ABSTRACT

CHRISTINE A. LA ROCCA. Effects of Diet on the Survival, Reproduction and Sensitivity of Ceriodaphnia dubia. (Under the direction of DR. DONALD E. FRANCISCO)

The effect of diet on the health and robustness of Ceriodaphnia dubia was investigated. C. dubia were raised on three diets for 19 generations to evaluate survival and reproduction. The three diets used to culture C. dubia were the green alga Selenastrum capricornutum plus a mixture of yeast, Cerophyl^R, and trout chow (YCT), the green alga Chlamydomonas reinhardtii plus YCT, and a combination of the two species of algae plus YCT. C. dubia were also subjected to various copper concentrations to evaluate the relative sensitivity to toxicants of animals raised on different diets. The levels of survival and reproduction of C. dubia raised on all three diets satisfied the North Carolina Division of Environmental Management Mini-chronic Pass/Fail Ceriodaphnia Effluent Toxicity Test minimum standards for control animals used in a chronic toxicity test. Survival was not significantly different in any diet tested. Reproduction was higher in the S. capricornutum/C. reinhardtii/YCT diet than in the other diets. C. dubia raised on the S. capricornutum/C. reinhardtii/YCT diet were also less sensitive to copper than animals raised on the

single algae diets. The results suggest that a diet that includes multiple species of algae is nutritionally superior to one that includes a single species of algae.

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INTRODUCTION

Background

Early in the history of U.S. environmental policy, state agencies bore the primary responsibility for the formulation, implementation and enforcement of water pollution control laws and regulations, with the federal role limited to matters of interstate pollution (Lamb, 1985). Recognizing the inability of the states to effectively control water pollution, Congress passed a series of laws designed to shift the responsibility of cleaning the nation's surface waters from the state governments to the federal government. The passage of the Water Quality Act of 1965 (P.L. 87-234) increased federal involvement in water pollution control, although the principal responsibility for establishing and enforcing standards remained with the states. One important provision of the act was the classification of streams according to their anticipated maximum beneficial use, which theoretically, forces states to reduce discharges into streams and prevents the further degradation of surface waters (Vesilind, Peirce and Weiner, 1990).

Through the Federal Water Pollution Control Act of 1972 (P.L. 92-500) Congress attempted to increase federal responsibility for establishing, implementing, and enforcing standards. A system was developed whereby minimum effluent standards are set universally for all dischargers, and these standards are modified based on the actual effect the discharge will have on the receiving water (Vesilind, Peirce and Weiner, 1990). This policy is implemented through the National Pollutant Discharge Elimination System (NPDES) which prohibits the discharge of pollutants into any public waterway unless authorized by a permit. NPDES permits contain effluent limits that require baseline use of treatment technologies regardless of the impact on receiving waters. The interim goal of the 1972 Federal Water Pollution Control Act was to provide for "...the protection and propagation of fish, shellfish, and wildlife, and...for recreation in and on the waters..." (Macek, 1985), with the ultimate goal being the attainment of zero discharge of wastes into streams by 1985 (Lamb, 1985). Also included in the legislation was the statement that "...it is the national policy that the discharge of toxic pollutants in toxic amounts be prohibited." It is this statement that enables chemical and toxicity limits to be set for effluents from point-source dischargers through permit programs administered by either EPA or authorized state agencies (Burkhard and Ankley, 1989).

The basic regulatory approach adopted in the 1977 amendments to the Clean Water Act (P.L. 95-217) required dischargers to use the highest available and affordable technology to treat their effluents. The emphasis on technology-based standards was intended to control toxics in wastewaters, yet toxicity to aquatic organisms was never a significant criterion for evaluating alternative technologies (Macek, 1985). Traditionally, EPA has pursued a chemical-specific approach to regulate discharges of toxic pollutants, which requires industrial and municipal dischargers to analyze their wastewaters for a number of common toxic compounds known as the "priority pollutants". However, by limiting priority pollutant concentrations in effluents, compliance with the Clean Water Act is still not guaranteed since many chemicals other than priority pollutants cause toxicity (Burkhard and Ankley, 1989).

EPA has recently recognized limitations in the chemical-specific approach. Data on the toxicity of substances to aquatic organisms are available for only a limited number of compounds. Also, additive, antagonistic and synergistic effects between toxic substances in complex effluents are not indicated by traditional chemical testing (EPA, 1985). Since it is not economically feasible to determine the toxicity of each of the thousands of potentially toxic substances in complex effluents or to conduct exhaustive chemical analyses of effluents, EPA has determined that effluent toxicity testing with aquatic

organisms is the most direct and cost-effective approach to the measurement of the toxicity of effluents.

A toxicity test is designed to measure the degree of response of an organism to a specific concentration of a substance or effluent. Aquatic toxicity tests are used to identify effluents that have adverse effects on aquatic organisms (Rand and Petrocelli, 1985). In order to be considered a valid and effective indicator of toxicity, a toxicity test must meet certain criteria. To keep the tests cost-effective, test organisms should be simple and inexpensive to maintain in the laboratory. The organisms chosen as indicators must be sensitive enough to toxicants to protect the aquatic environment and should be representative of organisms in the environment (Rand and Petrocelli, 1985). Tests must also be reproducible, with minimal inter- and intralaboratory variation. Interlaboratory variation results from the use of different populations of organisms, test conditions and procedures between laboratories. Intralaboratory variation is usually the result of temporal changes in the health of test organisms due to inadequate diet, dilution water or culturing techniques (EPA, 1990). Test procedures should have a sound statistical basis and should be quantifiable through graphical interpolation or statistical analysis (Rand and Petrocelli, 1985).

Through the Complex Effluent Toxicity Testing Program (Wall and Hanmer, 1987) and the 1984 national policy

statement, "Policy for the Development of Water Quality Based Permit Limitations for Toxic Pollutants" (Warren-Hicks and Parkhurst, 1990) EPA recommended that whole effluent toxicity tests be used in the NPDES permitting program. EPA further emphasized whole-effluent toxicity testing in the "Technical Support Document for Water Quality-Based Toxics Control" and recommended the 7-day life-cycle test with Ceriodaphnia dubia for chronic toxicity testing (EPA, 1985). This whole effluent approach for controlling toxic pollutants is referred to as the water quality-based approach. The goal is not to replace chemical-specific testing with whole effluent testing, but to integrate the two approaches (EPA, 1985).

Although C. dubia do not have a long history of use in toxicity testing, the development of a toxicity test protocol for C. dubia has definite advantages over test methodologies using other species. The use of other Cladocerans such as Daphnia magna for chronic testing requires longer, and therefore more expensive tests. It is also believed that Ceriodaphnia are more widely distributed than other species used in toxicity testing and are, therefore, more representative of organisms that appear in typical receiving waters (Berner, 1986). Perhaps the most important factor that makes C. dubia a more desirable indicator than other species is the greater sensitivity to both organic and inorganic toxicants that it repeatedly exhibits (Cowgill, Takahashi and Applegath, 1985;

Elnabarawy, Welter and Robideau, 1986; Winner, 1988). Using a more sensitive species as an indicator of toxicity assures a more greater protection of receiving waters.

Methodology for toxicity testing with C. dubia first appeared in the EPA manual "Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms" (Peltier, 1978). This manual, revised in 1985, describes acute toxicity tests, which are used to determine the effluent concentration, expressed as a percent volume, that is lethal to 50% of the organisms within the prescribed period of time (LC_{50}), usually a period of four days or less. In order to detect the response to the toxicant, the indicator organisms are exposed to a serial dilution of the chemical or effluent, and are examined at specified time intervals to determine mortality. Since acute toxicity tests are short-term, they can detect only gross responses to a toxicant, such as mortality or immobility. These tests, which are relatively quick and inexpensive, are conducted as part of compliance sampling inspections, compliance biomonitoring inspections, performance audits, and special investigations in accordance with the goals outlined in the "Policy for the Development of Water Quality Based Permit Limitations for Toxic Pollutants" (EPA, 1985).

Recognizing that acute toxicity tests do not always detect the more subtle, low-level, long-term adverse effects of effluents on aquatic organisms, such as reduction in growth and reproduction, EPA developed a protocol for

chronic toxicity testing, which is published in the manual "Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms" (Horning and Weber, 1985; EPA, 1989). The objective of chronic aquatic toxicity tests with whole effluents is to estimate the highest "safe" or "no-effect concentration" of wastewaters using a sublethal endpoint, such as hatchability, gross morphological abnormalities, survival, growth, or reproduction. As in the acute test, organisms are exposed to a serial dilution of the chemical or effluent. The duration of the exposure varies depending on the organism used, but should cover much of the reproductive life cycle. Unlike acute tests, the organism must be fed to allow the long-term survival and reproduction required to detect the more subtle chronic test endpoints. The food and culture water specified in the chronic test protocol are intended to satisfy all the nutritional requirements of C. dubia. Two terms used to describe the chronic test endpoints are No-Observed-Effect-Concentration (NOEC) and Lowest-Observed-Effect-Concentration (LOEC). The NOEC is the highest concentration of the toxicant to which organisms are exposed that causes no observable statistically significant adverse effects on the test organisms. The LOEC is the lowest concentration of the toxicant to which organisms are exposed which causes statistically significant adverse effects on the test organisms (EPA, 1989).

In an effort to reduce the complexity and expense of the EPA chronic testing methodology, the North Carolina Division of Environmental Management has developed the North Carolina Ceriodaphnia Chronic Effluent Bioassay Procedure which describes the Mini-chronic Pass/Fail Ceriodaphnia Effluent Toxicity Test. Instead of using an expanded dilution series to calculate the NOEC, the Mini-chronic Pass/Fail Test compares a single dilution of wastewater to a control. The objective of the test is to determine whether the exposure of the test population to an effluent concentration equal to the concentration in the stream during low flow events has significant detrimental impact upon reproduction as compared to the control population. The effluent concentration used in the test must equal the in-stream effluent concentration that occurs during the 7Q10, which is the minimum 7-day flow that occurs in the receiving water once in 10 years (NCDEM, 1989). Dischargers in North Carolina are required to conduct chronic toxicity tests quarterly. Failures can result in requirements for more frequent testing, or even the initiation of a Toxicity Reduction Evaluation (TRE) to eliminate the source of the toxicity (NCDEM, 1989). A TRE is a site specific study designed to identify the causative agents of effluent toxicity, isolate the sources of toxicity, evaluate the effectiveness of toxicity control options, and then confirm the reduction in effluent toxicity (EPA, 1985). Currently, it is EPA's policy to interpret a single exceedence of a

whole effluent toxicity limit as a violation of the NPDES permit (EPA, 1990).

