A Daphnia magna 4-day Survival and Growth Test Method

Lazorchak, J.M., Smith, M.E., Herrin, L. and Kneipp, A.

Introduction

As part of an ongoing search into shorter duration methods for use in evaluating the toxicity of effluents, discharges and receiving waters, an effort was undertaken to develop a short- term subchronic toxicity testing for use with the freshwater inverebrate, *Daphnia magna*. The typical chronic survival and reproduction test with *D. magna* requires a 21 to 28 day test duration, making it logistically difficult to conduct. The goal for this study was to develop a method that used a two to four day test duration, a temperature of 25°C and a small volume of test water, less than 100 ml. These parameters made a reproduction endpoint unreasonable, but the growth rate of *D. magna* at 25°C made growth of the animals (as measured by mean dry weight) a reasonable endpoint to use. The basic method was developed using standard reference toxicant materials. Once a 4-day *D. magna* growth and survival method was standardized, tests were conducted comparing the results from *D. magna* tests to results from other standard toxicity testing methods, specifically the Ceriodaphnia dubia 7-day chronic survival and reproduction testing method.

Materials and Methods

Culture methods

The *Daphnia magna* cultures providing animals for this study were maintained using an in-house SOP based on the USEPA Acute Testing Methods Manual (USEPA, 1993). The culture water was composed of well water, dechlorinated tap water, and Super-Q® deionized

water, mixed to a hardness of 90 to 120 mg/l as $CaCO_3$. Cultures were maintained in glass beakers containing 1L of culture water and fifteen animals per beaker. Water was changed and the young were removed on Monday, Wednesday, Friday, and Sunday. The water was also changed the day prior to each test set-up, so that all young were removed. Cultures were changed between 6 and 7 PM the day prior to a test and then checked for young the following day between 6 and 7 AM. This provided young released within a 12 hour time period. Daily culture feeding consisted of 4 ml of *Selenastrum capricornutum* ($100*10^6$ cells/ml), 3 ml of blended alfalfa, and 3 ml of digested FFAY.

The *S. capricornutum* (concentration $100*10^6$ cells/ml) was cultured according to the procedures described for *Ceriodaphnia dubia* culture and testing (USEPA, 1994). The alfalfa extract was prepared by blending 7.5 gm of alfalfa in 1 L of Super-Q® deionized water for 5 minutes. This mixture was placed in the refrigerator overnight to settle, and then the top 500 to 600 ml of supernatant was poured off and kept as the alfalfa extract. The flake food, alfalfa, and yeast (FFAY) also known as (YCT) was prepared according to the procedure described for *C. dubia* and *D. magna* culture methods (USEPA,1993).

Test design

The *D. magna* used for this testing were < 24-hour-old, and supplied from the in-house culture. It is recommended that the young be collected so that the difference in age for the test animals is 12 hours or less.

All tests were conducted using moderately hard reconstituted water (MHRW) supplemented with selenium, as recommended for *C. dubia* culture and testing (USEPA, 1994). A series of five dilutions and a control were used for each test. Four reference toxicants were tested with this method. The high concentrations for each are as follows: $ZnSO_4*7H_2O$ =500

µg/L, Phenol= 10 mg/L, NH_4Cl= 400 mg/L, and KCl= 1000 mg/L. To mix the test dilutions, 1L of the high concentration was prepared and then 0.5 serial dilutions were made using the toxicant and MHRW.

After allowing all solutions to reach 25 (±1)°C, 50 ml of control water or test solution was dispensed into properly labeled centrifuge tubes or plastic cups, with four replicates per dilution. Once the tubes or cups were filled, 5 test animals were randomly assigned to each container. After all tests were setup, the animals were fed a combination of 0.3 ml *S. capricornutum*, and 0.2 ml blended alfalfa extract. These test foods were prepared following the same methods previously described for the culture foods. The routine chemical parameters (pH, D.O., Conductivity, and Temperature) were measured and recorded for each dilution. At this time, 4 sets of 10 animals were randomly selected for initial dry weights. These animals were dried and weighed using the same procedures described for drying and weighing the animals at the completion of the test. These can be weighed using a 0.00001 gm balance. This number of animals is needed to insure an accurate dry weight average.

