Abstract: The effects of four diets [yeast-Cerophyl^{R}-trout chow (YCT) and YCT plus three concentrations of supplemental *Selenastrum capricornutum*] on the vitality and fecundity of five life-span generations of *Ceriodaphnia dubia* were determined. There was no significant difference among the diets for the five generations. Trends, however, indicated an increase of young production, especially during the first seven days, for successive generations. A subsequent seven generation, seven-day test was conducted to confirm young production trends observed in the life-span test for the YCT diet and the YCT plus 10^7 cells/ml algae diet. Although intensive individual culturing could account for much of the improvement in young production, the algae supplemented diet of 10^7 cells/ml had significantly greater young production than the YCT diet.

INTRODUCTION

The United States Environmental Protection Agency's (USEPA) National Pollutant Discharge Elimination System (NPDES) permitting program authorizes limitations on effluent discharges into a receiving water. Initially, these permits contained limits on specific, identifiable effluent constituents. Recognizing that the toxic effects of the combined waste stream (whole effluent) may be different than the additive effects of single effluent constituents, the USEPA determined that whole effluent biological testing was a useful regulatory parameter, perhaps more appropriate in some instances than specific chemical limits. In September 1985, the USEPA issued a document that emphasizes whole effluent biological testing and provides guidance for incorporating toxicity testing requirements into NPDES permits (USEPA, 1985). An increasing number of states, including North Carolina, have followed the USEPA's recommendation and have implemented toxicity testing requirements in new and renewed NPDES permits. Permit holders are required to comply by conducting tests in-house or by using outside contractors.

Four short-term chronic tests have been suggested by the USEPA for evaluating effluent toxicity in freshwater receiving streams, one of which is the seven-day static-renewal survival and reproduction test using the cladoceran *Ceriodaphnia dubia*. In this USEPA methodology, the *C. dubia* are exposed for seven days to a range of effluent concentrations and their survival and reproduction are used to calculate effluent toxicity. In March 1989, the USEPA modified its test termination criterion from the seven-day exposure to the time period (usually 6 days) when 60% of the surviving females in the control have had their third brood (brood referring to the total number of young released during one event from one individual). The short-term chronic test for *C. dubia* was renamed the three-brood

static-renewal test. Even though many feel the knowledge of the cladoceran's life cycle, especially reproduction, is incomplete, this test specifies minimum criteria for survival and reproduction. Many permit holders and testing laboratories are having limited success in consistently meeting the minimum criteria (80% survival and at least 10 young per female in the control) for toxicity tests (DeGraeve and Cooney, 1987; Cooney et al., 1988). *Ceriodaphnia dubia* should not die in seven-day tests; if the test control animals die in a seven-day test, then the testing laboratory should investigate their physical and chemical conditions (especially diet) (Rodgers, 1989).

Although it has been suggested that each testing laboratory is different and techniques that work well in one laboratory do not necessarily work well in another, a standard water and diet that works well over varying, but similar, laboratory conditions would make the tests more reliable and acceptable to permit holders. DeGraeve et al. (1989) reported that NPDES permit holders can expect that randomly selected laboratories may not be able to complete >56% of the scheduled *Ceriodaphnia dubia* seven-day, chronic survival and reproduction toxicity tests. Tests were commonly unsuccessful because the laboratory was unable to initiate the test, primarily because the proper aged *C. dubia* were unavailable (DeGraeve et al., 1989). In this intra- and interlaboratory study, only 78% of the tests which were initiated were successfully completed (DeGraeve et al., 1989). This low success rate should concern the permit holder especially when the inability to comply to permit requirements has the potential to result in imposed fines, bad public relations, and harsher discharge restrictions.

The inherent variability of any toxicity test should be recognized by both permittee and regulator. The overall interlaboratory coefficient of variation (CV) presented by

DeGraeve et al. (1989) was 29.8-37.9% for survival and 28.9-39.0% for reproduction. These ranges fall well within published variability ranges for other toxicity tests [fathead minnow larval and growth test (DeGraeve et al., 1988) and *Daphnia magna* 48-hour acute test (Grothe and Kimale, 1985)] which are accepted tests for estimating toxicity.

While it has been six years since Mount and Norberg (1984) presented the methodology for conducting the short-term chronic toxicity tests using *C. dubia*, many questions of culturing/testing water and diet are still unresolved. Prior to March 1989, the USEPA recommended using moderately-hard reconstituted water and a diet of Yeast-Cerophyl®-Trout Chow (YCT) when the objective of the test is to estimate the inherent chronic toxicity of the effluent (Horning and Weber, 1985). The inconsistent results within and between labs using the originally recommended (YCT) diet have spurred researchers to look for a dietary supplement or substitute diet. In their original methodology, Mount and Norberg (1984) suggested a diet of yeast in aged water with a possible addition of Cerophyl®. Since this original manuscript, many researchers have studied *C. dubia* diet focusing on algae and algae supplemented diets. The single algae diet of *Ankistrodesmus convolutus* was one recommended diet (Cowgill et al., 1985a; Cowgill et al., 1985b). Combinations of two algae were also suggested. *Selenastrum capricornutum* and *Chlamydomonas reinhardti* was suggested by Cowgill et al. (1985a). Even a three-algae combination diet was suggested by Belanger et al. (1989) who suggested that this diet (*Chlamydomonas reinhardti*, *Ankistrodesmus falcatus*, and *Chlorella vulgaris*) was superior to the YCT diet, among others. With the evidence that including at least one algae in the diet improves *C. dubia* health, the USEPA, in March 1989, revised its diet recommendation to the YCT plus *Selenastrum capricornutum* supplement (Weber et al., 1989).

Adequate culturing/testing water and diet continue to be concerns in the culturing and testing with *C. dubia* (Buikema et al., 1980; Buikema, 1983; Kraus and Kornder, 1987; Cooney et al., 1988; Cooney et al., 1989). The Carolina Power & Light Company Biomonitoring Laboratory experienced difficulties meeting test young production criteria when using the recommended moderately-hard reconstituted water and the YCT diet. The purpose of this experiment was to determine if algal-supplemented diets would increase *C. dubia* young production in toxicity tests.

To evaluate potentially optimal diets, multi-generation young production by *C. dubia* grown in the YCT and YCT plus algae-supplemented diets was observed. Consistent with previous laboratory test protocol and the USEPA protocol as revised in March 1989, each successive generation is composed of the third brood neonates of the preceding generation. The 3.0-3.5 x 10^7 cell/ml algal concentrate supplement recommended by the USEPA was included as a test diet. Two growth experiments were conducted to determine which tested diet produced consistently healthy animals for use as controls in chronic effluent toxicity testing. The first test, a five generation life-span test, was to determine if the algae supplemented diets improved vitality and young production. Particular attention was given to the first seven days of each generation. The second test, a seven generation seven-day test, was conducted to confirm the first seven days young production trends indicated in the first test.

MATERIALS AND METHODS

Two growth experiments were conducted to determine the effects of algae-supplemented diets on the vitality and fecundity of multiple generations of *Ceriodaphnia dubia* in individual cultures and as controls in chronic effluent toxicity tests. The four tested diets were Yeast-Cerophyl®-Trout Chow (YCT), and YCT plus the algae *Selenastrum capricornutum* at three concentrations. The first experiment was a five generation life-span test conducted between June 28, 1989 and August 25, 1989 during which each test animal was monitored throughout its life. The second was a seven generation seven-day growth experiment conducted between November 3, 1989 and December 18, 1989 during which each test animal was monitored for only seven days.

Ceriodaphnia dubia used in this study were originally obtained from the North Carolina Department of Environment, Health, and Natural Resources (Division of Environmental Management) and were cultured according to Carolina Power & Light Company's (CP&L) Standard Operating Procedures (Appendices A and B), which are based on the USEPA and the State of North Carolina Division of Environmental Management recommendations (Horning and Weber, 1985; NCDEM, 1985).

Ten days before the initiation of the life-span test, 25 adults were removed from a mass culture maintained in a 1000 ml beaker (Appendix A). This culture was fed 3 ml YCT daily (except weekends) and was renewed every week by placing 5-10 adults into fresh culture water, and discarding the remainder. The culture water used was surface water from Harris Lake, North Carolina which was pumped through a 5 μm filter and adjusted to a hardness of 30-50 mg/l. The adults removed from this mass culture were

placed 2 per 30 ml plastic beaker with 20 ml culture water and fed 0.13 ml YCT daily (except weekends). Water was renewed every other day (except weekends) until the tests were initiated.

Twenty-four hours before the first test began, these isolated adults were placed individually into the 30 ml beakers and checked every 4 hours until at least 40 neonates were hatched. These neonates of known age (less than 24 hours old and all hatched within a 4 hour period) were used to start the life-span test.

When the seven generation seven-day test was initiated, individual cultures of *C. dubia* had been maintained since September 1989. The cultures were renewed using the 3rd or 4th brood neonates from the previous generation for nine generations (Appendix B). The *C. dubia* were fed daily a combination diet of 0.10 ml YCT plus 0.10 ml *S. capricornutum* at a concentration of 3.0-3.5 x 10^7 cells/ml (Appendix B). These individual cultures were renewed when young were produced with the same culture water described above. The culture vessels where checked every 4 hours after the second brood so known-age neonates (<24 hours and hatched within a 4 hour period) would be available to initiate the seven-day test. This culturing practice was recommended by the USEPA for culturing test animals and is similar to the techniques used during this study.

Both tests in this study were initiated with cohorts of ten neonates. Each consecutive generation for each diet was started when at least sixty percent of the surviving females in the cohort had hatched their third brood. Third brood neonates of the preceding generation were combined to randomly select the next cohort of ten replicates. Only broods that contained at least eight neonates were used.

The *C. dubia* were maintained individually in a static-renewal system with 15 ml of test water in 30 ml disposable plastic beakers. The tests were conducted in an environmental chamber on a 16 hours light and 8 hours dark photoperiod under fluorescent light at an intensity of 50-100 foot-candles and the temperature maintained at 25±2°C.

When the objective of the test is to estimate the chronic toxicity of the effluent in an uncontaminated receiving water, the USEPA allows the use of a surface water instead of the moderately-hard reconstituted water (Horning and Weber, 1985). Therefore, a surface water that provided more consistent young production in previous tests was used in this study. The test water, collected from a 0.5 hectare pond near the research laboratory, was pumped through a 5 μm filter, adjusted to 30-50 mg/l hardness, and stored in a Nalgene carboy in a 0-4°C refrigerator for the test period. All water used for each test was collected the day before the test began.

Daily, each *C. dubia* in the individual beaker was fed, observed for vitality, and checked for young production. Any young produced were counted and removed. Each *C. dubia* was transferred into fresh test water on Monday, Wednesday, and Friday until it died (for the life-span test) or until the end of the seven days (for the seven-day test). The M-W-F renewal times were chosen due to time and budgetary constraints of renewing more often as recommended by the USEPA methodology of daily renewals. The NCDEM methodology requires a renewal only on Days 2 and 5 for their chronic pass-fail test.

Four diets were tested in the life-span test. One diet consisted of 0.10 ml YCT. The other three diets were 0.10 ml YCT plus 0.10 ml of the *S. capricornutum* at three different concentrations. The three algal concentrates were 8.6 x 10^6 cells/ml (cell

concentration used by the State of North Carolina; NCDEM, 1985), 3.5 x 10^7 cells/ml (the USEPA recommended concentration; Weber, et al., 1989), and 2.3 x 10^8 cells/ml (cell concentration recommended by Battelle Labs; Cooney, et al., 1988).

Two diets were tested in the seven-day test. One diet was 0.10 ml YCT and the other diet was 0.10 ml YCT plus 0.10 ml of the algal concentrate 3.0-3.5 x 10^7 cells/ml.

The YCT suspension was prepared according to the CP&L's SOP (Appendix A) using the USEPA formulation (Horning and Weber, 1985) with Fleischmann's active dry yeast, Zeigler Brothers fish food pellets (1/8 inch) and Cerophyl® (dehydrated rye cereal grass leaves from Sigma Chemical Company, Catalog No. G7141). The YCT was frozen in 30 ml plastic screw-top containers. The aliquots were thawed, dispensed by an automatic pipette into the test vessels, and any unused food discarded. Each test used YCT from the same prepared batch.

The algae, *Selenastrum capricornutum*, were cultured in the Marine Biological Laboratory (MBL) culture media (prepared according to Cooney et al., 1989) which is recommended by USEPA for the culturing of this algae. A new algae culture was started biweekly by inoculating approximately 250 ml of media with *S. capricornutum* from agar slants (from Carolina Biological Supply Company, Burlington, North Carolina, Catalog No. 152520). A vitamin supplement (prepared according to Goulden et al., 1982) was added to the media during the exponential growth phase of the algae. The algae were maintained at room temperature on a 16 hours light and 8 hours dark photoperiod under cool white fluorescent light. Each batch of *S. capricornutum* was harvested after 10-14 days by centrifuging the media and resuspending the cells in the *C. dubia* test water. New batches of algae were harvested every 4-7 days. The cell concentration of the algal concentrate was

determined by counting cells with a Neubauer Counting Chamber. Final algal concentrate cell concentrations were achieved by either re-centrifuging or diluting with additional test water. The concentrates were stored in a refrigerator between 0-4°C and discarded after two weeks.

