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Acute Toxicity of Ammonia to *Ceriodaphnia dubia* and a Procedure to Improve Control Survival

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A question often raised during routine toxicity testing of effluent from waste water treatment plants is how large a portion of the observed toxicity is caused by ammonia. One organism, *Ceriodaphnia dubia*, is used extensively as test organism for Whole Effluent Toxicity (WET) testing under the National Pollutant Discharge Elimination System (NPDES). However, limited information has been published on the toxicity of ammonia to *C. dubia.* A literature search of several data bases revealed only two reports dealing specifically with the acute toxicity of ammonia to *C. dubia* (Nimmo *et al.* 1989; Cowgill and Milazzo 1991).

In aquatic systems, ammonia may exist in both the un-ionized (NH_3) and the ionized (NH_4^+) form. The toxicity of ammonia to aquatic organisms has primarily been linked to the un-ionized form (Emerson *et al.* 1975, Nimmo *et al.* 1989, Ankley *et al.* 1990) with a few reports linking it to total ammonia or the ammonium ion (Borgman 1994). The concentration of un-ionized ammonia is dependent on pH, temperature and total ammonia species. At a given concentration of total ammonia, pH has a greater influence than temperature on the concentration of un-ionized ammonia (Emerson *et al.* 1975). Under test conditions of pH = 8.00 and temperature = 25°C a pH change of 1.5% will cause a change in the un-ionized ammonia concentration ranging from 24 to 30%, whereas a temperature change of 1.5% will cause a change ranging from 2 to 4%.

The first of the two LC50 values for acute toxicity of ammonia to *C. dubia* in the literature is reported by Nimmo *et al.* (1989) with a 48-h value of >1.43 mg/L un-ionized ammonia. The other LC50 value is by Cowgill and Milazzo (1991) who graphically show a 48-h LC50 of 9 mg/L total ammonia. Both LC50 values are based on survival rates in 7-day reproduction tests with *C. dubia* that were conducted under chronic test conditions (EPA 1989). The two reports give limited information on the two environmental factors, pH and temperature, controlling the levels of un-ionized ammonia. The objectives for the present study were to 1) measure the 48-h acute toxicity of ammonia to *C. dubia* under well-defined acute test conditions (EPA 1993), 2) to improve the estimate of the acute toxicity of ammonia to this important WET test organism, and 3) to develop a procedure for improving control survival in acute tests with *Ceriodaphnia*.

MATERIALS AND METHODS

The test material was ammonium chloride (NH_4Cl) (Fisher, Lot No. 965553) with a reported purity of 99.5%. The nominal concentrations for the dilution series were 0,0.6, 1.2, 1.8, 2.4 and 3.0 mg/L un-ionized ammonia. The appropriate amount of

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ammonium chloride was weighed out for each concentration based on a pH of 8.0 and a temperature of 25°C. Addition of ammonium chloride caused a decrease in pH which was re-adjusted to approximately 8.0 with a potassium hydroxide solution (maximum 1 ppm) prior to testing. Lake Washington water has a very limited buffer capacity; hence no pH adjustment was performed on the 0 mg/L un-ionized ammonia concentration (control).

The water used in the tests was collected by handdipping from a site at the middle of Lake Washington, Seattle, WA. The water was stored at 4°C and filtered to 0.45 µm before use. The lake water has been used extensively by this laboratory for several years. Mean values (± SD) for water quality parameters during testing were: pH 7.92 ± 0.34; hardness 38.1 ± 1.9 mg/L as $CaCO_3$; alkalinity 35.6 ± 1.7 mg/L as $CaCO_3$; and conductivity 96.0 ± 3.7 µmhos/cm. The metals content of the lake water was below the reported detection limits (Cd < 0.015, Cr < 0.025, Cu < 0.02, Pb < 0.15, Zn < 0.025 mg/L). Annual measurements of priority pollutant organic compounds in the lake water showed two of 109 compounds above the reported detection limits: bis(2-ethylhexyl) phthalate (5.15 µg/L) and diethyl phthalate (0.98 µg/L).

Total ammonia concentrations measured as ammonia nitrogen (NH_4^+-N + NH_3-N) were determined using the phenate method (Chaney and Marbach 1962). During testing, analyses were performed on all concentrations within the first 8 hours after addition of test organisms. The analyzed ammonia concentration, the pH and the water temperature measurements were used to calculate the respective un-ionized ammonia (NH_3-N) concentrations.