Because a TRE is both complicated and expensive to perform, a high level of confidence must be placed in the Mini-chronic Pass/Fail Ceriodaphnia Effluent Toxicity Test outcome. However, problems have arisen with the application and interpretation of chronic bioassays with C. dubia. Many investigators who culture and test with C. dubia periodically encounter problems maintaining healthy populations of animals in the laboratory (DeGraeve and Cooney, 1987). Other sources of uncertainty in toxicity tests include the inter- and intralaboratory variation in culturing techniques, test methods, and organism sensitivity that may affect toxicity test results (Warren-Hicks and Parkhurst, 1990). The acceptability of the levels of precision observed in inter- and intralaboratory studies has been examined. Round-robin studies have shown that the variability of the 7-day Ceriodaphnia test is similar to the variability observed with other toxicity tests and analytical measurements (EPRI, 1989; Anderson and Norberg-King, 1991). However, some researchers have argued that the results of these round-robin studies demonstrate an unacceptably high level of variation (Warren-Hicks and Parkhurst, 1990). One of the factors suspected of causing variability in toxicity testing and poor survival and reproduction in the laboratory is the diet used for the routine culturing of daphnids (Cowgill, Keating and

Takahashi, 1985; Winner, 1989). Further investigation into an adequate diet for C. dubia is warranted.

Literature Review

Although laboratories have performed toxicity tests with C. dubia for almost a decade, proper culturing techniques and feeding regimes are still widely debated. The earliest culturing procedures for Ceriodaphnia recommended feeding each animal 250 μ g of baker's yeast daily. The investigators reported average reproductions of 15 young for three broods with no loss of fecundity over seven generations (Mount and Norberg, 1984). They speculated that the actual source of nutrition was not the yeast, which settled quickly on the bottom of the test vessel, but the bacteria that grew on the yeast film. A modified version of this diet which includes yeast, Cerophyl^R and trout chow (YCT) appears in the first edition of the manual "Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms" (Horning and Weber, 1985) as the prescribed diet for the culturing of C. dubia. EPA added the Cerophyl^R and trout chow components to the diet presumably at the suggestion of other laboratories that anecdotally reported greater success with YCT than with yeast alone (Mount and Norberg, 1984).

As previously noted, many laboratories conducting toxicity testing with Ceriodaphnia reported poor success with the synthetic YCT diet (DeGraeve and Cooney, 1987), and it became necessary to develop a more adequate laboratory culturing protocol than the one suggested by Mount and Norberg (1984) to maintain the organism for extended periods of time. In a round-robin study conducted by the Electric Power Research Institute, it was found that only 47% of the participating laboratories could effectively initiate a toxicity test according to the criteria outlined in the 1985 edition of "Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms" (EPRI, 1989). It was noted in this study that successful test initiation and completion was most common in laboratories that regularly maintained healthy cultures of animals. In other words, when animal health is marginal, tests are marginal, and are therefore less desirable in a regulatory compliance setting. If control survival and control reproduction could be improved, a greater number of toxicity tests could be successfully initiated and completed (EPRI, 1989). One way to improve the health of control organisms is to improve the diet for Ceriodaphnia.

Many researchers using other daphnid species, particularly Daphnia magna and Daphnia pulex, had consistently noted considerably greater survival and reproduction in cultures fed an algal diet rather than a synthetic diet. For instance, D. magna sustained on the

green alga Chlamydomonas reinhardtii lived longer but had smaller broods than animals reared on trout chow and Cerophyl^R (Winner, et al., 1977). Winner attributed the larger brood sizes of animals fed trout chow to the higher caloric content of the diet rather than its overall nutritional adequacy. Since this study was conducted on cohorts of animals rather than multiple generations of animals, long-term effects of the diets are uncertain. In a multi-generational study comparing a two algae diet, a single alga diet and a trout chow/yeast diet, it was shown that both algal diets supported adequate survival and reproduction of D. magna over many generations while the synthetic diet did not (Goulden, et al., 1982). Goulden points out that daphnids used in toxicity testing are planktonic organisms and, therefore, an algal diet more closely resembles the organism's natural feeding regime than a synthetic diet. Keating's study conducted on D. pulex suggested that animals maintained on multiple algal diets reproduce better than animals maintained on single alga diets (Keating, 1985). Cowgill expands this idea in the following statement:

"Daphnids in nature derive the majority of their nutrition from the consumption of a variety of algae. Algae...contain 55 detectable elements in their tissues, at least 18 amino acids and a substantial array of saturated and unsaturated fatty acids. Although feral daphnids have been collected and analyzed for their chemical composition...no deficiency symptoms have been noted. Furthermore, the chemical composition of feral daphnids

is similar to algal-reared daphnids but not to trout chow-reared daphnids."

(Cowgill, 1987)

One can conclude that an organism would prefer to eat in the laboratory what it eats in its native habitat.

Choosing acceptable algae for the laboratory culture of daphnids is not a simple task. Goulden (1982) reviews research on this subject and notes that blue-green algae, filamentous algae, algae species larger than 50 μm and species that are enclosed in gelatinous sheaths are unsuitable foods for daphnids (Goulden, et al., 1982). Furthermore, the culturing of the algae can change its nutritional quality. For instance, animals fed a diet of Selenastrum capricornutum grown on MBL (Marine Biological Laboratory) medium proved a better food source than the same alga raised in the medium recommended by EPA for the algal bottle bioassay test (Goulden, et al., 1982). In another study, D. pulex fed algae grown in a vitamin-enriched medium produced healthy, viable offspring while animals fed algae grown in a vitamin-free medium produced very few live progeny (Keating, 1985).

Some investigators have indicated that nutritional deficiencies cannot always be remedied by changing the food source since daphnids uptake certain elements, particularly trace nutrients, preferentially from the water used to culture the animals rather than the food. In one study using D. magna, partial correlation techniques comparing the chemical compositions of the animals, food and culture water

indicated that the rearing water was more important in determining the chemical composition of the organisms than the algal food (Cowgill, et al., 1986). Evidence based on work with zinc and selenium suggests that, at least for these elements, nutritional requirements are not readily met by solid foods (Keating, Caffrey and Schultz, 1989; Keating and Dagbusan, 1984). However, it is clear that foods of poor nutritional quality do adversely affect survival and reproduction of daphnids (Goulden, et al., 1982; Cowgill, Keating and Takahashi, 1985). In fact, Winner believes that food is more important than dilution water as long as the food contains a proper balance of essential nutrients. He notes that animals fed a diet of either Chlamydomonas or Selenastrum/YCT survived and reproduced more than adequately for multiple generations in a dilution water containing only four salts (Winner, 1989).

Unfortunately, very few dietary studies have been conducted specifically on Ceriodaphnia, and some of the results of experiments that do examine Ceriodaphnia nutritional requirements are uncertain. For instance, Cowgill, Keating and Takahashi reported that upon offering Ceriodaphnia 17 different algal diets, Ankistrodesmus convolutus resulted in the highest reproductions (Cowgill, Keating and Takahashi, 1985). However, the investigators drew this conclusion from data gathered by using only four replicate animals per food type, one in each of four different types of water. Furthermore, the diet identified

as the best food source resulted in unacceptably low reproduction in two of the four culturing waters. A multi-generational study conducted by the Electric Power Research Institute subsequently demonstrated that the algal diet A. convolutus was unacceptable for the long-term culturing of Ceriodaphnia, as were the other algal diets C. reinhardtii and S. capricornutum (EPRI, 1988). Winner, however, found that C. reinhardtii cultured in a medium enriched with vitamins and selenium proved an adequate diet for maintaining healthy cultures of Ceriodaphnia over multiple generations (Winner, 1989). These studies indicate that any evaluation of potential diets for daphnids should cover multiple generations. It is easy to draw erroneous conclusions about the acceptability of a particular diet based on data taken from a single generation.

Nutritional studies using S. capricornutum in combination with YCT have shown that the diet provides adequate nutrition for the long-term culturing of Ceriodaphnia (EPRI, 1988; Winner, 1989). This diet was subsequently included in the second edition of "Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms" (EPA, 1989). Although no other algae have been specifically tested in conjunction with YCT, it is plausible that other species could replace Selenastrum as the algal component of the diet. Many laboratories maintain Selenastrum for use in the

algal bottle bioassay, so this alga may have been chosen by EPA for the sake of convenience.

While the recommended diets for the culturing of Ceriodaphnia have been examined in terms of longevity and reproduction, few have been evaluated with regard to the effects they might have on the sensitivity of the organism to toxicants. As Winner states:

"There seems to be a general, but unwarranted, assumption that there is, necessarily, a positive relationship between fecundity and robustness of cladoceran populations. Since brood sizes are proportional to caloric intake, large broods do not necessarily indicate healthy animals."

(Winner, 1989)

Some researchers suggest that the culturing techniques employed in the laboratory do have an effect on the sensitivity of cladocerans to toxicants. For example, selenium deficiency seems to sensitize daphnids to copper (Winner, 1984), and to cadmium (Winner and Whitford, 1987). It has also been shown that D. magna maintained on a vitamin-enriched algae were less sensitive to copper stress than animals maintained on a trout-granule diet (Winner, et al., 1977). In a review of factors affecting the variability of toxicity testing results, Cowgill compiled data from six sources that illustrated D. magna's greater resistance to assorted toxicants when fed algal diets versus synthetic diets (Cowgill, 1987). Since this information is based on data collected from different studies and investigators, it is difficult to determine whether other

factors such as handling, culture water or temperature affected the results, but it does illustrate a trend. In a study using Ceriodaphnia, Belanger, et al. found that animals fed a combination of three algae were 1.5 times more resistant to copper than animals reared on synthetic diets (Belanger, Farris and Cherry, 1989). It is clear that when evaluating the acceptability of a diet, one should examine not only longevity and reproduction, but also toxic response. The robustness of test organisms may not be immediately apparent when evaluated in terms of reproduction, but might be indicated by the response to a toxicant. Clearly, if a toxicity test fails due to the poor health of the culturing animals, the test is probably not valid for that toxicant.

Objectives

The objectives of this experiment are as follows:

1. To identify a diet that maximizes survival and reproduction of control organisms used in the Mini-Chronic Pass/Fail Effluent Toxicity Test over multiple generations.
2. To determine if a multi-algal/YCT diet provides a greater resistance to toxicants than a single-alga/YCT diet.

MATERIALS AND METHODS

General Culture Maintenance

All C. dubia used in this experiment originated from animals provided by the North Carolina Division of Environmental Management in February, 1988. Animals were grown and maintained in both mass cultures and individual cultures according to the guidelines described in "Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms" (Horning and Weber, 1985; EPA, 1989). A general overview of laboratory culturing and maintenance procedures is provided below.

Mass cultures were maintained continuously in order to provide animals for acute toxicity testing in ongoing research projects. They also served as a "backup" reservoir of animals in case of an unanticipated drop in reproduction due to conditions such as low dissolved oxygen in the culture vessels, poor food quality or breakage of a culture dish. A mass culture consisted of 50 animals plus their offspring in an 8" diameter culture dish (Carolina Biological) containing one liter of natural surface water. Dishes were started each weekday (Monday through Friday)

using neonates less than 24 hours old from other culture dishes. Adults were transferred to fresh water twice a week for 14 days. On days of transfer, neonates that were not required to initiate new mass culture dishes were discarded. After 14 days, all of the animals remaining in the culture dish were discarded. On days of transfer, adults were counted and mortality was noted. Neonates were not enumerated. These cultures were fed daily and aerated gently with laboratory air three times a week.