RESULTS AND DISCUSSION

Life-span Test

The objective of this study was to determine if any of the tested diets resulted in improved culture health as determined by vitality and reproduction. Each *Ceriodaphnia dubia* in the five generation, four diet, life-span test was observed daily throughout its life. Survival, total young production, and the USEPA young production criteria for toxicity testing were monitored.

Statistically, there was no significant difference for any of the parameters tested among the four diets. However, the following observations of the life-span test were used to formulate the subsequent seven-day test.

Survival

There was no significant difference for maximum or median survival among diets or within generations (Table 1). The median survival is that survival value for which 50% of the observations, when arranged in order of magnitude, lie on each side. Using the

median gives a more informative central value since the maximum survival was skewed towards fewer survival days because of early mortality, especially in the earlier generations.

The maximum survival means increased with decreasing supplemental algae concentration with the greatest survival in the YCT diet (Table 1). There was no trend of increasing maximum survival over the five generations for any diet.

The greatest median survival mean occurred in the YCT plus 10^6 cells/ml algae diet and the lowest occurred in the YCT plus 10^5 cells/ml algae diet (Table 1). The lowest median survival means occurred in the two diets with the highest concentrations of supplemental algae. The YCT plus 10^5 cells/ml algae diet had the only increasing trend of median survival over the five generations.

Total Young Production

There was no significant difference among diets for total young production as measured by: 1) total broods per generation; 2) total young per generation; 3) mean total young per female; 4) mean brood size; and 5) mean young per producing female per day. Although not significantly different, the highest mean values for all survival and total young production parameters, except mean brood size and mean young per day, occurred in the YCT and YCT plus 10^5 cells/ml algae diets.

The greatest total number of broods per generation was in the YCT and YCT plus 10^6 cells/ml algae diets (Table 2). The greatest number of broods for the algae-supplemented diets occurred in Generation 4 and the greatest number of broods for the YCT diet occurred in Generation 3.

Total young per generation varied greatly within each diet. There was no significant difference among diets (Table 2). The means ranged from 805.8 young in the YCT plus 10^8 cells/ml algae diet to 947.8 young in the YCT diet. The greatest total number of young per generation occurred in Generation 3 of the YCT diet.

There was a significant difference among generations in mean total young per female for the two diets with the highest concentrations of supplemental algae (Table 3). The greatest mean total young per female for the YCT plus 10^7 cells/ml algae diet occurred in Generation 4, the only peak in the otherwise declining young production trend for this diet (Figure 1). A trend of increasing young production occurred in the YCT plus 10^8 cells/ml algae diets with a high of 116.6 young per female in generation 5. The highest mean of total young produced per female was in the YCT diet.

The largest mean brood size and the largest brood size average occurred in the YCT plus 10^7 cells/ml algae diets (Table 4). There was a significant difference among generations for this diet but not an increasing trend over the generations. The YCT and YCT plus 10^8 cells/ml algae diet had increasing mean brood size over the generations. Generation 5 was significantly greater than Generations 1 and 2 for both diets. The difference between the smallest and largest mean brood size was less than 0.5 young.

The mean number of young produced per producing female per day showed similar trends for each diet (Figure 2). No young were produced on Days 1 and 2 and very few young were produced on Day 3. Almost all females produced their first brood on Day 4. Few females produced young on Day 6. The peak young production per producing female occurred on Day 9 for the two highest concentrations of supplemental algae diets and on Day 11 for the YCT and YCT plus 10^6 cells/ml algae diets (Figure 2).

There was a significant decrease in mean young production per female after the peak in all diets. The intermittent peaks primarily towards the end of the life-span for each diet were calculated based on less than 5 producing adults and in most cases based on only 1 producing female.

Abortions of neonates were common among all diets for all generations in the life-span test. In this study, an abortion was defined as any dead young or undeveloped eggs remaining in the shed carapace. Abortions were counted as a brood when calculating both total broods per generation and three-brood averages. The lowest mean number of abortions over the five generations in this test occurred in the YCT diet (15.2 abortions) and the highest mean number of abortions occurred in the YCT plus 10^7 cells/ml algae diet (25.6 abortions). The mean number of abortions decreased to fewer than 0.5 for the two diets in the seven-day test. Having frequent abortions may suggest a stressor was present (possibly the test water or the batches of algae for the first test) that could have diminished the ability to examine the effects of the diets.

Young Production Test Criterion

Prior to March 1989, the USEPA young production test criterion for the *C. dubia* chronic toxicity test considered the total number of young produced per female during the first seven days (usually totalling 10-30 young for controls). In March 1989, the young production criterion was changed to a minimum number of 15 young per surviving adult and at least 60% of the surviving females in the control must have had three broods. For comparison to data collected prior to March 1989, both parameters are discussed.

There was no significant difference in young production among diets for either the seven-day or the three-brood average young per female (Tables 5 and 6). The highest mean young production occurred in the YCT plus 10^7 cells/ml algae diet and the lowest mean young production occurred in the YCT diet for both parameters.

There was, however, a significant difference of young per female within generations for all diets except for the YCT plus 10^8 cells/ml algae diet for both parameters (Figure 3). There were ascending trends of average young per female over the five generations for the YCT and YCT plus 10^8 cells/ml algae diets and a descending trend of average young per female for the YCT plus 10^6 cells/ml algae diet.

The YCT plus 10^7 cells/ml algae diet had a significant difference at the $P < 0.001$ level among generations for both the three-brood average and the seven-day average young per female. The seven-day average young per female of 39.3 in Generation 4 for the YCT plus 10^7 cells/ml diet was significantly greater than averages in all other generations in all diets (Table 5). There was not a clear trend of average young per female for this diet (Figure 3).

The mean value within each diet was above the USEPA minimum young production criterion of 15 young per female. There were three generations in the three-brood average parameter, however, that would not have passed a toxicity test based on this revised criterion. The three-brood average young production for Generation 1 in the YCT and YCT plus 10^8 cells/ml algae diets was below the USEPA minimum test criterion (Table 6). The young production for Generation 3 in the YCT plus 10^7 diet was also below the USEPA minimum young production criterion.

Seven-day Test

The seven-day test compared the USEPA young production test criterion parameters of the seven-day and three-brood average young per female of the YCT and YCT plus 10^7 cells/ml algae diets. The YCT plus 10^7 cells/ml algae diet was selected because this diet produced the greatest three-brood average young per female in the life-span test and because it is recommended by the USEPA. Seven generations were monitored for seven days for the two diets. There was a significant difference between the diets and among the generations for both parameters (Table 7).

Seven-day Average Young Production

The mean seven-day average young per female for the YCT diet was significantly less than the mean for the YCT plus 10^7 cells/ml algae diet (Table 7). The seven-day average young per female ranged between 21.3 to 33.0 for the YCT diet. For the YCT plus 10^7 cells/ml algae diet, the seven-day average young per female ranged between 31.2 and 49.3.

There was also a significant difference among the generations within each diet. After the first two highest producing generations in the YCT plus 10^7 cells/ml algae diet, the average young per female dropped to the lowest value in Generation 3 and then leveled off for the last 4 generations (Figure 4). The average young production over the seven generations for the YCT diet was relatively stable.

Three-Brood Average Young Production

There was a significant difference between diets and among generations for three-brood average young per female (Table 7). The YCT plus 10^7 cells/ml algae diet was significantly greater than the YCT diet. The difference among the four highest averages in the YCT diet was less than 1.0 young per female. The difference among the three lowest averages for this diet was also approximately 1.0 young per female. The average young production over the seven generations for the YCT diet showed no increasing or decreasing trend (Figure 4). For the YCT plus 10^7 cells/ml algae diet, there appears to be a slightly ascending trend over the seven generations (Figure 4).

Discussion

The purpose of this study was to evaluate various diets for maximizing young production within the first three broods or within the first seven days of the test to meet or exceed the toxicity test young production criterion established by the USEPA. There was no significant difference in the toxicity test young production criterion among diets in the life-span test (Tables 5 and 6). However, there was a significant difference in young production in the subsequent seven-day test between the YCT and YCT plus 10^7 cells/ml algae diets (Table 7).

The ranges of the young production of this study were greater than the USEPA criterion. The three-brood average young production in the life-span test ranged between 17.5 and 21.4 young per female; the three-brood average young production in the seven-day test ranged between 24.5 and 32.2 young per female. As an average, 15 young per

female in three broods were reported in the original *C. dubia* toxicity test methodology (Mount and Norberg, 1984). The USEPA 1985 document (Horning and Weber, 1985) suggested a total of 10-30 young per female should be produced in the first three broods. Waller and Lazorchak (1986) reported a minimum total of 18 young per female mean in the first three broods. More recently, DeGraeve et al. (1989) presented data on 11 laboratories whose combined mean young per female for the first seven days was 20.7. The grand mean three-brood average young production in this study (22.5 young/female) was greater than the DeGraeve et al. (1989) mean value.

Both diets showed an increase in young production for the seven-day and three-brood averages from the first test to the second test in this study, indicating a more prolific and presumably healthier culture developed between tests. The seven-day average means increased 54.5% for the YCT diet and 73.9% for the YCT plus 10^7 cells/ml algae diet. The three-brood average means increased 40.0% for the YCT diet and increased 50.5% for the YCT plus 10^7 cells/ml algae diet. The higher mean percentage increase of young production in the YCT plus 10^7 cells/ml algae diet (a difference of 10.5%) indicates a real difference between these two diets. However, since both diets showed increases in young production, factors other than diet alone may have also influenced the results.

Four possible confounding factors that should be considered are: 1) healthier *C. dubia* may have developed during the life-span test because of the change from mass culturing to individual culturing for providing the test animals (each test vessel could be considered an individual culture); 2) the test animals for the 7-day test were obtained from individual cultures being fed the YCT plus 10^7 cells/ml algae diet whereas the test animals used to initiate the 7-day test came from a mass culture being fed YCT; and 3)

both the life-span test and the 7-day test used a surface water (collected at the initiation of each test). Since animal performance in a given test reflects both prior and current maintenance of the animal (Rodgers, 1989), healthy cultures should provide healthy test animals. Any improvement in culture health (i.e. increase in young production) would improve the chances that laboratories meet the minimum young production test criterion and consistently complete valid effluent toxicity tests.

If earlier young production and brood size (especially in the first three broods or seven days) are indicators of *C. dubia* culture health, then culture health was improved not only by culture technique but also by the addition of algae to the diet. In the life-span test there were only four incidences, one in each diet, where the three-brood average young per female was less than the seven-day average young per female. This difference indicates that 20% of the surviving females had produced three broods before seven days. These incidences occurred in Generations 4 and 5 (Tables 5 and 6).

In the seven-day test, there were eleven times out of fourteen (79%) when the three-brood average was less than the seven-day average. The females had produced their third brood before Day 7 in four generations in the YCT diet (57%) and in all seven generations in the YCT plus 10^7 cells/ml algae diet (100%).

Brood size is known to vary depending on food quality and quantity (Rodgers, 1989). The mean brood size for the first three broods in this study ranged from 7.2 in the YCT diet to 8.0 in the YCT plus 10^7 cells/ml algae diet (Table 8). This range is well above the 5.7 young per brood mean brood size reported by Cowgill et al. (1985a). Winner (1989b) reported a life-span mean brood size of 8.9 young per brood in the YCT diet which is greater than the 7.7 young per brood mean brood size reported in this study.

Values as low as 3.3 young per brood in the single-algae diet (*S. capricornutum*) were also reported by Winner (1989b). A larger brood size, especially in the first hatches, suggests a healthier culture since the *C. dubia* energy can be expended for young production instead of solely for self maintenance.

Fifteen percent (three out of twenty) of the generations in the life-span test did not meet the 15 young per female criterion for a successful toxicity test. In comparison, 100% (28) of the generations in the seven-day test met the criterion. If these generations were actual tests under the restrictions of the USEPA young production test criteria, these failures could result in a 15% loss in time and resources for permit holders required to perform and successfully complete this toxicity test. A minimal goal for any effluent toxicity testing laboratory is to pass the criteria established by regulatory agencies to eliminate these problems.

More prolific organisms may be "healthier" and thus be more resistant to certain effluent toxins (Belanger et al., 1989). However, since laboratory animals are generally not as healthy as animals in the wild, a truer indicator of the natural population would be represented by a healthier test culture. This hypothesis could be examined by testing these diets with a reference toxicant to see if there is any increased resistance.

Ceriodaphnia dubia demonstrated a decline in productivity with age regardless of diet type (Figure 2). The decline of mean young per producing female for the YCT diet was more gradual than the decline of young production in the algae-supplemented diets. This unsupplemented diet had the lowest peak production and lowest total production for the first seven days. Since a concern for chronic toxicity testing is to produce the most young within the first seven days (or three broods), the YCT diet would not be the best

diet of the ones tested. The YCT plus 10^7 cells/ml algae diet produced the greatest mean young per female and produced the most young within the first seven days.