Each day fresh dilutions were made, and routine chemistries were taken. The test animals were then transferred into fresh test solution, and the number of live/dead recorded. To do this, the test solution was poured from the cup or centrifuge tube, into a petri dish. The cup or tube was then refilled with fresh test solution. Using a 4 mm bore glass or plastic pipette, the animals were gently pulled into the pipette and released below the surface of the test solution in the cup or tube. Once all animals were transferred, the correct amount of each food was added to each test container. The old test solution was saved for determining routine final chemistries.

To end the test, the number of live/dead were counted and recorded. The remaining live animals were removed and placed into labeled aluminum weigh pans. All surviving animals

from each replicate were placed into the same pan, and weighed together to give a replicate weight. The animals were dried for 24 hours at 60°C and placed in the desiccator for at least one hour, to cool. A balance (Mettler AE 163) capable of reading 0.00001 gms (± 0.00001 gms) was used to measure the weights. These animals can be weighed by using pre-tared weighing pans, or by transferring all animals from each replicate onto a zeroed balance weighing pan. To be acceptable a test must have 90% or greater control survival, and the weight of the control animals must be at least 10 times (10X) that of the animals used to start the test.

For each test, the following endpoints were analyzed: the no observed effect concentration (NOEC), the lowest observed effect concentration (LOEC), the 25% inhibitory concentration (IC25), the 50% inhibitory concentration (IC50) and the 50% lethal concentration (LC50). These endpoints are determined using the same procedures and statistical methods/programs used for determining these endpoints for the *Pimephales promelas* chronic survival and reproduction toxicity test (USEPA, 1994). For this study, the statistical package TOXIS 2.4A (EcoAnalysis Inc.) was used to make these determinations.

TABLE 1. TEST METHOD FOR *Daphnia magna* SHORT-TERM GROWTH AND SURVIVAL TEST

TEST PARAMETER	CONDITION
Test Type	static-renewal
Test Duration	4 day
Temperature	25°C (±1°C)
Photoperiod	16 h light: 8 h dark
Test Chamber Size	60 ml
Test Solution Volume	50 ml
Renewal of Test Solution	daily
Age of Test Organisms	< 24 hrs old (12 hour age range)
No. Organisms/Test Chamber	5
No. Replicate Test Chambers	4
No. Organisms/concentration	20
Feeding Regime	0.3 ml algae & 0.2 ml alfalfa
Test Solution Aeration	None
Dilution Water	Moderately Hard Water + Selenium
Test Concentration	5 plus control
Dilution Series	0.5
Endpoint	Survival and Mean Dry Weight
Test Acceptability	90% or greater control survival/ control growth 10X initial weight

Results

Tests were conducted using four reference toxicant materials and two environmental samples. Five separate tests were conducted with three of the reference toxicant materials, zinc sulfate, ammonium chloride and potassium chloride. A total of six tests were conducted using the reference toxicant phenol. The two environmental samples were supplied by USEPA Regions VI(sample BS) and IX (sample PM). These tests were conducted concurrently with a *Ceriodaphnia dubia* 7-day Survival and Reproduction test, to provide a measure of the reliability and sensitivity of the *Daphnia magna* 4-day Survival and Growth test method.

The levels of toxicant reported below are nominal measurements for zinc (Zn^{++}). Four of the five tests with zinc sulfate (Table 1) had consistent results. The results of the fifth test are considerably higher than those of the other four, due mainly to the lack of mortality in the two highest test concentrations. No explanation for this difference is known. The survival No Observable Effect Concentration (NOEC) was 125 µg/l for these four tests, while the growth NOEC for these tests were 62.5 µg/l and 125 µg/l. The fifth test had a survival NOEC of 500 µg/l and a growth NOEC of 250 µg/l. The LC50 values (survival) for the four tests ranged from 154 µg/l to 177 µg/l and the IC25 values (growth) ranged from 88 µg/l to 122 µg/l. The LC50 value for the fifth test was >500 µg/l and the IC25 value was 444 µg/l. Literature values available from the Zinc criteria document (USEPA, 1987) report a soft water *D. magna* species mean acute value of 355µg/l and a soft water chronic value of 140 µg/l for *D. magna*. The moderately hard water *D. magna* species mean acute value is 525 µg/l and the chronic value is 48 µg/l. Zinc data generated in this laboratory for the *C. dubia* 7-day survival and reproduction test (five tests) show LC50 values for the *C. dubia* 7-day test are in the 180 to 200 µg/l range, compared to 154 to 177 µg/l (one value >500 µg/l) for the *D. magna* 4-day test. The *D. magna*

4-day tests had growth NOEC values of 62.5 µg/l, 125 µg/l and 250 µg/l, compared to reproduction NOEC values of 125 µg/l for the *C. dubia* 7-day test. The *C. dubia* reproduction IC25 values range from 130 to 150 µg/l and the 4-day *D. magna* growth IC25 values range from 88 to 122 µg/l (one value of 444 µg/l).