Mass cultures were fed 2.0 mL of a mixture of yeast, Cerophyl^R and trout chow (YCT) per liter of culture water daily, including weekends. They were also fed daily 0.4 mL per liter of culture water of a 1.4×10^7 cells/mL concentrate of S. capricornutum algae. The YCT was made according to EPA protocol (1989). The yeast, Cerophyl^R and trout chow components of the YCT were distributed by Fleischmann's Yeast Inc., Sigma Chemical Company, and Carolina Biological, respectively. YCT was prepared in 2 L batches and frozen in 80 mL aliquots until needed. Thawed YCT was kept at 4 °C in the dark. Unused thawed portions were discarded after one week. Algae culturing is described elsewhere in this report. The daily concentration of YCT given to mass cultures was approximately 2.0×10^{-3} mL YCT/mL of culture water. The concentration of algae given to mass cultures yielded a cell concentration in the culture dish of 5.7×10^4 cells/mL of culture water.

The water used for mass cultures came from Botany Pond, a 3.5 acre pond located in the Mason Farm Biological Reserve, a protected wooded watershed in Chapel Hill, North Carolina. Water was stored in a 55 gallon high density polyethylene tank (Nalgene, Inc.) and was continuously aerated with laboratory air using a Silent Giant aquarium pump (Aquarium Pump Supply, Inc.). Approximately 10 gallons of water were collected weekly off the end of a 30 foot dock using a plastic pump and a 5 μ m polypropylene wound filter (Carborundum Corp.). The point of collection was approximately 5 feet below the surface of the pond. The hardness of the water was adjusted to 33 mg-CaCO₃/L using equal weights of reagent grade calcium sulfate (Mallinckrodt, Inc.) and magnesium sulfate (MCB Manufacturing Chemists, Inc.). Water was aerated for at least 24 hours and hardness was verified before use.

Mass cultures were kept in a temperature controlled room at $25 \pm 2^{\circ}\text{C}$. The temperature was checked and recorded each weekday. An automatic timing device kept the room on a 16 hour light/8 hour dark cycle. During the light cycle, 65 foot candles of illumination reached the culture dishes. Illumination was provided by overhead fluorescent lights.

In addition to mass cultures, animals were also maintained regularly in individual cultures. These animals provided a constant source of animals for chronic toxicity tests. Animals were held singly in 30 mL disposable plastic cups (Plastics, Inc.) containing 15 mL of a 10% PerrierTM

90% distilled deionized water mixture. The set of animals kept in individual cultures originated from mass culture animals. Subsequently, individual culture cups were initiated from other individual culture animals. Twelve new cups were started each Monday, Wednesday and Friday with neonates of known origin less than 24 hours old. On those days, adults were transferred to new solution and the neonates counted and recorded. A Bausch and Lomb dissecting microscope placed on top of a fluorescent light table was used to count neonates. Animals were discarded after 14 days.

Animals used in both individual cultures and chronic tests were fed 100 μ L of a mixture of YCT and S. capricornutum daily using a micropipeter to facilitate feeding. A food in which the algae component is mixed with the YCT component prior to feeding is referred to as a "combination" food. The mixture fed to the individual cultures was half YCT and half algae by volume. The algae component of the combination food was prepared using the formula $X = (1.71 \times 10^7)(V)/(C)$ where X (mL) is the volume of algae concentrate used in the combination food, 1.71×10^7 (cells/mL) is the desired cell concentration in the algal portion of the combination food, V (mL) is the desired volume of the algal component of the combination food, and C (cells/mL) is the cell concentration in the algal concentrate. Preparation of algal concentrates is described elsewhere in this report. The algal concentrates used in

food preparation were diluted to the desired volume using 10% PerrierTM. An equal volume of freshly thawed YCT was added to the combination food. Therefore, the combination food was 50% YCT and 50% algae at a concentration of 1.71×10^7 cells/mL by volume. The concentration of YCT given to each cup daily was 3.3×10^{-3} mL of YCT/mL of culture water. The concentration of algae given to each cup daily was 5.7×10^4 cells/mL of culture water. Combination foods were kept at 4°C in the dark. Unused portions were discarded after one week.

The 10% PerrierTM solution used to culture the animals was made using distilled-deionized water (Corning Megapure System DI Deionizer using a Corning High-Capacity Inorganic Deionizer and a Corning AGII Still). The solution was aerated continuously with a Whisper 800 aquarium pump (Second Nature) and for at least 18 hours before use. The hardness of the water remained consistently between 39.5 and 40.3 mg- CaCO_3 /L. The conductivity was 91 to 94 $\mu\text{mho/cm}$ as determined with a Fisher Scientific Digital Conductivity Meter. 10% PerrierTM was made fresh every three days and was held in a 20 L high density polyethylene (Nalgene, Inc.) container.

Animals grown in individual cultures and any animals being used in chronic toxicity tests were kept in a Precision Dual Program Illuminated Incubator (CGA Corp.). The temperature was checked each weekday and remained at $25 \pm 1^{\circ}\text{C}$. The fluorescent lights in the incubator provided 37

foot candles of illumination. An automatic timing device kept the incubator on a 16 hour light/8 hour dark cycle.

Algal Cultures

The two species of algae used in this experiment were S. capricornutum and C. reinhardtii. Both of these cultures originated from slants obtained from the Starr Collection at the University of Texas in Austin. Their reference numbers were UTEX 1638 and UTEX 90 respectively. Both algae were grown in Marine Biological Laboratory (MBL) media (Stein, 1973) with a vitamin enrichment (Goulden and Henry, The Academy of Natural Sciences). S. capricornutum was also used as the algal component of the laboratory maintenance diet for Ceriodaphnia. The two species of algae were cultured separately and no cross-contamination occurred between species throughout the course of the experiment. The culturing methods for all algae were identical whether grown for experimental purposes or for general laboratory maintenance unless otherwise noted.

Two types of algal culture systems were used in the laboratory. Algae grown on agar containing MBL nutrient medium were kept for long periods of time, often up to six months, in screw capped test tubes at 4°C in the dark. This was a convenient method for maintaining algae in case batch cultures became contaminated with bacteria. Batch cultures consisted of approximately 125 mL of medium in a 250 mL

Erlenmeyer flask capped with a gauze stopper. Flasks, gauze stoppers and media were autoclaved prior to use. The vitamin solution was added after the media was autoclaved and immediately prior to the introduction of the algae inoculum into the flask. After inoculation, the flasks were placed on a G10 Gyrotory Shaker table (New Brunswick Scientific) set at approximately 100 revolutions per minute. Algae were cultured under fluorescent lights that provided continuous illumination of approximately 225 foot candles. Six flasks of each type of algae were inoculated every week with approximately 0.1 mL of the culture from the previous week using sterile technique. If batch cultures had become contaminated with bacteria, flasks were inoculated from slant cultures using sterile technique. Each set of six flasks were grown for seven days. On day seven, new cultures were inoculated and the algae were harvested.

Algae were harvested at a cell concentration just below maximum density to insure good food quality. S. capricornutum was harvested at a cell density of approximately 1×10^7 cells/mL. C. reinhardtii was harvested at a cell density of approximately 3×10^6 cells/mL. In the first step of the harvesting procedure, the six flasks of algae were combined and mixed in a 1000 mL graduated cylinder. Two drops of the combined algae were pipetted onto a Bright-Line Hemacytometer (American Optical) to determine cell density. Ten replicate squares on the hemacytometer were counted under a Bausch and Lomb compound

microscope and averaged. The average cell concentration was determined with the formula $C = (A \times 10^6)/4$, where C (cells/mL) is the cell concentration of the combined flasks, A is the average cell count per hemacytometer square, and 4 and 10^6 are constants to convert the cell count to cells/mL. The volume of the combined flasks was noted and recorded.

In order to use the algae for food, it had to first be separated from the algal nutrient medium and resuspended in 10% PerrierTM solution. The algae culture was evenly distributed into twelve 50 mL glass centrifuge tubes. Tubes were capped with aluminum foil and centrifuged at approximately 1400 revolutions per minute for 30 minutes. The supernatant was discarded, and the algae were rinsed into four tubes using approximately 100 mL of 10% PerrierTM. The tubes were recapped and centrifuged for 20 minutes. Finally, the algae were rinsed into two tubes and centrifuged for 20 minutes. After centrifugation, the cells were resuspended in 10% PerrierTM solution. S. capricornutum were resuspended to a concentration of approximately 1.4×10^8 cells/mL. The C. reinhardtii cells were more difficult to retrieve from the centrifuge tubes and required more solution to wash the cells free from the sides of the tubes. Therefore, the C. reinhardtii was stored at a lower cell density which was usually between 3×10^7 and 5×10^7 cells/mL. After resuspension, the algal concentrates were stored in the dark at 4°C . During this experiment, algae were used within one week of harvesting.

For the routine feeding of laboratory cultures, algae were used within 30 days of harvesting.

Long-term Culture Experiment

Multiple generations of C. dubia were raised on three different diets to determine the effect of diet on long-term survival and reproduction. The three diets examined in this experiment were YCT plus the algae S. capricornutum, YCT plus the algae C. reinhardtii, and YCT plus S. capricornutum and C. reinhardtii. The combination YCT plus C. reinhardtii food was prepared using the same formula that was used to prepare the combination YCT plus S. capricornutum food for the individual cultures. The combination YCT plus S. capricornutum and C. reinhardtii food was prepared so that the algal component of the food contained 50% S. capricornutum and 50% C. reinhardtii based on cell count. The cell counts of all foods used in the experiment were verified before use. All combination foods used in the experiment were prepared on the same day, using algal concentrates that were less than 48 hours old and freshly thawed YCT. Foods were used for one week and then discarded.

All animals used in this experiment were reared in 10% PerrierTM. Neonates used to initiate the experiment were the progeny of adult animals raised in individual cultures. Approximately 18 hours before the start of the long-term

experiment, adult animals that were likely to have their third or latter broods within 8 hours were noted and observed. The experiment was initiated while the neonates were less than 24 hours old. Neonates were used for the experiment only if they came from a brood that had eight or more neonates.

To start the test, one neonate was pipetted into each of twelve replicate cups per diet. The twelve replicate cups for each diet were labelled A through L. Neonates placed in cups of the same letter came from the same brood. Therefore, the neonate placed in the A cup that was fed Selenastrum/YCT came from the same brood as the neonate placed in the A cup that was fed Chlamydomonas/YCT. The animals were raised for seven days, a period which allowed each animal to produce at least three broods. On the seventh day, the progeny were enumerated and one neonate from each cup was used to initiate the next week of the experiment. Neonates were transferred to cups with the same letter. By transferring neonates in this manner, progeny originating from a single C. dubia were followed throughout the experiment. Animals originating from the same adult and fed the same diet are referred to as a culture line. The experimental design is illustrated in Figure 1.