Continuous in-house cultures of *Ceriodaphnia dubia* were raised at 25 ±1°C in static-renewal mass cultures, in 1-L crystallizing dishes filled with 900 mL Lake Washington water (LWW), filtered to 0.45 µm. The cultures were illuminated with full-spectrum fluorescent lights on a 16-h light : 8-h dark cycle at 538- 1076 lux. The stock culture in each dish was fed daily with the green algae *Selenastrum capricornutum* (1 mL @ $36x10^6$ cells per mL) and a diet of YT with a final solids concentration of 7-12 mg/L (EPA 1989). Each culture was transferred to new water every four days. At transfer the culture received the usual amount of diet but the algae were increased to 3 mL @ $36x10^6$ cells per mL. To obtain test organisms < 24 hours old, gravid adults (3rd brood or older) from the mass culture were placed individually into 30-mL plastic cups with standard diet on the day prior to the test. Two hours before test set-up the adults were taken out of the cups and the neonates were again fed the standard diet.

Tests were conducted in accordance with the EPA guidelines for standard acute WET testing with *C. dubia* (EPA 1993). The tests were conducted using 50-mL glass beakers containing 50 mL of the appropriate test solution. The beakers were sealed with parafilm to minimize ammonia loss and CO_2 exchange and placed in the incubator overnight to stabilize pH and temperature. Four replicate groups of five neonates were exposed to each of the five test concentrations and the control. The five neonates in each test beaker were selected so that each of the five came from a different brood, and with the same brood composition across treatments within a replicate. Two additional beakers were employed at each concentration for measurements of pH, temperature, dissolved oxygen and total ammonia. The test beakers were maintained in an incubator at 25 ±l°C with a light cycle of 16-h light : 8-h dark at 538- 1076 lux. Temperature,

dissolved oxygen and pH were measured at test initiation, 24-h and 48-h (test end). Mortality was recorded after 24-h and 48-h. As required by the EPA method (EPA 1993), the organisms were not fed during the test. The two environmental factors were controlled throughout the tests with variations in pH of ≤ 1.4% based on log unit changes and variations in temperature of ≤ 2.8%. The total ammonia concentrations used in the tests ranged from 0 to 56 mg/L. The dissolved oxygen concentrations ranged between 7.4 and 8.7 mg/L in all tests.

The concentration of un-ionized ammonia was calculated for each test concentration based on the total ammonia level, the average pH and the average temperature measured in the test concentration during the tests (Emerson *et al.* 1975). The 24-h and 48-h LC50 values and the 95% confidence intervals were determined by Probit or Moving Average analysis for the 48-h static acute toxicity tests using the statistical programs Toxcalc (Tidepool Scientific Software) and EPA LC50 program (EPA 1986).

RESULTS AND DISCUSSION

In measuring the acute toxicity of a chemical, the objective is to determine as precisely as possible the range of chemical concentrations that cause mortality in the test organisms over a certain time period. The acute toxicity of ammonia to aquatic organisms is primarily caused by the un-ionized form, the concentration of which is dependent on pH, temperature and total ammonia concentration. In the present study temperature and pH were controlled to levels below incubator specification of 25±1°C and pH variability of 1.7% based on in-house control chart. The combined maximum variation of un-ionized ammonia, based on the mean values for pH and temperature in Table 1, was 9% during the tests.

Table 1. Mean values of measured total ammonia concentrations, calculated un-ionized ammonia concentrations, pH, temperature and mortality in the five acute toxicity tests with *Ceriodaphnia dubia*. The controls had 100% survival in all tests.

Total Ammonia Mean Concentration, mg/L ± SD	pH Mean ± SD	Temperature Mean °C ± SD	Unionized Ammonia Mean Concentration, mg/L ± SD	Mortality ± SD (% Mortality) 24-h	Mortality ± SD (% Mortality) 48-h
0.03 ± 0.04	8.14 ± 0.05	24.7 ± 0.1	0.00 ± 0.00	0.0 ± 0.0 (0%)	0.0 ± 0.0 (0%)
9.31 ± 0.17	8.07 ± 0.01	24.8 ± 0.1	0.56 ± 0.03	0.8 ± 1.3 (4%)	1.6 ± 1.8 (8%)
16.91 ± 2.04	8.04 ± 0.01	24.8 ± 0.1	0.99 ± 0.11	0.4 ± 0.5 (2%)	4.6 ± 3.4 (23%)
27.42 ± 1.37	8.02 ± 0.01	24.8 ± 0.1	1.53 ± 0.10	6.2 ± 4.5 (31%)	14.2 ± 3.5 (71%)
35.48 ± 2.45	8.02 ± 0.02	24.8 ± 0.0	1.96 ± 0.18	13.2 ± 3.1 (66%)	18.6 ± 1.1 (93%)
43.55 ± 2.79	8.01 ± 0.02	24.8 ± 0.1	2.36 ± 0.19	16.8 ± 2.9 (84%)	19.8 ± 0.4 (99%)

A separate ammonia study without organisms (data not shown) was conducted to investigate the variation of ammonia within the replicates and the reduction in concentrations over the test period. The variation within replicates of each concentration was ≤8.3% (SD 4.6%). Total ammonia measurements taken at test initiation and termination showed a mean reduction of 17.4% (SD 8.7%) over the 48 hours. The reduction in measured ammonia ranged from 2.2% to 23.8% with no correlation between concentration and % reduction. Considering these variations in the controlling

parameters and the reduction in total ammonia over the 48-h the estimates of the LC50 value given here were considered optimum for the tests conducted according to WET testing protocols (Table 2).