The five tests conducted using ammonium chloride (Table 2) showed good consistency. The values reported below are nominal measurements of ammonium chloride, unless otherwise noted. The survival NOEC values for these five tests were either 100 or 200 mg/l, with growth NOEC values of 25, 50 and 100 mg/l. The LC50 values range from 223 mg/l to 278 mg/l, with IC25 values of 120 to 148 mg/l. When converted to reflect unionized ammonia (NH_3), these tests show nominal NH_3 LC50 values in the 2.0 to 3.0 mg/l range, in MHRW at a temperature of 25°C. The Ammonia Criteria Document (USEPA, 1985) reports NH_3 LC50 values of 2.0 to 2.6 mg/l at a pH range of 7.9 to 8.1 and a temperature range of 22°C to 25°C , which is within the pH ranges of these tests. Data from five inter-laboratory tests using ammonium chloride show survival NOEC values for the *C. dubia* 7-day test are 50 to 100 mg/l with LC50 values ranging from 100 to 200 mg/l. The *C. dubia* reproduction NOEC values are in the 50 to 100 mg/l range, with reproduction IC25 values ranging from 75 to 125 mg/l.

Six (6) tests were conducted using phenol (Table 3). All values listed below are nominal. These tests also showed reasonable consistency, with the survival NOEC values being 2.5 mg/l and 5.0 mg/l. The survival LC50 values ranged from 5.4 mg/l to 6.8 mg/l. The growth NOEC values were somewhat variable, with values of 0.625, 1.25 and 2.5 mg/l. The growth IC25 values ranged from 1.1 mg/l to 2.7 mg/l. The only data available from the USEPA Criteria Document (USEPA, 1990) for phenol reports acute LC50 values in the 9 to 10 mg/l range. Using this data, it appears that the method described here is more sensitive to phenol. This would be

expected, since the vast majority of toxicity work with *D. magna* has been conducted using temperatures around 20°C, compared to the 25°C test temperature used for this study. The reproduction NOEC values for *C. dubia* 7-day survival and reproduction tests conducted using phenol were 2.5 mg/l, with IC25 values of 3 to 4 mg/l.

The results from the potassium chloride (Table 4) tests were the most consistent of the four reference toxicant materials tested. The results reported are below are nominal values. The survival NOEC values were the same for all five tests, 500 mg/l. The growth NOEC values were also the same, >500 mg/l. The survival LC50 values ranged from 652 mg/l to 707 mg/l, while the IC25 values ranged from 593 mg/l to 625 mg/l. The Criteria Document for Chloride (USEPA, 1988) gives a species mean acute value of 1500 mg/l and a species mean chronic value of 370 mg/l for *D. magna* in moderately hard water. When testing using KCL, the survival NOEC values for the *C. dubia* 7-day survival and reproduction test range from 250 to 500 mg/l, while the reproduction NOEC value for the *C. dubia* test is 250 mg/l. The *C. dubia* LC50 values range from 325 mg/l to 650 mg/l, while the IC25 values range from 200 to 350 mg/l.

Results from the test using the PM effluent from USEPA Region IX show a survival NOEC of >100% for the *C. dubia* 7-day test, versus a survival NOEC of 50% for the *D. magna* 4-day test. The survival LC50 was 100% for the *D. magna* test, while the mortality in the *C. dubia* test was insufficient to generate an LC50. The reproduction NOEC for the *C. dubia* test was 50%, while the growth NOEC for the *D. magna* test was >50%. The IC25 values for each test were similar, the *C. dubia* 7-day test producing a value of 68.3% versus a value of 66.4% for the *D. magna* 4-day test. The results from the BS effluent from USEPA Region XI shows a *C. dubia* survival NOEC value of 42.2%, with an LC50 of 48.26%. The reproduction NOEC value was >42.4%, with an IC25 value of 37.9%. The *D. magna* survival and growth NOEC