The experiment was carried out for 19 weeks. At the end of every week, one neonate from the third brood of the adult animal in the cup was placed in a cup of the same letter and fed the same diet, thereby initiating the next

Figure 1. Diagram of the long-term culture experiment. Each letter represents one cup in which one adult *C. dubia* is cultured. Each arrow (↓) represents one neonate that is transferred into a cup of the same letter to begin the next generation.

week of the experiment. Normally, neonates on day 7 were from the third brood and were less than 24 hours old. On rare occasions, neonates from the fourth brood were used to initiate the test for a certain week in order to maintain a similar age structure for the animals used in the experiment. Some neonates were slightly older because of a faster rate of growth and development on certain diets. Neonates were never more than 48 hours old at the time of initiation. If an adult C. dubia died or did not produce a third brood, a neonate was taken from a different culture line. The use of neonates from broods other than the third brood or from different culture lines was noted. Temperature, photoperiod and feeding were carried out as described for the individual cultures. Changes of culture water occurred two days and five days after initiation of the test for a given week. When culture water was changed, any neonates in the cup were enumerated and discarded. On the seventh day, the adults and unused neonates were discarded and one neonate from each cup was placed in fresh dilution water to carry out the next week of the test.

Copper Toxicant Experiments

After raising C. dubia on the three diets for eight weeks, tests were performed with copper nitrate to determine if animals raised on the different diets had different levels of resistance to the effects of the toxicant.

Neonates used to conduct these experiments came directly from weeks 9 through 19 of the long-term culture experiment. The concentrations of copper used were 2 $\mu\text{g/L}$ as copper, 4 $\mu\text{g/L}$ as copper and 8 $\mu\text{g/L}$ as copper. Nominal concentrations of copper were prepared using a 1000 mg-Cu/L reference solution (Fisher Scientific). A 1 mg-Cu/L solution was prepared using 1 mL of the 1000 mg-Cu/L reference solution in 1 L of distilled-deionized water. 2, 4 or 8 mL of the 1 mg-Cu/L stock solution were added to 1 L of 10% PerrierTM to make 2, 4 or 8 $\mu\text{g-Cu/L}$ solutions, respectively. Volumetric pipets and flasks were used to prepare all solutions.

The decision to use copper in this experiment was based on the fact that copper nitrate had been used previously in the laboratory to conduct acute reference toxicant tests. This acute data simplified the choice of copper concentrations that would result in chronic toxicity. It was hoped that the concentrations of copper chosen would exhibit increasing levels of chronic toxicity. EPA (1989) recommends the use of copper sulfate as a reference toxicant, so their procedures also provided some guidance. Dates on which the copper tests were performed can be found in Appendix A.

Tests with copper were initiated simultaneously with new weeks of the long-term culture experiment. The neonates that were chosen to carry out the next week of the long-term experiment were placed in fresh 10% PerrierTM solution. An animal from the same brood was then placed in a cup

containing 10% PerrierTM plus the desired concentration of copper. The animals in the copper solutions were fed the same diet as the animal from the same brood that was not subjected to copper. The animals in the copper solution were not kept for multiple generations. After one week, the animals in the copper solutions were discarded and new copper tests were reinitiated with animals from the long-term culture experiment.

RESULTS

Long-Term Culture Experiment

Mortality of adults was minimal among C. dubia raised on all three diets tested. Since the long-term culture experiment was not designed as a life-span test, measures of mortality only refer to the 7-day period during which each adult C. dubia was cultured. Average 7-day survival in the three diets over the course of the 19 week experiment is illustrated in Figure 2. A Friedman Two-Way ANOVA was used to determine that the difference in mortality between the three diets was not significantly different ($p > 0.05$). Of the 228 adult C. dubia that were raised on each diet during the 19 week period, 4 animals died in the Selenastrum/YCT diet, 5 animals died in the Chlamydomonas/YCT diet, and 1 animal died in the Selenastrum/Chlamydomonas/YCT diet. Percent mortality in the three diets was 1.2%, 0.6% and 0.4% respectively. Average weekly survival in each diet is illustrated in Figures 3 through 5. The death that occurred in the seventh week of the experiment in the Selenastrum/Chlamydomonas/YCT diet was the result of injury during transfer to new culture water, and was therefore the fault of the handler, not the diet. All of the mortality in

Figure 2. Average 7-day survival of C. dubia raised on three different diets during the Long-Term Culture Experiment.

Figure 3. Number of surviving adult *C. dubia* raised on *Selenastrum*/YCT during each generation of the Long-Term Culture Experiment.

Figure 4. Number of surviving adult *C. dubia* raised on *Chlamydomonas*/YCT during each generation of the Long-Term Culture Experiment.

Figure 5. Number of surviving adult *C. dubia* raised on *Selenastrum*/*Chlamydomonas*/YCT during each generation of the Long-Term Culture Experiment.

the Selenastrum/YCT diet and the Chlamydomonas/YCT diet occurred during the 14th week of the experiment or later. The figures also illustrate the North Carolina Division of Environmental Management Mini-chronic Pass/Fail Ceriodaphnia Effluent Toxicity Test requirement for 80% survival in a chronic test control. In a 12 replicate control, greater than 9.6 of the organisms must survive for 7 days in order to be considered a valid control (NCDEM, 1989). All three diets result in a regular achievement of this minimum standard. Survival data is presented in Appendix B.

Three-brood reproduction was monitored for each adult C. dubia raised on each diet throughout the long-term culture experiment. In cases where an animal did not produce three broods in a week, for reasons such as death of the adult or abortion of a brood, the total reproduction during one week was substituted for the three-brood reproduction. In the few cases where an animal produced more than three broods in one week, only the three-brood reproduction was considered. The 19-week three-brood averages of animals raised on Selenastrum/YCT, Chlamydomonas/YCT and Selenastrum/Chlamydomonas/YCT were 19.86, 17.66 and 20.72 neonates/adult/week respectively. A total of 4529 neonates were produced by animals fed the Selenastrum/YCT, 4027 neonates were produced by animals fed the Chlamydomonas/YCT diet and 4724 neonates were produced by animals fed the Selenastrum/Chlamydomonas/YCT diet. Data

on three-brood reproductions is presented in Appendix C. The 19-week averages are illustrated in Figure 6.

Temporal variation in three-brood reproduction during the long-term culture experiment was high. Differences in three-brood reproduction varied more over time than between diets. It was also noted that the average weekly reproduction seemed to decrease over the course of the experiment in all diets. Average weekly reproduction in all diets is illustrated in Figure 7. The figure also illustrates the North Carolina Division of Environmental Management Mini-chronic Pass/Fail Ceriodaphnia Effluent Toxicity Test requirement for an average three-brood reproduction of at least 15 neonates per adult in order to be considered a valid control (NCDEM, 1989).

Over the course of the long-term culture experiment periods of low reproduction and high reproduction occurred during cyclical intervals. While no predictable pattern emerged over the 19 generations, it did appear as though low and high periods of reproduction lasted for multiple weeks at a time. For instance, periods of high reproduction occurred in all diets during the first four weeks and during the eleventh through fourteenth weeks of the experiment. Periods of low reproduction occurred during the fifth through seventh weeks and during the fifteenth through seventeenth weeks of the experiment. Although the average reproductions in each diet during the course of the long-term experiment satisfied the North Carolina Division of

Figure 6. Average three-brood reproduction of *C. dubia* raised on three different diets during the Long-Term Culture Experiment.

Figure 7. Average reproduction of *C. dubia* raised on all three diets during the Long-Term Culture Experiment.

Environmental Management minimum standards, failure to meet these standards during a given week of the experiment occurred sporadically in all diets. Failures occurred twice in the Selenastrum/YCT diet, seven times in the Chlamydomonas/YCT diet and four times in the Selenastrum/Chlamydomonas/YCT diet. Variability during each week of the experiment was high within the particular diets. This variability is illustrated in Figures 8 through 10. The degree of variability in reproduction within a particular generation did not substantially increase or decrease during the course of the long-term culture experiment. The degree of variability in reproduction was also not substantially different between animals raised on the three different diets.

Periods of low reproduction during the long-term culture experiment resulted in the loss of integrity of certain culture lines. When an adult C. dubia failed to produce viable third brood offspring, neonates from other broods were substituted in order to maintain the multi-generational aspect of the experiment. Mortality of adult organisms was rare, and, therefore, did not often result in the loss of a culture line. The heredity of animals raised in the long-term culture experiment is presented in Appendix D. Not all of the culture lines continued unbroken for 19 generations. Figures 11 through 22 illustrate the three-brood reproduction of C. dubia raised in the various culture lines in all diets. Note that the graphs of reproduction in

Figure 8. Average three-brood reproduction of *C. dubia* raised on *Selenastrum*/YCT. Bars represent + and - one standard deviation.

Figure 9. Average three-brood reproductions of *C. dubia* raised on *Chlamydomonas*/YCT. Bars represent + and - one standard deviation.

Figure 10. Average three-brood reproduction of C. dubia raised on Selenastrum/Chlamydomonas/YCT. Bars represent + and - one standard deviation.

Figure 11. Three-brood reproduction of Culture Line A raised on all three diets during the Long-Term Culture Experiment.

Figure 12. Three-brood reproduction of Culture Line B raised on all three diets during the Long-Term Culture Experiment.

Figure 13. Three-brood reproduction of Culture Line C raised on all three diets during the Long-Term Culture Experiment.

Figure 14. Three-brood reproduction of Culture Line D raised on all three diets during the Long-Term Culture Experiment.

Figure 15. Three-brood reproduction of Culture Line E raised on all three diets during the Long-Term Culture Experiment.

Figure 16. Three-brood reproduction of Culture Line F raised on all three diets during the Long-Term Culture Experiment.

Figure 17. Three-brood reproduction of Culture Line G raised on all three diets during the Long-Term Culture Experiment.

Figure 18. Three-brood reproduction of Culture Line H raised on all three diets during the Long-Term Culture Experiment.

Figure 19. Three-brood reproduction of Culture Line I raised on all three diets during the Long-Term Culture Experiment.

Figure 20. Three-brood reproduction of Culture Line J raised on all three diets during the Long-Term Culture Experiment.

Figure 21. Three-brood reproduction of Culture Line K raised on all three diets during the Long-Term Culture Experiment.

Figure 22. Three-brood reproduction of Culture Line L raised on all three diets during the Long-Term Culture Experiment.

each culture line illustrate the reproduction only for the period during which the culture line remained unbroken. The temporal variability that was observed for the average reproductions over the course of the long-term culture experiment is also apparent when the culture lines are examined singly. For easy examination, the three-brood reproduction in each culture line in each diet is presented in Appendix E. Summary statistics of reproduction data in each culture line appear in Appendix F. The large standard deviations of the mean reproductions observed in each culture line reflect the high level of temporal variation observed throughout the experiment. It is also apparent from the data on minimum and maximum reproduction in each culture line that all culture lines, regardless of the diet on which the C. dubia were raised, experienced periods of low reproduction and high reproduction. These periods were not erratic episodes, but seemed to be cyclical in nature. The periods of high reproduction seemed to occur for several weeks at a time, followed by periods of low reproduction.

