were higher than 60% of air saturation, which is the validity of the test in OECD test guideline 203.

Hardness was 146-159 mg/L $CaCO_3$ (test solution) in China, 28 mg/L $CaCO_3$ (dilution water) in

Japan, and 38 mg/L $CaCO_3$ (test solution) in Korea, respectively, and the value in China was moderate, while those in Japan and Korea were relatively low. Also, the values in the three countries were between 10 and 250 mg/L $CaCO_3$, which is the recommended value in the OECD test guideline 203. In addition, 3,4-dichloroaniline is not an ionic substance which is known to be affected by hardness. Therefore, it is considered that the difference in the value of hardness is not related to the toxicity in the three countries.

Biomass loading rate in China was 0.427 g fish/L (0.128 g×10 fish/3 L) and that in Korea was less than 1.0 g fish/L. These values were appropriate to the recommendations (1.0 g fish/L) for static and semi-static tests of OECD test guideline 203. Loading in Japan was adequately low because the flow-through system was adopted.

Photoperiod in China and Japan was 16-hour light/8-hour dark, and 12-hour light/12-hour dark in Korea. These conditions are appropriate to the recommendation (12 to 16 hours photoperiod daily).

Overall, conditions of exposure in the three countries were within the OECD test guideline 203, and it is considered that the difference in these did not affect toxicity values.

4.4 LC_{50}

During the exposure, abnormal responses were recorded in the three countries as shown in annex Tables 1-3. In China, fish lying on side or back were observed. In Japan and Korea, reduced activity and loss of equilibrium were mainly observed. No abnormalities were observed in the control group in the three countries.

The concentration-cumulative mortality curves at 96 hours in the three countries are shown in Figure 1. The figure indicates that the curves in the three countries are almost same. Although the curve in China is slightly apart from those of the other two countries, the slopes of the curves of the three countries are almost same.

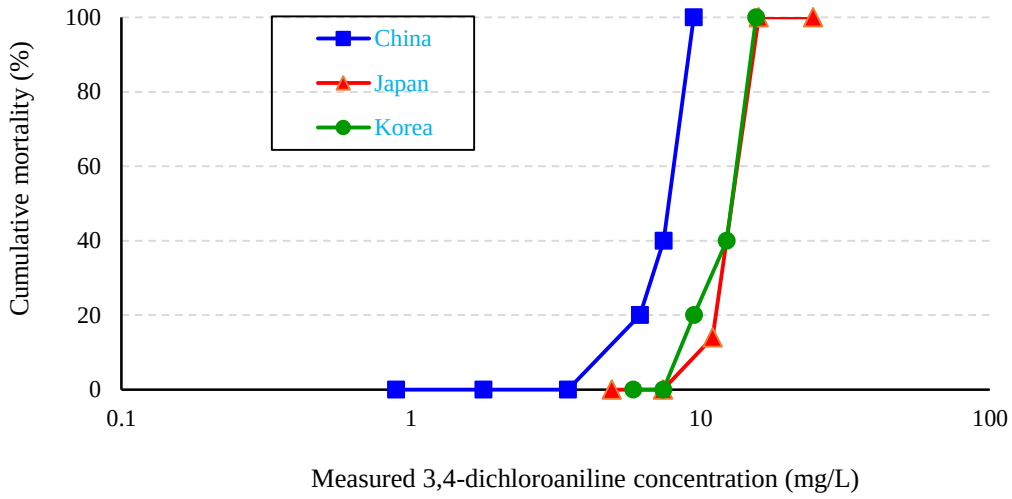

Figure 1　Concentration-cumulative mortality curves at 96 hours in the three countries.

LC_{50} was calculated by Trimmed Spearman-Karber Method (Version 1.5, USEPA) in China, Binomial test (Microsoft Excel) in Japan, and Probit method (SPSS Ver. 12.0) in Korea. Although the calculating methods were different in the three countries, these methods are common and scientifically acceptable.

The 96h LC_{50} of 3,4-dichloroaniline for zebrafish was 7.15 mg/L (measured concentration) in China, 12.9 mg/L (nominal concentration) and 12.6 mg/L (measured concentration) in Japan, and 12.013 mg/L (nominal concentration) and 12.054 mg/L (measured concentration) in Korea, respectively. The difference in 96h LC_{50}s in the three countries was within two times the amount of each respective test, indicating that these toxicity levels were very close to each other. Therefore, it is considered that the differences in the 96h LC_{50}s of the three countries are in a level which does not cause any problems.

There are several existing data on 96h LC_{50} of 3,4-dichloroaniline for recommended test fish of OECD test guideline 203. The 96h LC_{50} values of 3,4-dichloroaniline for medaka (*Oryzias latipes*), fathead minnow (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*) are 11.0 mg/L[5)], 6.99 mg/L[5)], and 1.94 mg/L[7)], respectively. The 96h LC_{50} values of 3,4-dichloroaniline for zebrafish in this study are 7.15-12.9 mg/L, showing that the toxic value of zebrafish is very close to those in medaka and fathead minnow.