Table 2. Acute toxicity (LC50 at 24-h and 48-h with 95% CI) of un-ionized ammonia to *Ceriodaphnia dubia.*

	24-h LC50 mg/L (C.I.)	48-h LC50 mg/L (C.I.)
	1.74 (1.56 - 1.98)	1.03 (0.86 - 1.18)
	2.07 (1.91 - 2.29)	1.19 (1.07 - 1.30*)
	1.76 (1.55 - 2.05*)	1.31 (1.12 - 1.51)
	1.52 (1.27 - 1.80)	1.12 (0.91 - 1.32)
	1.57 (1.45 - 1.68)	1.25 (1.07 - 1.39)
Mean LC50 ± SD	1.73 + 0.19	1.18 + 0.10

* Moving Average Analysis, all other values analyzed by Probit

The LC50 values for 24-h and 48-h exposure are shown in Table 2. The mean 48-h LC50 value of 1.18 mg/L un-ionized ammonia is within the general range reported in the literature for other *Ceriodaphnia* species (Table 3).

Table 3. EC50 and LC50 values for *Ceriodaphnia* species reported in the literature.

Species	Source	Endpoint	Un-ionized Ammonia, mg/L
Ceriodaphnia dubia	Nimmo et al. (1989)	48-h EC50	>1.43[a]
Ceriodaphnia dubia	Nimmo et al. (1989)	48-h EC50	0.46[b]
Ceriodaphnia dubia	Cowgill & Milazzo (1991)	48-h EC50	1.00[c]
Ceriodaphnia acanthina	Gersich et al. (1986)	48-h LC50	0.60
Ceriodaphnia acanthina	EPA (1985)	48-h EC50	0.63
Ceriodaphnia vetulus	Arthur et al. (1987)	48-h LC50	1.04[d]

[a] Tested at 25°C and median pH 7.8
[b] Tested at 7°C and median pH 8.2
[c] LC50 value calculated from an LC50 value of 9 mg/L ammonia, mean temperature of 26.4°C and mean pH of 8.3
[d] Tested at 20.4°C and pH 8.1

The LC50 value of >1.43 mg/L (Nimmo *et al.* 1989) was derived from a temperature value of 25°C and a median pH value of 7.8. Using the mean temperature and pH values of 26.4°C and 8.3 reported by Cowgill and Milazzo (1991), a LC50 value of 1.0 mg/L un-ionized ammonia can be calculated from the graphically displayed 9 mg/L total ammonia. The value of 1.0 mg/L is close to values reported in this work and to the mean value of 1.18 mg/L (Table 2). Values of 0.60 to 1.04 mg/L for other species of *Ceriodaphnia* are also close to the values reported in this work, given differences in experimental conditions. This indicates that the referenced data along with those from the present work provide a consistent estimate of the acute toxicity of un-ionized ammonia to *Ceriodaphnia* sps. Ammonia was found to be more toxic to C. *dubia* at a lower temperature (LC50 = 0.46 mg/L un-ionized ammonia, 7°C) a phenomenon also seen with other test organisms (Nimmo *et al.* 1989).

The EPA acceptability criterion of 90% survival in the control during an acute test (EPA 1993) can be achieved fairly easily in a 24-h test, but is more difficult to achieve in a

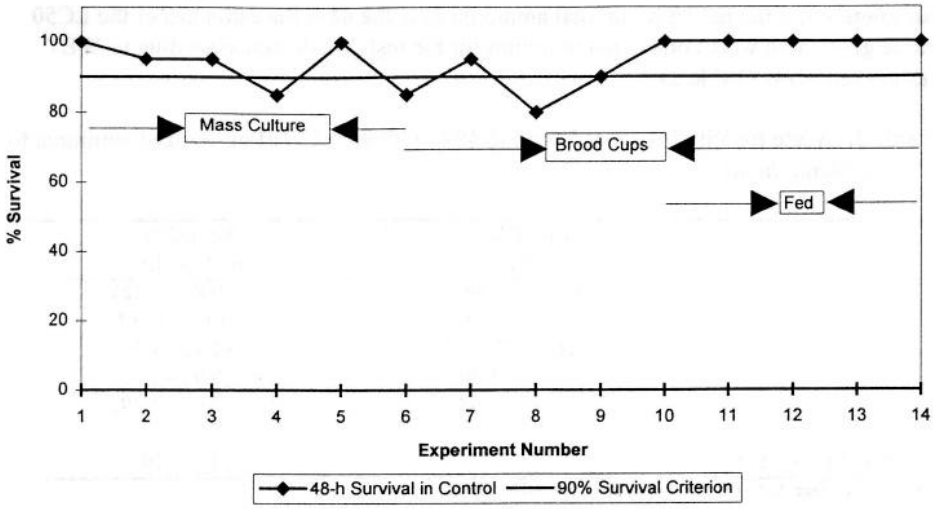

Figure 1. Control survivorship after 48-h with neonates taken from mass cultures and brood cups. The neonates were fed 2-h prior to test initiation in experiments 10-14.