Lines were fitted to the reproduction data in each of the culture lines and slopes were calculated. This information is presented in Appendix G. Virtually all of the slopes were negative, indicating that three-brood reproduction generally decreased over the course of the experiment in all culture lines. A Friedman Two-Way ANOVA was used to determine that the slopes of the lines fitted to the reproduction data in each culture line were not

significantly different between the three diets ($p > 0.05$). The mean reproduction in each culture line in each diet was calculated, and is illustrated in Figure 23. The data used to construct this figure is presented in Appendix F. In general, the reproduction was highest in the Selenastrum/Chlamydomonas/YCT diet, slightly lower in the Selenastrum/YCT diet, and quite a bit lower in the Chlamydomonas/YCT diet. A Friedman Two-Way ANOVA was used to compare the mean reproductions in each culture line in each diet. In other words, the average reproduction of C. dubia in culture line A that were raised on Selenastrum/YCT was compared to the average reproduction of C. dubia in culture line A that were raised on Chlamydomonas/YCT and the average reproduction of C. dubia in culture line A that were raised on Selenastrum/Chlamydomonas/YCT. It was determined that three-brood reproductions were significantly different between diets ($p < 0.05$). Subsequent Friedman Two-Way ANOVA of reproduction data for only two diets at a time indicated that the reproduction in the Selenastrum/Chlamydomonas/YCT diet was significantly greater than reproduction in the Selenastrum/YCT diet ($p < 0.016$). It was also determined that reproduction in the Selenastrum/YCT diet was significantly greater than reproduction in the Chlamydomonas/YCT diet ($p < 0.016$). Three-brood reproductions were analyzed only for those individuals that arose from the original adult C. dubia in order to maintain the genetic integrity of the organisms during the

Figure 23. Average reproduction in each culture line during the Long-Term Culture Experiment.

statistical analysis. Lowering the p value to 0.016 allowed for multiple comparisons to be made between the different diets.

Mortality among neonates had a fairly low occurrence in all three diets. Neonate mortality varied slightly between the three diets tested. The numbers of dead neonates born to animals raised on Selenastrum/YCT, Chlamydomonas/YCT and Selenastrum/Chlamydomonas/YCT were 54, 23 and 0, respectively. Percent neonate mortalities in the Selenastrum/YCT diet and the Chlamydomonas/YCT diet were 1.2% and 0.6% respectively. On certain occasions, broods were aborted by the adult C. dubia before the neonates had fully developed in the brood chamber. Abortions occurred 3 times in the Selenastrum/YCT diet, 5 times in the Chlamydomonas/YCT diet and 6 times in the Selenastrum/Chlamydomonas/YCT diet.

Copper Toxicant Experiment

The decrease in survival of animals cultured in copper solutions compared to the controls depended upon the concentration of copper tested. Average mortality in the different concentrations of copper is illustrated in Figures 24 through 26. Culturing the animals in 2 $\mu\text{g-Cu/L}$ did not significantly reduce the level of survival of C. dubia raised on any of the diets, as determined by a Friedman Two-Way ANOVA ($p < 0.05$). Culturing C. dubia in 4 $\mu\text{g-Cu/L}$

Figure 24. Average mortality of *C. dubia* subjected to 2 ug-Cu/L. Data from four tests have been pooled.

Figure 25. Average mortality of *G. dubia* subjected to 4 ug-Cu/L. Data from seven tests have been pooled.

Figure 26. Average mortality of *C. dubia* subjected to 8 ug-Cu/L. Data from two tests have been pooled.

caused a decrease in survival as compared to the controls only in those organisms reared on Selenastrum/YCT and Chlamydomonas/YCT. A Friedman Two-Way ANOVA confirmed that the difference between the levels of survival observed in 4 $\mu\text{g-Cu/L}$ compared to the controls was significantly different between organisms raised on the different diets ($p < 0.05$). An 8 $\mu\text{g-Cu/L}$ concentration of copper caused a decrease in survival for organisms reared on all diets. This decrease in survival was not significantly different between the different diets ($p > 0.05$).

Three-brood reproduction was also affected by copper. Figures 27 through 30 illustrate the average three-brood reproduction of C. dubia raised on each of the diets in each concentration of copper as compared to the control. The control reproduction was actually the average three-brood reproduction in each diet only during the weeks in which copper tests were performed. Control reproduction does not refer to the mean reproduction in each diet over the course of the long-term culture experiment. These figures indicate that the C. dubia raised on Selenastrum/Chlamydomonas/YCT were not as sensitive to the copper stress as the C. dubia raised on the other diets.

The average weekly reproduction was determined in each culture line at each concentration of copper. Figures 31 through 39 illustrate the average reproduction in each culture line in each concentration of copper compared to the controls. As in the analysis of the long-term culture

Figure 27. Average reproduction of *C. dubia* subjected to 2 ug-Cu/L as compared to the control. Data from four tests have been pooled.

Figure 28. Average reproduction of *C. dubia* subjected to 4 ug-Cu/L as compared to the control. Data from seven tests have been pooled.

Figure 29. Average reproduction of *C. dubia* subjected to 8 ug-Cu/L as compared to the control. Data from two tests have been pooled.

Figure 30. Average reproduction of *C. dubia* subjected to 2, 4 and 8 ug-Cu/L as compared to the control.

Figure 31. Average reproduction of *C. dubia* in each culture line raised on *Selenastrum*/YCT in 2 ug-Cu/L as compared to the controls. Data is presented only for those culture lines that were intact at the time of the copper test.

Figure 32. Average reproduction of *C. dubia* in each culture line raised on *Chlamydomonas*/YCT in 2 ug-Cu/L as compared to the controls. Data is presented only for those culture lines that were intact at the time of the copper test.

Figure 33. Average reproduction of *C. dubia* in each culture line raised on *Selenastrum/Chlamydomonas/YCT* in $2 \mu\text{g-Cu/L}$ as compared to the controls. Data is presented only for those culture lines that were intact at the time of the copper test.

Figure 34. Average reproduction of *C. dubia* in each culture line raised on *Selenastrum*/YCT in 4 ug-Cu/L as compared to the controls. Data is presented only for those culture lines that were intact at the time of the copper test.

Figure 35. Average reproduction of *C. dubia* in each culture line raised on *Chlamydomonas*/YCT in 4 ug-Cu/L as compared to the controls. Data is presented only for those culture lines that were intact at the time of the copper test.

Figure 36. Average reproduction of *C. dubia* in each culture line raised on *Selenastrum*/*Chlamydomonas*/YCT in $\bar{4}$ ug-Cu/L as compared to the controls. Data is presented only for those culture lines that were intact at the time of the copper test.

Figure 37. Average reproduction of *C. dubia* in each culture line raised on *Selenastrum*/YCT in 8 ug-Cu/L as compared to the controls. Data is presented only for those culture lines that were intact at the time of the copper test.

Figure 38. Average reproduction of *C. dubia* in each culture line raised on *Chlamydomonas*/YCT in 8 ug-Cu/L as compared to the controls. Data is presented only for those culture lines that were intact at the time of the copper test.

Figure 39. Average reproduction of *G. dubia* in each culture line raised on *Selenastrum/Chlamydomonas/YCT* in 8 ug-Cu/L as compared to the controls. Data is presented only for those culture lines that were intact at the time of the copper test.

reproduction information, data on reproduction in copper solutions was analyzed only for those individuals in which the original culture line remained intact. Figures 31 through 39 suggest that the animals raised on the Selenastrum/Chlamydomonas/YCT diet were less sensitive to the copper than the animals raised on the other diets. The difference between the reproduction in the controls and in the copper solutions in each culture line is illustrated in Figures 40 through 42. The data is presented in Appendix H. Large values indicate a greater difference between reproduction in the controls and in the copper solutions and therefore indicate a less desirable outcome of the test. Negative differences indicate that the reproduction was better in the copper solution than in the control. A Friedman Two-Way ANOVA was used to determine that the differences in each culture line between the reproduction in the controls and in the copper solutions was significantly different between the diets at all concentrations of copper tested ($p < 0.05$). Subsequent analyses were performed on the reproduction data gathered for C. dubia in each culture line that were raised on different diets and subjected to different concentrations of copper. The differences between the reproduction in the control and in the copper solutions were paired according to diet. Friedman Two-Way ANOVA was performed on each pair of diets at each concentration of copper. A comparison of the Selenastrum/Chlamydomonas/YCT diet and the Chlamydomonas/YCT diet determined that the

Figure 40. Difference in reproduction between the controls and 2 ug-Cu/L in each culture line. Negative values indicate that reproduction was better in 2 ug-Cu/L than in the control.

Figure 41. Difference in reproduction between the controls and 4 ug-Cu/L in each culture line. Negative values indicate that reproduction was better in 4 ug-Cu/L than in the control.

Figure 42. Difference in reproduction between the controls and 8 ug-Cu/L in each culture line. Negative values indicate that reproduction was better in 8 ug-Cu/L than in the control.

difference in reproduction between C. dubia subjected to copper and the controls was not significantly different at any concentration of copper ($p < 0.016$), although qualitatively, the C. dubia raised on the Selenastrum/Chlamydomonas/YCT diet were more resistant to the copper. C. dubia raised on the Selenastrum/YCT diet did not show any significant difference from animals raised on the Selenastrum/Chlamydomonas/YCT diet at $2 \mu\text{g-Cu/L}$. However, a significant difference in reproduction was exhibited at $4 \mu\text{g-Cu/L}$ and $8 \mu\text{g-Cu/L}$, indicating that the animals raised on the Selenastrum/Chlamydomonas/YCT diet were more resistant to the copper ($p < 0.016$). C. dubia raised on the Selenastrum/YCT diet did not show any significant difference from animals raised on the Chlamydomonas/YCT diet at $2 \mu\text{g-Cu/L}$. However, a significant difference was observed between the two diets at $4 \mu\text{g-Cu/L}$ and $8 \mu\text{g-Cu/L}$, indicating that the animals raised on the Chlamydomonas/YCT diet were more resistant to the copper ($p < 0.016$) than animals raised on the Selenastrum/YCT diet.

DISCUSSION

The North Carolina Division of Environmental Management Mini-chronic Pass/Fail Effluent Toxicity Test requires that the animals used as controls in a chronic toxicity test must meet certain minimum standards in order to be considered a valid control. A minimum of 80% of the adult C. dubia used in the control must survive for the duration of the 7 day test, and they must produce an average of at least 15 neonates/adult during that period (NCDEM, 1989). These minimum standards are intended to ensure that the animals used in a chronic test are healthy enough to place a high level of confidence in the outcome of the test. Since these standards are requirements of the Mini-chronic Pass/Fail Test, it is desirable for a laboratory to choose a culturing regimen that results in a regular achievement of these minimum standards. Laboratories that frequently report an inability to meet these chronic test criteria lose time and money repeating tests that fail due to inadequate control survival and reproduction (DeGraeve and Cooney, 1987). It is in their best interests to improve the culturing techniques for C. dubia.