5) Ministry of Health, Labour and Welfare, Ministry of Economy, Trade and Industry, Ministry of the Environment. Japan Chemical Collaborative Knowledge database (J-CHECK)

5) Call, D.J., S.H. Poirier, M.L. Knuth, S.L. Harting, and C.A. Lindberg (1987): Toxicity of 3,4-Dichloroaniline to Fathead Minnows, *Pimephales promelas*, in Acute and Early Life-Stage Exposures. Bull.Environ.Contam.Toxicol. 38(2):352-358.

7) Hodson, P.V. (1985): A Comparison of the Acute Toxicity of Chemicals to Fish, Rats and Mice. J.Appl.Toxicol. 5(4):220-226.

Annex Table 1 Abnormal responses observed during definitive test in China

Nominal Conc. (mg/L)	Measured Conc. (mg/L)	Visual Observations					
		3h	6h	24h	48h	72h	96h
0 (Blank Control)	ND	10NB	10NB	10NB	10NB	10NB	10NB
1.00	0.888	10NB	10NB	10NB	10NB	10NB	10NB
2.00	1.78	10NB	10NB	10NB	10NB	10NB	10NB
4.00	3.48	10NB	10NB	10NB	10NB	10NB	10NB
6.00	6.18	9NB& 1SR	7NB& 3SR	7NB& 3SR	5NB& 5SR	5NB& 4SR& 1Dead	5NB& 3SR& 2Dead
8.00	7.45	4NB& 6SR	4NB& 6SR	2NB& 8SR	2NB& 7SR& 1Dead	7SR& 3Dead	6SR& 4Dead
10.0	9.48	10SR	10SR	4SR& 6Dead	2SR& 8Dead	10Dead	10Dead

Note: NB-Normal behaviors; SR-Fish lying on side or back.

Annex Table 2 Abnormal responses observed during definitive test in Japan

Nominal concentration (mg/L)	Result of observation (Left column: Number of affected fish/Total survival number, Right column: Symptom detail)									
	3h		24h		48h		72h		96h	
Control	0/7	N	0/7	N	0/7	N	0/7	N	0/7	N
4.94	0/7	N	0/7	N	1/7	HEM(1) [a)]	1/7	HEM(1) [a)]	2/7	HEM(1) [a)] SP(1)
7.41	3/7	PLE(3) RA(3)	4/7	CLE(2) PLE(2) RA(1)	4/7	CLE(1) PLE(2) RA(3)	4/7	PLE(4) RA(3)	3/7	PLE(2) SP(1)
11.1	7/7	CLE(7) RA(3)	7/7	CLE(7) RA(7)	7/7	CLE(7) RA(7)	7/7	CLE(7) RA(7) HEM(1) [b)]	6/6	CLE(6) RA(6) SP(2)
16.7	7/7	CLE(7) RA(1)	7/7	CLE(7) RA(7)	6/6	CLE(5) LETH(1) RA(5)	1/1	CLE(1) RA(1)	-	-
25.0	7/7	CLE(7) RA(7)	1/1	CLE(1) RA(1)	-	-	-	-	-	-

N: Normal (No abnormal response)

- : No observation due to 100% mortality

Value in parentheses expresses the number of individuals that showed symptoms.

Abbreviation of symptoms

CLE: Complete loss of equilibrium

HEM: Hemorrhage [observed point: a) base of pectoral fin, b) edge of mouth]

LETH: Lethargic

PLE: Partial loss of equilibrium

RA: Reduced activity

SP: Scale protrusion

Annex Table 3 Abnormal responses observed during definitive test in Korea

Nominal conc. (mg/L)	Measured conc. (mg/L)	24h	48h	72h	96h
0.000	N.D.[1)]	N(10)[2)]	N(10)	N(10)	N(10)
5.821	5.855	N(6), LE[3)](4), RA[4)](2)	N(6), LE(4), RA(3)	N(6), LE(4), RA(3)	N(6), LE(4), RA(3)
7.451	7.445	LE(10), RA(8)	LE(10), RA(8)	LE(10), RA(8)	LE(10), RA(9)
9.537	9.507	LE(10), RA(9)	LE(10), RA(10)	LE(9), RA(8), I[5)](1), D[6)](1)	LE(8), RA(6), I(2), D(1)
12.207	12.303	LE(10), RA(10)	LE(9), RA(9), D(1)	LE(7), RA(7), D(2)	LE(8), RA(6), I(2), D(1)
15.625	15.583	LE(10), RA(10)	LE(6), RA(6), D(4)	D(6)	-

1) N.D.: Not detected, 2) N: Normal, 3) LE: Loss of equilibrium, 4) RA: Reduced activity

5) I: Immobilization (Unable to swim in spite of movements of gills or fins), 6) D: Death