Winner (1989a) suggested that there should be no decrease in fecundity with increasing age of the daphnid if the animals are adequately nourished. If Winner's observation is correct then none of the tested diets over the test period provided adequate nutrition for the *C. dubia* in these five generations. The test water and/or the YCT components might have been contaminated or possibly, another diet combination needs to be formulated. Winner may have been wrong.

In the life-span test, there appeared to be an oscillation of high and low young production within generations among diets for the two test criterion parameters (Figure 3). The means of Generations 2 and 4 were significantly greater than the means of Generations 1 and 3 for both parameters over all diets. An oscillation was not apparent in the seven-day test. The observed oscillations probably indicated a culture overcoming an adversity such as nutritional deficiency or overcrowding. An increase in food supply in quantity or quality affects the rate of development of the population by increasing fecundity (number of eggs per brood) but since this process is not immediate, population oscillations occur (Wetzel, 1975).

The *Ceriodaphnia dubia* in the YCT plus 10^8 cells/ml algae diet were difficult to observe and count. The culture media in the test vessels for this diet turned very green from the dense concentration of algal cells which were suspended in the media and that had settled on the bottom of the vessel. Often the test animals became trapped on bubbles that developed on the bottom and sides of the vessels. The young were easily missed among the bubbles which may account, in part, for the lower young counts in the

first generations. Though not significantly different, the seven-day and three-brood averages for the YCT plus 10^5 cells/ml algae diet increased over the five generations in the life-span test. Since the monitoring of the *C. dubia* being fed this diet was discontinued after this test, it is unknown at what generation the mean young production would have stabilized. Further testing of this diet after many generations is recommended to determine if the YCT plus 10^5 cells/ml algae diet is better for culturing *C. dubia* than the other diets tested.

Rodger (1989) explained that longevity appears to be the greatest in cultures where the cladoceran are poorly fed and they do not reproduce regularly, especially if the diet has an inadequate supply of calories. Longevity is therefore affected by food availability and commonly increases with a decrease in food consumption, short of starvation (Wetzel, 1975). Winner (1989b) reported a mean life-span of 39.3 days in the YCT diet, 34.0 days in a *S. capricornutum* diet, and 36.4 days in a *C. reinhardti* diet. Winner's longevity values are in line with the values presented in this study (Table 1). The maximum survival in this study (32.6 days) was far less, however, than the maximum longevity of *Ceriodaphnia dubia* reported by Cowgill et al. (1985a). of 125 days at 20°C. Although longevity is inversely related to temperature (Wetzel, 1975), a 5°C difference in test water temperature could not account for this great difference between this study's longevity values and the values presented by Cowgill et al. (1985a). Cowgill et al. (1985a) also reported that 29 regular broods were produced over the 125 days life of the *C. dubia.* This brood number is far less than the mean number of broods (95.4-112.4 broods for the four diets) presented in this study (Table 2). Therefore, the longevity value reported by Cowgill et al. (1985a) seems extreme and may reflect a culture that was poorly fed.

Although in the life-span test there was no difference that could be attributed to the basic diet and/or the supplements, the algae-supplemented diets did enhance the use of *C. dubia* for toxicity bioassays since it increased the 3-brood average young production.

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

1. The Yeast-Cerophyl^{R}-Trout Chow (YCT) diet, and the YCT plus 10^7 and 10^8 cells/ml *Selenastrum capricornutum* supplemented diets, yielded sufficient reproduction and were adequate for long-term culturing of *Ceriodaphnia dubia* in surface water under the test conditions.

2. The YCT plus 10^7 cells/ml algae diet produced the greatest three-brood average young per female (21.4 young/female in the life-span test and 32.2 young/female in the 7-day test) of the four diets tested. The YCT plus 10^7 cells/ml algae diet had a significantly greater three-brood average young production than the YCT diet in the 7-day test.

3. Third-brood young production occurred earlier in the YCT plus 10^7 cells/ml algae diet than in the YCT diet for the 7-day test. 100% of the *C. dubia* in the YCT plus 10^7 cells/ml algae diet had their third brood on Day 6 whereas only 57% of the *C. dubia* in the YCT diet had their third brood on Day 6.

4. The *C. dubia* in the algae-supplemented diets produced larger brood sizes (especially in the first three broods) than the *C. dubia* in the YCT diet. Mean brood size was 7.1 young for the YCT diet and 8.1 young for the YCT plus 10^7 cells/ml algae diet -- the lowest and the highest mean brood sizes of the diets tested in the life-span test.

5. There was a seven-day (and three-brood) average young production oscillation within generations among all diets especially in the first few generations in the life-span test. These oscillations were not apparent in the 7-day test.

6. After peak young production between Days 9 and 11, the *C. dubia* demonstrated a decline in fecundity with age for all diets tested in the life-span test.

7. Longevity is not a good indicator of how well the *C. dubia* will perform in a toxicity test. The YCT diet had the greatest longevity but one of the lowest three-brood young production averages.

Recommendations

1. Seven-day tests using known effluents and/or reference toxicants would help determine if any of the diets tested produced robust *C. dubia* that may be more resistant to certain effluent toxins.

2. Further study on individual culturing under more controlled testing criteria (i.e. synthetic water and a control) should be investigated to confirm that individual culturing provides "healthier" test animals.

3. Further life-span studies should be conducted to determine whether *C. dubia* decline in fecundity with age regardless of diet.

4. Culturing and testing with the YCT plus 10^5 cells/ml algae diet for more than five generations should be monitored to determine where the increasing trend for the three-brood average young per female stabilizes.

5. The development of a synthetic water that is consistent and reliable within and among laboratories is important to standardize toxicity testing with *C. dubia*. The inherent variabilities of surface water may confound test results.

6. If effluent testing is to be incorporated into a NPDES permit, it would be prudent for the permit holder to evaluate several culturing/testing options to ensure the optimal conditions for their testing laboratory.

REFERENCES

Belanger, S.E., J.L. Farris, and D.S. Cherry. 1989. Effects of diet, water hardness, and population source on acute and chronic toxicity to *Ceriodaphnia dubia*. Arch. Environ. Contam. Toxicol. 18(4):601-11.

Buikema, A.L., Jr. 1983. Inter- and intralaboratory variation in conducting static acute toxicity tests with *Daphnia magna* exposed to effluents and reference toxicants. American Petroleum Institute Pub. 4362, American Petroleum Institute, Washington, D.C. 95 pp.

Buikema, A.L., J.G. Geiger, and D.R. Lee. 1980. *Daphnia* toxicity tests. pp. 48-69. In: Aquatic Invertebrates Bioassays, ASTM STP 715, A.L. Buikema, Jr. and J. Cairns, Jr. Eds., American Society for Testing and Materials, Philadelphia, PA.

Cooney, J.D., G.M. DeGraeve, E.L. Moore, B.J. Lenoble, T.L. Pollock, and G.J. Smith. 1989. Effects of environmental and experimental design factors on culturing and testing of *Ceriodaphnia dubia*. Final Report No. 2368-2, Battelle Columbus Division, Columbus, OH. EPRI EN-6468. 267 pp.

Cooney, J.D., G.M. DeGraeve, E.L. Moore, T.L. Pollock, and W.D. Palmer. 1988. Effects of food and water quality on the culturing and toxicity testing of *Ceriodaphnia dubia*. Final Report No. 2368-2, Battelle Columbus Division, Columbus, OH. EPRI EA-5820. 177 pp.

Cowgill, U.M., D.P. Milazzo, and C.K. Meagher. 1988. New diet for *Ceriodaphnia dubia*. Bull. Environ. Contam. Toxicol. 41(2):304-9.

Cowgill, U.M., K. Keating, and I. Takahashi. 1985a. Fecundity and longevity of *Ceriodaphnia dubia/affinis* in relation to diet at two different temperatures. J. Crustacean Biol. 5:420-429.

Cowgill, U.M., I. Takahashi, and S. Applegath. 1985b. A comparison of the effect of four benchmark chemicals on *Daphnia magna* and *Ceriodaphnia dubia/affinis* tested at two different temperatures. Environ. Toxicol. Chem. 4:415-422.

DeGraeve, G.M., J.D. Cooney, B.H. Marsh, T.L. Pollock, and N.G. Reichenbach. 1989. Precision of the EPA seven-day *Ceriodaphnia dubia* survival and reproduction test: intra-and interlaboratory study. Final Report No.2368-2, Battelle Columbus Division, Columbus, OH. EPRI EN-6469. 177 pp.

DeGraeve, G.M., J.D. Cooney, T.L. Pollock, N.G. Reichenbach, J.H. Dean, M.D. Marcus, and D.O. McIntyre. 1988. Intra- and interlaboratory study to determine the reproducibility of the seven-day fathead minnow larval survival and growth test. American Petroleum Institute Publication Number 4468. American Petroleum Institute, Washington, DC. 200 pp.

DeGraeve, G.M. and J.D. Cooney. 1987. *Ceriodaphnia*: an update on effluent toxicity testing and research needs. Environ. Toxicol. Chem. 6:331-333.

Goulden, C., R.M. Comotto, J.A. Hendrickson, Jr., L.L. Horning, and K.L. Johnson. 1982. Procedures and recommendations for the culture and use of Daphnia in bioassay studies. Aquatic Toxicology and Hazard Assessment: Fifth Conference, ASTM STP 766. J.G. Pearson, R.B. Foster, and W.E. Bishop Eds. American Society for Testing and Materials. pp. 139-160.

Grothe, D.R., and R.A. Kimerle. 1985. Inter- and intralaboratory variability in *Daphnia magna* effluent toxicity test results. Environ. Toxicol. Chem. 4:189-192.

Horning, W.B., II, and C.I. Weber. 1985. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Environmental Monitoring and Support Laboratory -- Cincinnati, U.S. Environmental Protection Agency, Cincinnati, OH. EPA/600/4-85/014. 162 pp.

Kraus, G. and S. Kornder. 1987. Experience with EPA chronic bioassay methods. Tappi J. 70(8):62-6.

Mount, D.I. and T.J. Norberg. 1984. A seven-day life-cycle cladoceran toxicity test. Environ. Toxicol. Chem. 3:425-434.

NCDEM. 1985. North Carolina *Ceriodaphnia* chronic effluent bioassay procedure. Revised June 1988. 9 pp.

Rodgers, J.H., Jr.. 1989. Species tolerance for NPDES bioassays: volume I -- freshwater species. American Petroleum Institute Publication Number 4483. American Petroleum Institute, Washington, DC. pp. 105-16.

U.S. Environmental Protection Agency. 1985. Technical support document for water quality-based toxics control. USEPA, Washington, D.C.

Waller, T. and J. Lazorchak. 1986. Proposed new standard guide for conducting partial life-cycle toxicity tests with *Ceriodaphnia affinis* (Draft 3). Amer. Soc. Testing & Materials, Philadelphia.

Weber, C.I., W.H. Peltier, T.J. Norberg-King, W.B. Horning, II, F.A. Kessler, J.R. Menkedick, T.W. Neiheisel, P.A. Lewis, D.J. Klemm, Q.H. Pickering, E.L. Robinson, J.M. Lazorchak, L.J. Wymer, and R.W. Freyberg. 1989. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 2nd Edition. U.S. Environmental Protection Agency, Cincinnati, OH. EPA/600/4-89/001. 249 pp.

Wetzel, R.G. 1975. Limnology. W.B. Saunders Company, Philadelphia. 743 pp.

Winner, R.W. 1989a. An evaluation of some myths concerning the culture of daphnids. Aquatic Toxicology, Vol. 14, pp. 323-330.

Winner, R.W. 1989b. Multigeneration life-span tests of the nutritional adequacy of several diets and culture waters for *Ceriodaphnia dubia*. Environ. Toxicol. Chem. 8:513-520.

Table 1 Maximum and median survival for the life-span test.

	Diet	Generation	Survival [Days]	Mean[1] Survival [Days]	ANOVA[2] among Generation	ANOVA[2] among Diet
MAXIMUM SURVIVAL	YCT alone	1	33	32.6(1.0)	NS	NS
		2	35			
		3	34			
		4	32			
		5	29			
	YCT plus 10^6 cells/ml algae	1	35	30.6(1.2)	NS	
		2	31			
		3	29			
		4	29			
		5	29			
	YCT plus 10^7 cells/ml algae	1	29	27.4(0.8)	NS	
		2	26			
		3	29			
		4	25			
		5	28			
	YCT plus 10^8 cells/ml algae	1	26	27.4(2.2)	NS	
		2	22			
		3	35			
		4	25			
		5	29			
MEDIAN SURVIVAL	YCT alone	1	23.0	24.9(1.4)	NS	NS
		2	25.5			
		3	30.0			
		4	24.0			
		5	22.0			
	YCT plus 10^6 cells/ml algae	1	29.0	25.8(0.9)	NS	
		2	25.0			
		3	24.0			
		4	25.5			
		5	24.5			
	YCT plus 10^7 cells/ml algae	1	27.0	22.7(1.1)	NS	
		2	21.0			
		3	21.5			
		4	22.5			
		5	21.5			
	YCT plus 10^8 cells/ml algae	1	20.0	21.6(0.5)	NS	
		2	21.0			
		3	22.0			
		4	22.5			
		5	22.5			

1 Standard error in parentheses
2 ANOVA alpha level = 0.05 for type I error rate
NS = no significant difference

Table 2 Total broods and total young per generation for the life-span test.

