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Inter- and Intra-Laboratory Testing of the *Daphnia magna* IQ Toxicity Test™

K. R. Hayes, W. S. Douglas, J. Fischer

Aqua Survey Inc., 499 Pt Breeze Road, Flemington, New Jersey 08822, USA

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The United States Environmental Protection Agency (USEPA), through the Clean Water Act has mandated toxicity testing as part of routine monitoring of effluent discharges. Many state agencies have implemented these regulations and included toxicity limits in discharge permits and penalties for non-compliance. Although standardized bioassay protocols have been utilized to assess compliance (USEPA guidance), results of toxicity tests are usually not available for between 48 and 96 hours following sample collection. Recently, the *Daphnia magna* IQ Toxicity Test™ has been proposed as a technique for obtaining toxicity information in as little as 75 minutes following sample collection (ASTM, 1993). The IQ Toxicity Test™ assesses toxicity by observing, *in vivo*, the cleavage of the fluorometric biomarker methylumbelliferyl galactoside (MUF). Animals are exposed to a toxicant for one hour and then a solution of biomarker substrate is added directly to the exposure chamber. Those organisms that feed normally and with functional galactosidase enzyme systems are able to cleave the marker from the substrate. The fluorescent marker is freed to the hemolymph of the organism and is readily observed visually using long wave UV light. When this biomarker test is correlated to standard 48-hour toxicity tests, agreement has been shown to be greater than 95 % (Janssen and Persoone 1993, Hayes *et al.* 1993).

Inter-laboratory tests have been recognized as an important aspect of test method validation by both the USEPA and ASTM. Although during test development, problems with intra-laboratory variation were not observed (unpublished data), inter-laboratory tests have not been reported. Factors which may contribute to inter- and intra-laboratory test precision include organism health, age, test temperature, variations in culture and handling, and feeding (Rue *et al.* 1988). Although controlling these factors would likely increase the precision of any toxicity test, the *Daphnia magna* IQ Toxicity Test™ was intended to be used in a variety of conditions, with cultured or shipped organisms, and with little or no training. The effects of these factors on test precision have not been assessed. To determine the precision of the *Daphnia magna* IQ Toxicity Test™, 16 laboratories were selected representing academia, government agencies, and industry, to perform the test under conditions typical of test usage.

Correspondence to: K. R. Hayes

MATERIALS AND METHODS

The IQ Toxicity Test™ is designed to be a simple, rapid toxicity test that requires little or no previous experience to conduct, therefore no previous experience was required to participate in the round robin. Sixteen laboratories agreed to participate in the study, these included six industrial, four governmental, three academic, and three independent testing laboratories. Each laboratory was asked to perform five IQ Toxicity Tests™ on a single toxicant, copper sulfate. The first two tests were to be practice tests, the last three tests would be definitive tests. This design allowed for evaluation of the test on two levels; both intra-laboratory variability and overall inter-laboratory variability.

A single batch of the selected toxicant, pentahydrate copper sulfate, was prepared to a nominal concentration of 500 mg/L $CuSO_4 \cdot 5H_2O$ in a 1% w/v HNO_3 acidified solution, then dispensed to glass ampules and sealed. Five ampules were selected randomly and were analyzed for copper by atomic absorption spectrophotometry to verify the concentration and variability between ampules. A separate stock solution was prepared for each definitive test. First, a 1:1000 dilution in culture water was prepared using a new ampule of toxicant. This resulted in a 0.5 mg $CuSO_4 \cdot 5H_2O$/L working stock solution. At all but one facility, a 0.5 factor concentration series was prepared from this stock, to provide 0.50, 0.25, 0.125, 0.0625 and 0.03125 mg $CuSO_4 \cdot 5H_2O$/L. At one facility (number 16) the highest concentration was 0.25 and the lowest concentration was 0.0156 mg $CuSO_4 \cdot 5H_2O$ per L. This lower range was established based on results of the practice tests.

All materials and supplies needed for the testing were sent to each participating laboratory as kits produced under the trade name, *Daphnia magna* IQ Toxicity Test™. In addition to the test kits, each laboratory was also provided five copper sulfate toxicant ampules and detailed round robin testing instructions. Two ampules were to be used on two practice tests and the remaining three ampules were for the definitive tests.