values, as well as the LC50 and IC25 values, were >100%. It should also be noted the reproduction in the *C. dubia* control was marginally acceptable at 15.2 young/female. Comparing the reproduction of the 17.8 % effluent dilution, the lowest test concentration (25.3 young/female) to the reproduction in the 42.2% effluent dilution (18.4 young/female) using a t-test ($\alpha = 0.05$) shows the reproduction in the 42.2% sample is statistically different from the 17.8% sample, $p = 0.044$. The only mortality in the *D. magna* test was in the 100% sample, survival = 80%. The growth in the control sample was 147 µg, with the growth in the effluent dilutions ranging from 136 to 160 µg, none of which were statistically different from the control.

Growth in the tests (Table 5) was measured using mean dry weights for both the initial test animals and the animals surviving at the end of the test. Initial weights were varied from test to test, with the range being 6.2 µg to 13.7 µg. The coefficient of variance (CV) for the individual weights ranged from 4.2% to 13.1%. Final dry weights for the control samples also varied from test to test, with a range of 73 µg to 167 µg. The CV's for these weights also varied, with a range of 2.6% to 19.8%.

Table 1. Results from Zinc Sulfate Tests

µg/l	Survival			Growth			Cnt Grw
Test #	NOEC	LOEC	LC50	NOEC	LOEC	IC25	µg/sur
1	500	>500	>500	250	500	443.8	113
2	125	250	176.8	62.5	125	121.9	124
3	125	250	170.8	62.5	125	106.3	140
4	125	250	176.8	62.5	125	93.8	165
5	125	250	153.9	125	>125	88.4	117

Table 2. Results from Ammonium Chloride Tests

mg/l	Survival			Growth			Cnt Grw
Test #	NOEC	LOEC	LC50	NOEC	LOEC	IC25	µg/sur
1	200	400	246.2	100	200	145.1	111
2	100	200	246.6	50	100	121.4	129
3	200	400	277.7	100	200	147.5	126
4	200	400	246.2	100	200	119.7	136
5	100	200	223.2	25	50	125.8	121

Table 3. Results from Phenol Tests.

mg/l	Survival			Growth			Cnt Grw
Test #	NOEC	LOEC	LC50	NOEC	LOEC	IC25	µg/sur
1	5.0	10.0	6.8	2.5	5.0	2.7	128
2	5.0	10.0	5.7	1.25	2.5	1.6	120
3	5.0	10.0	6.0	0.625	1.25	1.2	128
4	2.5	5.0	5.4	1.25	2.5	1.8	110
5	2.5	5.0	5.6	0.625	1.25	1.1	131
6	2.5	5.0	5.7	1.25	5.0	1.5	121

Table 4. Results from Potassium Chloride Tests

mg/l	Survival			Growth			Cnt Grw
Test #	NOEC	LOEC	LC50	NOEC	LOEC	IC25	µg/sur
1	500	1000	651.7	500	>500	614.2	76
2	500	1000	683.0	500	>500	625.0	96
3	500	1000	707.1	500	>500	625.0	112
4	500	1000	688.7	500	>500	593.0	89
5	500	1000	694.7	500	>500	620.9	86

Table 5. Initial and Final Dry Weights

Test #	Toxicant	Initial _ Dry Wt. (µg)	C.V. %	Final _ Dry Wt. (µg)	C.V. %
1	Phenol	11.1	9.9	128	4.6
2		9.2	6.7	120	8.2
3		12.1	8.2	128	9.8
4		7.1	4.2	110	15.3
5		10.1	8.1	131	4.6
6		9.9	7.7	121	12.5
7	NH_4Cl	8.2	9.7	112	12.5
8		8.7	7.6	129	5.4
9		11.3	10.2	126	6.1
10		13.1	11.1	136	7.1
11		12.0	13.1	121	5.9
12	Zinc	10.2	6.4	113	9.7
13		9.8	5.7	124	9.2
14		13.7	9.9	140	5.9
15		13.1	7.8	167	5.4
16		10.3	8.4	117	19.8
17	KCl	6.2	4.7	73	19.2
18		8.4	6.1	96	15.4
19		10.1	9.9	112	11.4
20		7.6	7.3	89	2.6
21		7.4	10.2	86	7.3

Chronic Toxicity Test Methods
Daphnia magna
NPDES/Sediment Elutriates

1. SCOPE AND APPLICATION

This method estimates the chronic toxicity of whole effluents, receiving water and elutriates to the freshwater invertebrate, ***Daphnia magna,*** four-day, static renewal test. The following method is used at the USEPA AWBERC Cincinnati, Ohio facility.