	Diet	Generation	Total No. Broods or Young	Mean No.[1] Broods or Young	ANOVA[2] among Diet
TOTAL BROODS PER GENERATION	YCT alone	1	75	110.8(11.4)	NS
		2	116		
		3	146		
		4	112		
		5	105		
	YCT plus 10^6 cells/ml algae	1	110	112.4(5.8)	
		2	113		
		3	111		
		4	132		
		5	96		
	YCT plus 10^7 cells/ml algae	1	71	91.8(9.2)	
		2	87		
		3	98		
		4	124		
		5	79		
	YCT plus 10^8 cells/ml algae	1	55	95.4(12.6)	
		2	82		
		3	97		
		4	121		
		5	79		
TOTAL YOUNG PER GENERATION	YCT alone	1	581	947.8(123.0)	NS
		2	915		
		3	1356		
		4	951		
		5	936		
	YCT plus 10^6 cells/ml algae	1	890	920.0(94.6)	
		2	938		
		3	840		
		4	1255		
		5	677		
	YCT plus 10^7 cells/ml algae	1	591	808.8(132.7)	
		2	806		
		3	793		
		4	1299		
		5	555		
	YCT plus 10^8 cells/ml algae	1	400	805.8(144.2)	
		2	609		
		3	764		
		4	1090		
		5	1166		

1 Standard error in parentheses
2 ANOVA alpha level = 0.05 for type I error rate
NS = no significant difference

Table 3 Mean total young per female for the life-span test.

	Diet	Generation	Young[1]	Mean[1] Young	ANOVA[2] among Generation	Duncan's Multiple Range Test within Generation	ANOVA[2] among Diet
MEAN TOTAL YOUNG PER FEMALE	YCT alone	1	72.6(21.7)	99.8(10.3)	NS		NS
		2	91.5(20.5)				
		3	135.6(18.3)				
		4	95.1(16.4)				
		5	104.0(8.9)				
	YCT plus 10^6 cells/ml algae	1	111.2(12.9)	96.5(10.1)	NS		
		2	93.8(14.3)				
		3	84.0(15.4)				
		4	125.5(16.5)				
		5	67.7(12.8)				
	YCT plus 10^7 cells/ml algae	1	84.4(17.8)	86.0(12.1)	*	Generation: 4 1 2 3 5	
		2	80.6(8.4)			— ———	
		3	79.3(17.0)				
		4	129.9(12.3)				
		5	55.5(15.9)				
	YCT plus 10^8 cells/ml algae	1	57.1(19.3)	87.1(11.5)	*	Generation: 5 4 3 2 1	
		2	67.7(14.0)			———	
		3	84.9(18.8)			———	
		4	109.0(12.4)			———	
		5	116.6(10.2)				

1 Standard error in parentheses
2 ANOVA alpha level = 0.05 for type I error rate
NS = no significant difference
* = $0.01 < P < 0.05$

Table 4 Mean brood size for the life-span test.

	Diet	Generation	Young[1]	Mean[1] Young	ANOVA[2] among Generation	Duncan's Multiple Range Test within Generation	ANOVA[2] among Diet
MEAN BROOD SIZE	YCT alone	1	5.3(1.4)	7.7(0.4)	*	Generation: 5 3 4 2 1	NS
		2	6.5(1.1)				
		3	8.9(0.6)				
		4	8.0(0.6)				
		5	9.3(0.5)				
	YCT plus 10^6 cells/ml algae	1	8.1(0.5)	7.7(0.3)	NS		
		2	8.3(0.8)				
		3	6.8(0.7)				
		4	9.2(0.6)				
		5	6.5(0.8)				
	YCT plus 10^7 cells/ml algae	1	7.5(0.9)	8.1(0.4)	**	Generation: 4 2 1 3 5	
		2	9.2(0.5)				
		3	7.4(0.9)				
		4	10.4(0.7)				
		5	6.1(1.2)				
	YCT plus 10^8 cells/ml algae	1	6.0(1.2)	7.8(0.4)	*	Generation: 5 4 3 2 1	
		2	6.5(1.1)				
		3	7.1(1.0)				
		4	8.9(0.6)				
		5	9.7(0.7)				

1 Standard error in parentheses
2 ANOVA alpha level = 0.05 for type I error rate
NS = no significant difference
* = $0.01 < P < 0.05$
** = $0.001 < P < 0.01$

Table 5 Seven-day average young per female for the life-span test.

	Diet	Generation	Young[1]	Mean[1] Young	ANOVA[2] among Generation	Duncan's Multiple Range Test within Generation	ANOVA[2] among Diet
7-DAY AVERAGE YOUNG PER FEMALE	YCT alone	1	10.3(3.0)	17.8(1.4)	**	Generation: 5 3 2 4 1	NS
		2	16.6(2.5)				
		3	19.1(1.3)				
		4	16.2(3.8)				
		5	26.2(2.8)				
	YCT plus 10^6 cells/ml algae	1	20.9(2.0)	20.6(1.0)	*	Generation: 2 4 1 3 5	
		2	25.6(1.8)				
		3	17.7(2.4)				
		4	22.4(1.4)				
		5	16.4(2.4)				
	YCT plus 10^7 cells/ml algae	1	20.1(3.3)	23.4(1.9)	***	Generation: 4 2 1 5 3	
		2	26.3(2.7)				
		3	14.4(2.9)				
		4	39.3(2.0)				
		5	15.9(3.7)				
	YCT plus 10^8 cells/ml algae	1	13.0(3.6)	20.3(1.6)	NS		
		2	20.4(4.2)				
		3	17.9(3.0)				
		4	22.0(2.1)				
		5	25.7(3.6)				

1 Standard error in parentheses
2 ANOVA alpha level = 0.05 for type I error rate
NS = no significant difference
* = $0.01 < P < 0.05$
** = $0.001 < P < 0.01$
*** = $P < 0.001$

Table 6 Three-brood average young per female for the life-span test.

	Diet	Generation	Young[1]	Mean[1] Young	ANOVA[2] among Generation	Duncan's Multiple Range Test within Generation	ANOVA[2] among Diet
3-BROOD AVERAGE YOUNG PER FEMALE	YCT alone	1	10.3(3.0)	17.5(1.3)	*	Generation: 5 3 2 4 1	NS
		2	16.6(2.5)				
		3	19.1(1.3)				
		4	16.2(3.8)				
		5	24.7(1.8)				
	YCT plus 10^6 cells/ml algae	1	20.9(2.0)	20.3(1.0)	*	Generation: 2 4 1 3 5	
		2	25.6(1.8)				
		3	17.7(2.4)				
		4	21.2(1.8)				
		5	16.4(2.4)				
	YCT plus 10^7 cells/ml algae	1	20.1(3.3)	21.4(1.5)	***	Generation: 4 2 1 5 3	
		2	26.3(2.7)				
		3	14.4(2.9)				
		4	30.0(1.0)				
		5	15.9(3.7)				
	YCT plus 10^8 cells/ml algae	1	13.0(3.6)	19.1(1.4)	NS		
		2	20.4(4.2)				
		3	17.9(3.0)				
		4	22.0(2.1)				
		5	20.5(3.0)				

1 Standard error in parentheses
2 ANOVA alpha level = 0.05 for type I error rate
NS = no significant difference
* = $0.01 < P < 0.05$
*** = $P < 0.001$

Table 7 Seven-day and 3-brood average young per female for the seven-day test.

	Diet	Generation	Young[1]	Mean[1] Young	ANOVA[2] among Generation	Duncan's Multiple Range Test within Generation	ANOVA[2] between Diet
7-DAY AVERAGE YOUNG PER FEMALE	YCT alone	1	29.2(3.0)	27.5(0.9)	***	Generation: 4 7 1 3 5 2 6	***
		2	21.7(0.8)				
		3	27.5(1.2)				
		4	33.0(2.9)				
		5	27.1(0.9)				
		6	21.3(1.7)				
		7	32.9(2.9)				
	YCT plus 10^7 cells/ml algae	1	48.1(3.8)	40.7(1.2)	***	Generation: 2 1 7 4 6 5 3	
		2	49.3(3.3)				
		3	31.2(1.1)				
		4	39.3(1.3)				
		5	37.0(1.1)				
		6	39.3(3.2)				
		7	40.4(3.1)				
3-BROOD AVERAGE YOUNG PER FEMALE	YCT alone	1	20.4(2.4)	24.5(0.7)	***	Generation: 7 3 4 5 6 1 2	***
		2	20.2(0.9)				
		3	27.5(1.2)				
		4	27.4(1.8)				
		5	27.1(0.9)				
		6	21.3(1.7)				
		7	27.8(1.1)				
	YCT plus 10^7 cells/ml algae	1	28.6(2.8)	32.2(0.9)	***	Generation: 4 5 7 2 6 1 3	
		2	33.8(1.7)				
		3	23.8(2.9)				
		4	37.2(1.7)				
		5	36.1(1.0)				
		6	32.2(2.3)				
		7	33.9(2.1)				

1 Standard error in parentheses
2 ANOVA alpha level = 0.05 for type I error rate
*** = $P < 0.001$

Table 8 Mean young per brood for the first three broods (standard error in parentheses).

Diet	Generation	1st Brood (mean yg/ brood)	2nd Brood (mean yg/ brood)	3rd Brood (mean yg/ brood)	Grand Mean
YCT	1	4.0(2.3)	6.3(2.7)	7.7(4.2)	
	2	3.4(1.1)	8.1(2.8)	7.8(2.1)	
	3	4.2(2.4)	8.0(2.1)	8.5(3.4)	
	4	4.4(1.9)	10.0(3.1)	7.7(2.7)	
	5	5.3(1.1)	8.9(3.4)	11.8(3.6)	
	Mean	4.3(0.7)	8.3(1.4)	8.7(1.8)	7.1(2.4)
YCT plus 10^6 cells/ml algae	1	3.5(0.8)	8.4(2.4)	10.3(3.8)	
	2	4.9(1.9)	8.4(2.2)	12.3(4.4)	
	3	4.0(1.6)	8.1(3.8)	8.0(2.6)	
	4	4.1(2.3)	6.5(3.0)	11.9(3.9)	
	5	3.9(1.4)	7.3(3.6)	8.7(2.5)	
	Mean	4.1(0.5)	7.7(0.8)	10.2(1.9)	7.4(2.9)
YCT plus 10^7 cells/ml algae	1	3.3(0.5)	6.0(1.8)	11.7(5.5)	
	2	5.2(2.0)	9.3(2.3)	15.8(4.0)	
	3	4.6(1.6)	4.7(2.4)	8.8(3.8)	
	4	3.8(1.0)	9.2(1.7)	17.0(2.4)	
	5	3.4(1.6)	6.8(3.3)	11.8(5.3)	
	Mean	4.1(0.8)	7.2(2.0)	13.0(3.3)	8.1(4.4)
YCT plus 10^8 cells/ml algae	1	2.6(1.4)	5.2(3.3)	12.0(2.0)	
	2	4.6(1.9)	7.1(4.8)	12.9(3.9)	
	3	5.1(1.2)	3.9(1.6)	11.1(2.2)	
	4	4.4(1.3)	8.7(3.7)	11.1(1.9)	
	5	4.7(1.6)	9.0(1.8)	11.0(4.1)	
	Mean	4.3(1.0)	6.8(2.2)	11.6(0.8)	7.6(3.4)

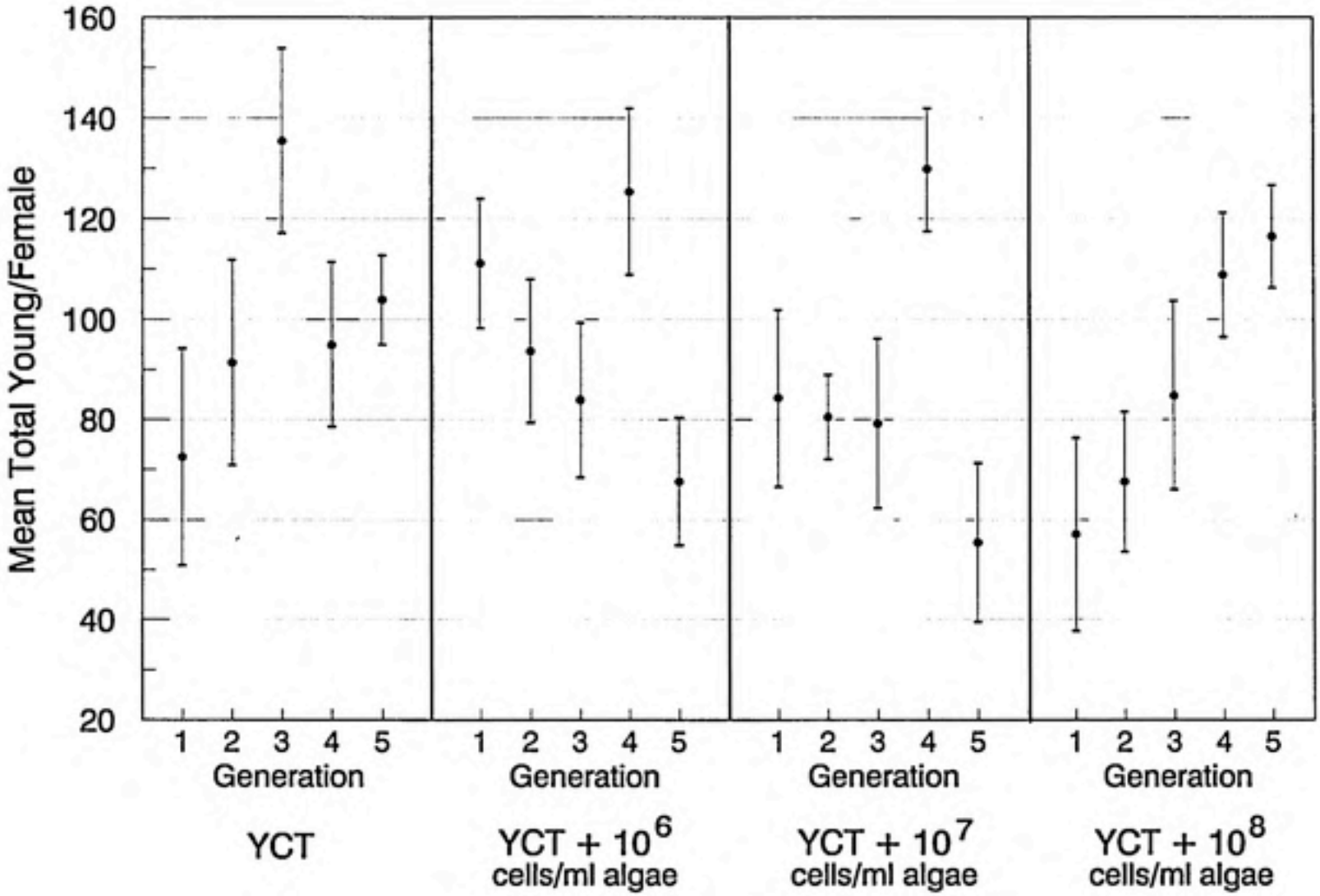


Figure 1 Mean total young/female for the life-span test (mean±standard error bars).

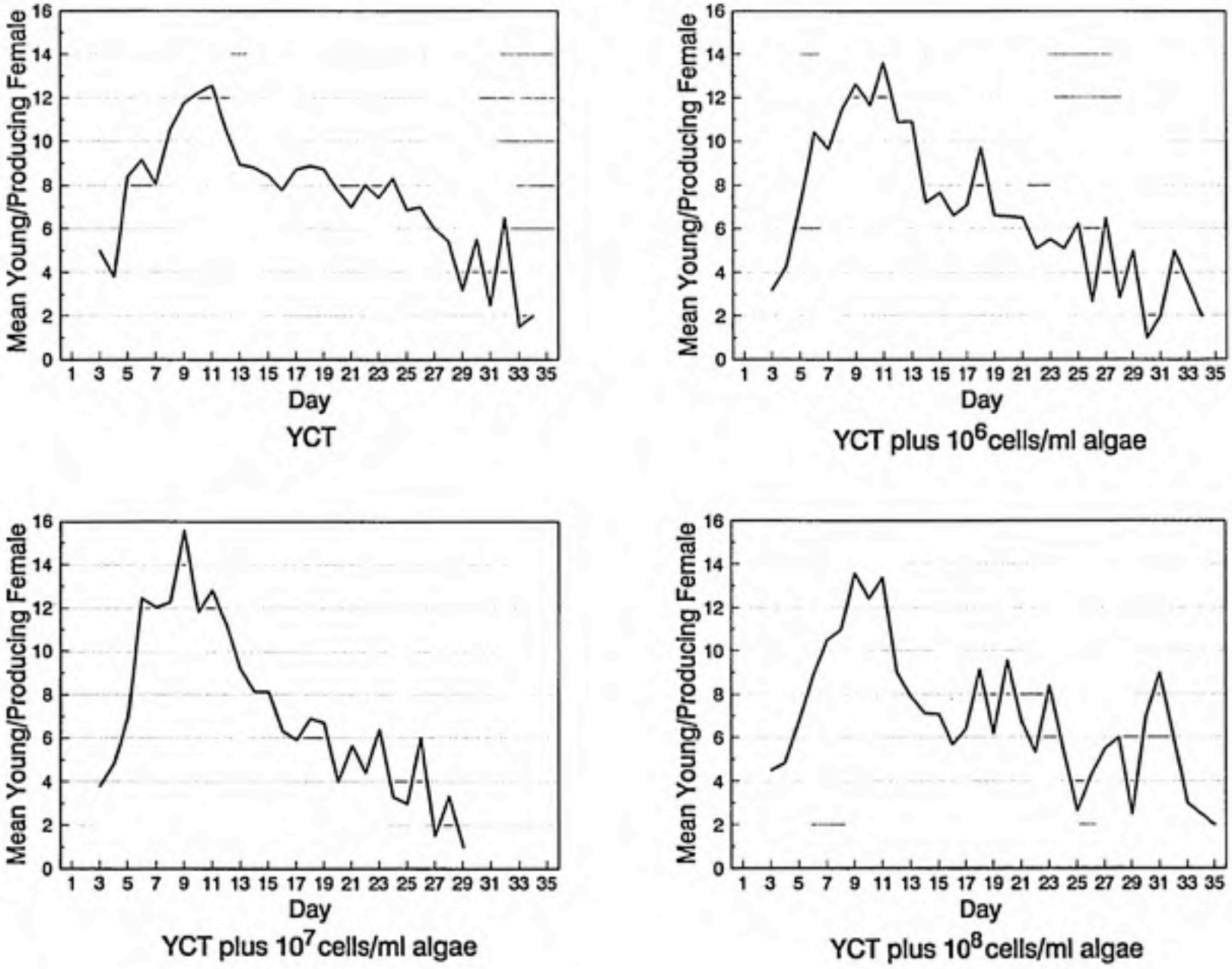
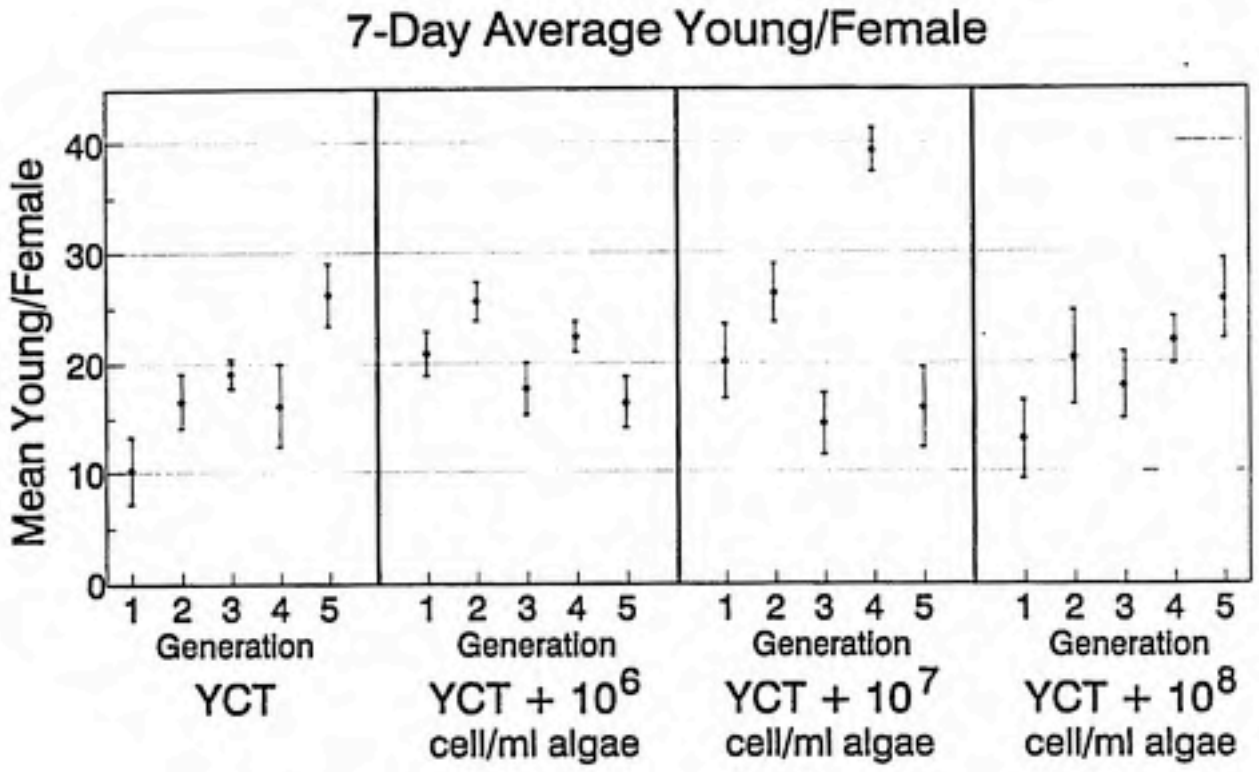


Figure 2 Mean young per producing female per day for the life-span test.



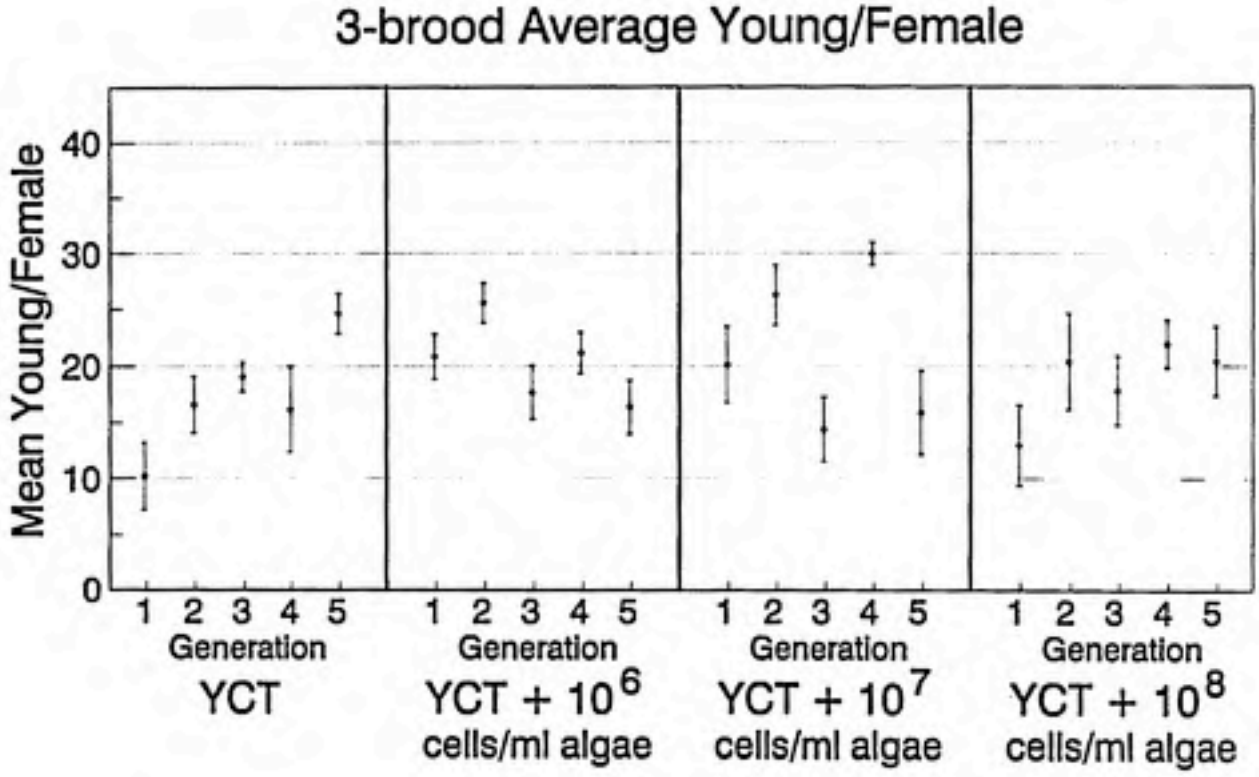
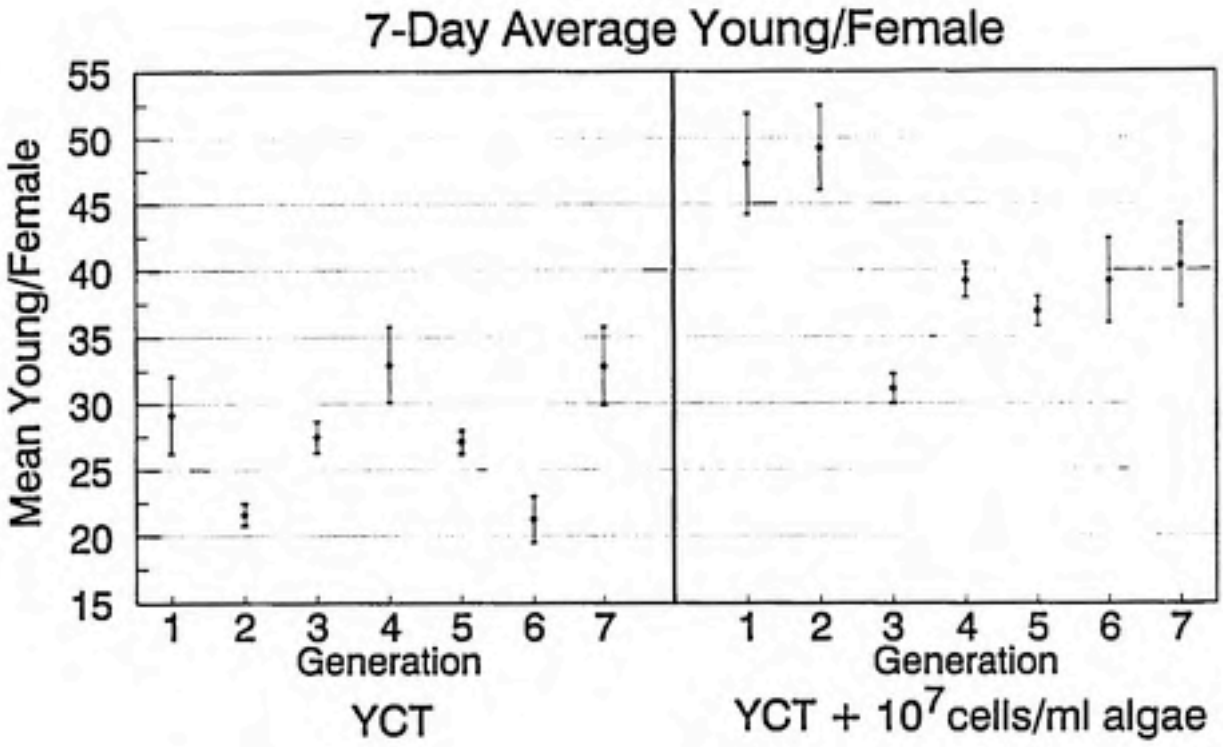


Figure 3 Seven-day and three-brood average young/female for the life-span test (mean ±standard error bars).



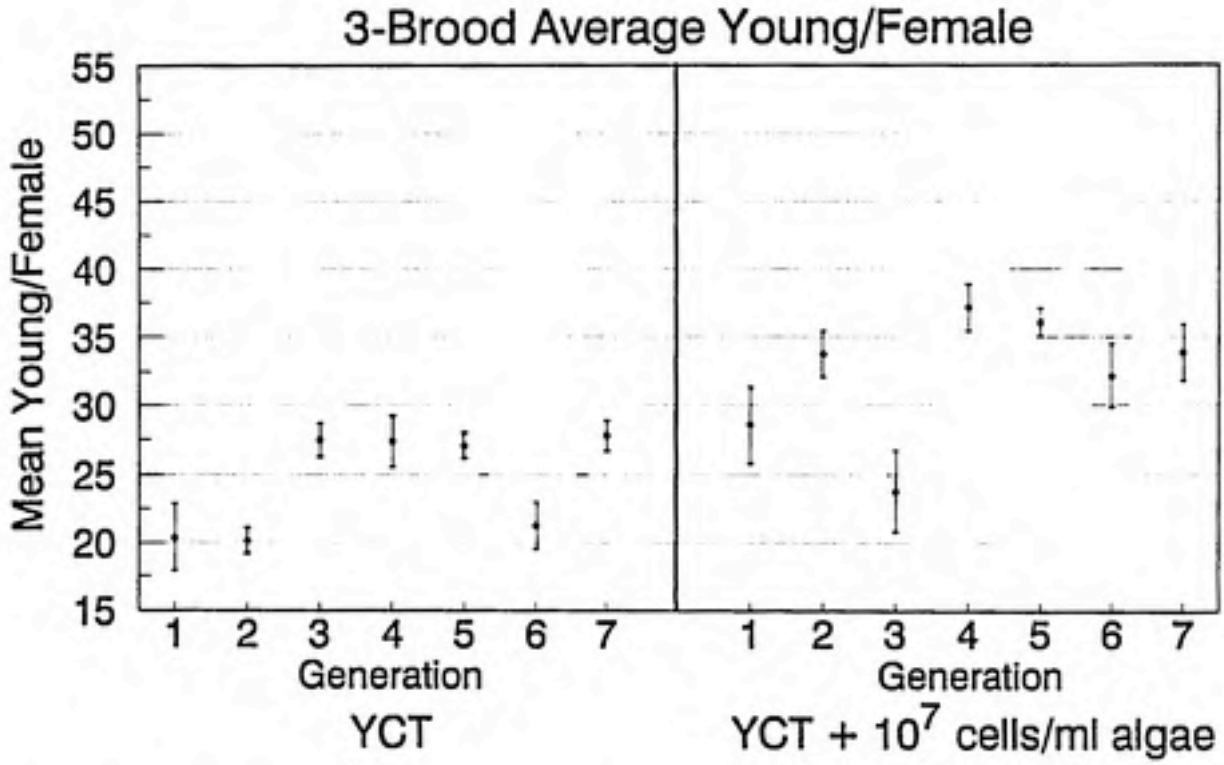


Figure 4 Seven-day and three-brood average young/female for the seven-day test (mean±standard error bars).

APPENDIX A

Carolina Power & Light Company's
Standard Operating Procedure for
Ceriodaphnia Culturing

10/28/88 – 09/27/89

SUBJECT: Ceriodaphnia Culturing and Health Check

1.0 Purpose

To ensure the standardization of culture techniques and compliance with applicable state guidelines.

2.0 Scope and Frequency

As outlined in Section 7.0 of this procedure.

3.0 Summary of Methods

There are four types of *Ceriodaphnia* cultures to ensure long-term culture viability and near-term neonate production. These are fed, water changed, renewed, and identified as follows:

The mass cultures. These are two 10-gallon aquariums that are fed daily (except weekends), renewed every three weeks, and restarted yearly from a single, identified adult. These cultures are used to start the other cultures if needed.

The P1000 culture. This culture is maintained in a 1000-ml beaker, is fed daily (except weekends), and is renewed weekly. It is used to provide healthy adults for neonate production.

The P230 culture. This culture is used to monitor the young production of the *Ceriodaphnia* to determine if they can be used for "test neonate" production. The culture consists of *Ceriodaphnia* removed from the P1000 culture and placed two per 30-ml plastic cup with 20 ml of culture water. The culture is started 7 to 10 days before a test is scheduled. Feeding is daily and the water is changed every other day (except weekends) until termination.

The P130 culture. This culture is used to obtain neonates of known age for effluent or reference toxicant tests. The culture consists of P230 adults that are separated to one per 30-ml cup. They are fed daily and the water is changed when young are produced.

4.0 Equipment or Apparatus

4.1 Two 10-gallon glass aquariums.

4.2 1,000-milliliter glass beaker.

4.3 30-milliliter plastic cups.

4.4 150-watt aquarium heaters with controllers.

4.5 Countertop environmental chamber with two 40-watt fluorescent lights on a 24-hour timer, and the ability to maintain 25 degrees Celsius plus or minus 2 degrees.

4.6 80-watt fluorescent lamp with 24-hour timer.

4.7 20 or less micron filter.

4.8 5 3/4-inch long Pasteur glass pipettes.

4.9 3-ml plastic pipettes.

4.10 20-ml vials.

4.11 Dissecting microscope.

4.12 YSI dissolved oxygen meter.

4.13 *Ceriodaphnia* food.

4.14 *Ceriodaphnia* culture maintenance log sheet (EXHIBIT A, B, or C).

4.15 Assorted glassware.

4.16 Stirring plate and bar.

4.17 50-ml titrating pipettes and stand.

4.18 pH meter.

4.19 2-liter Erlenmeyer flask.

4.20 20-liter Nalgene carboy.

4.21 5-gallon plastic bucket.

4.22 Hatching jar.

4.23 Air pump.

5.0 Reagent List

5.1 Calcium sulfate power (analytical reagent grade).

5.2 Zeigler Bros. fish food pellets (1/8 inch size).

5.3 Cerophyl (Sigma Chemical Co. No. G7141).

5.4 Dry yeast.

5.5 Standard EDTA.

5.6 Hardness buffer.

5.7 pH 4.0 and 7.0 buffers.

5.8 Commercial detergent.

6.0 Limitations, Precautions, and Interferences

6.1 Temperature of cultures must be maintained at 25°C ± 2°C.

6.2 Light must be maintained at 16 hours light and 8 hours dark.

6.3 *Ceriodaphnia* food must be kept frozen until use.

6.4 Culture water should be between 30- and 50-mg/l hardness.

6.5 Culture water should be between 6.0 and 8.0 pH.

7.0 Procedure

7.1 Culture/Dilution Water. The culture and dilution water is surface water from Harris Lake. The water is pumped through a 20-micron (or less) filter. Once filtered, the hardness is adjusted to between 30 and 50 mg/l.

A. Stock solution for adjusting the hardness of Harris water. This is a modification of the USEPA's synthetic freshwater procedure on page 22 of Methods for Measuring Acute Toxicity of Effluents to Freshwater and Marine Organisms. (1) In a 2-liter flask, add 2 liters of deionized water (DI water) and 1.2 grams of calcium sulfate. Place it on the stirring plate and dissolve the calcium sulfate. This is difficult and heating the solution will aid in dissolving. (2) Harris water averages approximately 16 mg/l of hardness. (3) To 18 liters of Harris water, add 1 liter of the stock solution. (4) This should bring the hardness to approximately 46 mg/l. (5) After adding the stock solution, conduct a hardness test. If not in the 30-50 mg/l range, add stock solution or dilute with Harris water. Recheck the hardness after each adjustment until the desired value is obtained.

B. After the hardness is acceptable, measure the pH and dissolved oxygen (DO). The pH should be between 6.0 and 8.0. The DO should be greater than 6 ppm. If not, aerate the water.

C. When all values are acceptable, this becomes the culture/dilution water.

D. The stock solution may be mixed in 20-liter volumes and stored in the 20-liter Nalgene carboy labeled for this use.

E. The culture/dilution water is stored in a marked 5-gallon bucket and used as needed.

F. The DO of the culture water should always be checked before using.

G. An analysis of Harris Lake water is conducted on a bimonthly basis by the CP&L Analytical Laboratory.

7.2 *Ceriodaphnia* Food. The food used by CP&L is the Yeast-Cerophyl-Trout Chow (YCTC) diet as described on page 63 of Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms.

A. Place 10 grams of Zeigler Bros. fish food in a hatching jar and add 2 liters of DI water. Aerate enough to keep the food in suspension. Ferment for 7 days.

B. Six days after starting the fish food, combine 10 grams of Cerophyl and 2 liters of DI water in an Erlenmeyer flask and stir for 24 hours.

C. On Day 7, add 3 grams of yeast to 600 ml of DI water and stir for an hour.

D. On Day 7, shut off the air to the food and allow to settle. Pour off 600 ml. Allow the Cerophyl to settle and pour off 600 ml. Combine all the ingredients for a total of 1800 ml of *Ceriodaphnia* food. Discard the dregs of the fish food and Cerophyl.

E. Mix the food well and pour approximately 15 ml per 20-ml vial until all the food is distributed.

F. Freeze these vials and thaw as needed.

G. Start the next batch of food when approximately 40 vials of the previous food remains.

7.3 Culture Origin. All four *Ceriodaphnia* cultures come from a single source. The record of the source is maintained in the laboratory culture logbook located at the Biomonitoring Laboratory.

7.4 Culture Health.

A. Ensure culture health and performance by monitoring the response of control animals during tests and by conducting

reference toxicant tests minimally two times per month with cadmium chloride. These reference toxicant tests are conducted using reagent-grade cadmium chloride, but at least three times per year, the reference tests are performed in duplicate using the reagent-grade cadmium chloride and EPA cadmium chloride reference standards for comparison.

B. Perform the reference tests using acute toxicity testing procedures for 24-hour exposure in moderately hard reconstituted water with neonate *Ceriodaphnia* (see Procedure 7.9.3). Neonates are obtained as discussed in Sections 7.6.3 and 7.6.4 of this procedure.

C. Use accumulative results of reference tests to calculate cusum (control) charts (see Reference 10.2) following each reference test conducted. If the culture is healthy and correct procedures are followed, estimated individual LC50s should fall within the control values. If the estimated LC50 falls out of the control limit range, the following steps must be taken:

1. Check test quality data (e.g., temperature, DO) for excursions or procedural discrepancies. If causal factors are found, the reference test is discarded and a new test is performed as soon as possible. If no causal factors can be identified or if the repeat reference test is also out of range, the laboratory supervisor must be informed and investigation of possible culturing and/or procedural problems is initiated.

2. The laboratory supervisor or designee determines which required effluent tests may have been affected by poor culture health/procedural problems and informs the appropriate responsible plant officials and Environmental Compliance Unit. Environmental Compliance Unit personnel inform the state laboratory. The laboratory supervisor or designee reschedules tests for those effluents as soon as the culture/procedural problems are corrected, and a reference test is completed within range results.

7.5 Culture Identification. A small number of animals are sampled, identified to species using a key(s) (see References) and compound microscope, and preserved from the P1000 culture once per month by laboratory personnel. The preserved specimens are maintained at the Bioassay Laboratory as a reference collection of the species utilized in effluent toxicity tests.

7.6 Culturing.

A. Mass cultures.

1. Vessels--The mass cultures are maintained in two 10-gallon aquariums designated as No. 1 and No. 2.

2. Food--The cultures are fed 8 ml of YCTC daily except on weekends.

3. Light--16 hours light/8 hours dark. Light is provided by a 80-watt (total) fluorescent lamp controlled by a 24-hour appliance timer.

4. Temperature--Temperature is controlled by a 150-watt aquarium heater. Temperature is maintained at 25 degrees Celsius plus or minus 2 degrees. It is monitored daily and recorded on the log sheet (EXHIBIT A).

5. Renewal--The cultures are renewed every three weeks. The water and *Ceriodaphnia* are removed and the aquarium is washed with detergent and rinsed with Harris Lake water. Ten gallons of culture water is added and 15 ml of food. The temperature is allowed to adjust to 25 degrees Celsius and 20 to 30 young *Ceriodaphnia* from the P1000 culture are introduced. The record of the renewal in entered on maintenance log (EXHIBIT A). The mass cultures are restarted yearly from a single identified adult.

6. DO--The DO is monitored daily and recorded on log sheet (EXHIBIT A). If the DO falls below 6.0 ppm, the culture should be aerated by using gentle aeration.

7. Aeration--If it is needed, it is provided by a standard aquarium air pump and air stone.

B. P1000. This culture is maintained in the environmental chamber.

1. Vessel--1000-ml glass beaker.

2. Food--The culture is fed 3 ml of YCTC daily except weekends.

3. Light--16 hours light/8 hours dark provided by the environmental chamber's 80-watt fluorescent lamp controlled by a 24-hour timer.

4. Temperature--Temperature is maintained at 25 degrees Celsius plus or minus 1 degree controlled by the environmental chamber.

5. Renewal--The culture is renewed on Monday of each week. A clean beaker is filled with culture water, temperature

adjusted to 25 degrees and 3 ml of food added. The water from the old P1000 culture is poured in stages into a 30-ml cup and observed under a microscope. Ten to twelve egg-bearing adults are pipetted from the old culture water and placed in the new culture. After the number is obtained, the remaining old culture is discarded and the new culture is placed in the environmental chamber. The renewal is noted on the log sheet (EXHIBIT B). The old beaker is washed and stored.

6. DO--The DO is monitored daily and recorded on the log sheet. If the DO falls below 5.5 ppm, the culture is discarded and a new one is started with *Ceriodaphnia* from the mass culture. The DO is recorded on the log sheet.

7. Aeration--The culture is aerated with single-bubble aeration provided by the aquarium pump and a pipette.

C. P230. This is two *Ceriodaphnia*/30-ml cup. The culture is maintained in the environmental chamber. This culture is activated 7 to 10 days before a test is scheduled.

1. Vessel--30-ml cups.

2. Food--Culture is fed 0.13 ml of YCTC food daily except on weekends.

3. Light--16 hours light/8 hours dark controlled by the environmental chamber.

4. Temperature--Temperature is maintained at 25 degrees Celsius plus or minus 1 degree controlled by the environmental chamber.

5. Starting--This culture is started with young *Ceriodaphnia* from the P1000 culture. The P1000 culture needs to be at least 4 days old to start this culture. Fill 30 to 50 30-ml cups (depends on the number of neonates needed). As a rule of thumb, use at least the number of P1000 *Ceriodaphnia* young as neonates needed. For example, if you need 60 neonates to start the test then use at least 30 cups (= 60 young). Fill 30-ml cups with 20 ml of culture water and add 0.13 ml of food. Pour a small amount of P1000 water into a cup and place under a microscope. Pipette 2 young/ prepared cup until the desired number is reached. Return the removed water to the P1000 culture and place it in the chamber (unless it is Monday, then renew it). Place the P230s in the chamber.

6. Renewal--The culture is renewed every other day except weekends (unless personnel are scheduled for test work). Fill clean cups with 20 ml of culture water and add 0.13 ml of food. Adjust the temperature to 25 degrees Celsius. Place each old P230 cup under the microscope and pipette the 2 adults into the fresh cup. Discard old water and any young produced. Continue this until all cups are renewed. Return the new cups to the chamber and wash the old cups. Record the renewal on the log sheet.

7. Aeration--None.

D. P130. The P130 culture is used to obtain "known age" neonates for testing. It is the P230 adults separated in individual 30-ml cups and is initiated at least 2 days before a test begins. It is maintained in the environmental chamber.

1. Vessel--30-ml cups.

2. Food--Culture is fed 0.13 ml of YCTC food daily.

3. Light--16 hours light/8 hours dark provided and controlled by the environmental chamber.

4. Temperature--Temperature 25 degrees Celsius plus or minus 1 degree controlled by the environmental chamber.

5. Starting--The culture is started by separating the P230 adults into single 30-ml cups. Add 20 ml of culture water and 0.13 ml of food to clean 30-ml cups and adjust the temperature to 25 degrees Celsius. Place a P230 cup under the microscope and pipette an adult into the fresh cup. Repeat with the second adult and discard the P230 water. Continue until all P230 adults are in single cups. Place the P130s in the environmental chamber. Wash the old cups.

6. Renewal--Renewal depends on the females that are producing the neonates. The purpose of this culture is to produce neonates for a *Ceriodaphnia* test which requires neonates less than 24 hours old hatched within a 4-hour span. Twenty-four hours before the test is scheduled to start, check all cups and remove any that have neonates. Fill clean cups with 20 ml of culture water and 0.13 ml of food. Pipette the adults that have produced neonates into the fresh solution and place all P130s back into the chamber. Discard the old water and wash the cups. Starting at the time the cups were initially checked, check them every 4 hours, separating the cups with neonates. Continue this until the correct number of

neonates are hatched within a 4-hour span. If the correct number is not hatched within 24 hours, repeat the process. After the correct number is collected, the P130s can be discarded if another test is not scheduled within the next 3 days. If a test is scheduled, continue the above renewal until 24 hours before the test and then repeat the neonate collection process. The renewal is noted on the log sheet.

7. Aeration--None.

8.0 Calculations

Cusum charts are calculated as described in Reference 10.2.

9.0 Results

N/A

10.0 Reference and Definitions

10.1 Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms USEPA. EPA/600/4-85/014.

10.2 Methods for measuring acute toxicity of effluents to freshwater and marine organisms USEPA. EPA/600/4-85/013.

10.3 Biological laboratory certification guidance manual for effluent toxicity testing NCDEM.

10.4 Scourfield, D. J., and J. P. Harding. 1966. A key to the freshwater Cladocera. Freshwater Biological Association, Scientific Publication No. 5.

10.5 USEPA. 1986. Taxonomy of Ceriodaphnia (Crustacea: Cladocera) in USEPA Cultures. EPA 600/4-86/032.

10.6 Neonate--Young *Ceriodaphnia* less than 24 hours old.

11.0 Quality Control

11.1 *Ceriodaphnia* identification is verified monthly.

11.2 *Ceriodaphnia* reference collection is maintained at the Biomonitoring Laboratory.

11.3 Reference toxicity tests are conducted two times monthly.

Uncontrolled Document

EXHIBIT A

CERIODAPHNIA MASS CULTURE LOG

Aquarium #_____ CDDP/7-20-86 2nd

Date	Renewal	Temp.	D.O.	Hardness	Name	Comments

ACCORDING TO PROCEDURE 7.9.5

CERIODAPHNIA "P" CULTURE LOG

P1000

Date	Date Renewed	Temp.	D.O.	Hard-ness	Name	Comments

Uncontrolled Document

EXHIBIT C

CERIODAPHNIA "P" CULTURE LOG

PROCEDURE 7.9.5

P230

Date	Start Date	Temp	Hard-ness	Date Discontinued	Comments

P130

Date	Start	Temp	Hard ness	Date Discont.	Time of Young	# of Young	Test #	Name

Uncontrolled Document

Rev. No.	Rev. Date
0	OCT 2 8 1988

Rev. 0 Reviewed by: [redacted]

Approved by: [redacted]

APPENDIX B

Carolina Power & Light Company's
Standard Operating Procedure for
Ceriodaphnia Culturing

09/28/89 – 01/01/90

SUBJECT: *Ceriodaphnia* Culturing and Health Check

1.0 Purpose

To ensure the standardization of culture techniques and compliance with applicable state guidelines.

2.0 Scope and Frequency

As outlined in Section 7.0 of this procedure.

3.0 Summary of Methods

There are three types of *Ceriodaphnia* cultures to ensure long-term culture viability and near-term neonate production. These cultures are fed, renewed, and identified as follows:

The mass culture. A 10-gallon aquarium is fed daily (except weekends), renewed every three weeks, and restarted yearly from a single, identified adult. This culture is used to start the other cultures if needed.

The P1000 culture. This culture is maintained in a 1000-ml beaker, is fed daily (except weekends), and is renewed weekly. It is used to provide healthy adults for neonate production.

The P130 culture. This culture is used to obtain neonates of known age for effluent or reference toxicant tests. They are fed daily and renewed when young are produced.

4.0 Equipment or Apparatus

4.1 10-gallon glass aquarium.

4.2 1,000-milliliter glass beaker.

4.3 30-milliliter plastic cups.

4.4 Aquarium heater with controller.

4.5 Countertop environmental chamber with two 40-watt fluorescent lights on a 24-hour timer, and the ability to maintain 25 degrees Celsius plus or minus 2 degrees.

4.6 80-watt fluorescent lamp with 24-hour timer.

4.7 20 or less micron filter.

4.8 5 3/4-inch-long Pasteur glass pipettes and bulbs.

4.9 3-ml plastic pipettes.

4.10 20-ml vials.

4.11 Dissecting microscope.

4.12 YSI dissolved oxygen meter.

4.13 *Ceriodaphnia* food: YCT and algae.

4.14 *Ceriodaphnia* culture maintenance log sheets (EXHIBITS A, B, and C).

4.15 Assorted glassware.

4.16 Stirring plate and bar.

4.17 50-ml titrating pipettes and stand.

4.18 pH meter.

4.19 2-liter Erlenmeyer flask.

4.20 20-liter Nalgene carboy.

4.21 5-gallon plastic bucket.

4.22 Hatching jar.

4.23 Air pump.

4.24 1/2-gallon jugs or 2000-ml flasks.

4.25 4-liter aspirator bottle.

4.26 1-ml syringe.

4.27 Whirlpaks.

4.28 Centrifuge and tubes.

4.29 Neubauer counting chamber or fluorometer.

4.30 Automatic pipette.

4.31 Telethermometer.

4.32 Electronic chart thermometer.

4.33 Balance.

4.34 Conductivity meter.

5.0 Reagent List

5.1 Calcium sulfate power (analytical reagent grade).

5.2 Zeigler Bros. fish food pellets (1/8-inch size).

5.3 Cerophyl (Sigma Chemical Co. No. G7141).

5.4 Dry yeast.

5.5 Deionized (DI) water.

5.6 Reagents for hardness tests (see Procedure 7.9.7).

5.7 pH 4.0 and 7.0 buffers.

5.8 Commercial detergent.

5.9 Reference toxicant solutions.

5.10 Vitamin stock solution (see Reference 10.2G).

5.11 Nutrient media solutions (see Reference 10.2B).

5.12 *Selenastrum capricornutum* or other algae species.

6.0 Limitations, Precautions, and Interferences

6.1 Temperature of cultures must be maintained at 25°C ± 2°C.

6.2 Light must be maintained at 16 hours light and 8 hours dark.

6.3 *Ceriodaphnia* food (YCT) must be kept frozen until use.

6.4 Culture water should be between 30- and 50-mg/l hardness.

6.5 Culture water should be between 6.0 and 8.0 pH.

6.6 Algae cultures are easily contaminated by other microorganisms.

6.7 YCT and algal concentrate should be thoroughly mixed by shaking before dispensing.

7.0 Procedure

7.1 Culture/Dilution Water. The culture and dilution water is local surface water. The water is pumped through a 20-micron (or less) filter. Once filtered, the hardness is adjusted to between 30 and 50 mg/l.

A. Stock solution for adjusting the hardness of surface water. This is a modification of the USEPA's synthetic freshwater procedure on page 22 of Methods for Measuring Acute Toxicity of Effluents to Freshwater and Marine Organisms. (1) In a 2-liter flask, add 2 liters of deionized water (DI water) and 1.2 grams of calcium sulfate. Place it on the stirring plate and dissolve the calcium sulfate. This is difficult and heating the solution will aid in dissolving. (2) The local (New Hill area) surface water averages approximately 16 mg/l of hardness. (3) To 14 liters of surface water, add 1 liter of the stock solution. (4) This should bring the hardness to approximately 46 mg/l. (5) After adding the stock solution, conduct a hardness test (Procedure 7.9.7). If not in the 30-50 mg/l range, add stock solution or dilute with surface water. Recheck the hardness after each adjustment until the desired value is obtained.

B. After the hardness is acceptable, measure the pH and dissolved oxygen (DO). The pH should be between 6.0 and 8.0. The DO should be greater than 6 ppm. If not, aerate the water.

C. When all values are acceptable, this becomes the culture/dilution water.

D. The stock solution may be mixed in 20-liter volumes and stored in the 20-liter Nalgene carboy labeled for this use.

E. The culture/dilution water is stored in a marked 5-gallon bucket and used as needed.

F. The DO of the culture water should always be checked before using.

G. An analysis of the surface water is conducted on alternate months by the CP&L Analytical Chemistry Laboratory.

7.2 *Ceriodaphnia* Food. The food used by CP&L is the Yeast-Cerophyl-Trout Chow (YCT) diet as described in Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms and algae.

YCT

A. Place 5 grams of Zeigler Bros. fish food in a hatching jar and add 1 liter of DI water. Aerate enough to keep the food in suspension. Ferment for 7 days.

B. Six days after starting the fish food, combine 5 grams of Cerophyl and 1 liter of DI water in an Erlenmeyer flask and stir for 24 hours.

C. On Day 7, add 1.5 grams of yeast to 300 ml of DI water and stir for an hour.

D. On Day 7, shut off the air to the food and allow to settle. Pour off 300 ml. Allow the Cerophyl to settle and pour off 300 ml. Combine all the ingredients for a total of 900 ml of *Ceriodaphnia* food. Pour off an additional 300 ml of trout food and Cerophyl and freeze. When a fresh batch of food is required, thaw and combine with 300 ml of yeast water. Discard the dregs of the fish food and Cerophyl.

E. Mix the food well and pour approximately 15 ml per 20-ml vial until all the food is distributed.

F. Freeze these vials and thaw as needed.

G. Start the next batch of food when approximately 40 vials of the previous food remains.

Suitability of New Lots of YCT

H. The suitability of new lots of YCT should be determined in side-by-side tests using two treatments with four replicates per treatment. In this test, the response of control test organisms fed with new food is compared with response of organisms fed a previously used, satisfactory food.

I. The dry weight of solids of each new batch of YCT should be measured before use. The food should contain 1.7-1.9 grams solids per liter. Use the following method:

1. Heat a clean 150-ml beaker for at least one hour in a 103°-105°C oven.

2. Cool in a desiccator for at least one hour and weigh.

3. Mix the sample. Using a graduated cylinder, transfer 100 ml of sample to the preweighed beaker. Rinse the graduated cylinder with deionized water and transfer to the sample beaker.

4. Dry at 103°-105°C in a drying oven or until completely dry.

5. Cool in a desiccator for at least one hour and reweigh.

Algae

J. Prepare nutrient medium solutions for algal cultures as described in Reference 10.2B.

K. Cultures are illuminated with ambient laboratory light and sequenced to 16 hours light, 8 hours dark.

L. Fill acid-washed culture vessels to no more than 20 percent of nutrient medium. Innoculate with 1 ml vitamin stock solution (prepared according to Reference 10.2G) per liter of nutrient medium.

M. Add algae either from a prepared slant or from existing stock cultures. Swirl twice daily (except weekends) by hand to keep algae cells suspended. The algae is harvested every four to seven days. Centrifuge and resuspend the algal pellet in *Ceriodaphnia* culture water to make an algal concentrate.

N. Count the number of cells/ml in the algae concentrate with a Neubauer counting chamber or fluorometer. Dilute or concentrate further to achieve the final desired cell count. Record on the *Ceriodaphnia* food chart at the Biomonitoring Laboratory.

O. Algal concentrate may be stored in the refrigerator for one month.

7.3 Culture Origin. All *Ceriodaphnia* cultures come from a single source. The record of the source is maintained in the laboratory culture logbook located at the Biomonitoring Laboratory.

7.4 Culture Health.

A. Ensure culture health and performance by monitoring the response of control animals during tests and by conducting reference toxicant tests. Reference toxicant acute tests should be conducted minimally two times per month with cadmium chloride reference standards from EPA. Reference toxicant chronic tests should be conducted minimally one time per month with sodium chloride from laboratory stock solutions.

B. Perform the acute and chronic reference tests using acute toxicity testing procedures for 48-hour exposure and chronic toxicity testing procedures for 7-day exposure in moderately hard reconstituted water or mineral water diluted to moderate hardness (see Procedure 7.9.3 or Procedure 7.9.1, respectively). Use neonate *Ceriodaphnia*. Neonates are obtained as discussed in Sections 7.6.3 and 7.6.4 of this procedure.

C. Use accumulative results of reference tests to calculate cusum (control) charts (see References 10.2 B and C for chronic and acute reference test procedures and analyses) following each reference test conducted. A minimum of five reference tests within control limits are required to calculate an adequate cusum chart. If the culture is healthy and correct procedures are followed, estimated individual LC50s and no observed effect concentrations (NOEC) should fall within the control values. If the estimated LC50 or NOEC fall out of the control limit range, the following steps must be taken:

1. Check test quality data (e.g., temperature, DO) for excursions or procedural discrepancies. If causal factors are found, the reference test is discarded and a new test is performed as soon as possible. If no causal factors can be identified or if the repeat reference test is also out of range, the laboratory supervisor must be informed and investigation of possible culturing and/or procedural problems is initiated.

2. The laboratory supervisor or designee determines which required effluent tests may have been affected by poor culture health/procedural problems and informs the appropriate responsible plant officials and Environmental Compliance Unit. Environmental Compliance Unit personnel inform the state laboratory if appropriate. The laboratory supervisor or designee reschedules tests for those effluents as soon as the culture/procedural problems are corrected, and a reference test is completed within range results.

7.5 Culture Identification. A small number of animals are sampled, identified to species using a key(s) (see References) and compound microscope, and preserved once per month by laboratory personnel. The preserved specimens are maintained at the laboratory as a reference collection of the species utilized in effluent toxicity tests.

7.6 Culturing.

A. Mass culture.

1. Vessel--The mass culture is maintained in a 10-gallon aquarium.

2. Food--The culture is fed 8 ml of YCT and 3 ml of algae daily (except weekends).

3. Light--16 hours light/8 hours dark. Light is provided by a 80-watt (total) fluorescent lamp controlled by a 24-hour timer.

4. Temperature--Temperature is controlled by an aquarium heater. Temperature is maintained at 25 degrees Celsius plus or minus 2 degrees. It is monitored daily (except weekends) and recorded on the log sheet (EXHIBIT A).

5. Renewal--The culture is renewed every three weeks. The water and *Ceriodaphnia* are removed and the aquarium is washed with detergent and rinsed. Ten gallons of culture water is added with YCT and algae. The temperature is allowed to adjust to 25 degrees Celsius and 20 to 30 young *Ceriodaphnia* from the P1000 culture are introduced. The record of the renewal is entered on the maintenance log (EXHIBIT A). All cultures are restarted yearly from a single identified adult.

6. DO--The DO is monitored daily (except weekends) and recorded on the log sheet (EXHIBIT A). If the DO falls below 6.0 ppm, the culture should be aerated by using gentle aeration.

7. Aeration--If needed, aeration is provided by a standard aquarium air pump and air stone.

B. P1000. This culture is maintained in the environmental chamber.

1. Vessel--1000-ml glass beaker.

2. Food--The culture is fed 3 ml of YCT and 3 ml algal concentrate daily (except weekends).

3. Light--16 hours light/8 hours dark provided by the environmental chamber's 80-watt fluorescent lamp controlled by a 24-hour timer.

4. Temperature--Temperature is maintained at 25 degrees Celsius plus or minus 2 degrees controlled by the environmental chamber.

5. Renewal--The culture is renewed each week. A clean beaker is filled with culture water, temperature adjusted to 25 degrees and food added. Ten to twelve egg-bearing adults are pipetted from the old culture water and placed in the new culture. After the number is obtained, the remaining old culture is discarded and the new culture is

placed in the environmental chamber. The renewal is noted on the log sheet (EXHIBIT B). The old beaker is washed and stored.

6. DO--The DO is monitored daily (except weekends) and recorded on the log sheet. If the DO falls below 5.5 ppm, the culture is discarded and a new one is started with *Ceriodaphnia* from the mass culture. The DO is recorded on the log sheet.

7. Aeration--The culture is aerated with single-bubble aeration provided by the aquarium pump and a pipette.

C. P130. The P130 culture is used to obtain "known age" neonates for testing. It is started as young separated in individual 30-ml cups and is initiated weekly. It is maintained in the environmental chamber.

1. Vessel--30-ml cups.

2. Food--Culture is fed 0.13 ml of YCT food and 0.1 ml algae daily.

3. Light--16 hours light/8 hours dark provided and controlled by the environmental chamber.

4. Temperature--Temperature 25 degrees Celsius plus or minus 2 degrees controlled by the environmental chamber.

5. Starting--The culture is started with neonates from the third or forth brood from the existing P130 culture. Use only neonates from broods containing eight or more young. Add 20 ml of culture water and food to clean 30-ml cups and adjust the temperature to 25 degrees Celsius. Place a cup under the microscope and pipette a neonate into the fresh cup. Continue until all cups have a neonate. Place the P130s in the environmental chamber. Wash the old cups.

6. Renewal--Renewal depends on the females that are producing the neonates. The purpose of this culture is to produce neonates for a *Ceriodaphnia* test which requires neonates less than 24 hours old hatched within an 8-hour span and from broods with eight or more young. Twenty-four hours before the test is scheduled to start, check all cups and remove any that have neonates. Fill clean cups with 20 ml of culture water and add food. Pipette the adults that have produced neonates into the fresh solution and place all P130s back into the chamber. Discard the old water

and wash the cups. Starting at the time the cups were initially checked, check them every 8 hours or sooner, separating the cups with neonates. Continue this until the correct number of neonates are hatched within an 8-hour span. If the correct number is not hatched within 24 hours, repeat the process. After the correct number is collected, the P130s can be discarded or continued for 14 days. The renewal is noted on the log sheet.

7. Aeration--None.

8.0 Calculations

Cusum charts are calculated as described in References 10.2 B and C.

9.0 Results

N/A

10.0 References and Definitions

10.1 Definitions

Neonate--Young *Ceriodaphnia* less than 24 hours old.

10.2 References

A. Peltier, W. H., and C. I. Weber. (eds.) 1985. Methods for measuring the acute toxicity of effluents to freshwater and marine organisms. U.S. Environmental Protection Agency, Cincinnati, OH. EPA/600/4-85/013.

B. Weber, C. I., W. H. Peltier, T. J. Norgerg-King, W. B. Horning, II, F. A. Kessler, J. R. Menkedick, T. W. Neiheisel, P. A. Lewis, D. J. Klemm, Q. H. Pickering, E. L. Robinson, J. M. Lazorchak, L. J. Wymer, and R. W. Freyberg. 1989. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 2nd Edition. U.S. Environmental Protection Agency, Cincinnati, OH. EPA/600/4-89/001.

C. Horning, W., and C. I. Weber. (eds.) 1985. Methods for estimating the chronic toxicity of effluents to aquatic organisms. U.S. Environmental Protection Agency, Cincinnati, OH. EPA/600/4-85/014.

D. Scourfield, D. J., and J. P. Harding. 1966. A key to the freshwater Cladocera. Freshwater Biological Association, Scientific Publication No. 5.

E. USEPA. 1986. Taxonomy of Ceriodaphnia (Crustacea: Cladocera) in USEPA cultures. EPA 600/4-86/032.

F. NCDEM. 1989. Biological laboratory certification guidance manual for effluent toxicity testing.

G. Goulden, C., R. M. Comotto, J. A. Hendrickson, Jr., L. L. Horning, and K. L. Johnson. 1982. Procedures and recommendations for the culture and use of Daphnia in bioassay studies. Aquatic Toxicology and Hazard Assessment: Fifth Conference, ASTM STP 766. J. G. Pearson, R. B. Foster, and W. E. Bishop, (eds.). American Society for Testing and Materials. pp. 139-160.

11.0 Quality Control

11.1 *Ceriodaphnia* identification is verified monthly.

11.2 *Ceriodaphnia* reference collection is maintained at the laboratory.

11.3 Reference toxicity acute tests are conducted minimally two times monthly and a reference toxicity chronic test is conducted minimally once per month.

Uncontrolled Document

CERIODAPHNIA MASS CULTURE LOG

Aquarium #_____

Date	Renewal	D.O.	Temp	Hardness	Name	Comments

ACCORDING TO PROCEDURE 7.9.5

Uncontrolled Document

CERIODAPHNIA "P" CULTURE LOG

P100Q

Date	Date Renewed	D.O.	Temp	Hard-ness	Name	Comments

ACCORDING TO PROCEDURE 7.9.5

Uncontrolled Document

EXHIBIT C

CERIODAPHNIA "P" CULTURE LOG

PROCEDURE 7.9.5

P130

Date	Start Date	Temp	Hard-ness	Date Renew	Date Discon.	Age/# Yg.	Test #	Run #	Name/Comments

Uncontrolled Document

Rev. No.	Rev. Date
0	10/28/88
1	SEP 2 8 1989

Rev. 1 Initiated by: William D. Carter

Approved by: H. Booth

R. Lyatt

Bobby J. Ward