Adult *D. magna* were cultured in moderately hard reconstituted water (MHRW) (USEPA, 1991) and were shipped by overnight express to facilities that requested test organisms. Young collected from these adults were held in MHRW and fed daily a mixture of algae (*Selenastrum capricornutum* or *Ankistradesmus sp.*) and a mixture of yeast, cereal leaves, and trout chow (YCT). Prior to shipping, culture vessels containing the young *D. magna* were drained, refilled to rinse, and drained again to concentrate the organisms. Organisms were then transferred by pipet to plastic shipping containers containing MHRW lacking feed. The low-volume head space was oxygenated prior to overnight express shipment. Of the 16 participating laboratories, 7 requested that test organisms be shipped from the host laboratory, Aqua Survey, Inc. The remaining laboratories used in-house cultures. All tests were conducted with organisms from 1-5 days old and were cultured according to currently accepted methods (USEPA, 1991).

Once obtained by the participating laboratory, all *D. magna* were acclimatized to test conditions (laboratory culture water and ambient laboratory temperature and lighting) for at least 24 hours prior to testing. Organisms for any one test were within a one to two day age span. For six to 24 hours prior to testing, organisms were held in water lacking feed. This starvation period produces more consistent organism response and faster scoring (Aqua Survey Inc., 1989).

To assess overall suitability of the test organisms for use with the IQ Toxicity Test™, 18 individuals from a prepared batch of *D. magna* were placed in a glass tube or beaker containing 20 mL of dilution water. After approximately ten minutes, 0.5 mL of IQ Additive™ (a 2000 mg/L aqueous solution of 4-methylumbelliferyl β-D-galactoside, or MUG) was added to the beaker. After an additional fifteen minutes, the beaker was illuminated with a longwave ultraviolet light source. At least 15 of 18 *D. magna* individuals must fluoresce to have an acceptable pretest. *D. magna* used in the pretest were not used again in other testing.

Each IQ Toxicity Test™ was conducted in specially manufactured acrylic six-compartment test chambers according to directions provided with the kit (Aqua Survey, 1989). Dilutions were prepared directly in each compartment by adding, consecutively, dilution water to one designated line and the working stock of copper sulfate toxicant to the volume, 10 mL, line. Each test was conducted in triplicate; i.e., three six-compartment chambers were used, with each compartment stocked with six *Daphnia magna* (n = 18 per treatment). Organisms were quickly transferred to each compartment, and one hour after stocking, the fluorogenic substrate (0.25 mL of a 2000 PPM solution of MUG) was added to each compartment. Fifteen minutes later, all compartments were observed using a hand held longwave ultraviolet light. Scoring was conducted by comparing the fluorescence of individuals in a test solution compartment to the (average) fluorescence of control organisms. An organism not fluorescing at least as brightly as the control group was scored as "effected. " A test was considered valid if at least 15 of 18 organisms were glowing in the controls. Due to the fact that the test is provided in kit form, there was no attempt to further standardize test organism source, holding and acclimatization methods, or dilution water. The variability observed could then be assumed to be the variability expected during normal use of the kit.

In addition to the IQ Toxicity Tests™, a single standard 48-hour bioassay (USEPA, 1991) was also conducted by the host laboratory. This test utilized the same water, stock toxicant solution, and test organism source as used in the fluorescent test. Test volume was 50 mL per plastic chamber, stocked with 10 individuals in each of two chambers per treatment.

All EC_{50} and LC_{50} values were determined using an USEPA computer program which calculated the median effective concentration based on the probit, moving average or binomial methods as appropriate (Stephen, 1977). Coefficients of

variation were calculated on the reported EC_{50}s for replicate tests within each laboratory, for the average reported EC_{50} across laboratories using similar organism sources, and for the average reported EC_{50} across all laboratories.

RESULTS AND DISCUSSION

Laboratory analyses for copper in five toxicant ampules containing the nominal 500 mg $CuSO_4 \cdot 5H_2O$/L averaged 121.2 mg Cu/L (s = 10.6 mg Cu/L). The individual results are 104, 130, 122, 120, and 130 mg Cu/L. For simplicity, all results discussed below are in nominal concentration of pentahydrate copper sulfate (% Cu = 25.4).

The reported EC_{50}s for copper sulfate ranged from 0.029 to 0.259 mg/L with all sixteen laboratories were able to report at least one valid test (Table 1). Four laboratories produced only two valid tests, and one laboratory produced only one valid test. At least half of the poor control responses appear to be due to shipping stress on the test organisms. The average EC_{50} across all laboratories and all reported tests for copper sulfate pentahydrate was 0.113 mg/L (s = 0.059) (Table 1). This compares to a 48-hour LC_{50} of 0.082 mg/L using standard USEPA methodology. Although there was a noticeable (albeit statistically insignificant) difference in average EC_{50} for copper sulfate depending on the source of organisms used (0.074 mg/L for organisms supplied by Aqua Survey, 0.144 mg/L for organisms supplied by the participating laboratory), the coefficient of variation (CV) was almost identical between the two groups (41.5% for organisms supplied by Aqua Survey, 41.0% for organisms from the participating laboratory). The overall inter-laboratory CV was 52.4%. By dropping out the lowest and highest mean EC_{50}, he CV dropped to 41.6 % .

The inter-laboratory variation observed for the IQ Toxicity Test™ is within the range reported for aquatic bioassays. In a review of inter-laboratory data for aquatic toxicity tests, 85% of the CVs for *Dahnia spp* tests ranged from 050%, with 50% of the tests being within 0 and 20% (Rue *et al.*, 1988). The USEPA (1991) reported CVs of 166% for 12 laboratories exposing *D. magna* to silver chloride, 51% for 11 laboratories exposing *D. magna* to endosulfan, and 50% for 11 laboratories exposing *Ceriodaphnia dubia* to potassium chloride. Finally, in another test examining the effects of test temperature and duration, the CV of the 48-hour LC_{50} for three toxicants to *D. magna* ranged from 21 to 58 percent.

Irma-laboratory variability observed in this study ranged from a coefficient of variation (CV) of 4.7% to 58.4%, with an average of 22.0%. A summary of the distribution of intra-laboratory variability is provided in Table 2. Most (58.8%) of the facility data sets had intra-laboratory CVs of less than 20 percent. Fifteen of 17 facility data sets had intra-laboratory CVs below 40%. The intra-laboratory variation typically observed for *Daphnia spp.* tests is less than 20% (Rue *et al.*, 1988).

Table 1. Summary of results of IQ Toxicity Tests with pentahydrate copper sulfate. Results in nominal dose mg $CuSO_4.5H_2O$/L. ICR = Insufficient control response. NC = not calculated. NP = not provided.

Facility	Organism Source	Water Hardness	Results of IQ Test (EC_{50} in mg/L) Test #1	Test #2	Test #3	Average EC_{50} (mg/L)	Inter-laboratory CV (%)
16	A	104	0.04	0.03	0.03	0.029	19.0
7	A	114	0.06	0.03	0.05	0.043	40.1
9	A	124	ICR	0.06	ICR	0.059	NC
14	A	64	0.04	0.08	0.08	0.067	33.1
3	O	100	0.07	0.07	0.07	0.069	6.8
1	A	100	0.07	0.09	ICR	0.078	24.4
6	O	77	0.08	0.07	0.09	0.080	17.2
2	O	88	0.10	0.10	0.06	0.085	29.1
11	A	100	0.10	0.09	0.09	0.094	6.3
5	A	60	0.18	0.09	ICR	0.105	18.2
8	A	91	0.12	0.10	0.14	0.119	17.2
15	O	NP	0.18	0.13	0.14	0.127	9.1
10	O	96	0.15	0.17	0.08	0.134	35.9
9	O	124	0.15	ICR	0.17	0.156	10.2
13	O	100	0.15	0.17	0.16	0.159	9.3
8	O	91	0.13	0.23	0.14	0.167	35.7
12	O	75	0.22	0.20	0.20	0.206	4.7
4	O	130	0.16	0.35	ICR	0.259	58.4

Table 2. Cumulative distribution of variability in intra-laboratory response. Coefficients of variation in IQ Test response (n=3 for each lab except where noted).

Coefficient of Variation	Number of Labs	Cumulative Percent
0.1 to 10	5	29.4
10.1 to 20	5	58.8*
20.1 to 30	2	70.6**
30.1 to 40	3	88.2
40.1 to 50	1	94.1
Over 50	1	100*

* n = 2 for one lab
** n = 2 for two labs

These data indicate that the *Daphnia magna* IQ Toxicity Test™ has an inter-laboratory precision in the range of that of the standard 48-hour *D. magna* bioassay. Considering that little attempt was made to control for organism age, culturing technique, and laboratory conditions, the precision is much higher than expected. Further, it is reasonable to expect that with practice, experience, and refinement of technique, that intra-laboratory variation will decrease. In that regard, after as few as two practice tests (which applies to most of the facilities) the facilities yielded data in which ten of 17 data sets had CV's less than 20 percent. The average CV of these ten data sets is 11.8 percent, well within the reported CV of the standard *Daphnia* toxicity tests.

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