2. REFERENCES

USEPA 1989, Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Second edition. C.I. Weber (ed.) U.S. Environmental Protection Agency, Cincinnati, OH 45268. EPA-600/4-89-001.

3. ASSOCIATED SOPs

3.1 **Randomization and Color Chart** SOP No. 206

3.2 **Moderately Hard Reconstituted Water Prep** SOP No. 229

3.3 **Monthly Reference Toxicant Tests** SOP No. 213

3.4 **Mass Culture (*Daphnia magna*)** SOP No. 207

3.5 **Statistical Analysis Procedures** SOP No. 224

3.6 **Routine Chemistries** SOP No. 223

3.7 **Record Keeping** SOP No. 211

3.8 **Algal Culture (*Selenastrum capricornutum*)** SOP No. 238

4. DEFINITIONS

4.1 ***Daphnia magna***: A freshwater invertebrate (Crustacea: Cladocera) used for acute and chronic testing.

4.2 **Chronic toxicity tests**: A definitive test, designed to estimate the highest "safe" or "no-effect concentration".

4.3 **Reference toxicants**: Reagent grade chemicals used for quality control purposes, such as NaCl, KCl, $CdCl_2$, and $CuSO_4$.

4.4 **MHRW**: Moderately Hard Reconstituted Water; A water formulated from a specific formula to achieve a water hardness range of 80-100 mg $CaCO_3$/L.

4.5 **Static-Renewal test**: A test in which the test organisms are exposed to a fresh solution of the

same concentration of sample every 24 hr by transferring the test organisms from one test chamber to another.

4.6 **NOEC:** No-Observed-Effect-Concentration. The highest concentration of toxicant to which organisms are exposed that causes no observable adverse effects.

4.7 **LOEC:** Lowest-Observed-Effect-Concentration. The lowest concentration of toxicant to which organisms are exposed that causes adverse effects.

4.8 **IC25:** Inhibition Concentration. A point estimate of the toxicant concentration that would cause a 25% reduction in a non-quantal biological measurement such as fecundity or growth.

4.9 **LC50:** Lethal concentration 50%. The concentration of toxicant that would cause death in 50% of the test population.

4.10 **NPDES:** National Pollutant Discharge Elimination System Permits Program.

4.11 **Growth:** Size increase/difference in the test animals, over the 4-day test duration. Measured as mean dry weight.

5. PROCEDURE

5.1 SUMMARY

This test is a 4-day, static-renewal, chronic toxicity test. Test Criteria Specifications are enumerated in Table 5.1.1.

5.2 **Interferences:** Poor quality test organisms or dilution water can cause the test to fail. Observe *D. magna* cultures for the week prior to testing, monitoring reproduction and survival. Allow dilution water to age for 72 hr prior to testing and ensure that the water hardness is in the proper range.

5.3 Sample Handling and Safety

Follow GLP standards

5.4 Apparatus and Equipment

1L cubitainers
50 ml. plastic test cups or (glass beakers, if requested)
Styrofoam or plastic test boards to hold test cups
Plastic pipets (bore diameter 4mm)
Randomization and color chart
Data sheets
60cc syringes
Constant temperature incubator (set at 25°C)

5.5 Reagents and Consumable Materials

200, ≤24-hour-old, *D. magna* neonates.
10L of moderately hard reconstituted water (aged 72 hrs)

TEST CRITERIA	SPECIFICATIONS
Test Type	Static-Renewal
Test Duration	4 days
Temperature	25°C ± 1°C
Photoperiod	16 hour light/ 8 hour dark
Test Chamber Size	50 ml (plastic cups)
Test Solution Volume	50 ml
Renewal of Test Solution	Daily
Age of Test Organism	<24 hours, all released within an 8h period
Number of Organisms per Test Chamber	5
Number of Replicates per Concentration	4
Number of Organisms per Concentration	20
Feeding	Feed 0.3 ml of algae and 0.2 ml alfalfa per test chamber daily.
Dilution Water	Moderately Hard Reconstituted Water supplemented with Se.
Endpoint	Survival
Test Acceptability	≥90% survival in the controls
Endpoint	Growth
Test Acceptability	Mean dry weight of the control animals is 10 times greater than the mean dry weight of the initial test animals.