The long-term culture experiment evaluated the survival and reproduction of C. dubia raised on three different diets

for 19 weeks. Winner (1989) emphasized the importance of testing culture waters and diets for multiple generations. Culturing animals for a single generation can only indicate gross inadequacies in a diet or water, such as insufficient calories or macro-nutrients, inability of the organisms to assimilate the nutrients because of particle size, or toxicity present in one or more of the components of the diet or culture water. A multi-generational culturing test allows time for more subtle problems, such as trace nutrient deficiencies, to become apparent. Winner (1989) also emphasized the importance of evaluating the nutritional adequacy of a particular diet in a synthetic water or a groundwater rather than a natural surface water. Surface waters may contain supplementary food or nutrients that can confound the results of a nutritional study.

During the 19 week experiment, survival of C. dubia raised on all diets remained high. Average survival of C. dubia, which was above 98% in all diets tested, satisfied the North Carolina Division of Environmental Management Mini-chronic Pass/Fail Ceriodaphnia Effluent Toxicity Test requirement of 80% survival in the controls. During individual weeks of the experiment, survival fell below 80% once in the Selenastrum/YCT diet and once in the Chlamydomonas/YCT diet (Figures 3 through 5). Survival is probably not the best measure of the health of C. dubia since there were many occasions during the experiment when fecundity was quite low but survival remained unaffected.

Winner (1989) and Elias (1991) observed similar instances of high levels of survival and low levels of reproduction in their multi-generational experiments which suggests that the factors controlling survival and reproduction may be different.

Reproduction averaged above the North Carolina Division of Environmental Management standard of 15 neonates/adult in all of the diets tested. However, it is difficult to draw conclusions about the overall success of the three diets based on reproduction. Although the Selenastrum/Chlamydomonas/YCT diet was significantly better than the other diets in terms of reproduction, there were many occasions on which the diet did not support three-brood reproductions that could meet the minimum standard. Failure to meet the standard of 15 neonates/adult for a given week occurred sporadically in all of the diets tested, as illustrated in Figure 6. Variability in reproduction during a given week of the experiment, which is reflected in the standard deviations of the weekly averages, was high in all diets tested (Figures 8 through 10). Elias (1991) also noted large weekly standard deviations of three-brood reproduction in his dilution water study. Winner (1989) did not observe large standard deviations between replicates in his study, although he monitored reproduction over the life-span of each replicate, and it may be that brood size becomes more consistent as the animal matures. Since he

does not report three-brood reproductions, it is difficult to draw conclusions.

Temporal variability, or variability from one week to another, was also high during the course of the 19 week experiment. In general, periods of high reproduction and low reproduction followed the same pattern in all three diets, suggesting that a factor other than diet or a factor common to all three diets was affecting reproduction. One factor that may be responsible is the 10% PerrierTM used to culture the animals. Elias (1991), however, noted high temporal variability among many different culture waters, including 10% and 20% PerrierTM, two synthetic media, and a natural surface water, so it is unlikely that the culture water alone is responsible for the fluctuation in reproduction. It is possible that the laboratory air used to aerate the culture waters may change in quality and impart contaminants to the water at certain times. However, some experiments comparing waters that had been aerated using air that had passed through a carbon filter and air that had not passed through a carbon filter indicated that contaminants were not imparted to the culture water through the laboratory aeration system (unpublished data). It is also possible that the distilled-deionized water used to prepare the 10% PerrierTM changes in quality from time to time, although this would not explain observed temporal variation in natural surface water (Elias, 1991). Another reason for the observed variability might be the YCT.

Winner (1989) reports that different batches of trout chow and Cerophyl^R differ greatly in nutritional content and contaminant levels. However, only two lots of YCT were used throughout the course of the long-term culture experiment, so this too is an unsatisfying explanation for the observed variability. It is possible that C. dubia reproduction is inherently cyclical, although no information on the mechanisms that control temporal changes in reproduction has been published for this species.

One factor that possibly affected the outcome of the long-term culture experiment is the preparation of the combination algae and YCT foods. It was common practice in the laboratory to mix the algal and YCT components of the diet and use the mixture for one week. This practice was adopted based on the recommendation of the North Carolina Division of Environmental Management in the Mini-chronic Pass/Fail Ceriodaphnia Effluent Toxicity Test procedures. It is possible that YCT kept for a week at 4°C degrades in quality. It is also possible, although less likely, that mixing the algae and YCT components of the diet and leaving them combined for a week at a time may degrade the algae. This method of preparing combination foods was applied consistently to the three diets used in this experiment. If combining the algae and YCT did indeed lower the quality of the food at certain times, it may account for the temporal variability observed among animals raised on all diets. Whether or not food preparation or other factors were the

cause of the observed temporal variability, it is clear that the culturing techniques chosen in this study were unsuccessful at reducing it. Therefore, confidence in any single outcome of a Mini-chronic Pass/Fail Test using these animals as controls would remain low.

Measures of survival and reproduction in the long-term culture experiment suggest that the Selenastrum/Chlamydomonas/YCT diet is nutritionally superior to the single algae diets. Experiments with copper confirm that belief. The diet containing multiple species of algae conferred a greater resistance to the toxicant than the diets containing single species of algae. Survival and reproduction were higher among animals fed the Selenastrum/Chlamydomonas/YCT diet than among animals fed the Selenastrum/YCT diet and the Chlamydomonas/YCT diet in all concentrations of copper tested. Cowgill (1987) summarized the results of acute and chronic tests in which similar results were observed. Experiments were performed by six investigators in which the responses of daphnids to several organic and inorganic toxicants were dependent upon diet. Those animals that were fed YCT alone were more sensitive to toxicants than animals fed an algal diet. Animals fed single alga diets were more sensitive than animals fed multi-algal diets.

Survival and reproduction indicate that the Selenastrum/Chlamydomonas/YCT diet lowers the sensitivity of C. dubia to chronic copper stress. This could be due to a

nutritional deficiency in the single algae foods which imposes a nutritional stress in addition to the copper stress, or to differences in the concentration of toxic species of copper in the three culturing systems. All copper solutions used in this experiment were prepared nominally, and at no point were soluble copper concentrations measured directly. Despite the lack of measured data, a few lines of evidence suggest that the differences in sensitivity are due to nutritional inadequacy of the single algae diets. Data collected in this experiment is in agreement with data collected by other investigators (Winner, et al., 1977; Belanger, Farris and Cherry, 1989; Cowgill, 1987). Winner (1977) and Belanger (1989) measured the soluble copper in solutions after filtering out food particles and failed to note a significant difference in the copper content of solutions that had contained different types of food. If the algae used in this experiment did uptake significant amounts of copper, it is likely that one species of algae would uptake copper preferentially. Therefore, the results of the experiment would have shown that C. dubia were more resistant to copper when fed one of the single algae diets. This was clearly not the case. The Selenastrum/Chlamydomonas/YCT diet, which contained equal numbers of cells of each species, clearly provided C. dubia with a greater resistance to copper toxicity. It was also observed that the Selenastrum/Chlamydomonas/YCT diet

resulted in slightly higher survival, reproduction and neonate survival during the long-term culture experiment, suggesting a nutritional superiority to the other diets tested.

It was observed during the copper experiments that even the lowest concentration tested ($2 \mu\text{g-Cu/L}$) reduced the reproduction of C. dubia fed the Selenastrum/YCT diet and the Chlamydomonas/YCT diet. The highest concentration tested ($8 \mu\text{g-Cu/L}$) reduced reproduction in the animals fed the Selenastrum/Chlamydomonas/YCT diet. These levels of copper that exhibit chronic toxicity are lower than the levels that Belanger (1989) observed. He measured chronic copper toxicities of $7.9 \mu\text{g-Cu/L}$ and $10.1 \mu\text{g-Cu/L}$ in surface waters that had hardnesses of 94 and $170 \text{ mg-CaCO}_3/\text{L}$ respectively. Although the chronic toxicities of copper he observed were higher, the water that Belanger used to conduct his experiments had hardnesses substantially higher than 10% PerrierTM. The toxic concentrations of copper measured in this experiment were fairly low. However, it must be taken into consideration that data was collected for the offspring of all adults, whether or not the adult seemed to be healthy. Data was collected in this manner in order to maintain the genetic integrity of culture lines. The goal of this experiment was to measure relative differences in sensitivity to copper, not to maximize resistance to copper.

CONCLUSIONS AND RECOMMENDATIONS

1. Survival and reproduction of Ceriodaphnia dubia were above the minimum standards recommended by the North Carolina Division of Environmental Management in the Mini-chronic Pass/Fail Ceriodaphnia Effluent Toxicity Test procedure in all diets tested.
2. Variability in reproduction remained high in all diets tested and did not seem to be directly related to inadequacies in any particular diet. Animals raised on all diets experienced sporadic periods of high and low reproduction. If these animals were used as controls in a Mini-chronic Pass/Fail Ceriodaphnia Effluent Toxicity Test, confidence in a single outcome of the test would remain low.
3. Survival was not significantly different for C. dubia raised on any diet tested. On many occasions, survival remained high while reproduction was low. It is possible that the factors that affect survival and reproduction are not the same.
4. Reproduction was significantly different for C. dubia raised on the different diets. In terms of reproduction, the Selenastrum/Chlamydomonas/YCT diet proved slightly better than the Selenastrum/YCT diet and the Chlamydomonas/YCT diet. The Selenastrum/YCT diet proved slightly better than the Chlamydomonas/YCT diet.
5. The sensitivity to copper of animals raised on different diets was significantly different. C. dubia raised on the Selenastrum/Chlamydomonas/YCT were significantly more resistant to copper than C. dubia raised on the Selenastrum/YCT diet. C. dubia raised on the Selenastrum/Chlamydomonas/YCT were also more resistant to copper than C. dubia raised on the Chlamydomonas/YCT diet, although statistical significance was not noted. Measures of survival and reproduction may not be sufficient to evaluate the health and robustness of C. dubia. A more effective indicator is response to a toxicant.
6. The nutritional adequacy of the diet used to culture C. dubia does affect the outcome of chronic toxicity experiments. Diet must be taken into account when comparing the results of different experiments. In order to reduce

inter-laboratory variation, the diet for C. dubia used in toxicity tests should be standardized. Methods of algae culturing, sources of the components of YCT, food preparation and food storage should also be standardized since they may affect the quality of the food.

7. This experiment should be repeated without the inclusion of YCT in the diet. If it can be shown that C. dubia can be successfully cultured and maintained for multiple generations and remain resistant to toxicants without YCT in the diet, it should be eliminated since it is the largest source of contamination and nutritional inconsistency in the currently recommended diet for C. dubia.

8. The results of this experiment suggest that a multiple algal diet is a better choice for C. dubia than a single alga diet.

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Appendix A. Dates on which tests were initiated.

Generation	Date Test Started	Concentrations of Copper Tested (ug/L)
1	4/19/91	0
2	4/26/91	0
3	5/2/91	0
4	5/9/91	0
5	5/16/91	0
6	5/23/91	0
7	5/30/91	0
8	6/6/91	0
9	6/14/91	0
		2
10	6/21/91	0
		2
11	6/29/91	0
		2
12	7/6/91	0
		4
		8
13	7/13/91	0
		4
		8
14	7/20/91	0
		2
		4
15	7/27/91	0
		4
16	8/3/91	0
		4
17	8/10/91	0
18	8/17/91	0
		4
19	8/24/91	0
		4

Appendix B. Percent survival of adults raised on different diets.

Generation	Selenastrum/YCT (%)	Chlamydomonas/YCT (%)	Selenastrum/Chlamydomonas/YCT (%)
1	100	100	100
2	100	100	100
3	100	100	100
4	100	100	100
5	100	100	100
6	100	100	100
7	100	100	92
8	100	100	100
9	100	100	100
10	100	100	100
11	100	100	100
12	100	100	100
13	100	100	100
14	75	92	100
15	100	100	100
16	92	100	100
17	100	100	100
18	100	100	100
19	100	67	100

Appendix C. Reproduction of animals fed Selenastrum/YCT during the Long-Term Culture Experiment with mean reproduction (neonates/animal/week) and standard deviation (SD).

Generation	A	B	C	D	E	F	G	H	I	J	K	L	Mean	SD
1	27	26	27	27	27	21	27	29	28	25	21	25	25.83	2.52
2	28	26	26	26	27	21	24	25	25	27	17	23	24.58	3.06
3	25	21	24	23	22	23	24	23	19	24	24	29	23.42	2.39
4	19	23	19	26	21	21	20	26	26	18	22	20	21.75	2.90
5	24	16	24	19	16	19	19	24	23	18	16	19	19.75	3.19
6	13	22	20	16	19	17	17	17	19	12	16	15	16.92	2.84
7	22	23	18	11	17	21	20	23	18	21	17	20	19.25	3.36
8	22	18	23	28	21	25	22	22	23	19	14	22	21.58	3.50
9	15	14	19	20	17	23	19	18	23	21	15	19	18.58	2.97
10	16	19	19	13	13	15	10	18	22	13	12	19	15.75	3.65
11	24	22	26	22	24	25	26	24	24	22	25	30	24.50	2.24
12	21	16	23	27	18	22	22	23	26	21	22	26	22.25	3.19
13	26	24	24	24	20	25	24	23	20	20	25	21	23.00	2.17
14	21	12	2	15	0	0	19	23	4	13	20	19	12.33	8.66
15	15	20	11	11	21	18	19	24	21	19	16	16	17.58	3.96
16	0	0	9	16	15	17	17	15	16	13	6	16	11.67	6.39
17	24	18	23	16	20	19	21	18	18	21	21	16	19.58	2.54
18	13	21	16	18	21	18	16	23	23	24	17	15	18.75	3.57
19	18	18	22	17	22	17	28	23	17	19	18	25	20.33	3.63

Reproduction of animals fed *Chlamydomonas*/YCT during the Long-Term Culture Experiment with mean reproduction (neonates/animal/week) and standard deviation (SD).

Generation	A	B	C	D	E	F	G	H	I	J	K	L	Mean	SD
1	17	31	20	26	29	26	29	28	25	30	23	21	25.42	4.38
2	23	23	16	26	28	25	26	22	19	24	27	27	23.83	3.54
3	22	22	26	27	29	19	22	22	15	27	26	24	23.42	3.92
4	30	26	25	27	14	24	18	22	22	20	19	21	22.33	4.38
5	16	12	13	22	11	19	19	16	16	15	7	10	14.67	4.27
6	16	21	15	14	15	12	8	8	12	16	15	14	13.83	3.56
7	14	12	17	17	26	19	15	18	20	18	15	20	17.58	3.60
8	18	21	9	13	22	21	18	17	19	21	18	11	17.33	4.21
9	14	14	12	15	13	15	14	15	14	10	10	11	13.08	1.88
10	19	20	4	17	6	10	17	10	16	14	12	11	13.00	5.05
11	31	25	25	20	22	27	24	24	25	20	29	21	24.42	3.42
12	10	16	28	20	24	18	19	25	21	21	13	20	19.58	5.00
13	14	12	24	28	25	26	24	24	25	17	19	25	21.92	5.12
14	16	18	14	16	16	16	21	16	19	19	21	20	17.67	2.31
15	15	16	16	16	11	14	13	16	18	8	12	9	13.67	3.11
16	19	10	7	6	8	4	0	9	10	3	2	0	6.50	5.33
17	18	12	23	19	19	9	14	17	6	20	19	20	16.33	5.05
18	22	22	16	20	16	23	17	17	13	24	23	13	18.83	3.97
19	0	13	16	10	3	15	11	2	18	20	25	13	12.17	7.54

Reproduction of animals fed *Selenastrum*/*Chlamydomonas*/YCT during the Long-Term Culture Experiment with mean reproduction (neonates/animal/week) and standard deviation (SD).

Generation	A	B	C	D	E	F	G	H	I	J	K	L	Mean	SD
1	30	29	27	31	31	30	25	29	28	28	27	28	28.58	1.78
2	20	9	23	30	22	26	31	24	21	27	26	21	23.33	5.73
3	20	25	27	29	27	29	27	29	27	26	26	26	26.50	2.43
4	33	25	27	32	27	30	28	29	26	27	28	27	28.25	2.38
5	23	16	19	21	17	17	18	24	19	16	19	21	19.17	2.62
6	15	11	16	14	10	12	12	14	24	13	17	16	14.50	3.68
7	22	16	24	16	18	17	15	23	19	0	14	16	16.67	6.17
8	25	28	25	29	24	21	20	30	25	21	20	19	23.92	3.75
9	14	9	13	11	12	14	12	11	18	15	19	19	13.92	3.29
10	13	20	18	18	20	19	18	18	13	20	18	22	18.08	2.68
11	26	26	19	27	30	29	27	28	23	25	10	31	25.08	5.73
12	27	29	22	25	25	24	26	24	22	34	28	23	25.75	3.41
13	26	21	17	22	25	27	26	27	24	23	25	27	24.17	3.01
14	20	22	24	25	24	25	27	23	23	22	26	24	23.75	1.91
15	4	15	11	13	20	19	14	15	25	18	12	14	15.00	5.24
16	7	12	9	6	18	8	10	17	9	13	12	16	11.42	3.96
17	13	13	14	2	4	15	21	14	17	14	11	12	12.50	5.14
18	23	27	16	22	21	21	23	23	25	21	22	19	21.92	2.78
19	23	23	23	23	23	20	21	21	25	12	20	20	21.17	3.30

Appendix D. Heredity of animals fed Selenastrum/YCT during the Long-Term Culture Experiment.

Generation	A	B	C	D	E	F	G	H	I	J	K	L
1	A	B	C	D	E	F	G	H	I	J	K	L
2	A	B	C	D	E	F	G	H	I	J	K	L
3	A	B	C	D	E	F	G	H	I	J	K	L
4	A	B	C	D	E	F	G	H	I	J	K	L
5	A	B	C	D	E	F	G	H	I	J	K	L
6	A	B	C	D	E	F	G	H	I	J	K	L
7	A	B	C	D	E	F	G	H	I	J	K	L
8	A	B	C	D	E	F	G	H	I	J	K	L
9	A	B	C	D	E	F	G	H	I	J	K	L
10	A	B	C	D	E	F	G	H	I	J	K	L
11	A	B	C	D	E	F	G	H	I	J	K	L
12	A	B	C	D	E	F	G	H	I	J	K	L
13	A	B	C	D	E	F	G	H	I	J	K	L
14	A	B	C	D	E	F	G	H	I	J	K	L
15	A	B	A	D	G	G	G	H	I	J	K	L
16	A	B	A	G	G	G	G	H	I	J	K	L
17	A	G	A	G	G	G	G	H	I	J	L	L
18	A	G	A	G	G	G	G	H	I	J	L	L
19	A	G	A	G	G	G	G	H	I	J	L	L

Heredity of animals fed Chlamydomonas/YCT during the Long-Term Culture Experiment.

Generation	A	B	C	D	E	F	G	H	I	J	K	L
1	A	B	C	D	E	F	G	H	I	J	K	L
2	A	B	C	D	E	F	G	H	I	J	K	L
3	A	B	C	D	E	F	G	H	I	J	K	L
4	A	B	C	D	E	F	G	H	I	J	K	L
5	A	B	C	D	E	F	G	H	I	J	K	L
6	A	B	C	D	E	F	G	H	I	J	K	L
7	A	B	C	D	E	F	G	H	I	J	K	L
8	A	B	C	D	E	F	G	H	I	J	K	L
9	A	B	C	D	E	F	G	H	I	J	K	L
10	A	B	C	D	E	F	G	H	I	J	K	L
11	A	B	B	D	D	D	G	G	I	J	K	L
12	A	B	B	D	D	D	G	G	I	J	K	L
13	B	B	D	D	D	D	G	G	I	J	L	L
14	B	B	D	D	D	D	G	G	I	J	L	L
15	B	B	D	D	D	D	G	G	I	J	L	L
16	B	B	D	D	D	D	G	G	I	J	L	L
17	B	B	D	D	D	D	G	G	I	B	B	D
18	B	B	D	D	D	D	G	G	I	B	B	D
19	B	B	D	D	D	D	G	G	I	B	B	D

Heredity of animals fed *Selenastrum/Chlamydomonas/YCT* during the Long-Term Culture Experiment.

Generation	A	B	C	D	E	F	G	H	I	J	K	L
1	A	B	C	D	E	F	G	H	I	J	K	L
2	A	B	C	D	E	F	G	H	I	J	K	L
3	A	B	C	D	E	F	G	H	I	J	K	L
4	A	B	C	D	E	F	G	H	I	J	K	L
5	A	B	C	D	E	F	G	H	I	J	K	L
6	A	B	C	D	E	F	G	H	I	J	K	L
7	A	B	C	D	E	F	G	H	I	J	K	L
8	A	B	C	D	E	F	G	H	I	K	K	L
9	A	B	C	D	E	F	G	H	I	K	K	L
10	A	B	C	D	E	F	G	H	I	K	K	L
11	A	B	C	D	E	F	G	H	I	K	K	L
12	A	B	C	D	E	F	G	H	I	K	L	L
13	A	B	C	D	E	F	G	H	K	K	L	L
14	A	B	C	D	E	F	G	H	K	K	L	L
15	A	B	C	D	E	F	G	H	K	K	L	L
16	A	B	C	D	E	F	G	H	K	K	L	L
17	A	B	C	E	E	F	G	H	K	K	L	L
18	A	B	C	C	G	F	G	H	K	K	L	L
19	A	B	C	C	G	F	G	H	K	K	L	L

Appendix E

C. dubia raised on Selenastrum/YCT in Culture Line A.

C. dubia raised on Selenastrum/YCT in Culture Line B.

C. dubia raised on Selenastrum/YCT in Culture Line C.

C. dubia raised on Selenastrum/YCT in Culture Line D.

C. dubia raised on Selenastrum/YCT in Culture Line E.

C. dubia raised on Selenastrum/YCT in Culture Line F.

C. dubia raised on Selenastrum/YCT in Culture Line G.

C. dubia raised on Selenastrum/YCT in Culture Line H.

C. dubia raised on Selenastrum/YCT in Culture Line I.

C. dubia raised on Selenastrum/YCT in Culture Line J.

C. dubia raised on Selenastrum/YCT in Culture Line K.

C. dubia raised on Selenastrum/YCT in Culture Line L.

C. dubia raised on Chlamydomonas/YCT in Culture Line A.

C. dubia raised on Chlamydomonas/YCT in Culture Line B.

C. dubia raised on Chlamydomonas/YCT in Culture Line C.

C. dubia raised on Chlamydomonas/YCT in Culture Line D.

C. dubia raised on Chlamydomonas/YCT in Culture Line E.

C. dubia raised on Chlamydomonas/YCT in Culture Line F.

C. dubia raised on Chlamydomonas/YCT in Culture Line G.

C. dubia raised on Chlamydomonas/YCT in Culture Line H.

C. dubia raised on Chlamydomonas/YCT in Culture Line I.

C. dubia raised on Chlamydomonas/YCT in Culture Line J.

C. dubia raised on Chlamydomonas/YCT in Culture Line K.

C. dubia raised on Chlamydomonas/YCT in Culture Line L.

C. dubia raised on Selenastrum/Chlamydomonas/YCT in Culture Line A.

C. dubia raised on Selenastrum/Chlamydomonas/YCT in Culture Line B.

C. dubia raised on Selenastrum/Chlamydomonas/YCT in Culture Line C.

C. dubia raised in Selenastrum/Chlamydomonas/YCT in Culture Line D.

C. dubia raised on Selenastrum/Chlamydomonas/YCT in Culture Line E.

C. dubia raised on Selenastrum/Chlamydomonas/YCT in Culture Line F.

C. dubia raised on Selenastrum/Chlamydomonas/YCT in Culture Line G.

C. dubia raised on Selenastrum/Chlamydomonas/YCT in Culture Line H.

C. dubia raised on Selenastrum/Chlamydomonas/YCT in Culture Line I.

C. dubia raised on Selenastrum/Chlamydomonas/YCT in Culture Line J.

C. dubia raised on Selenastrum/Chlamydomonas/YCT in Culture Line K.

C. dubia raised on Selenastrum/Chlamydomonas/YCT in Culture Line L.

Appendix F. Summary of reproduction data gathered in each culture line for all diets tested. Listed are the number of successive generations that each culture line remained intact, the minimum and maximum reproductions observed for individuals in each culture line, the mean reproduction and the standard deviation observed in each culture line.

Diet: *Selenastrum*/YCT

	A	B	C	D	E	F	G	H	I	J	K	L
# of Generations	19	16	14	15	14	14	19	19	19	19	16	19
Minimum	0	0	2	11	0	0	10	15	4	12	6	15
Maximum	28	26	27	28	27	25	28	29	28	27	25	30
Mean	19.63	18.68	21.00	20.53	18.71	19.66	20.74	22.16	20.79	19.47	18.00	20.79
Standard Deviation	6.66	6.49	6.23	6.05	6.71	6.42	4.31	3.45	5.26	4.29	5.15	4.54

Diet: *Chlamydomonas*/YCT

	A	B	C	D	E	F	G	H	I	J	K	L
# of Generations	12	19	10	19	10	10	19	10	19	16	12	16
Minimum	10	10	4	6	6	10	0	8	6	3	7	0
Maximum	31	31	26	28	29	26	29	28	25	30	29	27
Mean	19.17	18.21	15.70	18.90	19.30	19.00	17.32	17.60	17.53	17.69	17.83	16.56
Standard Deviation	6.35	5.86	6.60	6.11	8.49	5.38	6.73	6.02	5.15	6.66	7.11	7.32

Diet: *Selenastrum*/*Chlamydomonas*/YCT

	A	B	C	D	E	F	G	H	I	J	K	L
# of Generations	19	19	19	16	17	19	19	19	12	7	11	19
Minimum	4	9	9	6	4	8	10	11	13	0	10	12
Maximum	33	29	27	32	31	30	31	30	28	28	28	31
Mean	20.21	19.79	19.68	21.81	20.82	21.21	21.11	22.26	22.08	19.57	20.36	21.11
Standard Deviation	7.56	6.94	5.57	8.00	7.20	6.46	6.30	5.90	4.34	10.50	5.78	5.15

Appendix G. Slopes of the lines fitted to the reproduction data in the Long-Term Culture Experiment.

Culture Line	Selenastrum/YCT		Chlamydomonas/YCT		Selenastrum/Chlamydomonas/YCT	
	Slope	R Squared	Slope	R Squared	Slope	R Squared
A	-0.554	0.219	-0.357	0.041	-0.511	0.144
B	-0.844	0.383	-0.574	0.304	0.002	0.000
C	-0.725	0.237	-1.691	0.567	-0.561	0.322
D	-0.561	0.172	-0.604	0.309	-0.954	0.322
E	-1.011	0.398	-1.861	0.441	-0.500	0.123
F	-0.457	0.089	-1.370	0.595	-0.433	0.142
G	-0.118	0.024	-0.591	0.244	-0.263	0.055
H	-0.244	0.158	-1.552	0.610	-0.437	0.174
I	-0.419	0.202	-0.312	0.117	-0.605	0.253
J	-0.207	0.074	-1.013	0.495	-4.357	0.803
K	-0.268	0.061	-0.706	0.128	-1.445	0.687
L	-0.274	0.115	-0.704	0.210	-0.354	0.150

Appendix H: Average reproduction (neonates/adult) in the control and 2 ug/L as copper. Combined data from four tests.

Diet: Selenastrum/YCT -			
Culture Line	Control	2 ug/L Copper	Control - 2 ug/L Copper
A	19.00	15.75	3.25
B	16.75	13.50	3.25
C	16.50	16.50	0.00
D	17.50	20.75	-3.25
E	13.50	16.00	-2.50
F	15.75	17.50	-1.75
G	18.50	19.00	-0.50
H	20.75	20.00	0.75
I	18.25	20.25	-2.00
J	17.25	16.75	0.50
K	18.00	19.75	-1.75
L	21.75	21.50	0.25

Diet: Chlamydomonas/YCT			
Culture Line	Control	2 ug/L Copper	Control - 2 ug/L Copper
A	21.33	21.00	0.33
B	19.25	20.50	-1.25
C	8.00	14.50	-6.50
D	17.00	15.75	1.25
E	9.50	17.00	-7.50
F	12.50	14.50	-2.00
G	19.00	18.25	0.75
H	12.50	13.00	-0.50
I	18.50	13.50	5.00
J	15.75	17.25	-1.50
K	17.00	20.67	-3.67
L	15.75	17.00	-1.25

Diet: Selenastrum/Chlamydomonas/YCT			
Culture Line	Control	2 ug/L Copper	Control - 2 ug/L Copper
A	18.25	19.75	-1.50
B	19.25	22.25	-3.00
C	18.50	22.50	-4.00
D	20.25	23.75	-3.50
E	21.50	22.00	-0.50
F	21.75	25.00	-3.25
G	21.00	21.75	-0.75
H	20.00	22.25	-2.25
I	18.00	21.67	-3.67
J	ND	ND	ND
K	15.67	21.00	-5.33
L	24.00	19.25	4.75

ND indicates that no data was collected for this culture line.

Average reproduction (neonates/adult) in the control and 4 ug/L as copper. Combined data from seven tests.

Diet: Selenastrum/YCT -			
Culture Line	Control	4 ug/L Copper	Control - 4 ug/L Copper
A	16.29	9.71	6.57
B	14.40	4.80	9.60
C	16.33	3.00	13.33
D	19.25	10.75	8.50
E	12.67	0.00	12.67
F	15.67	0.67	15.00
G	20.71	7.57	13.14
H	22.00	12.43	9.57
I	18.14	8.29	9.86
J	18.43	8.57	9.86
K	17.80	6.80	11.00
L	19.71	11.71	8.00

Diet: Chlamydomonas/YCT			
Culture Line	Control	4 ug/L Copper	Control - 4 ug/L Copper
A	10.00	9.00	1.00
B	15.29	9.00	6.29
C	ND	ND	ND
D	16.57	9.14	7.43
E	ND	ND	ND
F	ND	ND	ND
G	15.00	7.86	7.14
H	ND	ND	ND
I	17.71	9.14	8.57
J	13.60	13.20	0.40
K	13.00	12.00	1.00
L	14.80	11.00	3.80

Diet: Selenastrum/Chlamydomonas/YCT			
Culture Line	Control	4 ug/L Copper	Control - 4 ug/L Copper
A	18.57	17.29	1.29
B	21.29	16.29	5.00
C	17.43	21.71	-4.29
D	18.20	25.60	-7.40
E	22.40	19.40	3.00
F	20.57	20.57	0.00
G	21.00	22.00	-1.00
H	21.43	23.43	-2.00
I	22.00	21.00	1.00
J	ND	ND	ND
K	ND	ND	ND
L	20.43	26.43	-6.00

ND indicates that no data was collected for this culture line.

Average reproduction (neonates/adult) in the control and 8 ug/L as copper. Combined data from two tests.

Diet: Selenastrum/YCT			
Culture Line	Control	8 ug/L Copper	Control - 8 ug/L Copper
A	23.50	12.00	11.50
B	20.00	0.00	20.00
C	23.50	16.00	7.50
D	25.50	2.00	23.50
E	19.00	0.00	19.00
F	23.50	0.00	23.50
G	23.00	0.00	23.00
H	23.00	2.00	21.00
I	23.00	0.00	23.00
J	20.50	6.00	14.50
K	23.50	12.00	11.50
L	23.50	0.00	23.50

Diet: Chlamydomonas/YCT			
Culture Line	Control	8 ug/L Copper	Control - 8 ug/L Copper
A	10.00	7.00	3.00
B	14.00	15.50	-1.50
C	ND	ND	ND
D	24.00	6.00	18.00
E	ND	ND	ND
F	ND	ND	ND
G	21.50	11.50	10.00
H	ND	ND	ND
I	23.00	8.00	15.00
J	19.00	10.50	8.50
K	13.00	22.00	-9.00
L	22.50	13.00	9.50

Diet: Selenastrum/Chlamydomonas/YCT			
Culture Line	Control	8 ug/L Copper	Control - 8 ug/L Copper
A	26.50	25.50	1.00
B	25.00	15.50	9.50
C	19.50	12.50	7.00
D	23.50	8.00	15.50
E	25.00	14.00	11.00
F	25.50	14.00	11.50
G	26.00	14.00	12.00
H	25.50	14.50	11.00
I	22.00	21.00	1.00
J	ND	ND	ND
K	ND	ND	ND
L	25.00	12.00	13.00

ND indicates that no data was collected for this culture line.