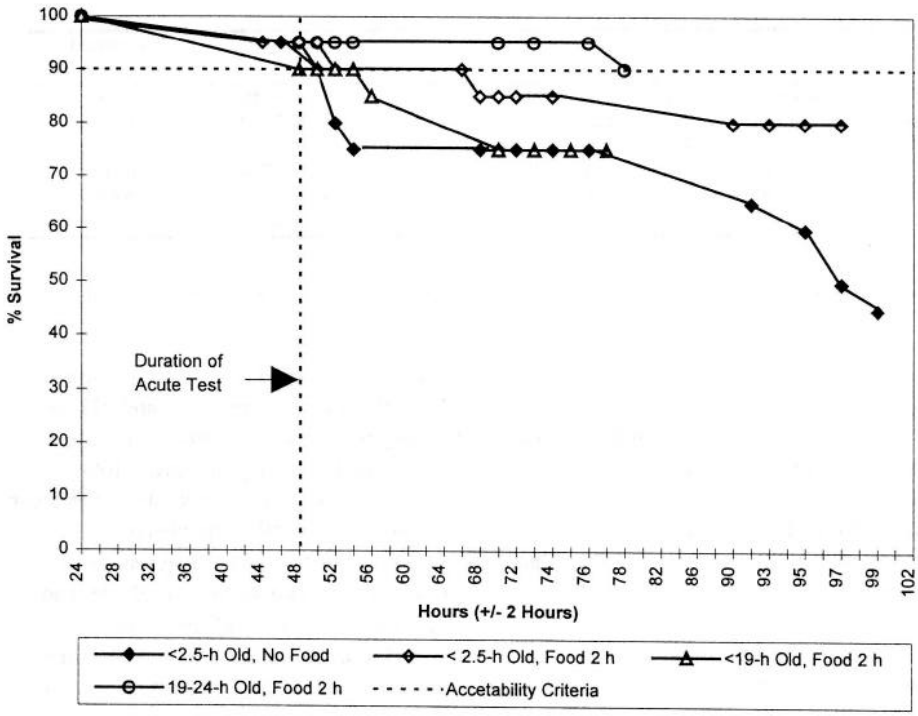

Figure 2. Survivorship of different age classes of neonates fed two hours before test initiation. The control was newly hatched neonates (< 2.5-h old) not fed.

48-h test based on experience of this laboratory. The addition of diet to test solutions improves control survival and has been employed in acute tests with *Ceriodaphnia* (Ankley and Burkhard 1992, EPA 1991). These reports that control survival decreases with test duration and improves with feeding during testing, raise the issue of nutritional status of test organisms. The effect of nutritional status as an important modifying factor in toxicity testing has been reviewed by Lanno *et al.* (1989). They point out that with small organisms, like *Ceriodaphnia* neonates, adequate pre-test nutrition will prolong the period after test initiation that the organism is in a positive energy balance. This will ensure that the organism is responding to the test material without the confounding effect of a progressive negative energy balance (starvation) as the test proceeds. To address the problem of adequate nutrition as it relates to control survival without adding diet during the test, which is contrary to WET test methodology (EPA 1993), the method of organism handling prior to test initiation was examined. Figure 1 shows results from 14 experiments conducted using three procedures for pre-test collection of neonates: 1) newly released neonates from mass culture, 2) released in brood cup and held with parent female until test initiation and 3) two hours prior to test initiation the adult females were removed from brood cups and the neonates (<19 h old) were fed the standard diet. As shown in Figure 1 (experiments 10-14), procedure 3 consistently resulted in 100% control survival. An experiment was conducted to determine the optimum age at test initiation (all < 24 h old) using procedure 3, described above. The best survivorship occurred when neonates were 19-24 h old and fed two hours before test initiation (Fig. 2). This means that the time safety margin between an acceptable and non-acceptable test can be extended considerably by removing the adult females and feeding the neonates 2 hours prior to test initiation. Based on these findings, the authors suggest that existing protocols for acute WET tests (EPA 1993) be amended in future editions to include the collection and feeding regime for obtaining neonates with optimal pre-test nutritional status described in Procedure 3 (above).

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