Table 5.1.1

5.6 Calibrations

Samples that are not at the test temperature of 25°C must be adjusted by heating or cooling. Dispense samples into 1L cubitainers for ease of handling. Label cubitainers with permanent marker to identify the different samples. Dilute samples by mixing 500 ml of 100% sample with 500 ml of MHRW to prepare the 50% sample. A 500 ml aliquot of 50% is mixed with 500 ml of MHRW to make the 25% sample. Continue until all necessary dilutions are made. If the test designates a concentration other than 100%, measure the correct amount of sample into a 1L graduated cylinder, then fill to the 1L mark with MHRW. Always use a 0.5 dilution factor, so the rest of the dilutions can be made using the 1:1 dilution technique described above. Once the dilutions are made, dispense the solutions into the appropriate randomly assigned test cups using a separate 60cc syringe for each concentration. For NPDES tests, a minimum of two (2) separate samples, collected two (2) days apart, are required. For elutriate tests, only one sample is collected/prepared.

5.7 Operation/Analysis

Set up two test boards, one randomized for the test and the other non-random for the change-over renewal cups. Follow color chart and label boards and cups accordingly. After randomizing and dispensing the test solutions, add animals with a plastic pipet, (bore diameter of 4 mm.) Pipet 5 animals gently into each test cup. Start in the lower corner of the test board and work up or down each row, rinsing the pipet with D.I. water between each cup. Feed and cover with plastic wrap and place in a 25°C incubator. Save 4 sets of 5 animals each, dry and weigh to determine the initial weight.

After each 24-hour exposure period, the test animals must be transferred into fresh test solution. Pour the cup, with the animals, into a clear glass dish. Refill the cup with fresh test solution. Count and pipet the animals back into the cup and record live/dead animals on the data sheets. Once the animals are transferred, feed and cover with plastic and place back in the incubator. Perform routine chemistries on both old (final) and renewal (initial) samples. (refer to SOP No 201.23)

After 4 days total, remove the cups from the incubator and count the live/dead animals and record on the data sheets. The animals are then placed onto dried preweighed aluminum weigh pans for mean dry weight determination. Pans are dried by placing into a 60°C oven for 24 hours. They are then cooled in a dessicator for 2 hours before weighing. This is done for determining the tare weight of the pan, as well as drying and weighing the animals at the end of the test. It is also acceptable to place the animals onto plastic or aluminum weigh pans to dry, then transfer the animals onto a tared balance pan using a fine tip, natural bristle brush.

5.8 Calculations/Data analysis

The endpoints of toxicity tests using *Daphnia* are based on the adverse effects on survival and growth. Point estimates, such as LC50 and IC25 are calculated using point estimation techniques. LOEC and NOEC values, for survival and growth, are obtained using a hypothesis test approach. Refer to the Statistical Analysis Procedures SOP for the correct procedure.

5.9 Quality Control

Test acceptability is ≥ 90% survival, and a control mean dry weight 10X the initial animal weight. Accuracy and precision in handling of organisms, effluent preparation, and data analysis, shall be used in all aspects of this test to meet GLP standards.

6.0 Nonconformance and Corrective Action

If a test fails it should be stamped on the front of the data sheet `FAILED' and all pertinent data placed in the notebook. The quality of the organisms and the dilution water shall be reexamined, and the test performed again if these issues are satisfactory. If there is a problem with the organisms or the dilution water, the problem shall be resolved before performing the test again. (Note: most effluents have a specified holding time that cannot be extended. If holding time has elapsed a new sample will have to be obtained if available).

7.0 Records Management and documentation

All data sheets including randomization, color charts, and final report shall be kept together, signed, dated, and placed in the designated notebook to be kept as a hard copy for an indefinite period. Any entry errors shall have one line drawn through, and initialed and dated.

Prepared by:	QA:	Approved by:	Date: