# DEVELOPMENT OF A THREE-TROPHIC LEVEL TOXICITY TEST UTILIZING AN ALGA (*Chlorella vulgaris*), ROTIFER (*Brachinous calyciflorus*), AND FISH (*Pimephales promelas*)

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(ABSTRACT)

In this research a test system was developed that is designed as a tool to evaluate the potential hazard of chemicals to aquatic ecosystems. The system developed is a linear three-trophic level food chain consisting of an alga (*Chlorella vulgaris*), rotifer (*Brachionus calyciflorus*), and fish (*Pimephales promelas*). The chemostat design used for the lower two trophic levels was crucial in being able to supply the top trophic level with sufficient food on a continuous basis. The system was initially evaluated using copper (Cu) and selenium (Se) as toxicants. In the copper experiments, results of a 7 day three-trophic level toxicity test were compared with a series of single species tests. The LOEC was 31.5 $\mu$g/L based on a temporary impairment of the algal population growth, with a corresponding NOEC of 16.2 $\mu$g/L. The algal population at all initially impaired treatment levels demonstrated recovery to control levels by the end of the test. Single species tests with the same

species showed impairment at treatment concentrations lower than the corresponding value from the three-trophic level test. The difference in sensitivity is attributable to the fact that most of the Cu in the single species tests was in the dissolved form (approximately 80 %), whereas in the trophic level test most of the Cu was not (< 15 % dissolved Cu). The three-trophic level Se experiment lasted for 25 days, with both short-term and long-term impacts evident. At the algal trophic level, growth was not impaired on a daily basis at any of the exposure levels (110.3, 207.7, and 396.1 $\mu$g/L Se). However, algal densities were slightly reduced at the 207.7 and 396.1 $\mu$g Se/L treatments, although not significantly different when the data was pooled across days. Rotifer populations were impaired at these same levels by day 4, and succumbed to the Se by day 7. Fathead minnow growth was also impaired at these two concentrations by day 7. In addition, sublethal impairment of rotifer and fish growth was evident at the 110.3 $\mu$g/L level after day 20 indicating a more subtle trophic impact. Bioconcentration factors ranged between 100 and 1000 $\mu$g/L and were found to be dependent on the species, treatment, and day.

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## ABSTRACT

The overall goal of this research is to develop a "trophic toxicity test" that links physically isolated populations of the algae and rotifers (*Brachionus calyciflorus*) to larval fathead minnow (*Pimephales promelas*). The flow-through test system toxicity test utilizes a chemostat design, which has a number of advantages as compared to standard algal bottle tests. Initial batch culturing experiments found that *Chlorella vulgaris* was a suitable alga to use in the system, whereas the rotifers were unable to utilize *Selenastrum capricornutum* as a food source. The final rotifer density after 7 days was found to be independent of the size of the test chamber and initial starting density, although time until stabilization of the population density was dependent on the starting density. Copper toxicity was evident within 1 day of adding the Cu to the algae at the three highest treatment levels of 50, 100, and 200 $\mu$g/L. The algal population completely died off at the 100 and 200 $\mu$g Cu/L by day 7. Addition of 50 $\mu$g Cu/L to the algal chemostats caused an initial decline in the algal population, but the population recovered to control levels by the seventh day. Results of an initial trial of the trophic test system demonstrates the feasibility of the concept. The system was found to be able to support a population of rotifers with a density of 120 females/ml and egg ratio 0.400. There was sufficient rotifer biomass to maintain 25 larval fathead minnows over 7 days from each chemostat. Fish dry weight was relatively high averaging 0.762 ± 0.057 mg.

## INTRODUCTION

There is an ongoing debate in the field of aquatic ecotoxicology concerning the role of single species versus multispecies tests. Historically, the majority of regulatory action has been based on single species testing (Buikema et al. 1982, Stephan et al. 1985), with only limited provisions to use multispecies tests. In general mutispecies tests have only been used after extensive single species testing has been completed. Single species tests, which rely on assessing the response of individual organisms, have the advantage of being reproducible, amendable to standardization, long history of past use, and are relatively inexpensive (Buikema et al. 1982, Rand and Petrocelli 1985). However, their environmental realism is low, and their relevance and predictive capacity with regard to actual ecosystems has been questioned (Kimball and Levin 1985, Cairns and McCormick 1991). The single species approach relies solely on structural aspects of an ecosystem and ignores the functional attributes. The current approach (Stephan et al. 1985, US EPA 1991) assumes that if enough single species or "sensitive species" are tested, the functioning of the ecosystem will be maintained.

In contrast, multispecies tests have the ability to evaluate ecosystem level interactions and their functional attributes (Giesy 1985, Cairns and McCormick 1991). The most common approach to multispecies testing is to set up a model ecosystem (often called a microcosm or mesocosm) that mimics the type of ecosystem targeted for protection. This approach has been

criticized as being too expensive and complicated to base regulatory decisions on, as well as not providing a sufficient increase in predictive capacity as compared to a suite of single species tests to justify the cost. Advocates argue that there is no strong evidence that single species tests can predict the response of multispecies tests or ecosystems accurately (Cairns 1983). Benefits include the ability to work with populations of organisms, not just individuals as in single species testing, and the interactions between these populations can be studied (Halbach 1984).

The goal of this research was to devise a toxicity evaluation scheme based on single species testing that evaluates potential impacts across trophic levels. To achieve this goal, an approach that is intermediate between single species and mesocosom tests is used. The test system is simpler and less expensive then mesocosm tests, with a clear endpoint for regulatory decision making. This approach relies on a simple unidirectional food chain that is an oversimplification of actual food chains that exist, but it allows one to study the impact of a chemical on this functional attribute of all ecosystems. To achieve this goal, three species suitable for toxicity testing have been coupled together in a simple three-level food chain (Figure 1). The role of the proposed trophic toxicity test is to examine the effect of a chemical on the ecosystem attribute of trophic transfer, and measure the biomagnification potential of the chemical. The trophic toxicity test system tracks the transfer of energy in the form of biomass along with associated xenobiotics up the trophic ladder. The test

system is designed to evaluate the impact of the toxicant on this process. The impact may be reflected at each trophic level by the reduction of population density, lethality, reduced growth, and reduced quality of food. At the top of the food chain these effects are summarized. One endpoint (fish viability) at the top summarizes the impact of the chemical on the whole process. In addition, by examining the response at each trophic level, the "weak link" in the food chain can be identified.

## EXPERIMENTAL DESIGN

The most difficult aspect of this test design was being able to produce sufficient biomass at the lower trophic levels to support the organisms at the higher end. Addressing this problem dictated the type of organisms chosen for the trophic toxicity test. The organisms chosen are an alga (*Chlorella vulgaris* or *Selanastrum capricornutum*), rotifer (*Brachionus calyciflorus*), and a fish (*Pimephales promelas*). The three species are linked using a continuous flow-through test system where the lower two levels function as a two-stage chemostat (Figure 2). In a chemostat the standing crop of the organism is maintained at a constant level by establishing the population growth rate at a level that equals the flow rate divided by the culture volume. The steady-state algal culture system will provide a physiologically consistent and stable algal population. The stability of the algal population should make detection of a toxicant induced response easier to detect.

There are a number of practical obstacles in developing a trophic toxicity test. This research deals primarily with developing the algal/rotifer continuous growth system and characterizing the population parameters of the algae and rotifers. This paper addresses the basic questions that need to be answered to develop the system as envisioned. These factors can be divided into four groups:

1) Species selection
2) Dilution water selection
3) Test system development
4) Endpoint selection

*Species selection*

The focus on which species to use for a trophic test system rapidly narrowed to an algae-rotifer-fish system for two reasons. Rapidly reproducing organisms that are necessary for the lower trophic levels to provide sufficient biomass to support the higher trophic levels. Choosing an alga for the first level was an obvious choice, but the second level was more difficult. To obtain rapid reproduction at this level a parathogenic organism with a short life cycle and ability to live in high densities was required. Daphnids were considered because of there wide use in toxicity testing, but because of the space needed to culture them, and their slower growth rate, rotifers were a better choice. A fish was chosen for the top level because of the wide spread importance generally placed on protecting them and because rotifers are an excellent larval fish food (Lauth et al. In prep, Lubzens 1987).

Selection of an alga for this research is based on the following characteristics: 1) This alga should be able to support a healthy population of rotifers and be suitable for toxicity testing. 2) The alga also needs to be conducive to continuous culture techniques. 3) It should be acceptable to the toxicity testing community.

It has generally been found that simple unicellular algae support the highest growth rate of *Brachionus calyciflorus*. There is a large body of literature (Stemberger 1981, Rothaupt 1985, Hirayama 1987, Lubzens 1987, Bennett and Boraas 1988, Boraas and Bennett 1988, James and Rezeq 1988) that clearly supports the use of *Chlorella* as food for *Brachionus* spp. in aquaculture. Although some individual researcher's (Lubzens 1987) have found other algae that can support higher growth rates than *Chlorella* spp., *Chlorella* does consistently support high growth rates of *Brachionus* spp. An additional benefit is that rotifers grown on *Chlorella* spp. have been shown to be nutritionally complete for larval fish culture (Scott and Baynes 1978, Watanabe et al. 1983, Rezeq and James 1987)).

There is a limited body of literature dealing with the use of *S. capricornutum* as *B. calyciflorus* food. Hutchinson (1967) reported on studies conducted by Pourriot in which the possible correlation between algal structure and rotifer growth was investigated. *S. capricornutum*, while unicellular, is coccoid in shape which would suggest that it is less suitable then *Chlorella*. In Pourriot's study *Chlorella pyrenoidosa* was found to be the best food for *B.*

*calyciflorus* of the algae used in the study (i.e., *Chlorella, Scenedesmus, Haematococcus, Selenastrum, Ankistrodesmus*, and *Pandorina* spp.). More recent literature (Scott and Baynes 1978, Stemberger 1981, Fulton 1988) has shown that rotifers in general can utilize a variety of algal food sources, although simple algae do appear to support the best growth in the laboratory.

*Dilution water selection*

Another problem that was addressed in the initial phase is the selection of a suitable growth medium. In choosing an appropriate dilution water for the trophic toxicity test, several factors were considered:

1) The medium should be suitable for maintaining all three organisms.

2) If possible the medium should be stable as concentrated stock solutions.

3) The medium must be able to support nutritionally complete organisms.

4) For toxicity testing purposes the fewer complex organic substances in a medium the better, so as not to interfere with a toxicant's toxicity.

This research evaluated the suitability of a synthetic water and a nutrient supplemented creek water for the trophic test system. The synthetic water used was Ecosystem Simulation Water or ESW (Lauth et al. 1990), which is a modified version of Guillard's Woods Hole Marine Biological Laboratory (MBL) algal culture medium (Nicholas 1973). The creek water used was collected from Sinking Creek, Newport, VA and supplemented with nutrients based on the MBL formula.

*Test system development*

Before proceeding with development of the trophic test system a number of basic decisions were needed concerning what media, and algal species to use. These decisions were made based on review of pertinent literature and a few simple bench top experiments. In addition, optimizing the rotifers' test and culture conditions was necessary since they are not a very commonly tested organism. While the literature does contain numerous accounts of culture systems for production of *Brachionus* spp. on a commercial basis (Lubzens 1987), and a limited amount of toxicity testing information (Buikema et al. 1974, Capuzzo 1979, Dad and Pandya 1982, Borgmann and Ralph 1984, Halbach et al. 1984, Schluter and Groeneweg 1985, Rao and Sarma 1986, Snell and Persoone 1989a, b), combining the two technologies was necessary for this research.

Chemostat technology is used in bacterial culture to provide stable cell lines. Application of this technology to algae, and even rotifers (Boraas and Bennett 1988) is not uncommon. The ability to maintain algae and rotifers at set growth rates using continuous culture techniques has been demonstrated by several researchers (Boraas 1983, Droop 1983, Hirata et al. 1983, Rothaupt 1985, Boraas and Bennett 1988). Note that the initial media input rate controls the flow rate throughout the system, while the size of the test chamber in conjunction with the flow rate controls the turnover or dilution rate which is

equal to the population growth rate. The turnover rate is the rate at which the population replaces itself or doubles.

The algae from the first stage is pumped into the second level to provide a consistent food source for continuous steady-state cultures of rotifers. Similarly to the algae, rotifer population dynamics will be stabilized at a set-point allowing inferences to be made when deviations occur under the influence of a toxicant. Measurement of population dynamics for the rotifers and algae can be achieved via sample ports.

*Endpoint selection*

Endpoints were evaluated based on the efficiency to measure them relative to the amount of information gained. Determination of the best endpoints for toxicity evaluation is crucial for acceptance of the trophic toxicity test system. The endpoints should reflect how each trophic level is responding and incorporate any cumulative effects that are passed up the food chain. At the highest trophic level the three parameters measured are fish survival, dry weight, and bioconcentration of the toxicant in the organisms.

Because the algae and rotifers are grown under chemostat culture conditions, the parameters used focus on measuring the density of various components of the population. In the chemostats, all these parameters should be stable, unless perturbed by the toxicant. A number of different rotifer population parameters were investigated as toxicity endpoints. Possible

parameters are direct counts of the density of adult female rotifers, males, juveniles, amitic egg production, mitic egg production, and egg ratio. The standing crop of algae was measured by three techniques: direct cell counts, measurement of relative chlorophyll content using a fluorometer, and measurement of the dry weight.

## METHODS

### *Trophic System Design*

There are four levels to the trophic test system, linked together in series (Figure 2). The top level consists of the test solution reservoirs and a peristaltic pump with an eight-channel pump head. A typical test would have four reservoirs, one for each treatment level, feeding into eight algal chemostats. The reservoirs consist of 25-L polypropylene carboys with two lines fitted with one-way check valves for delivery of the test solution via the peristaltic pump. The test solution is aerated vigorously to promote mixing using air that passes through a 0.2-$\mu$m filter. The carboys are plugged with cotton bungs and wrapped in aluminum foil to prevent light from causing any changes in the test solution. The whole apparatus is sterilized before use.

Test solution is prepared from Sinking Creek (Newport, VA) water which is collected within a month of testing. The water is first filtered through a 1.6-$\mu$m filter to remove suspended solids, and then autoclaved to sterilize it. An appropriate volume of sterile water is then added to each of the four sterilized

25-L polypropylene carboys. Nutrients are then added from sterilized nutrient stocks while the water is aerating. The nutrient stocks used are based on the Woods Hole MBL algal media (Nicholas 1973). Because it is critical that all the treatment levels receive exactly the same amount of nutrients, the media is exchanged between the four carboys until the solution is uniform in composition. At this point appropriate volumes of concentrated stock of the chemical of interest are added to the reservoirs.

The algal chemostat portion of the system uses custom made glass cylinders that taper to a point at the bottom (Figure 2). The cylinders are designed to hold at least 2 L and are approximately 10 cm in diameter and 40 cm long; the bottom 10 cm forms a cone. A short piece of glass tubing (2 cm x 6 mm OD x 2 mm ID) is attached to the bottom and serves as the air and media inlet. It is important to aerate vigorously to keep the algae well mixed. The conical bottom, with the air bubbling up from the center prevents the algae from settling out. Illumination is provided by 20-watt cool white fluorescent lights providing 4300 ± 400 lux at the algal chemostat. The bottom third of the chemostats sit in a water bath to maintain temperature at 25 ± 0.2°C.

On the top of the chamber is a short neck designed to hold a 40-mm silicon stopper. A small J-shaped glass (6 mm OD x 4 mm ID) tube passes through the stopper and is the outlet for the system. The height of the tube in the chamber is adjusted so that the chamber holds the desired, 2-L volume. There is also a smaller hole on top that is plugged with a 15-mm septum and

is the sample port for the system. The system is air tight, with the air pressure forcing excess algae out on a continuous basis. Media inflow is approximately 0.7 ml/min and is delivered from the reservoirs using a peristaltic pump. Approximately a liter of fresh test solution enters the algal chemostat daily, with an equal volume of the algal culture being forced through the overflow by air pressure to the next trophic level. This results in a turnover rate for the algal chemostats of about once every 2 days, and is designed to maintain an algal population in late log phase with a density between 3 and 6 x $10^6$ cells/ml. The whole system, including the silicone outflow tubing to rotifer chemostats, is sterilized before use. Inoculation of the system is achieved by batch culturing 16 L in a large carboy until late log phase of growth (~7 days) and then transferring the algae directly to the test vessels. Toxicant is directly added to the algal chemostats at the beginning of the test.

The rotifer chemostats are similar in design to the algal chemostats, but are made from 1-L polypropylene sample bottles with caps. Holes are drilled in them to accommodate glass tubes for aeration, media in/out, and an overflow (Figure 2). One-way check valves are placed on the inlet and outlet to prevent back contamination. The chambers are wrapped in aluminum foil since the rotifers prefer a dark environment. Again vigorous aeration is necessary to keep the population of *B. calyciflorus* well mixed. *C. vulgaris* from the first level is continually forced into the rotifer chamber by air pressure from the algal

chemostat. The overflow is positioned to maintain a volume of 950-ml in each chamber, with the spill-over dripping into the fish containers.

Unlike the algal chemostats, the overflow is not the principal exit port for the rotifer chemostats. Because rotifers are mobile animals, they have the potential to avoid a slow flowing overflow. To overcome this problem rotifers are pumped out at a high flow rate on a periodic basis using a peristaltic pumped controlled by a timer. Typical outflow parameters are: pump on 1 minute out of every 30 with a flow of 18 ml/min. The pumped out enters into the bottom of the fish chambers. The turnover rate is approximately once per day for the rotifer chemostats. The chambers are sampled with a syringe by removing the cap.

The larval fathead minnows are held in 350-ml polystyrene chambers with an inlet consisting of the outflow from the rotifer chemostats entering at the bottom. The overflow is a whole cut into the side of the chamber, screened with 100-$\mu$m nitex to keep the organisms in. The chambers are cleaned daily to remove any waste. Aeration is provided at 100 bubbles/min via narrow bore glass tubes. The larval fatheads used are 24 hours old, with up to 40 placed in each chamber depending on the sampling strategy of the test.

*Batch culture testings*

Routine culturing of the rotifers and algae was achieved using a system originally developed for use in small scale aquaculture (Florida Aqua Farms,

Dade City, FL). The system consists of a PVC frame designed to suspend 5-L plastic bags in front of two 20-watt cool white fluorescent bulbs providing 4000 lux of continuous illumination. The media used to grow the algae and rotifers in the bags is either a synthetic (Lauth et al. 1990) or a natural water (Sinking Creek water, Newport, VA) supplemented with MBL nutrients. Batch cultures with *Chlorella vulgaris* as the food source were maintained to determine what quantity of algae would support certain densities of rotifers. It was estimated from this work that a rotifer population with a density of 100 females/ml could be maintained by halving the rotifer cultures on a daily basis and replacing that volume with *C. vulgaris* culture at a density of $6 \times 10^6$ cells/ml. Rotifers were initially hatched from cysts (Florida Aqua Farms, Dade City, FL) to start a culture.

Culture health and rotifer density were determined from direct counts using a Sedgwick-Rafter counting cell. The number of females/ml was used as an estimate of population density, while the egg ratio was used to judge how quickly the culture was growing. The egg ratio is equal to the number of eggs divided by the number of females and can range for this species from 0 to 1.4. A high value indicates that the population is reproducing very rapidly, while a low value indicates no reproduction. Typical values for routine culturing range from 0.3 to 0.6.

Batch cultures with the green alga, *S. capricornutum,* were set up to address whether this alga could be used instead of *C. vulgaris* in the trophic

test system. *S. capricornutum* was fed to *B. calyciflours* directly hatched from cysts and from populations that had been started on *C. vulgaris*.

Since most of the work with culturing *Brachionus* spp. has been developed for the aquaculture industry, there is a limited amount of information on culturing at the smaller scale required for the trophic test system culture. A bench-top experiment designed to address the following issues was used to optimize rotifer culture conditions:

1) Does the size of the culture vessel affect the growth of *B. calyciflorus*?

2) How does the starting density of the rotifer culture affect the final density after 7 days of growth?

Rotifers were grown in polypropylene sample bottles using Sinking Creek water with MBL nutrients as the media for 7 days under vigorous aeration. The rotifers were fed *C. vulgaris* at a density of $6 \times 10^6$ cells/m. The 7 treatments used are listed in Table 1 and consist of combinations of three different bottle sizes and four different starting densities. The parameter used to measure growth was direct counts of the number of females/ml in preserved samples using a Sedgwick-Rafter counting cell.

*Algal Chemostat Trials*

Development of the algal chemostats for the trophic test system involve an innumerable series of trials to optimize test conditions. Test vessel size and shape, pumping conditions, environmental control, and other factors were all

explored in these early trials. The system described in the "Trophic System Design" section of this paper is a result of those trials. In this paper the variability and system characteristics of the algal chemostats are examined. Of paramount importance is the long-term stability and replicablility of the system.

The results of two algal chemostat trials are presented in this paper. The first experiment involved running two algal chemostats side by side for 35 days to determine the variation and stability of the system. The two algal chemostats were inoculated from a 7-day old culture of *C. vulgaris* grown in MBL media at a staring density of $1.1 \times 10^6$ cells/ml. Renewal of the media reservoir was done every 5 to 7 days using an axenic technique. Algal density was tracked by direct cell counts. Flow rate was approximately 0.7 mls/min.

In the second experiment *C. vulgaris* was exposed to four different levels of copper for 7 days. The dilution water used was sterilized Sinking Creek (Newport, VA) water with MBL nutrients added. The goal of this initial experiment was to examine how quickly the algal chemostats would respond to copper. Treatment levels were 0, 25, 50, 100, and 200 $\mu$g Cu/L, with two replicate chemostats per treatment. Dry weight, fluorometer reading, and cell counts were all used to track algal growth in this experiment. Dry weight was used as the definitive parameter because cell counts were too labor intensive when running 8 chemostats; and fluorometer readings were found to decline at times when cell density was constant. Test conditions and design were as described previously.

*Trophic Trial*

After the algal portion of the test system was developed, the linked system was initially evaluated without any toxicant added. Four replicates, each consisting of one algal-rotifer-fish series, were set up with the goal of evaluating the following factors: stability of the system, suitability of test parameters, and if sufficient biomass would be produced to support an adequate number of fish at the highest trophic level. Also examined was the relationship between fluorometer reading and cell counts. One reservoir was used for all four replicate lines and was renewed every 5 days. The algal-rotifer system was allowed to stabilize for 5 days before the fish chambers were added, and the test lasted for a total of 12 days. Each fish chamber contained approximately 30, 24-hr old fathead minnows. The rotifer population was measured by cell counts of preserved samples (70 % ethanol) in a Sedgewick-Rafter cell. The number of adult females, juveniles, amitic eggs, males, and mitic eggs was enumerated for each sample by counting the total slide. Fluorometer readings and cell counts were used to track the algal population, while mortality and dry weight were used to assess fish health.

## RESULTS & DISCUSSION

*Batch culture testings*

Results of the batch culture trials with *S. capricornutum* were disappointing but definitive. The rotifer *B. calyciflorus* could not utilize this alga

as a food source. In the trials where the food was slowly changed from *C. vulgaris* to *S. capricornutum* over the course of a week, the rotifer populations egg ratios decreased as the amount of *S. capricornutum* increased. The rotifer populations simply died when *S. capricornutum* was the principal food source.

The goal of rotifer scale experiment was to evaluate the role of vessel size and starting density in culturing and testing *B. calyciflorus*. While the issue of scale tends to be ignored by many laboratory researchers, a real potential exists for it to influence test results. The biology of *B. calyciflorus* suggests that the size of the culture vessel could play a role.

In evaluating test vessel size with constant density, three vessel sizes (0.2, 1, and 2 L) with a starting density of 50 females/ml were used. No differences larger then the expected variation inherent in counting the rotifers throughout the experiment was observed (Table 1). A similar analysis holds for the other rotifer densities that had multiple sizes.

The starting density in this experiment ranged from 25 to 200 females/ml (Table 1). In the beginning of the experiment densities were highly variable, but by the end of 7 days all treatments were in the range of 89 to 122 females/ml. The closer the starting density to the final density, the quicker the culture appeared to stabilize. Another way to examine the data was to address the variability of all the treatments across a particular day. On day 2 the standard deviation was 49.6 females/ml, and by day 7 it was 11.7 females/ml. The

results suggested that the population would stabilize quicker if the culture was not over inoculated with rotifers.

*Algal Chemostat Trials*

It should be noted that a long term (30 days) growth trial that used ESW as the dilution water failed to maintain long term functioning of the chemostats. While ESW worked well in batch culture, there was a tendency for salts to precipitate out in the reservoir which negatively affected algal growth.

The 35-day algal chemostat trial with MBL media was very successful in illustrating the ability of the algal chemostats to maintain a stable population over an extended period of time (Figures 3a and 3b). During the first five days of the trial the algal density was increasing to the carrying capacity of the system as regulated by the nutrient supply, light intensity, and flow rate (Figure 3a). After the first 5 days algal density was stable around the set point of 2.5 x $10^6$ cells/ml. At this density the algal growth rate maintained pace with the rate of outflow.

Over the course of the last 30 days the mean algal density of the two replicates settled between 2 and 3 x $10^6$ cells/ml which indicated excellent stability of the system (Figure 3a). While the standard deviation of the mean appeared large at times, most of the variability could be accounted for by the counting technique used to estimate cell density. The size of the error bars in Figure 3b reflect the error in the measurement technique, whereas Figure 3a

reflects the overall error in the experiment. The size of the error bars are similar in both graphs, suggesting that most of the variability is due to the measurement technique and not actual differences between the replicates.

Prior to copper exposure, algae was added to the chemostats 3 days before dosing to examine any variability between the eight chemostats. The most important aspect of the results is the rapid response of the algal chemostats to copper exposure (Figure 4). Within 2 days, the three highest exposure levels (50, 100, 200 $\mu$g/L) were significantly impaired based on the ANOVA and Dunnett's multiple comparison at $p = 0.05$. The *C. vulgaris* population at the 200 $\mu$g/L level completely died off by day 4, while the algal chemostats at 100 $\mu$g/L level died by day 7. Algae exposed at the 50 $\mu$g/L treatment level were initial impacted, but recovered to control levels by day 7.

The recovery of the algal population may be due to a number of factors. The population may be adapting to the presence of the toxicant by selecting for more tolerant cells, or be due to a drop in the dissolved Cu levels, after the initial exposure. Chemical analysis of the test solution shows that there is practically no copper in the dissolved form after the 1st day. The results of this experiment show that the algal chemostats react rapidly to the introduction of a toxicant.

*Trophic Trial*

Behavior of the algal chemostat portion of the trophic trial during the 12-day trial was adequate, although less than ideal. In evaluating the performance of the algal portion of the system, a number of important factors came to light based on the two parameters (cell counts, fluorometer readings) used to estimate algal density. In general, they paralleled each other and had some revealing differences (Figure 5 and Table 2). Figure 5 illustrates that for the first 5 days cell counts were constant or increasing, while relative absorbance steadily declined.

A continuous decline in the fluorometer reading was detected in each chemostat, which was probably due to a decline in the amount of chlorophyll in each cell. This was related to a change in algal growth environment when cells from the inoculum culture are transferred to the chemostats. The chemostat environment favors rapid cell division and is characterized by high and uniform light intensity, consistent temperature control, plus ample and constant nutrient supply. In contrast the batch culture conditions are characterized by the same starting nutrient levels, but these levels decline as algal density increases. While the inoculum culture is grown at the same outside light intensity (4300 lux), self shading within the large carboy probably reduces the average light intensity that individual cells experience. Differences in the growth environment between batch and chemostat culture are supported

by the observation that cells grown in the chemostats are smaller and lighter in color than those grown in batch culture.

It should be noted that the increase in fluorometer reading at day 10 in replicates A and D is due to the addition of algae on day 9. This addition of algae was a mistake due to concern over insufficient algae being produced for the rotifers. At the time the researcher did not realize that algal density may have been significantly higher than suggested by the overall appearance of the cultures. This addition caused increases in the fluorometer reading (Table 2) and rotifer density (Table 3). The apparent "crashing" of the algal chemostats was a major disappointment, since previous work had shown them to be reliable. The crash most likely was due to either bacterial contamination or old nutrient stocks.

It is important to remember that the purpose of using algal chemostats was to provide food for the rotifer trophic level. From the rotifer population growth curves (Figures 6 and 7; Tables 3 and 4) it is apparent that sufficient algae was being supplied to maintain the rotifer population in the chemostats at a stable density. Rotifer density appeared to cycle around 120 females/ml (Table 3), with the egg ratio generally being above 0.400, indicating a healthy population (Table 4). The high value for the rotifer population on day 10 was causes by the addition of more food and rotifers to this replicate on day nine as described in the previous section. These parameters appeared to be the best way to summarize the population. In general the other population parameters

(i.e., # of males, etc.) were not sufficiently high to be useful for statistical purposes.

Also examined in this trial was the density of algae in the rotifer chambers. Algal abundance was expected to be extremely low if the rotifer population growth rate was equal to the dilution rate. Based on Table 5 it was apparent that the algae were consumed by the rotifers as fast as it entered the test chamber. This is the expected situation since food availability (algae) was controlling the rotifer density. Again, the only aberrant values are replicates A and D after the erroneous addition of outside algae. One should also note that a very low algal density was evident in the fish chambers.

The most encouraging section of this trial involved the fathead minnow portion of the trophic test. In examining Table 6 it became apparent that the fish responded well in the test system. Fish survival ranged from 84.4 % in replicate B to 100 % in replicate A with a mean of 92.8 %, which is well within toxicity test guidelines for chronic testing with fish (US EPA 1989, APHA 1992)). Dry weight of the fish after 7 days was encouraging, with a mean weight for all replicates of 0.762 mg, which is higher than typically seen in most standard toxicity tests with this species. In setting up this experiment, a high density of larval fathead minnows were added to each test chamber, to explore the limits of the system. The larval minnows exceeded expectations for growth considering their high density in the test chambers.

There were some differences between replicates in overall growth after 7 days (Table 6). The fish in Chamber A had the greatest mean weight of 0.827 mg with no mortality. Chamber B, had the lowest mean weight (0.701 mg) and was significantly different in growth based on ANOVA followed by Tukeys multiple comparison test at the $\alpha = 0.05$ level. Chamber B also had the highest mortality. Why the health of fish in Chamber B was relatively less then the other replicates is unknown. There is no evidence from the rotifer density data (Table 3) or the chemical/physical parameters (Tables 7 and 8) to explain why this replicate did not perform as well as the others, the results are adequate.

The high value for replicate A can be explained by examining the rotifer densities (Table 3). Again the high rotifer density on day 10 was caused by the operator interfering with the system. From this mistake, an interesting point emerged. It appeared that when the rotifer density doubled (days 9-10), the food supply to the fathead minnows was limiting growth. Therefore, if more food had been available by increasing rotifer density or decreasing fish densities, high growth rates would have been achieved.

## CONCLUSION

Based on these results, the trophic toxicity test can be achieved using the design described in this research. A number of additional conclusions can be drawn as well. The outflow from the algal chemostats was able to support

healthy rotifer populations. There were some disappointing problems with maintaining the proper algal density, but they were not inherent to the system design. The algal chemostats also demonstrated the ability to respond rapidly to a toxicant and suggested that the algal populations can recover and adapt to a sublethal chemical exposure.

Fluorometer measurements are not the best measure of algal population density, although they are useful for making comparisons between treatments on a daily basis. The use of direct biomass measurement is recommended as a more definitive measure of the algal population.

Rotifer populations were similar between replicates and the populations maintained themselves at stable, though somewhat cyclic levels (Figures 6 and 7; Tables 3 and 4). The only exception occurred when the system was not allowed to be operated as designed and additional algae was added to one of the chambers.

The rotifer population was sufficiently dense and nutritionally adequate to support the density of larval fathead minnows. The weight of individual fathead minnows was relatively high (averaging overall 0.762 ± 0.057 mg and ranging from 0.377 to 1.346 mg), indicating an adequate food source.

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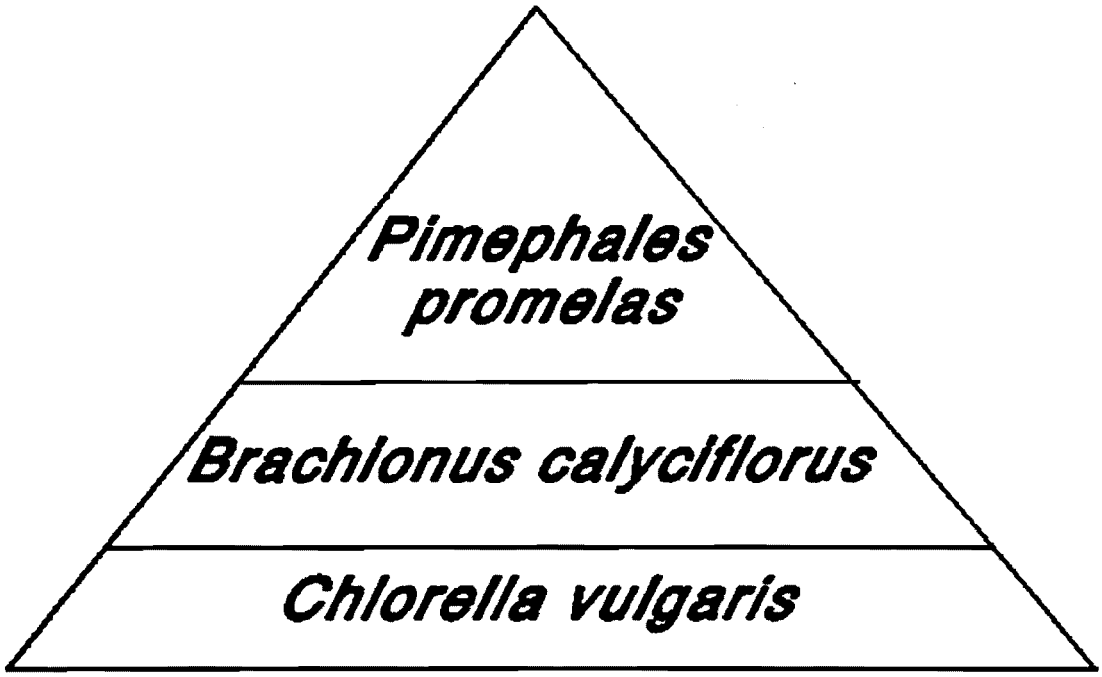


Figure 1: Three-level food chain used in "Trophic Toxicity Test"

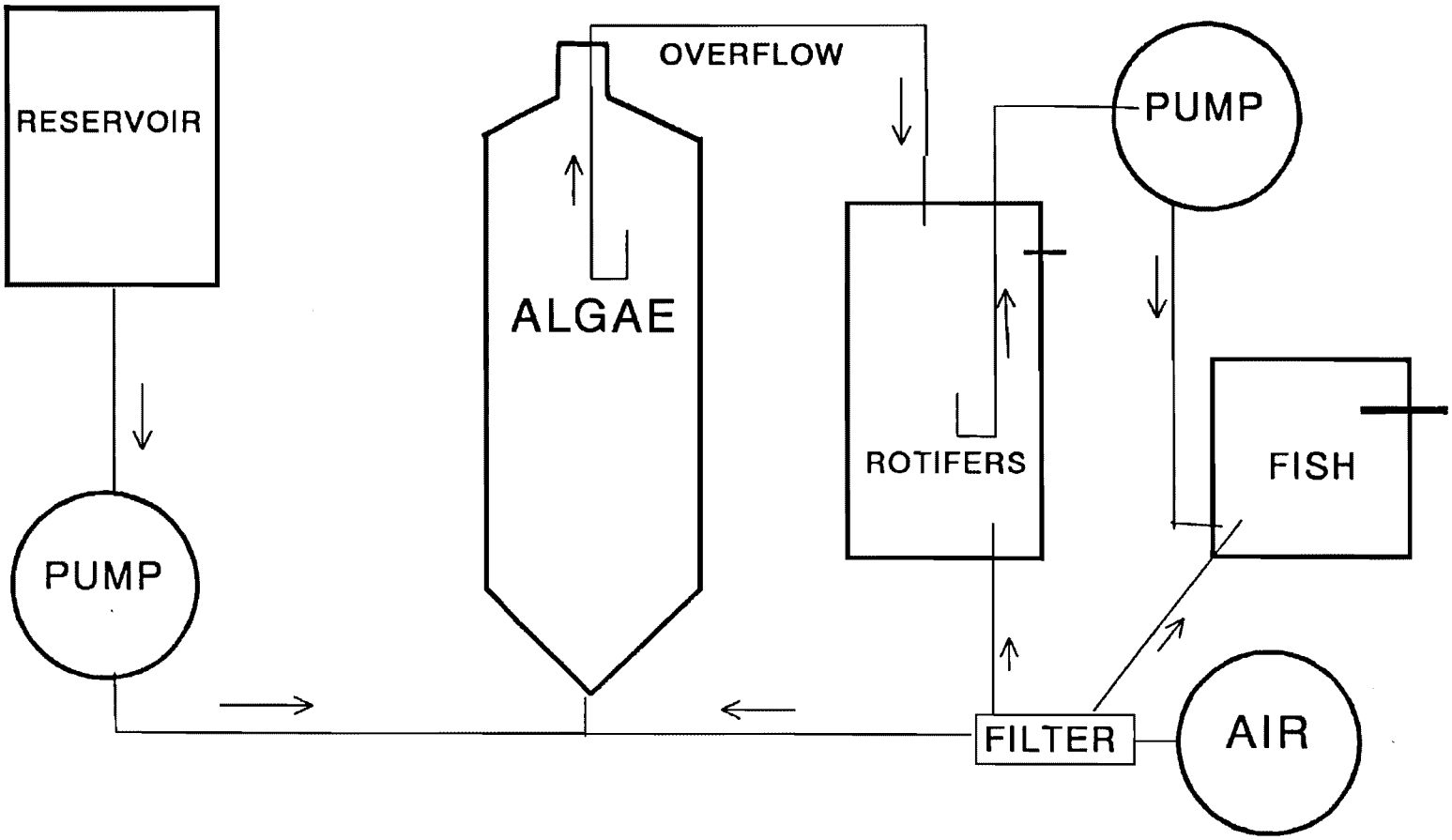


**Figure 2: Diagram of Trophic Test System**

Table 1. Rotifer Scale Experiment

| FLASK # | FLASK VOLUME (ml) | TARGET DENSITY | ROTIFER DENSITY (# FEMALES/MI) DAY 0 | 2 | 3 | 5 | 7 | MEAN | SD |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.2 | 50 | 51.0 | 80 | 174 | 121 | 108 | 121 | 39 |
| 2 | 1.0 | 25 | 25.5 | 48 | 150 | 127 | 117 | 111 | 44 |
| 3 | 1.0 | 50 | 51.0 | 72 | 174 | 116 | 97 | 115 | 43 |
| 4 | 1.0 | 100 | 102.0 | 123 | 162 | 118 | 113 | 129 | 22 |
| 5 | 1.0 | 200 | 204.0 | 191 | 165 | 104 | 89 | 137 | 49 |
| 6 | 2.0 | 25 | 25.5 | 54 | 132 | 113 | 122 | 105 | 35 |
| 7 | 2.0 | 50 | 51.0 | 83 | 162 | 131 | 101 | 119 | 35 |
| MEAN | - | - | 72.9 | 93.0 | 159.9 | 118.6 | 106.7 | | |
| SD | - | - | 63.2 | 49.6 | 14.8 | 9.0 | 11.7 | | |

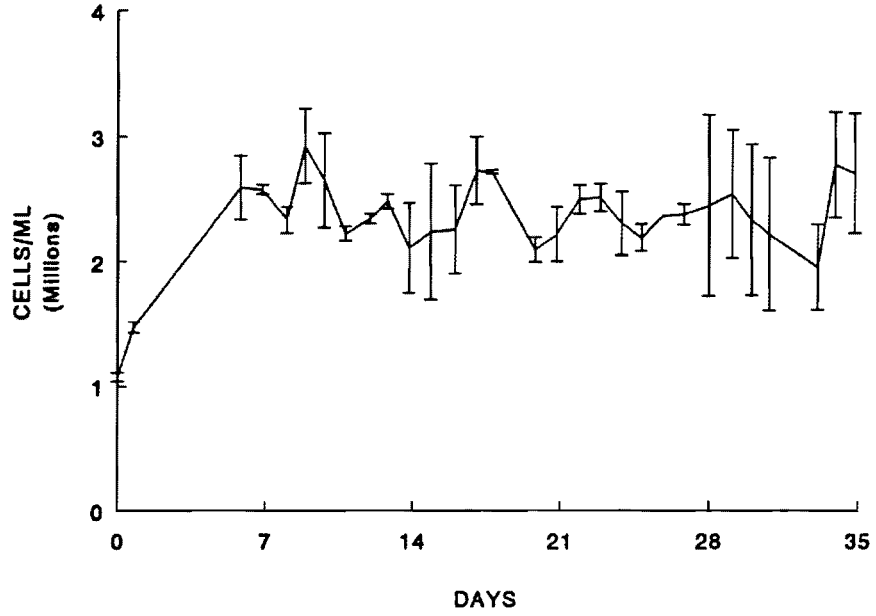


**Figure 3a: LONG-TERM ALGAL CHEMOSTAT GROWTH TRIAL (Mean Cell Density ± 1 SD)**

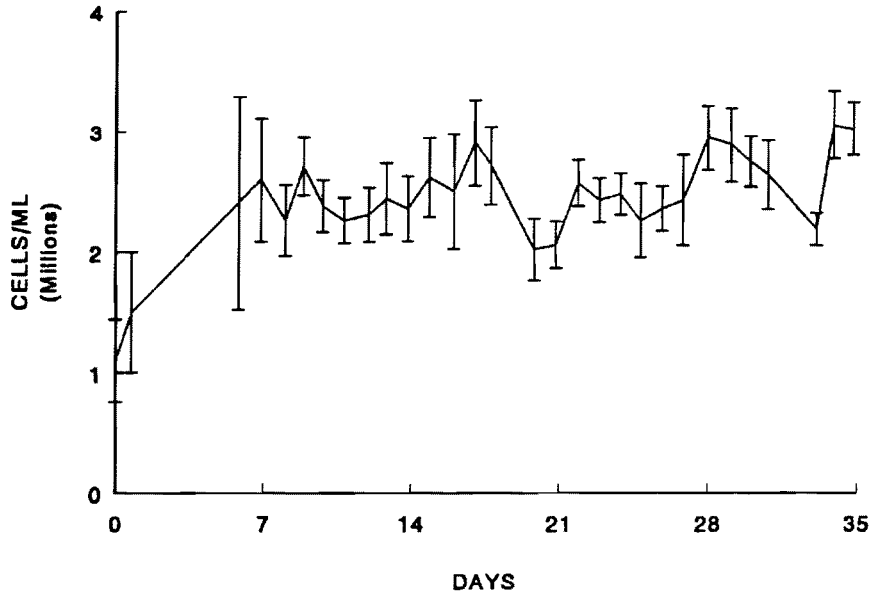


**Figure 3b: LONG-TERM ALGAL CHEMOSTAT GROWTH TRIAL Variation of counting technique (Mean density ± 1 SD)**

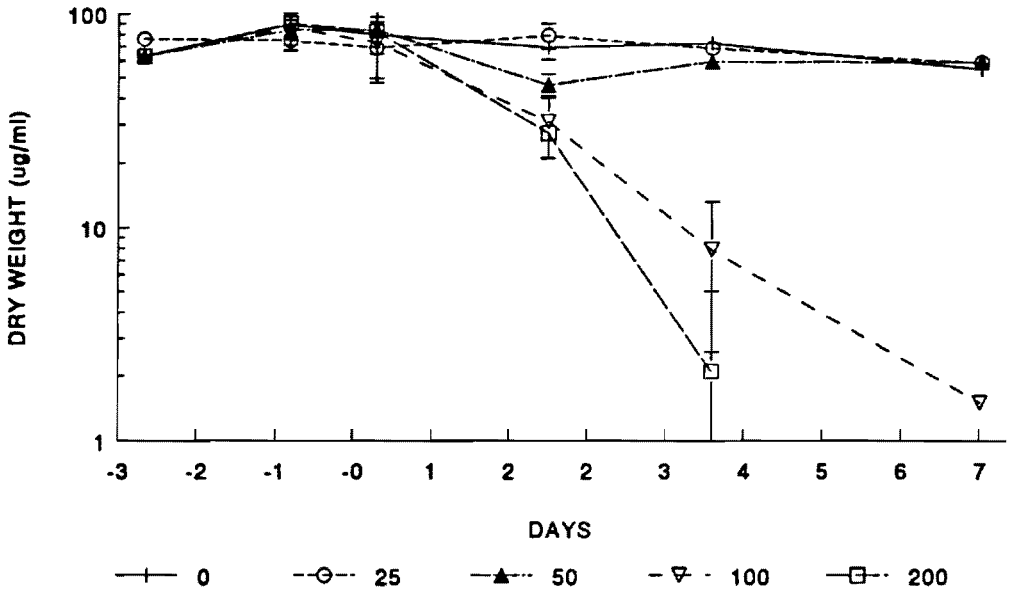


**Figure 4: *Chlorella vulgaris* Flow-Through Toxicity Test**
**(Dosing began on day 0 with the mean ± 1 sd shown)**

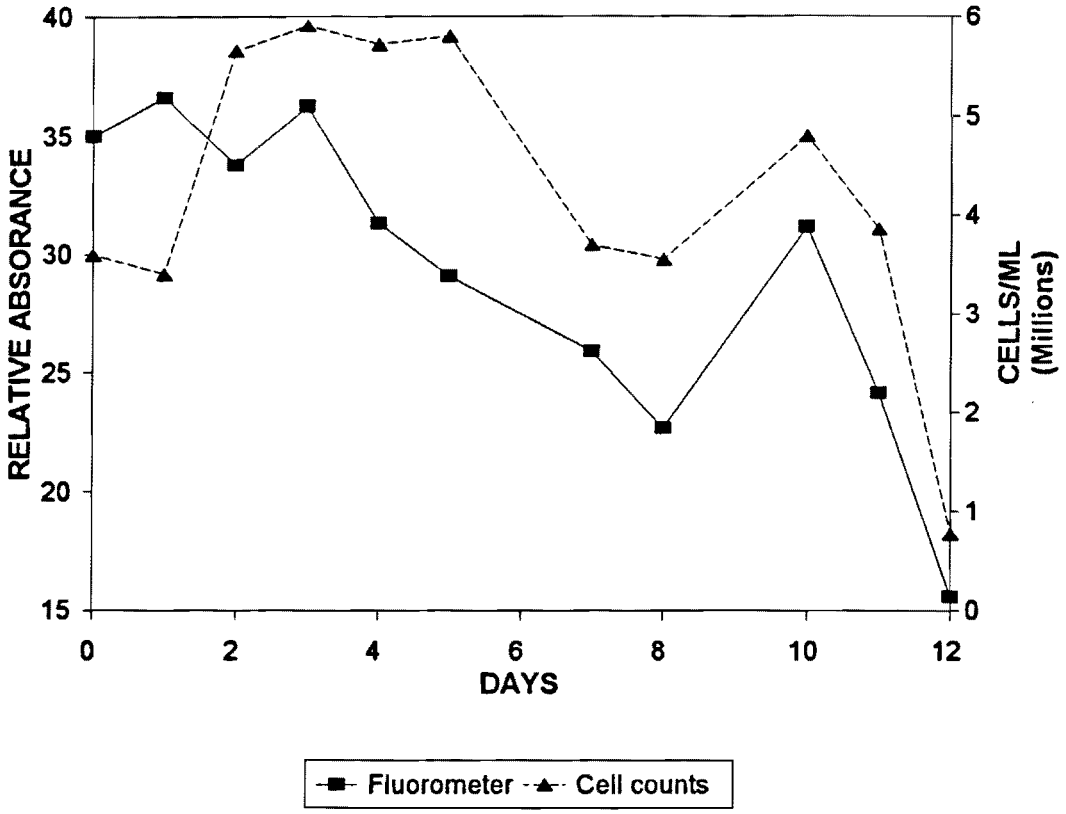


**Figure 5: Trophic Trial - Algal Density**
Relative Absorbance vs. Cell Counts

Table 2. Fluorometer readings from algal chemostats in trophic trial. All values reported as relative absorbance.

| DAY | Replicate A | B | C | D | DAILY MEAN | SD |
|---|---|---|---|---|---|---|
| 0 | 35.0 | 35.0 | 35.0 | 35.0 | 35.0 | 0.0 |
| 1 | 36.5 | 36.5 | 37.1 | 36.3 | 36.6 | 0.3 |
| 2 | 33.0 | 30.5 | 37.5 | 34.1 | 33.8 | 2.9 |
| 3 | 34.5 | 32.2 | 41.8 | 36.5 | 36.3 | 4.1 |
| 4 | 31.5 | 29.0 | 36.3 | 28.5 | 31.3 | 3.6 |
| 5 | 28.8 | 26.7 | 33.2 | 27.6 | 29.1 | 2.9 |
| 7 | 26.0 | 26.9 | 27.8 | 23.0 | 25.9 | 2.1 |
| 8 | 20.3 | 27.0 | 26.5 | 16.9 | 22.7 | 4.9 |
| 10 | 40.8 | 22.8 | 23.0 | 38.1 | 31.2 | 9.6 |
| 11 | 31.5 | 18.4 | 18.3 | 28.5 | 24.2 | 6.8 |
| 12 | 19.0 | 14.6 | 13.8 | 14.9 | 15.6 | 2.3 |
| Summary parameters for days 1 through 12: | | | | | | |
| MEAN | 30.2 | 26.5 | 29.5 | 28.4 | | |
| SD | 6.9 | 6.5 | 9.2 | 8.2 | | |
| Summary parameters for days 5 through 12: | | | | | | |
| MEAN | 27.7 | 22.7 | 23.8 | 24.8 | | |
| SD | 8.0 | 5.2 | 7.0 | 8.5 | | |

Table 3. Rotifer counts for trophic trial. All values reported as # females per ml.

| DAY | Replicate A | B | C | D | MEAN | SD |
|---|---|---|---|---|---|---|
| 0 | 110.6 | 110.6 | 110.6 | 110.6 | 110.6 | 0.0 |
| 1 | 102.6 | 94.6 | 125.3 | 110.6 | 108.3 | 13.1 |
| 3 | 114.6 | 136.0 | 118.0 | 94.0 | 115.2 | 17.2 |
| 5 | 181.3 | 160.0 | 132.0 | 153.3 | 156.7 | 20.3 |
| 7 | 141.3 | 153.3 | 124.0 | 125.3 | 136.0 | 14.0 |
| 10 | 214.0 | 102.6 | 116.0 | 114.0 | 136.7 | 51.9 |
| 12 | 148.0 | 108.0 | 73.3 | 130.7 | 115.0 | 32.3 |
| | Summary parameters for days 1 through 12: | | | | | |
| MEAN | 150.3 | 125.8 | 114.4 | 121.3 | 128.0 | |
| SD | 41.6 | 27.8 | 21.0 | 20.2 | 30.3 | |
| | Summary parameters for days 5 through 12: | | | | | |
| MEAN | 171.2 | 131.0 | 111.3 | 130.8 | 136.1 | |
| SD | 33.5 | 29.9 | 26.2 | 16.5 | 33.2 | |

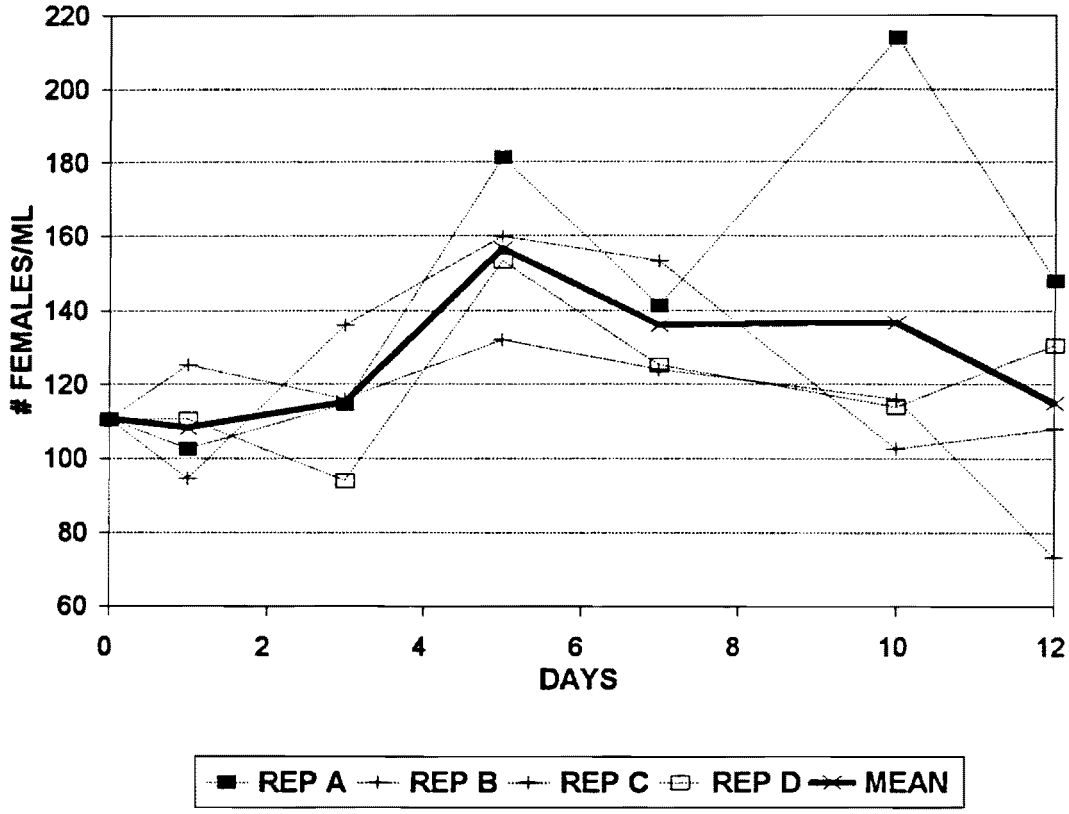


**Figure 6: Trophic Trial**
Rotifer Density (Females/ml)

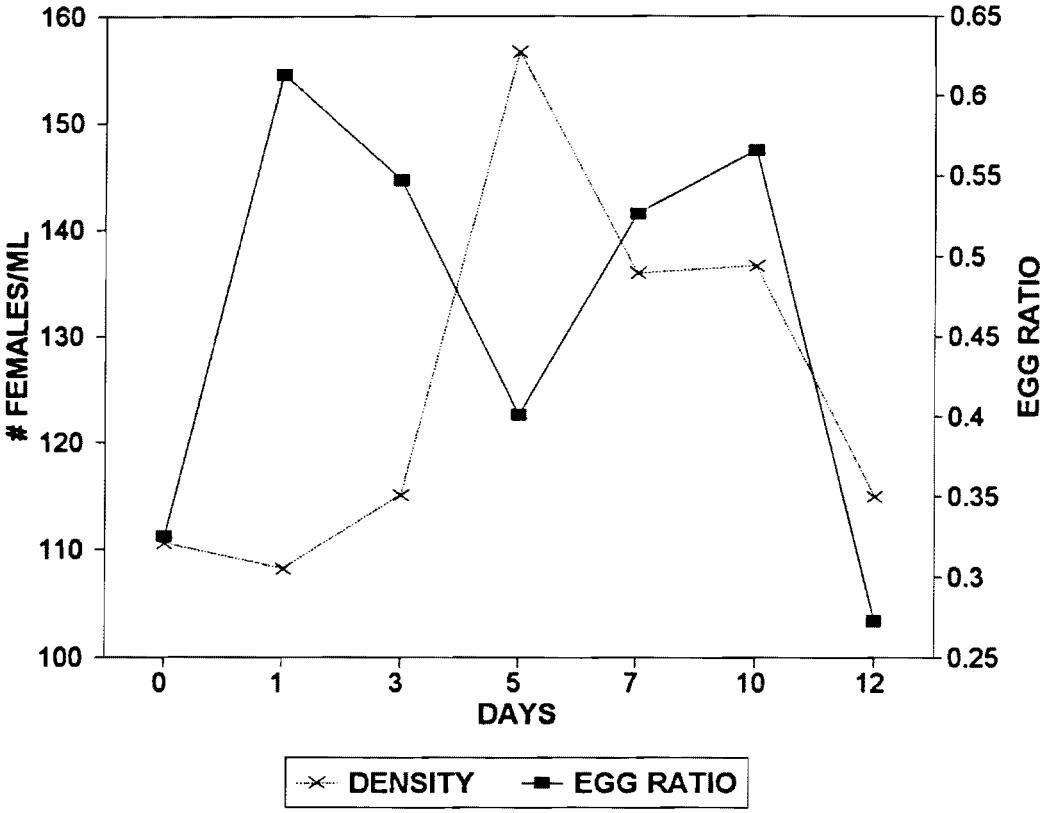


**Figure 7: Trophic Trial**
Rotifer Density (female/ml) and Egg Ratio

Table 4. Rotifer egg ratios for trophic trial. Egg ratio is equal to the # of eggs/# of females.

| DAY | A | B | C | D | DAILY MEAN | DAILY SD |
|---|---|---|---|---|---|---|
| 0 | 0.325 | 0.325 | 0.325 | 0.325 | 0.325 | 0.000 |
| 1 | 0.571 | 0.775 | 0.543 | 0.566 | 0.614 | 0.108 |
| 3 | 0.581 | 0.343 | 0.632 | 0.634 | 0.548 | 0.139 |
| 5 | 0.368 | 0.492 | 0.414 | 0.330 | 0.401 | 0.070 |
| 7 | 0.500 | 0.452 | 0.688 | 0.468 | 0.527 | 0.109 |
| 10 | 0.627 | 0.597 | 0.494 | 0.547 | 0.566 | 0.058 |
| 12 | 0.360 | 0.198 | 0.309 | 0.224 | 0.273 | 0.075 |
| | Summary parameters for days 1 through 12: | | | | | |
| MEAN | 0.501 | 0.476 | 0.513 | 0.462 | 0.488 | |
| SD | 0.114 | 0.200 | 0.140 | 0.156 | 0.147 | |
| | Summary parameters for days 5 through 12: | | | | | |
| MEAN 5-12 | 0.464 | 0.435 | 0.476 | 0.392 | 0.442 | |
| SD 5-12 | 0.126 | 0.169 | 0.160 | 0.144 | 0.139 | |

Table 5. Fluorometer readings from rotifer chambers, pumped outflow from rotifer chambers, and from the fish chambers in trophic trial. All values reported as relative absorbance.

| ID | DAY | Replicate | | | | Daily | |
|---|---|---|---|---|---|---|---|
| | | A | B | C | D | MEAN | SD |
| Rotifer | 1 | 15.8 | 17.4 | 18.3 | 17.1 | 17.15 | 1.03 |
| " | 3 | 13.0 | 8.4 | 10.1 | 12.4 | 10.98 | 2.12 |
| " | 5 | 5.05 | 5.60 | 7.19 | 5.62 | 5.87 | 0.92 |
| " | 7 | 3.15 | 3.60 | 4.45 | 3.75 | 3.74 | 0.54 |
| " | 10 | 21.9 | 3.18 | 3.30 | 32.2 | 15.15 | 14.38 |
| " | 12 | 3.10 | 2.70 | 2.31 | 3.90 | 3.00 | 0.68 |
| Pumped | 7 | 4.53 | 4.80 | 5.55 | 7.81 | 5.67 | 1.49 |
| " | 12 | 2.62 | 2.98 | 2.03 | 4.70 | 3.08 | 1.15 |
| Fish | 7 | 1.61 | 1.16 | 1.57 | 1.89 | 1.56 | 0.30 |
| " | 12 | 1.34 | 1.11 | 1.42 | 2.53 | 1.60 | 0.63 |

Table 6. Summary results of fathead minnow trophic trial after 7 days in the test system.

| Replicate | FISH SURVIVAL | | | FISH DRY WEIGHT | | |
|---|---|---|---|---|---|---|
| | Number Added | Number Surviving | % Survival | MEAN* (mg) | SD (mg) | STDERR (mg) |
| A | 32 | 32 | 100.0 | 0.827[a] | 0.221 | 0.039 |
| B | 32 | 27 | 84.4 | 0.701[b] | 0.176 | 0.034 |
| C | 25 | 24 | 96.0 | 0.790[ab] | 0.131 | 0.027 |
| D | 32 | 29 | 90.6 | 0.728[ab] | 0.140 | 0.026 |
| MEAN | 30.3 | 28 | 92.8 | 0.762 | - | - |
| SD | 3.5 | 3.4 | 6.8 | 0.057 | - | - |

* Values that share the same letter are not statistical different at the $\alpha = 0.05$ level based on ANOVA followed by Tukey's test.

Table 7. Summary of water quality parameters for trophic trial.

| | | TEMP (°C) | D.O. (mg/L) | pH | CONDUCTIVITY (μmhos/cm) | ALKALINITY (mg/L) | HARDNESS (mg/L) |
|---|---|---|---|---|---|---|---|
| RESERVOIR | MEAN | 24.7 | 7.4 | 7.93 | 346 | 68 | 116 |
| | SD | 1.0 | 0.3 | 0.49 | 12 | 7 | 5 |
| | N | 4 | 4 | 4 | 3 | 3 | 3 |
| ALGAL | MEAN | 24.9 | 7.7 | 8.42 | 332 | 117 | 41 |
| | SD | 0.2 | 0.1 | 0.11 | - | - | - |
| | N | 5 | 5 | 5 | 1 | 1 | 1 |
| ROTIFER | MEAN | 24.6 | 7.0 | 8.30 | 340 | 117 | 63 |
| | SD | 0.4 | 0.4 | 0.25 | - | - | - |
| | N | 13 | 13 | 13 | 1 | 1 | 1 |
| FISH | MEAN | 24.0 | 5.5 | 7.60 | - | - | - |
| | SD | 0.6 | 0.5 | 0.11 | - | - | - |
| | N | 16 | 16 | 16 | - | - | - |

Table 8. Flow rates (ml/min) of media to algal chemostats.

| DAY | Replicate | | | | MEAN | SD |
|---|---|---|---|---|---|---|
| | A | B | C | D | | |
| 0 | 0.702 | 0.722 | 0.716 | 0.721 | 0.715 | 0.009 |
| 1 | 0.701 | 0.721 | 0.717 | 0.721 | 0.715 | 0.010 |
| 2 | 0.688 | 0.709 | 0.707 | 0.714 | 0.704 | 0.012 |
| 3 | 0.718 | 0.717 | 0.712 | 0.725 | 0.718 | 0.005 |
| 4 | 0.733 | 0.738 | 0.739 | 0.738 | 0.737 | 0.003 |
| 5 | 0.700 | 0.703 | 0.711 | 0.705 | 0.705 | 0.005 |
| 6 | 0.685 | 0.693 | 0.697 | 0.685 | 0.690 | 0.006 |
| 7 | 0.706 | 0.715 | 0.723 | 0.711 | 0.714 | 0.007 |
| 8 | 0.713 | 0.718 | 0.727 | 0.716 | 0.718 | 0.006 |
| 9 | 0.730 | 0.742 | 0.728 | 0.725 | 0.731 | 0.008 |
| 10 | 0.730 | 0.745 | 0.724 | 0.723 | 0.730 | 0.010 |
| 11 | 0.735 | 0.755 | 0.728 | 0.732 | 0.737 | 0.012 |
| 12 | 0.743 | 0.767 | 0.737 | 0.739 | 0.746 | 0.014 |
| MEAN | 0.714 | 0.726 | 0.720 | 0.719 | - | - |
| SD | 0.019 | 0.021 | 0.012 | 0.014 | - | - |

# CHAPTER 2:

# A COMPARISON BETWEEN THE RESPONSE OF AN ALGA (*CHLORELLA VULGARIS*), ROTIFER (*BRACHIONUS CALYCIFLORUS*), AND FISH (*PIMEPHALES PROMELAS*) TO WATER BORNE AND DIETARY EXPOSURE TO COPPER

## ABSTRACT

The three-trophic level toxicity test is designed to evaluate whether the effect of a toxicant will be transmitted up the food chain, resulting in increased impairment at the higher trophic levels. The three organisms used in the test system are an alga (*Chlorella vulgaris*), rotifer (*Brachionus calyciflorus*), and a fish (*Pimephales promelas)*, each representing a different trophic level. The organisms were exposed in separate chambers that were linked in a continuous, flow-through system with each organism feeding on the trophic level below it. Copper was used as the toxicant to test the system. The three organisms were continually exposed for 7 days to 1.1, 16.2, 31.5, and 62.7 μg/L in creek water (hardness = 100 mg/L) supplemented with nutrients to sustain algal growth. Most of the copper in the test was in an undissolved form with the total to dissolved ratio always < 0.15. Impairment of the algal population was apparent within 1 day of dosing at the 31.5 and 62.7 μg/L levels; however, rapid acclimation of the algae resulted in recovery to near control levels by day 5. Rotifer population response to the toxicant in general paralleled the algal response, at the corresponding exposure, but deviations were of lesser magnitude and were not statistically different from the control. The larval fathead minnows (24 hr old) tested showed no significant reduction in growth after seven days, although there was a statistically significant increase in mortality at the 62.7 μg/L level. The results of the three-trophic level test were compared to single species tests using the same species. The single species

tests were more sensitive to Cu due to the higher levels of dissolved Cu in these tests. The principal route of Cu exposure in the three-trophic level test was through the food, whereas it was through the water in the single species tests. Direct comparison of the endpoints from the single species and trophic level tests is not valid because of the different routes of exposure.

## INTRODUCTION

The central theme of ecotoxicology is evaluation of the potential hazard of anthropogenic substances on the environment. The impact of a substance on an ecosystem is controlled by the interactions between the many abiotic and biotic elements that compose the ecosystem. Because of this complexity, evaluation of a substance's potential hazard to ecosystems is typically conducted by examining a very limited number factors. Evaluation of abiotic factors is essential to understanding a chemical's fate and how biological active it will be. Determining the impact of a chemical on biological systems can only be achieved by exposed test systems to the substance. Historically, the principal tool used to evaluate the biotic response to a substance has been the single species toxicity tests (Buikema et al. 1982, Rand and Petrocelli 1985). Single species toxicity tests focus on the response of the individual components (i.e., species) of ecosystems, but do not address responses at high levels of biological organization 1, 2. It is the interaction of the chemical with the various abiotic and biotic components, that determines its overall impact on the ecosystem. A major drawback of single species tests is that they do not address interactions between the different biological components that make an ecosystem greater then the sum of its parts.

Addressing how a chemical affects the interactions between organisms at different trophic levels is one of the most difficult problems in ecotoxicology. Most research that attempts to address trophic interactions focus primarily on

bioaccumulation and biomagnification of a chemical are just one element of the potential impact. Impacts can be more subtle and indirect, with effects to organisms at lower trophic levels having negative consequences for organisms further up the trophic ladder. This research is designed to investigate the impact of copper on the trophic interactions between species using the "Three-Trophic Level Toxicity Test" (Chapter 1).

Copper was chosen as the initial toxicant to evaluate the three-trophic level toxicity test system for a number of reasons. It bioconcentrates, but is generally toxic before this becomes a factor. Another reason is because is that copper is especially toxic to plant life as compared to animals (USEPA 1985, Nor 1987, Flemming and Trevors 1989). This allowed use of test concentrations which will impact the lower trophic level, but will not overtly wipe out the species at the higher ones. By stressing the lower trophic level, without direct toxicity at the upper levels, the system's potential can be more fully evaluated.

The three-trophic level test couples the alga, *Chlorella. vulgaris*, rotifer, *Brachionus calyciflorus,* and fathead minnow (*Pimephales promelas*), and is designed to examine two characteristics that most single species tests do not address. It is designed to detect impacts due to interactions between trophic levels and population level responses. In this research a series of standardized single species tests were conducted with copper as the toxicant to allow a direct comparison between single species tests and the three-trophic level

toxicity test). By comparing the results of the trophic test with the single species test, it should be possible to elucidate whether the impact is due to direct toxicity or a more subtle trophic effect. Three algal bottle tests, a rotifer population test, and a fathead minnow chronic test were conducted to form a database to compare the results of the three-trophic level toxicity test.

A series of algal bottle tests were conducted with the goal of proving a baseline value to compare the algal portion of the three-trophic level test too. The multiple tests were necessary to account for the different media used and to compare to standard species. Bottle tests were conducted with *C. vulgaris* and *Selenastrum capricornutum* in the same medium as the three-trophic level test, and with *S. capricornutum* in EPA algal media (US EPA 1989).

While methods for testing with algae are well defined (US EPA 1989, APHA 1992), procedures for using rotifers in toxicity testing are not as widely recognized. Some methods have been developed to examine the acute and chronic toxicity to rotifers (Snell and Persoone 1989), but these tests are not population level experiments. Since the three-trophic level test uses population level parameters, it was necessary to develop a population level static renewal rotifer test for this research. The final single species test used for comparison purposes was the 7-day fathead minnow (*Pimephales promelas*) larval survival and growth test (US EPA 1989).

It is hypothesized that the trophic test will be a more sensitive measure of copper toxicity than a series of single species tests because of its ability to detect population and trophic level impacts. The objectives of this study are:

1) Conduct a trophic toxicity test using Cu at concentrations that are directly toxic to the algae, but not the other species to investigate the trophic interactions.

2) Develop a database with Cu using single species tests in the same dilution water to compare with the trophic level toxicity test.

3) Quantify the amount of Cu dissolved and suspended in the water column, and in the test organisms.

Use of one trophic test versus a battery single species tests is more cost efficient. In addition, most single species tests do not operate on the population level or examine any functional components. Because of these advantages, the three-trophic level test may have more predictive capacity in evaluating the potential harm of a chemical, than the use of a series of single species tests.

## MATERIALS & METHODS

*Dilution water*

The water used for most tests was an unpolluted creek water collected from Sinking Creek, Newport, VA and supplemented with nutrients to sustain algal growth. Sinking Creek water was collected within a month of testing.

The water was first filtered through a 1.6 $\mu$m filter to remove suspended solids, and then autoclaved to sterilize it. Appropriate volumes of nutrient stocks based on the Guillard's Woods Hole Marine Biological Laboratory (MBL) algal culture medium (Nicholas 1973) as modified by (Stemberger 1981) were added to the sterilized water after it has cooled and been aerated overnight. Because the normal MBL formulation contains a calculated Cu level of 2.5 $\mu$g/L, the amount of Cu was reduced to 0.4 $\mu$g/L. It should be noted that Sinking Creek level contains no detectable levels of Cu ($<$ 1.0 $\mu$g/L). The nutrient stocks were sterilized by filtration before use, and were stored at 4° C for up to 6 months. This dilution water will be referred to as SC w/MBL throughout the rest of this paper. EPA algal media was prepared in a similar fashion, except sterilized distilled water and EPA nutrients were used (US EPA 1989).

*Algal Bottle Tests*

Procedures used for the algal bottle tests in general followed standard procedures (USEPA 1978, 1989, APHA 1992), except for modifications in the media and species used. Either sterile EPA algal media (without EDTA) or SC w/MBL was used as the dilution water. The test apparatus consisted of 125-ml erlenmeyer flasks capped with foam plugs (VWR Scientific, Altanta GA) and aluminum foil, and sterilized in an autoclave before use. Sterile test solutions were then add to the flasks using axenic technique. Flasks were placed on a rotary shaker at 100 rpm under constant illumination by 20 watt cool white

fluorescent bulbs at 4300 lux ± 400 at 25 ± 1°C. There were 3 replicates for each treatment level, shaken twice a day by hand. Each treatment level's position was randomly rotated on a daily basis. The algal cultures used to inoculate the tests were maintained in the same medium being used for testing for at least two weeks before use. Initial starter cultures were obtained form Carolina Biological Supply, NC. The cultures were 4 to 7 days since last renewal when used to start a test. A portion of the inoculum culture would be spun and washed with fresh media and then diluted to a cell density of 2.55x $10^6$ cells/ml. Inoculum density was determined using a hemocyctomer. One ml of the inoculum stock was then added to each test flask containing 50 mls of test solution, making the starting algal density 5 x $10^4$ cells/ml. Algal growth in the tests was measured daily using a fluorometer to measure relative absorbance and checked at the end of the test by direct cell counts and/or biomass measurements. The amount of total recoverable Cu was determined at the beginning of each test before the addition of the algae. Routine water chemistry measurements consisted of conductivity, D.O., and pH at all treatments levels, and hardness and alkalinity on the control and highest treatment level were made at the beginning of the tests. Initial pH was adjusted to 7.5 in the test media for all tests.

For the *C. vulgaris* tests in SC w/MBL the following nominal Cu concentrations were prepared by serial dilutions using axenic technique: 0, 1.25, 2.5, 5.0, 10.0, and 20 $\mu$g Cu/L. The *S. capricornutum* tests were

conducted in both EPA algal medium and SC w/MBL. The SC w/MBL test using *S. capricornutum* used nominal Cu concentrations of: 0, 15, 30, 60, 120, and 240 $\mu$g Cu/L. The test in EPA media followed closely established protocols (US EPA 1989), with nominal concentrations of 0, 7.5, 15, 30, 60, and 120 $\mu$g Cu/L.

*Rotifer Static Tests*

Test solutions were prepared using algal culture as the dilution water at twice the intended concentration. It is important to recognize that because *B. calyciflorus* is capable of very rapid growth, it is typically cultured in a thick suspension of its algal food supply (in this case *C. vulgaris)*, the food supply acts as the dilution water. Rotifers were suspended in *C. vulgaris* cultures maintained in SC w/MBL at algal densities between 2 and 3 x $10^6$ cells/ml. The rotifer inoculum was prepared by filtering a rotifer culture through a 153 $\mu$m plankton net to remove old algae then suspended in fresh algal culture at twice the density (i.e., 4 x $10^6$ cells/ml) intended to start the test. The test was begun by mixing equal volumes of the rotifer inoculum and the test solutions and then distributing the test solutions among the replicates. Test chambers consisted of 250 ml glass stock bottles filled with 200 ml of log phase rotifer culture and aerated vigorously. Environmental conditions were: temp = 25 $\pm$ 1°C; 16/8 light cycle at 500 to 1000 lux. To renew the test, chambers were siphoned to 80 % of original volume using a tube covered with 153$\mu$m Nitex

screening and then filled with algal suspension (2 to 3 x $10^6$ cells/ml) dosed with the appropriate levels of toxicant. On non-renewal days, algae was centrifuged and concentrated so that 1 ml of the suspension added to the 200 ml test volume produced an algal density in the test chambers of 2 to 3 x $10^6$ cells/ml. Counts of the number of rotifers and eggs were performed on preserved sample on days 0-4, and 7 using a Sedgewick-Rafter counting cell. The set up is designed so that the food supply limits population growth.

A 7-day non-renewal screening test was used to determine the optimal starting concentrations for the definitive population test. Nominal test concentrations were 1000, 500, 250, 125, 62.5, and 31.25 $\mu$g/L Cu, with only one test chamber per treatment level. Starting rotifer density was 41 females/ml in a volume of 200 ml. The definitive test consisted of three replicates at 6 treatment levels (80, 40, 20, 10, and 0 $\mu$g Cu/L) and lasted for 7-days. It was a static renewal test, with renewals occurring on days 3 and 5. The starting density for this test was 30 females/ml. Water samples for metal analysis were taken each time new test solution was used and directly before each renewal. Samples were filtered through a 153 $\mu$m Nitex screen, followed by a 0.45 $\mu$m membrane filter. The suspended and dissolved fractions were analyzed separately and combined to calculate the total recoverable copper levels.

*Fathead Minnow Static Renewal*

Procedures used in this test closely followed standard procedures (US EPA 1989). Fathead minnows (*P. promelas*) were exposed to Cu at the following nominal concentrations of 0, 10,20, 40, 80, 160 µg/L in 300-ml borosilicate glass culture dishes with 250 mls of test solution. Test solution temperature was maintained at 25 ± 1 °C, and light intensity between 500-1000 lux and a photoperiod of 16/8 light/dark. Test solution were prepared daily. The test was initiated with larval fathead minnows less than 24 h old by randomly assigning them to the test chambers until 10 fish have been allocated to each of the 4 replicate chambers per treatment. The fish were fed 0.1 ml of a concentrated suspension of newly hatched brine shrimp, *Artemis,* nauplii (approximately 700 to 1000 shrimp) three times per day at 4 h intervals. Brine shrimp were rinsed with fresh water prior to use. Fish were not fed during the last 12 hrs of the test.

At the time of daily renewal of test solutions, test chambers were cleaned of debris and dead fish by siphoning out approximately 80 to 90% of the test solution, and then adding fresh test solution. The sides of the test chamber were wiped with a rubber spatula and debris removed prior to adding new test solution. Water quality measurements of temperature, pH, and dissolved oxygen were performed daily on end of day samples and the dilution water (filtered Sinking Creek water). Conductivity, hardness, and alkalinity were measured on the dilution water on days 1, 4, and 7 in control and highest

treatment. An extensive metal sampling schedule was used to track the relative amounts of total and dissolved Cu during the test. Total and dissolved Cu were measured at the beginning and end of days 1, 5, and 7 for all treatment levels.

The test was terminated after seven days of exposure. At test termination, the surviving fish were counted, rinsed with clean water and killed with ethanol and transferred to a weigh boat. The fish were dried at 60° C for 24 hours, placed in a desiccator to cool and then each chamber was weighed as one group of 10 fish.

*Three-Trophic Level Toxicity Test*

Development of the three-trophic level test system has been previously described in detail (Chapter 1). It consists of the following three species linked together in a continuous flow-through system: *Chlorella vulgaris*, *Brachionus calyciflorus*, and the fathead minnow (*Pimephales promelas*). The system consisted of four different levels, linked together in series (Figure 1). The top level consisted of the four 25-L polypropylene carboys, one for each treatment level, that serve as the test solution reservoirs. The test solution was aerated vigorously to promote mixing using air that passes through a 0.2 $\mu$m filter. The carboys were plugged with cotton bungs and wrapped in aluminum foil to prevent light from causing any changes in the test solution. The whole apparatus was sterilized before use. An eight channel peristaltic pump was

used to continuously delivered test solution to the next level, which was composed of eight algal chemostats, two for each treatment.

The algal chemostat portion of the system used custom made glass cylinders that taper to a point at the bottom (Figure 1). The cylinders were design to hold at least 2-L and were approximately 10 cm in diameter and 40 cm long; the bottom 10 cm forms a cone. There was a inlet at the bottom where fresh media and air enters the chamber, and an overflow at the top of the chamber. The algal population was sampled using a sterile syringe through a sample port sealed with a septum.

Media inflow was approximately 0.7 ml/min and was delivered from the reservoirs using a peristaltic pump. Approximately a liter of fresh test solution entered the algal chemostats daily, with an equal volume of the algal culture being forced through the overflow by air pressure to the next trophic level (rotifer chemostats). This resulted in a turnover rate for the algal chemostats of about once every 2 days, and was designed to maintain an algal population in late log phase with a density between 3 and 6 x $10^6$ cells/ml. The whole system, including the silicone outflow tubing to rotifer chemostats, was sterilized before use. Inoculation of the system was achieved by batch culturing 16 L in a large carboy until late log phase of growth ( ~ 7 days) and then transferring the algae directly to the test vessels. Illumination was provide by 20 watt cool white fluorescent lights providing 4300 ± 400 lux at the algal chemostat. The bottom third of the chemostats sat in a water bath to maintain

temperature at 25 ± 0.2°C. Toxicant was directly added to the algal chemostats at the beginning of the test.

The rotifer chemostats were similar to the algal chemostats in design, but were made from 1-L polypropylene sample bottles with holes drilled in them to accommodate glass tubes for aeration, media in, media out, and an overflow (Figure 1). The chambers were wrapped in aluminum foil because the rotifers prefer a dark environment. *C. vulgaris* from the first level was continually forced into the rotifer chamber by air pressure from the algal chemostat. The overflow was positioned to maintain an volume of 950-ml in each chamber, with the spill over dripping into the fish containers.

Unlike the algal chemostats, the overflow is not the principal exit port for the rotifer chemostats. Because rotifers are mobile animals, they have the potential to avoid a slow flowing overflow. To overcome this problem, the rotifers were pumped out at a high flow rate on a periodic basis using a peristaltic pumped controlled by a timer. Outflow parameters were: pump on = 1 minute out of every 30; flow rate while on = 18 ml/min. The pumped out enters into the bottom of the fish chambers. The turnover rate was approximately once per day for the rotifer chemostats. The chambers were sampled with a syringe by removing the cap.

The larval fathead minnows were held in 350-ml polystyrene chambers with an inlet consisting of the outflow form the rotifer chemostats entering at the bottom. The overflow is a whole cut into the side of the chamber, screened

with 120 $\mu$m Nitex® to keep the organisms in. The chambers were cleaned daily to remove any waste. Aeration was provide at 100 bubbles/min via narrow bore glass tubes. The larval fatheads used were 15 ± 9 hours of age, with ~23 placed in each chamber.

Nominal concentrations for this test were 0, 17.75, 35.0, and 70.0 $\mu$g/L of copper. The algal-rotifer chemostat system was set up 3 days before the addition of the copper to allow the system to stabilize. On day 0, the algal and rotifer chemostats were directly spiked with appropriate volume of concentrated stock to bring them to desired Cu concentrations. The larval fathead minnows were then added to their test chambers that had been filled with the outflow from the rotifers.

*Statistical Analysis*

In the trophic test Analysis Of Variance (ANOVA) was used at each trophic level to make comparisons between the control and treatments on specific days. For the majority of the tests presented here, statistical analyses was confined to analysis on a daily basis for a response parameter. In the majority of tests, the response parameter cycles on a daily basis due to the test system design and the biology of the organisms. Therefore combining daily values produces a misleading value for the variance. Statistical analysis of the single species tests was perform based on the procedures outlined in US EPA 1989. The data was generally analyzed using ANOVA followed by Dunnett's

test. Either TOXSTAT® (Gulley 1993) or SAS® (SAS Institute Inc. 1989) computer programs were used to perform the statistical computations.

*Metal Analysis*

One of the questions the multi-trophic level test is designed to evaluate is how Cu is transferred up the food chain. This necessitated sampling each trophic level to determine the amount of Cu in the test organisms and the water column. In addition by measuring dissolved Cu in selected components of the system an estimate of the fraction of the total Cu that was bioavailable can be determined.

For all toxicity tests conducted total recoverable Cu was determined in the reservoirs and test solutions before addition of test organisms to base exposure values on and to use in the statistical calculations. In addition, total recoverable Cu, dissolved Cu, and/or the amount of Cu in the test organism was determined in some tests. All water samples (50 ml) were preserved by acidifying with 150 $\mu$l of 50% $HNO_3$ (0.5 % $HNO_3$). Samples for total recoverable Cu determination were preserved immediately upon collection, whereas samples for dissolved Cu analysis were persevered after filtration through a 0.45 $\mu$m membrane filter. Preserved samples were digested with concentrated $HNO_3$ before analysis as described in EPA method 220.2 (Kopp and McKee 1983, USEPA 1991). Biological samples were vigorously digested to determine the total amount of Cu in accordance with EPA method 220.2

(Kopp and McKee 1983). Algae were sampled by filtering through a 0.45 μm membrane filter. A Perkin-Elmer 1100 Atomic Adsorption Spectrometer equipped with a HGA-300 graphite furnace was used to measure Cu levels after sample digestion in accordance with EPA method 220.2). The detection limit for all matrixes was 1 μg/L Cu. Ten percent of the samples analyzed were quality control spikes and blanks for all matrixes used. Final values were corrected for matrix effects if necessary.

## RESULTS and DISCUSSION

*Algal Bottle Tests*

The endpoints used to summarize the algal response were the 4-day LOEC and NOEC for growth based on the measured total recoverable Cu concentrations from the beginning of the test (Tables 1 and 2). Of the three tests conducted, *C. vulgaris* in SC w/MBL was the most sensitive test with an LOEC of 12.2 μg/L and NOEC of 4.6 (Table 2). This result was unexpected when compared to the results of the test in EPA media with *S. capricornutum*, because of the extreme differences in hardness (Table 2). Hardness of the SC w/MBL was 100 mg/L, while the EPA medium was very soft with a value of 16 mg/L. In SC w/MBL the LOEC for *S. capricornutum* was 31.0 μg/L with a NOEC of 15.0 whereas the LOEC for the EPA media test was 55.2 μg/L with a corresponding NOEC of 27.8 μg/L.

The LOEC of 55.2 μg/L for *S. capricornutum* in EPA media, Table 2, closely agreed with the value of 50 and 85 μg/L reported in the literature (USEPA 1985). The value 12.2 μg/L reported here for *C. vulgaris* appears to be relatively low, since *C. vulgaris* LOEC values ranging from 62 to 200 μg/L have been reported (USEPA 1985). For the genus *Chlorella* in general, a board range of values, from 1 to 550 μg/L, have been reported by various researchers (USEPA 1985). In evaluating the differnces in algal toxicity data, it is important to note the important effect that test conditions, such as pH and media composition, can have on the results. This research suggests that algae exposed to Cu in the SC w/MBL media were more sensitive than when other, more standard test media were used.

*Rotifer Static Renewal*

Results (Table 3a and 3b) of the rotifer screening test suggest that copper was toxic to *Brachionus calyciflorus* at the nominal concentration of 125 μg Cu/L. At this level all individuals had died by day 2. At the next lowest concentration (62.5 μg/L), rotifer density appeared to be reduced. Since only one replicate was used, it was impossible to make daily statistical comparisons between treatments. ANOVA between treatments using each day as a data point failed to detect any significant differences at $\alpha = 0.05$. Since populations densities tend to cycle around a mean level, analysis across days resulted in a misleading increase in the variability. However, the mean rotifer density

(Table 3a) over the course of the experiment decreased with increasing concentration suggesting a dose dependent response. The 7-day mean density was 82.8 ± 21.8 for the control, and decreased to 64.8 ± 19.2 at the 62.5 µg Cu/L level. On day 1 population densities increased at levels below 62.5 µg/L, but decreased at this level. No impact was observed on the egg ratio (Table 3b) for those treatments that maintained population densities throughout the test. One other factor to consider is that since no renewals occurred during this test, Cu levels probably decreased to significantly below nominal as the test progressed, reducing toxicity.

Exposure levels for the definitive test were 5.6, 16.8, 23.5. 43.5, and 62.7 µg/L as estimated by adding the measured amount of Cu in the suspended and dissolved fractions at test renewals to determine total recoverable Cu concentrations (Table 4). These values may underestimate the total amount of Cu because the fraction of Cu associated with the rotifers was not included in the suspended fraction. Samples were taken to determine the quantity of Cu in the rotifers, but were contaminated in sample processing. Because these were begin of day samples taken immediately after adding fresh toxicant solution that was composed of an algal suspension in SC w/MBL, the amount of Cu associated with the rotifers was probably low. Also note that dissolved Cu levels dropped drastically as the test progressed (Table 4). The dissolved/total Cu ratio appears to have been around 0.20 for all treatments for

most of the test (Table 4). This low ratio was most likely due the high level of suspend material (i.e., algae and rotifers) in the test chambers.

In the definitive test, the *B. calyciflorus* population density was impaired at the 23.5, 43.5, and 62.7 µg/L levels on day 1, and at the 43.5 and 62.7 µg/L levels on day 7 (Table 5a, Figure 2). No impairment was evident at 16.8 µg/L. Other researchers have found rotifer impairment by Cu at 30 µg/L, with a NOEC of 20 µg/L (Snell and Moffat 1992). No statistically significant differences were observed for the population egg ratio (Table 5b). However, egg ratios were elevated on day 1 at the 62.7 µg/L level due to the extremely low population density (Table 5a) and not enhanced reproduction. With an average population density on day 1 for the 62.7 µg/L of 1.1 females/ml, and a correspondingly high egg ratio of 1.29, the rotifer population appears to be surviving the Cu insult mainly as eggs. In fact, one replicate had no females or amitic eggs present, only resting eggs. As dissolved Cu levels (Table 4) decreased during the test, the eggs hatched and the population rebounded (Figure 2).

The lack of any statistical significant differences on the intermediate days (i.e., days 3 and 4) was initially surprising. One possible explanation for the lack of statistical significance on days 3 and 4 was that the rotifer populations at the lower treatment levels were being limited by the food supply throughout the test, while at the higher treatments levels there was excess food early in test due to the decline in the rotifer population caused by the initial exposure

to the Cu. This theory assumes that the rotifer population have the ability to adapt to the Cu and recover from the initial exposure. Recovery of the rotifer population may be due to lower dissolved Cu levels as the test progressed (Table 4), or an actually selection of clonal lines that were resistant to Cu. By day 7, the rotifer density at all treatment levels was being limited equally by the food supply with the growth rate of rotifers at the higher levels slower when food was equally available. According to this theory the rotifers at the higher Cu levels grew slower then the controls when exposed to an equal food supply. An alternative explanation is that the initial population crash caused by the Cu was causing wide population swings.

This test began with very low population density at all treatment levels (Table 5a), and populations declined (Figure 3) initially due to the low egg ratio of the inoculum culture (Table 5b). The egg ratios were relative low for the second half of the test, reflecting that the algal supply was limiting population growth in all treatments (Table 5b). Overall, the data suggests that when food was equally limiting for all treatments, that the rotifers exposed to 43.5 and 62.7 $\mu$g/L Cu have a slower growth rate. It also suggests that a viable, although slower growing rotifer population can be maintained at these Cu levels. The slower growth rate may be due to a reduction in the filtration rate of the rotifers. In one study (Ferrando et al. 1993), Cu was found to reduce the filtration of the rotifer *Brachionus calyciflorus* by 50 % when exposed to 32

µg/L Cu for 5 hours. The relatively low levels of dissolved Cu may be why the values reported here were great than other researchers (Snell and Moffat 1992).

A number of interesting questions are raised from the results of this test. With dissolved levels of Cu being extremely low through most of the test, was the resulting toxicity due to the initial exposure period when dissolved copper levels were around 65%, or was Cu have a negative impact through the food? It is likely that while the initial Cu exposure did cause an acute response on day 1, but the long term toxicity in probably due to dietary exposure. If the suspended Cu was associated with the algae then ingestion would expose the rotifers to significant levels of Cu in the gut. Since digestion in rotifers is generally extracellular, the Cu associated with the algae may be solubilized in the gut increasing toxicity 3.

*Fathead Minnow Static Renewal*

Fish survival was impaired at the two highest test concentrations, while growth was impaired at the three highest levels (Table 6). There was a clear dose response relationship in the weight data, with all treatments less then the control. The LOEC for this test was 39.2 µg/L based on growth, with a corresponding NOEC of 23.0 µg/L, and CV of 30.0 µg/L expressed as total recoverable Cu. The USEPA reports a CV of 18.53 µg/L for an early life stage test and CVs ranging 13.97 to 27.71 µg/L for full life cycle tests (USEPA 1985). While the value derived in this research is slightly higher then the

literature values (USEPA 1985), it is comparable to what other researchers have found.

Results of copper analysis (Tables 7, 8a, and 8b ) found total recoverable values to be close to the nominal levels in contrast to the rotifer static renewal test. There was not a large difference between the beginning and ending total recoverable copper levels (Tables 8a and 8b) indicating that copper levels were maintained in the water column. There was, however, a drop in dissolved Cu levels over the course of a day (Tables 8a and 8b). The ratio of dissolved to total levels was generally above 0.90 (except for the highest level) for begin of day samples, and ranged from 0.72 to 0.83 for the end of day samples (Table 8a and 8b). Overall the proportion of dissolved Cu in solution stayed relatively high, averaging a ratio of 0.82 for the test as a whole. These data indicate that fathead minnows were exposed to a high proportion of dissolved Cu throughout the test. Dissolved Cu is generally believed to be most biologically active from, and is typically responsible for the majority of the toxicity (Harrison 1985, O'Donnel et al. 1985, Nor 1987, USEPA 1992). In this test the principal route of exposure was from the water column.

In addition to tracking survival and growth over the 7-days, the amount of copper concentrated in the whole bodies of the fish was analyzed (Table 9). There was a dose related response with more Cu being found at the higher treatment levels (Table 9). Bioconcentration factors (BCF) were highly variable and ranged from 1680 at the 13.2 $\mu$g/L level to 640 at 151.2 $\mu$g/L level (Table

9). The high value for the control indicates that the minnows contain a certain base level of copper in their tissues. The general decrease in BCFs with increasing Cu level is probably because 7-days is too short a time period for these fish to reach steady-state. Overall, the BCF appears to be around 1100 for this short term exposure, which is larger than the BCF of 290 after 30 days cited by the USEPA, 1985.

*Three-Trophic Level Toxicity Test*

The quantity of Cu was estimated in a number of different components of the three-trophic level test system (Tables 10-14). Total recoverable Cu was measured in the reservoirs, algal and rotifer chemostats, and fish chambers on 5 days during the test (Tables 10 and 11). In addition, dissolved Cu levels were measured in the algal chemostats (Table 12). The amount of Cu in the algae and fish was also measured (Tables 13 and 14), although not in the rotifers due to sample contamination. The concentration of total recoverable Cu in the reservoirs (i.e., 1.1, 16.2, 31.5, 62.7 $\mu$g/L) was used as the treatment levels for the three-trophic level toxicity test. The majority of the Cu in the reservoirs is most likely in the dissolved form, because the test media is filtered and therefor contains low quantities of particle matter.

The total recoverable Cu levels should have been the same in all parts of the test system, but were highest in the reservoirs and in general lower in the test chambers (Tables 10 and 11). Of the three trophic levels, the rotifer

portion had lower Cu levels then the algae and fish, although there was considerable variability in the data (Table 11). In general the amount of Cu in the test chambers decreased slightly with time. Dissolved Cu levels were extremely low in algal chemostats throughout the test (Table 12). This was most likely due to binding of the Cu to the algal cells. Since the algal suspension is pumped to the other trophic levels, it is likely that dissolved Cu levels were low at all trophic levels.

Of the three species exposed in the trophic test, the alga *C. vulgaris* was the most sensitive. In the *C. vulgaris* stage of the system there was an initial rapid decline in algal density at the higher treatment levels followed by recovery of the algal population to control levels (Figure 3 and Tables 15, 16). Algal density at the 62.7 µg/L Cu level was significantly below control densities for days 1 through 4. By day 7 the algal density at the 62.7 µg/L level was close to control densities. The algae at the 31.5 µg/L level were also initially impacted on day 1. The LOEC for this portion of the trophic test was 31.5 µg/L, with a corresponding NOEC of 16.2 µg/L based on fluorometer measurements of relative absorbance (Table 15). However, after this initial negative effect, growth was enhanced above control densities at the 31.5 µg/L level (Figure 3). Dry weight was also measured on selected days, but was a less sensitive (Table 16) indicating impairment on day 1 of the 62.7 µg/L treatment and enhancement of growth on day 7 at 31.5 µg/L. This algal recovery may be due to the extremely low dissolved Cu levels in the algal

chemostats, with the initial negative effect being caused by the direct addition of Cu to the chemostats on day 0 (Table 12). An alternative hypothesis is that the algal population is adapting to the Cu stress by selecting for resistant cell lines or compensating by making physiological changes. In support of this hypothesis is the fact while dissolved Cu levels were low, most of the Cu not in solution is bound to the algal cells. For Cu to effect the algal cells it seems reasonable that the first step would be binding to the cell surface.

Although Cu is know to enhance algal growth a low levels ($< 4$ $\mu$g/L), 31.5 $\mu$g/L is much higher then one would expect enhancement. In addition, if enhancement was occurring, it should also be evident at the 16.2 $\mu$g/L which shows no signs of growth enhancement (Figure 3). One dose related explanation for growth enhancement at the 31.5 $\mu$g/L level is that the initial die off of algal cells caused by the copper increased nutrient levels in these chemostats, stimulating growth of the remaining cells. However this does not explain why the higher algal densities were maintained until the end of the test. Other possibilities include differential bacterial contamination of the reservoirs, or differences in nutrient levels in the reservoirs.

Accumulation of Cu associated with algae was dose dependent with more Cu in the algae as the treatment level increased (Table 13a). Surprisingly Cu levels in the algae decreased as the test progressed. Bioconcentration factors were highly variable and appear to be around 11,000. The USEPA reports a value of 2000 for *C. regularis* exposed for 20 hours (USEPA 1985).

The differences may be because samples were not washed when filtering, so all associated Cu is included in the BCF. Because the focus of this research is on trophic transfer of the chemical, it is appropriate to included all Cu that is associated with a cell.

None of the fluctuations in the *Brachionus calyciflorus* populations at the second trophic level were significantly different from the control (Figures 4a, 4b, and Table 17). Rotifer population levels in general paralleled fluctuations in algal density, with the greatest declines at the highest levels. The rotifers appear to be responding directly to the food level available, although the response is delayed slightly and dampened. The apparent lack of direct toxicity is probably related to the low levels of dissolved Cu in the test. This is the type of effect the trophic test is designed to account for, but with the low levels of dissolved Cu, the effect of the food only exposure was not large enough to be significantly different from the control. The trophic interaction between the rotifers and algae illustrates the ability of a population at a higher trophic level to "weather" a stress on the lower level. Obviously, the ability of the algae to recover from the initial chemical insult was crucial in maintaining the integrity of the food chain. While the algal population recovery at the impacted concentrations may be due to reductions in the dissolved Cu levels (Table 12), the validity of these finding are no less valuable.

At the top trophic level fathead minnows survival was impaired at the highest exposure level of 62.7 $\mu$g/L (Table 18). No adverse impacts on growth

was observed, but at 31.5 $\mu$g/L Cu level the fathead minnows were significantly heavier then the control (Table 18). This is most likely due to increased food availability due to enhancement of algal and rotifer populations at this treatment level. The lack of an impact on growth can be attributed to the low dissolved levels of Cu and because the rotifer chemostats maintained a sufficient outflow of rotifers at all treatment levels throughout the test. The reduction in survival at the 62.7 $\mu$g/L level may be due to the reduced quality of the Cu tainted rotifers. The dissolved Cu levels (Table 12) were below where any survival impacts would be expected to occur. If an individual fish could tolerate the Cu associated with its food, then growth was normal. If the ingested Cu gave the fish "indigestion", then they died. Mortalities occurred relatively equally throughout the test, and appeared to be mainly small fish that were not growing at all. The fact that growth was lowest in the control is disappointing, but may be because more fish survived at this level, placing a higher demand on the rotifer supply available to them.

## SUMMARY

The hypothesis that this research attempted to address is that by linking three trophic levels together the effect of copper may be magnified up the simplified food chain and result in a detectable negative biological response at copper levels lower than those observed in single species tests with the same organisms. However, the results of single species and trophic tests indicated

that the single species tests were more sensitive (Table 19), although not necessarily more environmentally realistic.

This result was unexpected, but may be explained once the metal speciation data was examined (Tables 1,4, 8-14). In the trophic test, copper appeared to be rapidly bound to the algae in the first stage of the test, greatly reducing the dissolved copper concentrations in solution at all trophic levels to far below nominal. This was in contrast to the single species tests where the majority of the copper was in the dissolved form. In the algal bottle tests dissolved Cu levels were probably proportional to algal densities, with the majority of Cu in the dissolved form at the beginning. The rotifer static renewal had low dissolved Cu levels with average ratios ranging from 0.19 to 0.65 (Table 4) due to high amount of biomass in the chambers, but still not as low as in the three-level trophic test (total/dissolved Cu ratios $\leq$ 0.15). In the fathead minnow static renewal test dissolved Cu levels remained high (total/dissolved Cu ratio $>$ 0.79), in contrast to the trophic test where dissolved concentrations were low. In the trophic test the route of copper exposure was not water and food as planned, but through the food at the higher trophic levels. The route of exposure in the single species test was principally from the water. Copper in the diet appears to be less toxic than in the water column based on this research.

Comparisons between the single species and three-trophic level tests using Cu was really a comparison between the two different routes of

exposure. Because dissolved copper was almost totally absent in the trophic test, my original hypothesis was not addressed as planned. Despite the fact that the trophic test did not address the question for which it was designed, the results have value since they address the response of the organisms when the principal route of Cu exposure is through ingestion. This may represent a more environmentally realistic condition, because natural waters tend to have higher levels of organic material that remove metals from the dissolved fraction. Direct comparisons of the endpoints from the single species tests and the three-trophic level test are not valid because of the different routes of exposure (Table 19).

The three-trophic level test illustrated a number of responses to Cu that were not detected by the single species tests. As planned, the algal population was the most sensitive species in the trophic test system to Cu and responded rapidly to the addition of Cu. Initial declines in population density were observed at 31.5 and 62.7 $\mu$g/L, followed by recovery of the algal populations to or above the control levels by day 6. The transfer of the response of the algae to the other trophic levels is evident in a number of ways. The enhancement of algal growth at the 31.5 $\mu$g/L level from day 4 on, although unexplained, was transferred up the food chain resulting in higher rotifer densities and fish weights at this treatment level (Tables 17 and 18). Rotifer population density paralleled the fluctuations in algae density although they were not statically significant (Figures 3 and 4a). The ability of the higher

trophic levels to weather declines in the algal population can not be detected with single species tests and demonstrates the robustness of natural ecosystems. These examples demonstrate two valuable properties, although diametrically opposed, of the three-trophic level toxicity test that it shares with natural ecosystem. The transfer of a response to a chemical stressor up a trophic ladder, and the ability of an ecosystem to compensate for it, are two essential functional aspects of ecosystems not address in single species testing. This research has demonstrated that the three-level trophic toxicity test is a useful tool in evaluating how ecosystems respond to a chemical insult because it has the ability to detect effects at higher levels of biological organization.

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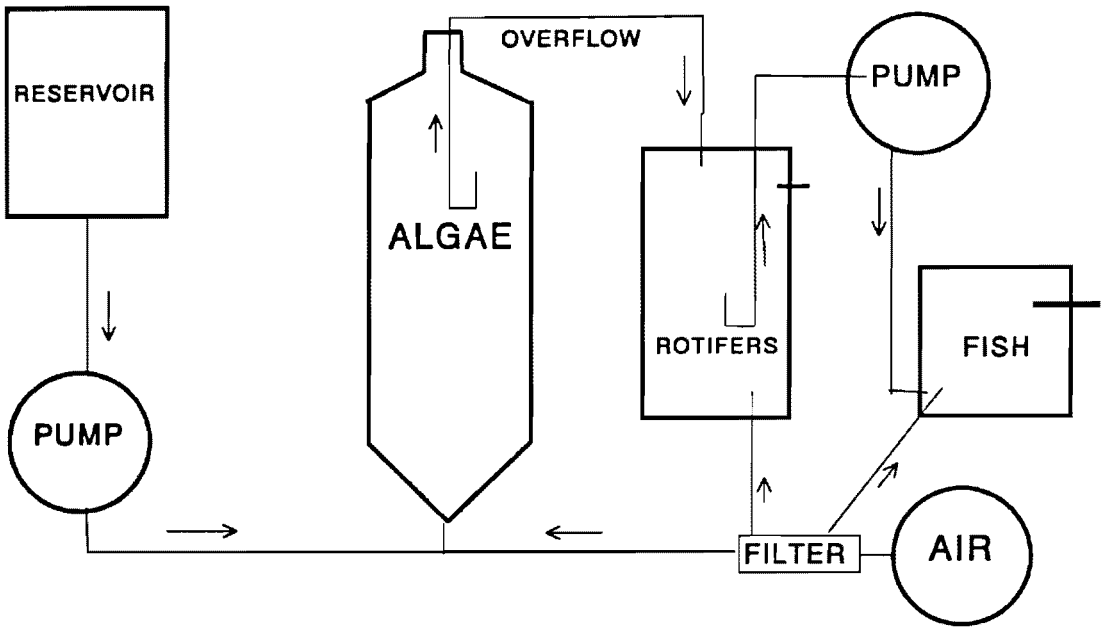


**Figure 1: Diagram of Trophic Test System**

Table 1. Results of total recoverable Cu analysis for algal bottle tests (all values μg/L).

| *C. vulgaris* in SCw/MBL | | *S. capricornutum* in SCw/MBL | | *S. capricornutum* in EPA | |
|---|---|---|---|---|---|
| Nominal Conc. | Measured Conc. | Nominal Conc. | Measured Conc. | Nominal Conc. | Measured Conc. |
| 0.00 | 1.6 | 0.0 | 1.2 | 0.00 | < 1 |
| 1.25 | 3.0 | 15.0 | 15.1 | 7.50 | 7.2 |
| 2.50 | 4.0 | 30.0 | 32.9 | 15.0 | 11.9 |
| 5.00 | 5.9 | 60.0 | 68.7 | 30.0 | 27.8 |
| 10.0 | 12.2 | 120 | 125.3 | 60.0 | 55.2 |
| 20.0 | 22.3 | 240 | 244.7 | 120.0 | 123.4 |

Table 2. Results of algal bottle tests with copper based on relative absorbance measurements of cell density.

| Species | Media | 4-Day Endpoint (μg Cu/L) | | Hardness (mg/L) |
|---|---|---|---|---|
| | | LOEC | NOEC | |
| *S. capricornutum* | EPA | 55.2 | 27.8 | 16 |
| " | SC w/MBL | 31.0 | 15.0 | 100 |
| *C. vulgaris* | " | 12.2 | 4.6 | 100 |

Table 3a. Rotifer densities in Cu screening test. All values are females/ml based on direct counts.

| Treatment level (µg Cu/L) | DAY 0* | 1 | 2 | 3 | 5 | 7 | 7- DAY MEAN | 7-DAY SD |
|---|---|---|---|---|---|---|---|---|
| 0 | 41 | 60 | 74 | 92 | 72 | 116 | 82.8 | 21.8 |
| 31.25 | 41 | 64 | 62 | 104 | 74 | 84 | 77.6 | 17.2 |
| 62.5 | 41 | 34 | 60 | 84 | 74 | 72 | 64.8 | 19.2 |
| 125 | 41 | 6 | 0 | 0 | 0 | 0 | - | - |
| 250 | 41 | 0 | 0 | 0 | 0 | 0 | - | - |
| 500 | 41 | 0 | 0 | 0 | 0 | 0 | - | - |
| 1000 | 41 | 0 | 0 | 0 | 0 | 0 | - | - |

* = All treatments were inoculated with same starting population and the mean of 8 counts is reported as the starting density.

Table 3b. Rotifer egg ratio values for Cu screening test. All values are the ratio of the # of amitic eggs to females based on direct counts.

| Treatment Level (μg Cu/L) | DAY 0 | 1 | 2 | 3 | 5 | 7 | 7-DAY MEAN | 7-DAY SD |
|---|---|---|---|---|---|---|---|---|
| 0 | 0.19 | 0.43 | 0.51 | 0.93 | 0.53 | 0.24 | 0.46 | 0.28 |
| 31.25 | 0.19 | 0.59 | 0.32 | 0.46 | 0.35 | 0.31 | 0.36 | 0.15 |
| 62.5 | 0.19 | 0.47 | 0.37 | 0.57 | 0.32 | 0.47 | 0.40 | 0.14 |
| 125 | 0.19 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - | - |
| 250 | 0.19 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - | - |
| 500 | 0.19 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - | - |
| 1000 | 0.19 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - | - |

* = All treatments were inoculated with same starting population and the mean of 8 counts is reported as the starting egg ratio.

Table 4. Metal analysis of begin of day samples in rotifer chronic test . Total recoverable values are the sum of the suspended and dissolved fractions. The amount of Cu in the rotifers themselves is not included in the suspended fraction due to sample contamination.

| NOMINAL CONC. | DAY | SUSPENDED | DISSOLVED | TOTAL | RATIO | RATIO | |
|---|---|---|---|---|---|---|---|
| | | | µg/L | | | MEAN | SD |
| 0 | 1 | 2.5 | 3.5 | 6.0 | 0.58 | 0.65 | 0.08 |
| 10 | 1 | 5.9 | 8.0 | 13.9 | 0.58 | | |
| 20 | 1 | 7.9 | 14.7 | 22.6 | 0.65 | | |
| 40 | 1 | 10.8 | 20.6 | 31.4 | 0.66 | | |
| 80 | 1 | 12.9 | 45.8 | 58.7 | 0.78 | | |
| 0 | 3 | 5.8 | 0.5 | 6.3 | 0.08 | 0.20 | 0.11 |
| 10 | 3 | 13.7 | 3.1 | 16.8 | 0.18 | | |
| 20 | 3 | 14.6 | 5.0 | 19.6 | 0.26 | | |
| 40 | 3 | 56.3 | 8.5 | 64.8 | 0.13 | | |
| 80 | 3 | 30.5 | 16.6 | 47.1 | 0.35 | | |
| 0 | 6 | 4.3 | 0.3 | 4.6 | 0.07 | 0.19 | 0.09 |
| 10 | 6 | 19.2 | 3.4 | 22.6 | 0.15 | | |
| 20 | 6 | 19.0 | 6.5 | 25.5 | 0.25 | | |
| 40 | 6 | 25.2 | 6.2 | 31.4 | 0.20 | | |
| 80 | 6 | 35.7 | 14.2 | 49.9 | 0.28 | | |

Table 5a. Rotifer static renewal summary of population density. All values expressed as # female rotifers/ml (n = 3) . The starting rotifer densities for all treatments was 31 females/ml.

| DAY | Treatment Levels (μg Cu/L) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 5.6 | | 16.8 | | 23.5 | | 43.5 | | 62.7 | |
| | MEAN | SD | MEAN | SD | MEAN | SD | MEAN | SD | MEAN | SD |
| 1 | 19.4 | 4.6 | 20.1 | 4.2 | 10.0[a] | 3.0 | 10.7[a] | 5.7 | 1.1[ab] | 1.2 |
| 3 | 43.5 | 20.4 | 47.8 | 16.5 | 48.8 | 17.3 | 38.3 | 13.7 | 23.8 | 14.2 |
| 4 | 86.5 | 7.3 | 85.4 | 25.5 | 89.0 | 6.3 | 80.2 | 22.7 | 64.2 | 18.2 |
| 7 | 57.1 | 6.9 | 60.0 | 11.7 | 52.6 | 16.2 | 35.0[a] | 6.0 | 20.8[a] | 10.8 |

[a] = Significantly different from the control at $\alpha$ = 0.05 based on ANOVA followed by Dunnett's test.
[b] Note: In one replicate there were only 2 resting eggs/ml; no females or amictic eggs present.

Table 5b. Rotifer static renewal summary of population egg ratios (n=3) . The starting rotifer egg ratio for all treatments was 0.05.

| DAY | Treatment Levels (μg Cu/L) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 5.6 | | 16.8 | | 23.5 | | 43.5 | | 62.7 | |
| | MEAN | SD | MEAN | SD | MEAN | SD | MEAN | SD | MEAN | SD |
| 1 | 0.93 | 0.31 | 0.96 | 0.25 | 0.82 | 0.49 | 1.30 | 0.34 | 1.29[b] | 0.40 |
| 3 | 0.61 | 0.25 | 0.56 | 0.26 | 0.60 | 0.22 | 0.44 | 0.14 | 0.48 | 0.18 |
| 4 | 0.27 | 0.28 | 0.35 | 0.11 | 0.40 | 0.11 | 0.44 | 0.32 | 0.48 | 0.22 |
| 7 | 0.26 | 0.08 | 0.21 | 0.09 | 0.20 | 0.04 | 0.22 | 0.14 | 0.43 | 0.06 |

[a] = Significantly different from the control at $\alpha$ = 0.05 based on ANOVA followed by Dunnett's test.
[b] Note: In one replicate there were only 2 resting eggs/ml; no females or amictic eggs present.

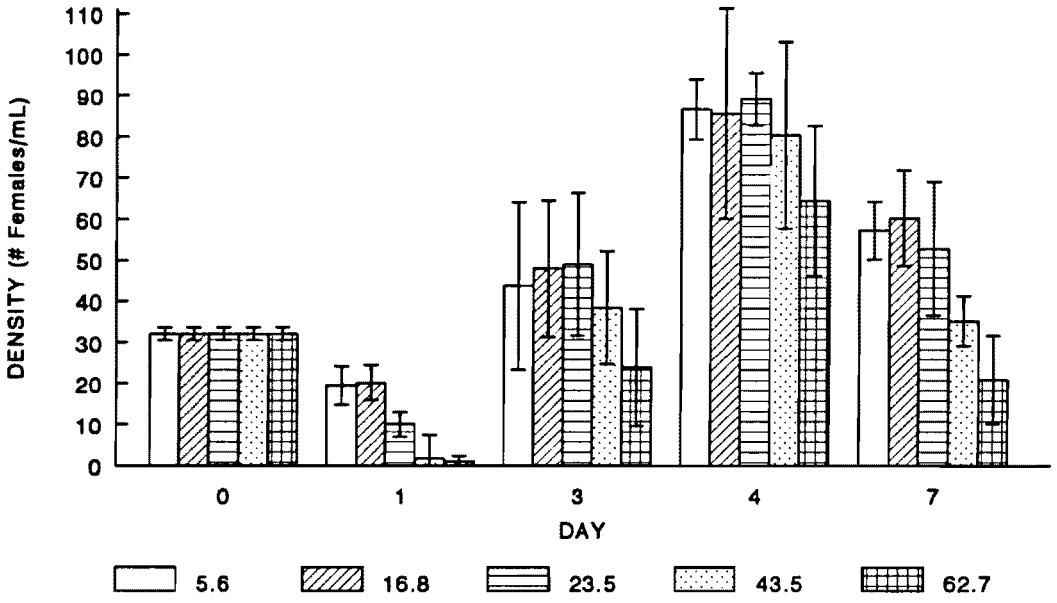


**Figure 2: Rotifer Cu Static Renewal**
**Mean population density ± 1 sd**

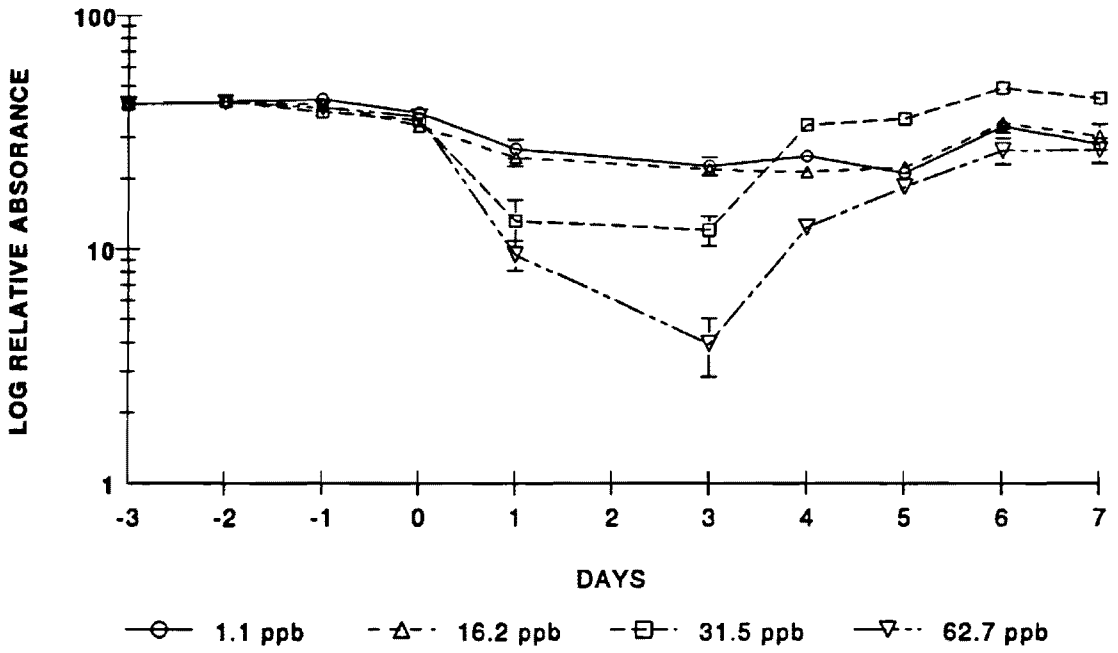


**Figure 3: Algal Population Response to Cu**
**Flow-through Chemostat test**

Table 6. Summary of 7-day fathead minnow static renewal test (n = 4).

| Treatment Level (μg/L) | % Survival | | DRY WEIGHT (mg) | |
|---|---|---|---|---|
| | MEAN | SD | MEAN | SD |
| 1.7 | 80.0 | 0.0 | 0.447 | 0.066 |
| 13.2 | 72.5 | 9.6 | 0.376 | 0.061 |
| 23.0 | 65.0 | 10.0 | 0.384 | 0.035 |
| 39.2 | 70.0 | 16.3 | 0.211* | 0.046 |
| 73.8 | 45.0* | 10.0 | 0.161* | 0.010 |
| 151.2 | 37.5* | 17.1 | 0.143* | 0.015 |

* = Significantly different from control at $\alpha$ = 0.05 based on ANOVA followed by Dunnett's tests.

Table 7. Summary of Cu levels in fathead minnow static renewal test (n = 3).

| NOMINAL CONC. (μg/L) | TOTAL (μg Cu/L) | | DISSOLVED (μg Cu/L) | | RATIO (DS/TOTAL) |
|---|---|---|---|---|---|
| | MEAN | SD | MEAN | SD | |
| 0 | 1.7 | 1.9 | 2.4 | 1.5 | 1.37 |
| 10 | 13.2 | 3.4 | 11.7 | 2.7 | 0.89 |
| 20 | 23.0 | 4.1 | 20.3 | 3.6 | 0.88 |
| 40 | 39.2 | 4.2 | 35.3 | 4.8 | 0.90 |
| 80 | 73.8 | 0.9 | 61.6 | 10.3 | 0.83 |
| 160 | 151.2 | 5.9 | 118.8 | 8.9 | 0.79 |

Table 8a. Begin of day measured Cu levels in fathead minnow static renewal test (n = 3).

| NOMINAL CONC. | μg Cu/L TOTAL | | DISSOLVED | | RATIO (DS/TOTAL) |
|---|---|---|---|---|---|
| | MEAN | SD | MEAN | SD | |
| 0 | 0.4 | 0.2 | 1.2 | 0.3 | 2.93 |
| 10 | 13.5 | 4.8 | 12.8 | 2.7 | 0.95 |
| 20 | 21.8 | 0.3 | 23.3 | 2.0 | 1.07 |
| 40 | 40.5 | 0.7 | 39.3 | 3.0 | 0.97 |
| 80 | 74.1 | 0.4 | 69.8 | 6.0 | 0.94 |
| 160 | 150.4 | 5.0 | 124.2 | 8.3 | 0.83 |

Table 8b. End of day measured Cu levels in fathead minnow static renewal test (n = 3).

| NOMINAL CONC. (μg/L) | μg Cu/L TOTAL | | DISSOLVED | | RATIO |
|---|---|---|---|---|---|
| | MEAN | SD | MEAN | SD | (DS/TOTAL) |
| 0 | 3.0 | 1.9 | 3.5 | 1.8 | 1.16 |
| 10 | 12.8 | 2.2 | 10.6 | 2.7 | 0.83 |
| 20 | 24.3 | 6.1 | 17.4 | 1.9 | 0.72 |
| 40 | 37.9 | 6.3 | 31.3 | 0.7 | 0.83 |
| 80 | 73.5 | 1.3 | 53.4 | 5.2 | 0.73 |
| 160 | 151.9 | 7.8 | 113.5 | 6.4 | 0.75 |

Table 9. Total Cu levels in fatheads minnows after 7-days exposure at various exposure levels for static renewal test (n = 4).

| WATER CONC. (µg/L) | ng Cu/mg Fish | | BCF | |
|---|---|---|---|---|
| | MEAN | SD | MEAN | SD |
| | (ppm) | | | |
| 1.7 | 7.9 | 3.7 | 4540 | 630 |
| 13.2 | 22.2 | 5.9 | 1680 | 450 |
| 23.0 | 27.9 | 6.0 | 1210 | 260 |
| 39.2 | 35.3 | 15.6 | 900 | 400 |
| 73.8 | 85.6 | 24.0 | 1160 | 330 |
| 151.2 | 96.6 | 26.3 | 640 | 170 |
| ALL SAMPLES | | | 1120 | 390 |

Table 10. Measured Cu concentrations in test solution reservoirs for three-trophic level toxicity test (n = 5).

| NOMINAL CONC. (µg/L) | MEAN | SD | STDERR | MAX | MIN |
|---|---|---|---|---|---|
| | | | (µg Cu/L) | | |
| 0.00 | 1.1 | 0.2 | 0.08 | 1.3 | 0.8 |
| 17.75 | 16.2 | 1.5 | 0.69 | 17.7 | 13.6 |
| 35.00 | 31.5 | 7.7 | 3.45 | 41.8 | 23.2 |
| 70.00 | 62.7 | 9.6 | 4.30 | 73.5 | 53.5 |

Table 11a. Total recoverable Cu concentrations in algal chemostats over 7 days. Copper analysis was performed on days 0, 1, 4, 6, and 7. All values are $\mu$g/L with N = 5.

| NOMINAL CONC. | MEAN | SD | STDERR | MAX | MIN |
|---|---|---|---|---|---|
| 0.00 | < 1.0 | - | - | 2.3 | < 1.0 |
| 17.75 | 16.4 | 7.3 | 3.7 | 24.4 | 9.9 |
| 35 .00 | 28.1 | 9.4 | 4.7 | 36.4 | 19.6 |
| 70.00 | 55.3 | 17.5 | 8.8 | 79.7 | 42.4 |

Table 11b. Total recoverable Cu concentrations in rotifer chambers over 7 days. Copper analysis was performed on days 0, 1, 4, 6, and 7. All values are $\mu$g/L with N = 5.

| NOMINAL CONC. | MEAN | SD | STDERR | MAX | MIN |
|---|---|---|---|---|---|
| 0.00 | 2.1 | 1.6 | 0.7 | 4.3 | 0.1 |
| 17.75 | 10.1 | 6.6 | 3.3 | 17.9 | 3.5 |
| 35.00 | 20.0 | 7.9 | 3.6 | 30.9 | 11.1 |
| 70.00 | 48.6 | 19.4 | 9.7 | 65.4 | 27.0 |

Table 11c. Total recoverable Cu concentrations in fish chambers over 7 days. Copper analysis was performed on days 0, 1, 4, 6, and 7. All values are μg/L with N = 5.

| NOMINAL CONC. | MEAN | SD | STDERR | MAX | MIN |
|---|---|---|---|---|---|
| 0.00 | 2.8 | 1.6 | 0.9 | 4.2 | 1.0 |
| 17.75 | 18.3 | 0.5 | 0.4 | 18.6 | 17.9 |
| 35.00 | 27.8 | 2.5 | 1.8 | 29.5 | 26.0 |
| 70.00 | 51.3 | 14.4 | 10.2 | 61.5 | 41.1 |

Table 12. Dissolved Cu levels in algal chemostats, based on 6 replicates.

| NOMINAL CONCENTRATION (μg/L) | MEAN | SD | STDERR (μg/L) | RANGE |
|---|---|---|---|---|
| 0.00 | < 1.0 | - | - | < 1.0 - 1.8 |
| 17.75 | 2.5 | 1.2 | 0.5 | 1.2 - 4.8 |
| 35.00 | 2.6 | 0.7 | 0.3 | 2.0 - 3.4 |
| 70.00 | 7.0 | 5.3 | 2.2 | 2.6 - 14.7 |

Table 13a. Algal Cu loading in trophic test.

| TREATMENT LEVEL (μg/L) | DAY | | | | | |
|---|---|---|---|---|---|---|
| | 1 | | 6 | | 7 | |
| | MEAN | SD | MEAN | SD | MEAN | SD |
| 1.1 | 22.4 | 3.9 | - | - | 3.0 | 2.8 |
| 16.2 | 187.9 | 95.2 | 229.2 | 34.3 | 189.1 | 72.5 |
| 31.5 | 384.3 | 11.9 | 270.6 | 54.6 | 213.3 | 17.8 |
| 62.7 | 965.2 | 37.7 | 805.5 | 74.7 | 606.5 | 87.7 |

Table 13b. Algal bioconcentration factors (BCFs) in trophic test.

| TREATMENT LEVEL (μg/L) | DAY | | | | | |
|---|---|---|---|---|---|---|
| | 1 | | 6 | | 7 | |
| | MEAN | SD | MEAN | SD | MEAN | SD |
| 1.1 | 20372 | 3530 | - | - | 2767 | 2514 |
| 16.2 | 11599 | 5876 | 14146 | 2119 | 11671 | 4476 |
| 31.5 | 12200 | 376 | 8592 | 1734 | 6773 | 566 |
| 62.7 | 15394 | 602 | 12847 | 1191 | 9674 | 1399 |
| ALL* | 13064 | 2040 | 11861 | 2905 | 9373 | 2463 |

* Control not included in overall means.

Table 14. Fathead minnow bioconcentration in trophic test

| Dosed Conc. (μg/L) | N | μg Cu/g fish | | BCF | |
|---|---|---|---|---|---|
| | | MEAN | SD | MEAN | SD |
| 1.1 | 2 | 16.6 | 1.1 | 15064 | 955 |
| 16.2 | 2 | 14.4 | 2.4 | 891 | 145 |
| 31.5 | 2 | 14.6 | 1.4 | 463 | 45 |
| 62.7 | 2 | 18.6 | 1.8 | 297 | 29 |

Table 15. Mean algal densities in algal chemostats over time (as relative absorbance).

| Dose Conc. (μg/L) | DAY | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 1 | 3 | 4 | 5 | 6 | 7 |
| 1.1 | 37.9 | 29.5 | 27.8 | 25.2 | 21.0 | 33.4 | 28.0 |
| 16.2 | 33.5 | 27.0 | 25.0 | 21.1 | 22.4 | 34.4 | 30.3 |
| 31.5 | 35.3 | 20.9* | 33.0 | 33.7@ | 35.7@ | 48.8@ | 44.0@ |
| 62.7 | 36.6 | 5.9* | 9.4* | 12.2* | 18.3 | 26.3 | 26.5 |

* = significantly < control, @ = significantly > control at $\alpha = 0.05$ based on ANOVA followed by Dunnett's multiple comparison.

Table 16. Algal dry weight in algal chemostats (mg/ml).

| Dose Conc. (μg/L) | DAY | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | -3 | | 1 | | 6 | | 7 | |
| | MEAN | SD | MEAN | SD | MEAN | SD | MEAN | SD |
| 1.1 | 0.145 | 0.0021 | 0.079 | 0.0017 | 0.056 | 0.0117 | 0.059 | 0.0021 |
| 16.2 | 0.145 | 0.0021 | 0.078 | 0.0074 | 0.060 | 0.0070 | 0.069 | 0.0171 |
| 31.5 | 0.145 | 0.0021 | 0.074 | 0.0037 | 0.083 | 0.0124 | 0.090* | 0.0078 |
| 62.7 | 0.145 | 0.0021 | 0.048* | 0.0071 | 0.058 | 0.0080 | 0.067 | 0.0103 |

* = Statistically different from daily control at $\alpha = 0.05$, based on ANOVA followed by Dunnett's test.

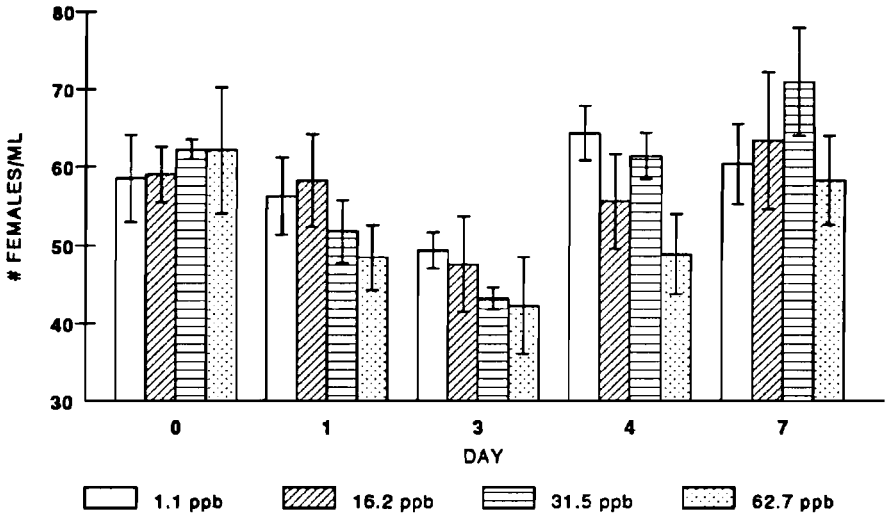


**Figure 4a: Flow-through Trophic Level Test**
**Mean rotifer population density ± 1 sd**

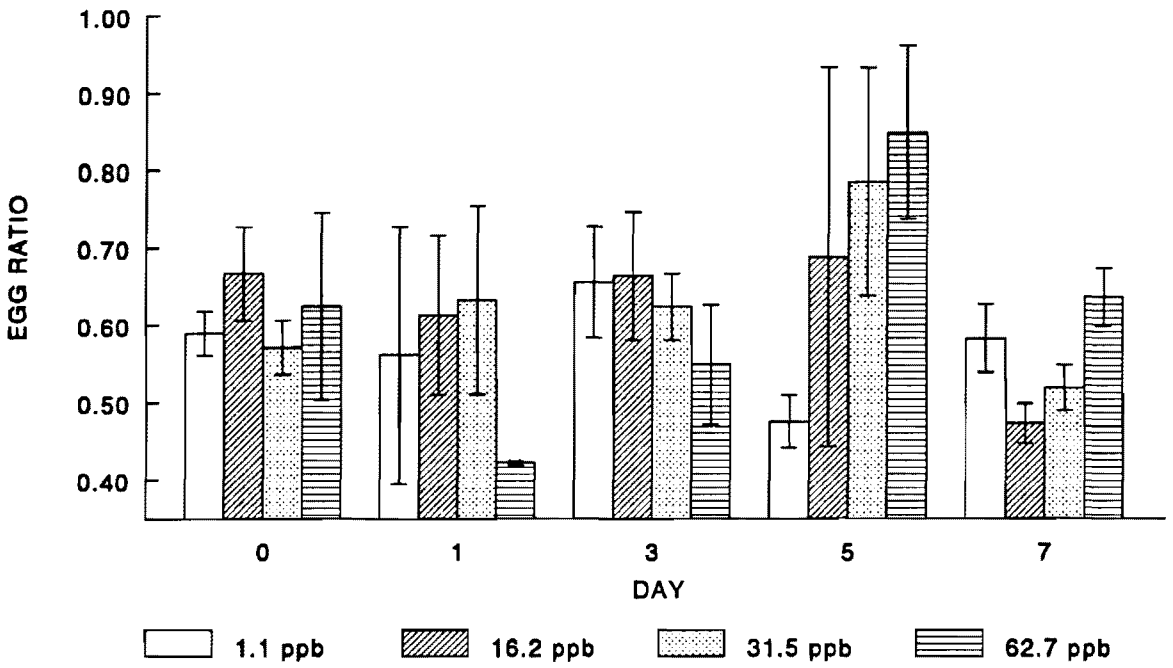


**Figure 4b: Flow-through Trophic Test**
**Mean rotifer egg ratio ± 1 sd**

Table 17. Rotifer population parameters for middle trophic level exposed copper. *Brachionus calyciflorus* densities are the mean of averages of the reps for each treatment on the days that measurements were taken. Rotifer population parameters were measured on days 0, 1, 3, 5, and 7.

| Dosed Conc. (µg/L) | N | DENSITY (females/ml) | | EGG RATIO (# eggs/# females) | |
|---|---|---|---|---|---|
| | | MEAN | STDERR | MEAN | STDERR |
| 1.1 | 5 | 57.7 | 2.47 | 0.576 | 0.030 |
| 16.2 | 5 | 56.7 | 2.63 | 0.609 | 0.037 |
| 31.5 | 5 | 57.8 | 4.75 | 0.633 | 0.096 |
| 62.7 | 5 | 52.2 | 3.55 | 0.613 | 0.155 |

Table 18. Fathead growth and survival in trophic test after 7 days. Fish individually weighed and the data pooled for both replicates. The percent survival is the mean of the replicates.

| Dosed Conc. (µg/L) | N | MEAN | SD (g) | STDERR | % SURVIVAL |
|---|---|---|---|---|---|
| 1.1 | 41 | 0.460 | 0.139 | 0.022 | 91.1 |
| 16.2 | 36 | 0.549 | 0.144 | 0.024 | 80.0 |
| 31.5 | 37 | 0.585* | 0.163 | 0.027 | 79.5 |
| 62.7 | 31 | 0.518 | 0.212 | 0.038 | 65.1@ |

* = Fish weight was significantly greater then the control at the $\alpha = 0.05$ level.
@ = Survival was impaired after 7 days as compared to the control ($\alpha = 0.05$).

Table 19. Summary of toxicity tests results with copper in Sinking Creek water supplemented with nutrients. The endpoints reported are the most sensitive response detected over the course of the individual tests. For test #1A the values reported are after 1 day, while for test #2 the result is after 4 days. The values reported for all other tests are after 7 days of exposure.

| Test # | Test Type | Test Species | LOEC | NOEC |
|---|---|---|---|---|
| | | | (µg Cu/L) | |
| 1A | Trophic | *C. vulgaris* | 31.5 | 16.2 |
| 1B | Trophic | *B. calyciflorus* | - | 62.7 |
| 1C | Trophic | *P. promelas* | 62.7 | 31.5 |
| 2 | Single Species | *C. vulgaris* | 12.2 | 5.6 |
| 3 | Single Species | *B. calyciflorus* | 23.5 | 16.8 |
| 4 | Single Species | *P. promelas* | 39.2 | 23.0 |

# CHAPTER 3:

## Toxicity and Biomagnification of Se to a Three Trophic Level Food Chain

## ABSTRACT

Three species, *Chlorella vulgaris*, *Brachionus calyciflorus*, and *Pimephales promelas*, were exposed to selenium (Se) for 25 days in a three trophic level test system. The organisms were linked in a continuous flow-through system in separate vessels with each organism feeding on the trophic level below it. These organisms were continually exposed for 25 days to 0, 110.3, 207.7, and 396.1 $\mu$g/L $Se^{+6}$ in natural creek water supplemented with nutrients to sustain algal growth. Dissolved levels of Se were maintained close to nominal concentrations in all the test chambers throughout the test. The standing crop of algae and rotifers and the growth of larval fathead minnows (initial age: 24-hours) were estimated by measuring dry weight throughout the test. Fathead minnow growth was impaired after 7 days at the 207.7 and 396.1 $\mu$g/L levels, with 100% mortality by day 16. Rotifer populations were impaired at these same levels by day 4, and succumbed by day 7. In addition, impairment to the rotifers and fish was apparent at the 110.3 $\mu$g/L level after day 20. The algal populations show evidence of reduced growth rates at 207.7 and 386.1 $\mu$g/L, but not at 110.3 $\mu$g/L in one test. In the other experiment growth rates were impaired at 81.7 and 1053.7 $\mu$g/L, with no effect at 8.5 $\mu$g/L. The amount of Se in whole organisms was measured throughout the experiment. Bioconcentration factors were found to be dependent on the species, treatment level, and length of exposure period and ranged between 100 and 1000.

## INTRODUCTION

The complex behavior of selenium in the environment poses some unique problems in estimating the risk posed by its release in to aquatic systems. Selenium is a metalloid with many properties similar to sulfur, and exists in three oxidation states in surface waters (selenide = -2, selenite = +4, and selenate +6). It is an essential micronutrient for many organisms which is both beneficial and toxic within a relatively narrow range to biological systems (Shamberger 1983). For example it has been estimated that dietary concentrations of < 0.1 $\mu$g Se/g dry weight for rainbow trout can lead to Se deficiency symptoms, whereas at levels above 10 $\mu$g Se/g toxic effects begin to manifest themselves (Hodson and Hilton 1983). Most water bodies are not selenium deficient with food organism generally containing between 0.1-4.7 $\mu$g Se/g dry wt. in unpolluted waters (Hodson and Hilton 1983).

The dichotomous nature has fascinated researchers in all fields of biology. There is epidemiological evidence in humans that areas with higher Se levels in the soils, have lower cancer rates (Shamberger 1983). Laboratory studies and clinical trials support the epidemiological evidence that Se can act as an anticarcinogen and antimutagen (Shamberger 1983, Bronzetti and della Croce 1993). In contrast, it can also be toxic, carcinogenic, and genotoxic (Bronzetti and della Croce 1993). There is also evidence that Se can mediate the toxicity of Cd and Hg to the body and aquatic organisms (Robberecht and Van Grieken 1982, USEPA 1987, Bronzetti and della Croce 1993, Rudd et al.

1983, Turner and Rudd 1983). Selenium deficiency in animals can lead to a variety of disease, including nutritional myopathies, liver necrosis, pancreatic fibrosis, and a number of others (Shamberger 1983). Too much selenium in the diet can also cause a variety of disorders that can lead to death (Shamberger 1983). A similar situation exists with plants, with both low and high levels of selenium in the diet causing negative impacts.

Se enters aquatic habitats from a number of natural and anthropogenic sources, including coal based power generation and irrigation waters. It is used in a number of industries (glass,and paint coloring, electrical) (Bennett 1983, Bronzetti and della Croce 1993) . The burning of coal is the principal source of environmental contamination (Bronzetti and della Croce 1993). It is ubiquitous in the earth's crust, although generally found only at trace levels. Surface waters generally contain < 1 $\mu$g/L of selenium (Shamberger 1983) with most having levels between < 0.1 and 0.4 $\mu$g/L (Hodson and Hilton 1983) (Bennett 1983). Elevated levels in aquatic systems can be found in regions where the soil is naturally rich in Se, especially if draining irrigated land or systems receiving inputs from fly ash piles or ponds associated with coal burning and production (Bronzetti and della Croce 1993, USEPA 1987, Gutenmann et al. 1976). Initial concern for contamination of water bodies by Se were raised after fish survival and reproduction in Belews lake, NC was linked to selenium concentrations of 10 $\mu$g/L in the system (Cumbie and VanHorn 1978, Lemly 1985b). During this same period, (Adams and Johnson 1981) proposed a

substantially higher value of 52 μg/L as an appropriate water quality criterion for selenium based on laboratory toxicity tests. The current recommend chronic water quality criterion for Se is 5 μg/L, which is primarily based on the results of the Belews Lake study and other field studies of contaminated sites (USEPA 1987). Most documented cases of Se contamination have been due to drainage from fly ash ponds or from irrigation water of Se rich soils (Lemly 1985a, USEPA 1987).

Selenium exists in surface waters in inorganic (selenite and selenate) and organic forms. The response of aquatic organisms is different depending on the form, with organic forms generally being more toxic and bioaccumulating faster then either selenite or selenate (Besser et al. 1993). Reports of selenium toxicity at levels that can occur in the environments include impairment of reproduction in fish and mortality (Lemly 1985a). Impacts due to selenium contamination of the environment appear to be due mainly to dietary toxicity and the accumulation of Se in the tissues (Lemly 1985b). Inorganic selenium has been found to be acutely toxic to some species as low as 65 μg/L, although most aquatic organism exhibit acute responses at levels above 1000 μg/l (USEPA 1987). The use of short term toxicity tests to evaluate Se toxicity has been challenged because of the importance of bioaccumulation in the response of the organisms (Lemly 1985b). The lowest adverse effects have principally been observed with fish at the high end of the food chain under long-term low level exposures from water and food (Lemly 1985b, Hermanutz et al. 1992).

The goal of this research is to quantify Se toxicity and bioconcentration using a test system that was specifically designed to examine trophic level interactions in the laboratory. The system is unique because it links three trophic levels in a continuous flow-through system able to maintain sufficient biomass to support all trophic levels. Other researchers have had to supplement the diet (Bertram and Brooks 1986), or accept weight lost (Besser et al. 1993)during the test because of the inherent difficulties in producing sufficient Se laden food for the highest trophic levels. Also in many research efforts, the food organism is no exposed to the same water borne Se concentrations as the organisms at the top trophic level as experienced in the environment. The system used in this research allows the lower trophic levels to come to equilibrium with Se in the water, making it a more environmentally realistic evaluation of selenium exposure. It is important that all trophic levels be exposed to the same water concentrations of Se so that the higher trophic levels receive a dose in the food which accurately reflects the water borne exposure conditions.

## MATERIALS & METHODS

To estimate the potential hazard that Se poses to aquatic ecosystems we utilized a three-trophic level test system that utilizes the alga, *Chlorella vulgaris*, the rotifer, *Brachionus calyciflorus*, and the fathead minnow, *Pimephales promelas* (Chapter 1). Two experiments were conducted. The first was a

screening test and consisted of exposing only the algal portion of the trophic test system to sodium selenate at 0, 10, 100, and 1000 $\mu$g Se/L for 7 days. The purpose of this experiment was to determine at what concentration the algae would be able to maintain sufficient density for the next trophic level, and whether Se would be in the dissolved form under these test conditions. An additional goal was to determine the lowest water borne Se concentration that would result in detectable Se concentrations in the algae using the analytical technique available.

The second experiment consisted of a full scale three-trophic level test with the food chain exposed to 0, 100, 200, and 400 $\mu$g Se/L as sodium selenate for 28 days. The treatment levels chosen were based on a number of factors. Based on a review of the pertinent literature (USEPA 1987), it appeared probably that an effect would be detectable at the highest trophic level during the relatively short duration of the experiment. Lower treatment levels were not used because effects on fish below 100 $\mu$g/L have only been detected in long term experiments (> 90 days to 1 year) and a water concentration of 10 $\mu$g/L resulted in levels in algae below the ability to detect. Note that the test was actually terminated after 25 days due to an unexplained contamination of the system by a fungus. Contamination appeared overnight so it is believed that the system was functioning properly up until this point.

*Dilution water*

The water used was a natural creek water collected from Sinking Creek, Newport, VA and supplemented with nutrients to sustain algal growth. Sinking Creek water was collected within a month of testing. The water was first filtered through a 1.6 $\mu$m filter to remove suspended solids, and then autoclaved to sterilize it. Appropriate volumes of nutrient stocks based on the Guillard's Woods Hole Marine Biological Laboratory (MBL) algal culture medium (Nicholas 1973) as modified by (Stemberger 1981) was added to the sterilized water after it has cooled and been aerated overnight. The typical water chemistry characteristics of the Sinking Creek water used were: pH, 8.21; conductivity, 250; hardness, 120 mg/L; alkalinity, 120; and no detectable levels of Se ($< 2$ $\mu$g/L). The nutrient stocks were sterilized by filtration before use and stored at 4° C for up to 6 months. This dilution water will be referred to as SC w/MBL throughout the rest of this paper.

*Three-Trophic Level Test*

Development of the three-trophic level test system has been previously described in detail (Chapter 1). It consists of the following three species linked together in a continuous flow-through system: *Chlorella vulgaris*, *Brachionus calyciflorus*, and the fathead minnow (*Pimephales promelas*). The system consists of four different levels, linked together in series (Figure 1). The top level consists of the four 25-L polypropylene carboys, one for each treatment

level, that serve as the test solution reservoirs. The test solution was aerated vigorously to promote mixing using air that passes through a 0.2 $\mu$m filter. The carboys were plugged with cotton bungs and wrapped in aluminum foil to prevent light from causing any changes in the test solution. The whole apparatus was sterilized before use. An eight channel peristaltic pump was used to continuously delivered test solution to the next level, which was composed of eight algal chemostats, two for each treatment.

The algal chemostat portion of the system used custom made 2-L glass cylinders that tapered to a point at the bottom. There was an inlet at the bottom where fresh media and air enters the chamber, and an overflow at the top of the chamber. The algal population was sampled using a sterile syringe through a sample port sealed with a septum.

Media inflow was approximately 0.7 ml/min and was delivered from the reservoirs using a peristaltic pump. Approximately a liter of fresh test solution entered the algal chemostat daily, with an equal volume of the algal culture being forced through the overflow by air pressure to the next trophic level (rotifer chemostats). This resulted in a turnover rate for the algal chemostats of about once every 2 days, and was designed to maintain an algal population in late log phase with a density between 3 and 6 x $10^6$ cells/ml. The whole system, including the silicone outflow tubing to rotifer chemostats, was sterilized before use. Inoculation of the system was achieved by batch culturing 16 L in a large carboy until late log phase of growth ( ~ 7 days) and then

transferring the algae directly to the test vessels. Illumination was provided by 20 watt cool white fluorescent lights providing 4300 ± 400 lux at the algal chemostat. The bottom third of the chemostats sat in a water bath to maintain temperature at 25 ± 0.1°C. Toxicant was directly added to the algal chemostats at the beginning of the test.

The rotifer chemostats were similar to the algal chemostats in design, but were made from 1-L polypropylene sample bottles with holes drilled in them to accommodate glass tubes for aeration, media in, media out, and an overflow (Figure 1). *C. vulgaris* from the first level was continually forced into the rotifer chamber by air pressure from the algal chemostat. The overflow was positioned to maintain an volume of 950-ml in each chamber, with the spill over dripping into the fish containers. Unlike the algal chemostats, the overflow was not the principal exit port for the rotifer chemostats. Because rotifers are mobile animals, they have the potential to avoid a slow flowing overflow. To overcome this problem, the rotifers were pumped out at a high flow rate on a periodic basis using a peristaltic pumped controlled by a timer. Outflow parameters were: pump on = 1 minute out of every 30; flow rate while on = 18 ml/min. The pumped out entered into the bottom of the fish chambers. The turnover rate was approximately once per day for the rotifer chemostats. The chambers were sampled with a syringe by removing the cap. Biomass determinations and metal samples were taken by removing 60 or 120 mls from each chamber and then filtering the suspension through a 120 $\mu$m Nitex® screen

and then rinsing the sample with glass distill water. Then rotifers were then transferred to a pre-weighed 20 $\mu$m membrane filter using a vacuum filter apparatus and dried at 60 ° C for at least 24 hours. The staring rotifer density for all treatment levels was 151.4 ± 7.7 females/ml, with an egg ratio of 0.517 ± 0.014.

The larval fathead minnows were held in 350-ml polystyrene chambers with an inlet consisting of the outflow form the rotifer chemostats entering at the bottom. The overflow was a whole cut into the side of the chamber, screened with 120 $\mu$m Nitex® to keep the organisms in. The chambers were cleaned daily to remove any waste. Aeration was provide at 100 bubbles/min via narrow bore glass tubes. The larval fatheads used were between 12 and 27 hours old, with 35 placed in each chamber.

*Statistical Analysis*

Analysis Of Variance (ANOVA) procedures were used at each trophic level to make comparisons between the control and treatment biomass. SAS® software procedures were used to perform all the statistical computations (SAS Institute Inc. 1989). If the data met assumptions of normality and homogeneity of variance then parametric procedures consisting of ANOVA followed by mean comparisons using Dunnett's test for ANOVAs that were significant at the desired $\alpha$ level. If the data did not meet the necessary parametric assumptions, then ANOVA procedures were performed on the ranked data. For daily

comparisons a simple two parameter model consisting of the response variable biomass and the treatment level was used. To determine the overall impact of the treatments at each trophic level the biomass data for each treatment was pooled for all days, except day 0. Because the biomass at each trophic level varies on a daily basis independently of dose, the following multiple parameter repeated measure model was used: biomass = treatment chemostat(treatment) day day *treatment.

*Metal Analysis*

One of the questions the multi-trophic level test is designed to evaluate is how Se is transferred up the food chain. This necessitated sampling each trophic level to determine the amount of Se in the test organisms and the water column. Because dissolved Se concentrations were the same as total in the algal screening test, dissolved levels were measured in all components of the trophic level test in addition to determining the concentration of Se in the test organisms. Samples for total recoverable Se determination were preserved immediately upon collection, whereas samples for dissolved Se analysis were persevered after filtration through a 0.45 $\mu$m membrane filter. All water samples (50 ml) were prepared by acidifying with 0.5 ml concentrated $HNO_3$ (1% $HNO_3$), and then adding 1 ml of 30% $H_2O_2$ and 1 ml of a 5% nickel nitrate solution according to EPA method 270.2 (Kopp and McKee 1983). Water

samples were analyzed directly by AAS when it was determined that digestion of the samples was not necessary.

Biological samples were vigorously digested to determine the total amount of Se in accordance with EPA method 200.3 and 200.9 (Kopp and McKee 1983, USEPA 1991). The digestion procedure involved adding concentrated $HNO_3$ and 30% $H_2O_2$ alternately until the sample was clear. Generally it was only necessary to do this twice. Once the same was clear 1 ml of 1% nickel nitrate was added to the sample and the volume was adjusted to 10 ml.

A Perkin-Elmer 1100 Atomic Adsorption Spectrometer (AAS) equipped with a HGA-300 graphite furnace and a selenium electrodeless discharge lamp was used to measure Se levels after sample preparation in accordance with EPA method 270.2 (Kopp and McKee 1983, USEPA 1991). The machine detection limit for all matrixes was 2 $\mu$g/L Se using a 20 $\mu$l injection volume and a pyrocoated graphite tube with a L'vov platform using the temperature program listed in Table 1. Ten percent of the samples analyzed were quality control spikes and blanks for all matrixes used. Results of the quality control samples found no Se above the limit of detection (2 $\mu$g/L) in any of the blanks (n = 24) and a average recovery of 91.5 $\pm$ 4.7 (n = 33) for all water spikes. Average recovery from the quality control samples for the biological matrices was 92.1 $\pm$ 7.4% (n = 26). Final values were corrected for matrix effects using the % recovery of the matrix.

## RESULTS and DISCUSSION

*Preliminary Algal Test*

Measured levels of selenium in this experiment were consistent although slightly below nominal at the 10 and 100 µg/L levels (Tables 2 and 3). For all metal samples taken, the concentration in the control was always below the detection limit of 2 µg/L. The ratio between the amount of dissolved and total Se was examined in the test reservoirs (Table 2) to determine whether it was in a bioavailable form (USEPA 1992). The speciation ratio was essentially 1 for all treatment levels with amount of dissolved Se equalling the total concentration (Table 2). The dissolved Se concentration in the algal chemostats (Table 3) was similar to what they received from the reservoirs (Table 2). At a nominal level of 10 µg/L dissolved Se concentrations averaged 8.7 ± 0.2 and 9.1 ± 0.4 µg/L; at 100 µg/L dissolved levels averaged 79.5 ± 1.8 and 82.6 ± 1.1 µg/L; at 1000 µg/L dissolved levels averaged 1045.2 ± 12.5 and 999.6 ± 18.7 µg/L, in the reservoirs and chemostats, respectively.

The water chemistry parameters were relatively stable throughout the test with no differences between treatments. The average water quality characteristics of the medium reservoirs were: hardness, 122 ± 3.5 mg/L (n = 17); alkalinity, 81.5 ± 13.6 mg/L (n = 17); conductivity, 383 ± 16 µmohms (n = 13); dissolved oxygen, 7.5 ± 0.4 mg/L (n = 13); pH, 8.38 ± 0.40 (n = 13). In the algal chemostats there were no dose related trends in the water quality parameters measured and the values averaged for all treatments from

the end of the test were: temperature, 25.3 ± 0.05 °C (n = 8) and pH, 8.24 ± 0.04 (n = 8). Alkalinity and hardness were not measured in the algal chemostats because the high amount of algal biomass interferes with the measurements.

There were no significant differences in algal dry weight between the treatment levels on any single day (Table 4). This was expected since the lowest reported literature value for *C. vulgaris* is 5,480 µg/L (USEPA 1987) exposed to selenite. However, algal biomass was consistently higher in the control compared to the other treatment levels (Table 4 and Figure 2). There is a clear, although subtle, dose related trend in the overall mean algal weight, with each higher treatment level having a slightly lower biomass then the preceding level. When post exposure weight data was pooled across days and ANOVA was performed using a repeated measure model, which resulted in a $\alpha = 0.0783$ for the hypothesis that there was a difference between the treatments. The 100 and 1000 µg/L treatments were statistically different from the control based on Dunnett's test if one accepts the less conservative results of the ANOVA (Table 4). With small sample size and clear trend in the data (Figure 2) it is acceptable to use a less conservative $\alpha$ level. Whether this is truly significant is difficult to determine with the current test design, but the Se may be subtly affecting the metabolism of the algae, reducing the growth rate. A reduction in growth rate would be expected, if Se was competing with sulfur in biochemical reactions within the cell (Shamberger 1983). Shrift (1954),

found that Se reduced the growth rate and final density of *C. vulgaris* exposed to different concentrations and ratios of sulfur and Se as selenate.

The bioaccumulation of Se in the algae could only be estimated for the 100 and 1000 μg/L treatment levels because concentrations at the other treatments were below the method detection limit (4 μg Se/g algae for the sample size used). The data for the two highest treatment level was very consistent with more Se bioaccumulating at the 1000, then 100 μg/L level (Table 5). The 1000 μg/L treatment accumulated 10 times more Se then the 100 μg/L treatment which correlated with the differences in the water concentrations. The amount of Se and the BCFs increased as the test progressed (Table 5, Figures 3 and 4). The bioconcentration factors were independent of dose (Table 4, Figures 3 and 4), but did vary by day. The BCFs were generally in close agreement for any specific day for the two treatment levels examined (Table 5, Figures 3 and 4). The algae had not yet reached equilibrium with the Se in the solution by the 7$^{th}$ day, although it appeared to be leveling out (Figure 3).

The continually increase of Se levels was surprising because the algae in the chemostats were doubling once every 2 days, and being flushed out at the same rate. A chemostat is designed so that chemical and biological components are constant in the media and within the cell (Droop 1983), so with constant in-flow of Se, the cell levels should stabilize quickly. One

explanation is that the algae is adapting to the Se and bioconcentrating higher levels as the test progressed.

*Three Trophic Level Test*

Selenium exposure levels were consistent during the 25-day experiment both within and between each trophic level (Tables 6-9). In the test solution reservoirs total Se levels were < 2, 108.1 ± 4.2, 202.4 ± 4.3, and 393.0 ± 4.7 µg/L for the control, 100, 200, 400 µg/L nominal treatment levels (Table 6). As in the screening test, the close agreement between the dissolved levels in the test chambers and the total values in the reservoirs support the conclusion that Se is principally in dissolved forms. Dissolved Se concentrations in the algal chemostats were < 2, 105.5 ± 2.6, 204.9 ± 2.2, 397.6 ± 2.8 µg/L (Table 7). In the rotifer chemostats dissolved Se levels were slightly more variable, but still in the general range of the other trophic levels with average values of < 2, 110.3 ± 5.6, 207.7 ± 3.8, and 396.1 ± 7.5 µg/L (Table 8). The value of 235.1 ± 15.6 µg/L at the middle treatment level of the fish chambers is high, but acceptable, compared to the measured levels of < 2, 106.8 ± 1.8, and 389.1 ± 8.5 µg/L at the other treatment levels (Table 9).

Any severe impacts on the base of the trophic level test will be transmitted up the trophic ladder, because if the algae die then the rotifers at the next trophic level do not have a food source. In this experiment algal population level were maintained at all treatment levels at sufficient density to

support the next trophic level (Table 10 and Figure 5). Algal biomass did not vary more then 20% on any specific day and there were no significant statistical differences on a daily basis (Table 10). However, there is a clear trend in data with the higher treatment levels generally being less then the control (Table 10 and Figure 5). When the data is pooled across days (leaving day 0 out) there were no statistically differences from the control at $\alpha = 0.05$, with the greatest difference between the means being less then 12 % of the control weight (Table 11). This results in a NOEC of 397.6 µg/L based on dissolved concentrations for the 25-day exposure. In the algal screening test dissolved Se concentrations of 82.6 and 9.1 µg/L were the LOEC and NOEC for a 7-day exposure of *Chlorella vulgaris* to selenate in a flow-through system.

The difference between the two tests may be explained by lower variability in the screening test because of the shorter duration, or perhaps the higher biomass (therefor slow growth rate) in the screening test is the reason for the greater sensitivity. There is a limited amount of data in the literature for *C. vulgaris* exposed to selenate. Shrift (1954) found a reduction in the growth rate and final cell density of *C. vulgaris* exposed to Se as selenate at 2,500 µg/L, and no impact at 1250 µg/L. A number of researchers have found slight reductions in algal growth with other species at Se concentrations ranging from 75 to 300 µg/L (USEPA 1987).

Accumulation of Se in the *C. vulgaris* cells was dose dependent, with higher levels found at the higher treatment levels (Table 12 and Figure 6).

Selenium was below the method detection limits (MDL) for the control in all tissue samples (MDL around 5 μg Se/g biomass). Levels of Se in the algae increased until day 11, and then declined to relatively stable levels (Figure 6). The distinct peak, followed by a decline in Se levels in the cells, was unexpected because bioaccumulation theory predicts increasing tissue levels until they stabilize at their maximum (Rand and Petrocelli 1985). A similar pattern was observed by Besser et al. (1993) with Se concentrations in *Chlamydomonas reinhardtii* peaking after 3 h of exposure and then declining. The algae appears to be regulating Se levels in the cell, as supported by the work of (Foe and Knight 1986, Kiffney and Knight 1990) with *Selenastrum capricornutum* and *Anabaena flos-aquae*.

Bioconcentration factors (BCFs) varied on a daily basis and were independent of dose (Table 13 and Figure 7). Fluctuations in BCFs closely followed changes in Se content in the cells with a rapid increase from day 0 to day 5 to about 400, followed by a peak on day 11 and subsequent decline back down to around 400 (Figure 7). The most striking result is how similar the BCFs were between treatments on any specific day. Stabilization of the BCF for *C.vulgaris* appears to be around 400 for an algal population exposed to constant Se levels as selenate. Comparison of these results with values in the literature (Foe and Knight 1986, USEPA 1987, Kiffney and Knight 1990, Besser et al. 1993) are difficult because of differences in technique. Standard algal toxicity tests are not well suited for determination of BCFs since they start with

a low cell density and are not renewed, resulting in a continuous decline in dissolved levels as the algal biomass increases exponentially. Some researchers have exposed a concentrated algal culture, with limited growth during exposure, but the same situation occurs with declines in chemical levels as the algae take up the compound (Besser et al. 1993). These are first long-term population level BCFs calculated for an alga based on a stable algal population and stable Se concentrations in solution.

At the rotifer (*Brachionus calyciflorus*) trophic level a number of different responses to Se exposure were observed. All treatment levels had lower biomass then the control during the experiment (Tables 14, 15 and Figure 8). At the 400 $\mu$g/L level the impact of the Se was swift, with population essentially dead by day 4, with a similar result at the 200 $\mu$g/L except the *B. calyciflorus* population was dead by day 6 (Table 14 and Figure 8). It should be noted that dry weights below 0.005 mg/ml are at the limits of the precision of the filtering and weighing technique used. A more subtle impact was observed at the 100 $\mu$g/L treatment level with no statistically difference on a daily basis until day 11 (Table 14). However, days 14 and 17 were not statistical different from the control, whereas days 20, 24, and 25 were depending on the significance level chosen (Table 14). A clearer picture is evident if the control and 100 $\mu$g/l treatments are compared by pooling the data from the different days and then using repeated measures ANOVA. The

biomass levels in the two treatments were overall significantly different ($\alpha = 0.05$) using this technique (Tables 14 and 15).

There is no laboratory data available on the toxicity of Se to rotifers in the literature to directly compare to our results; however, the lowest effect level for an invertebrate is 65.4 µg/L for the amhipod, *Gammarus pseudolimnaeus* exposed to selenate in an acute test (USEPA 1987). Salki, et al. (1985) used lake enclosures to examine what effect 1, 10, and 100 µg/L Se as selenate would have on the zooplankton community (dominated by crustaceans) and found no changes in the community. The LOEC effect level reported here for a reduction in population growth of B. calyciflorus at a measured concentration of 108.1 µg/L is among the lowest values observed for aquatic invertebrates (USEPA 1987).

The levels of Se in the rotifers almost exactly matched on a daily basis the levels found in the algae (Tables 12 and 16). Because of the rapid decline in the rotifer populations at the 200 and 400 µg/l levels, there is only limited data on loading for these treatments (Table 16 and Figure 9). The Se levels in the rotifers rapidly increased until day 11, and were relatively stable until day 20 when a rapid decline in tissue concentrations began (Table 16 and Figure 9). The life span of individual *B. calyciflorus* is approximately 7 days at 25 °C, so this decline appears to be unrelated to changes in the population. Rotifer density was decreasing at this time and this may be a factor. The levels do, except for day 25, follow closely those values observed in the algae at this

treatment level. The value on day 25 should be viewed with caution because the test was terminated then due to failure of the test system. It should be noted that the values reported here include any algae within the gut of the rotifers at the time of sampling. This is the most appropriate representation of the amount of Se in the rotifer as food for organism at the next trophic level. There is no evidence that dissolved Se levels declined at all during this period of the test.

BCFs in the algae paralleled the levels found in the tissues, ranging from 115 to 538 (Table 17 and Figure 10). It appears that BCFs were generally lower at the higher treatment levels then at the 100 $\mu$g/L, although the higher levels never reached equilibrium. BCFs were similar to those observed with the algae. There was an initial rapid rise in BCFs until day 11, where it stabilized around 500 and then declined on day 20.

With the rotifer population at the 200 and 400 $\mu$g/L levels rapidly declining (Figure 8), it is not surprising that the larval fathead minnows were also impacted at these levels (Table 18). At 400 $\mu$g/L the minnows did not grow appreciable and even lost weight between days 7 and 11. Because fish mortality rapidly increased at the 400 $\mu$g/L level from day 8 on, all the remaining individuals were sacrificed on day 11. A similar was pattern was observed at 200 $\mu$g/L. Some growth occurred during the first 7 days followed by declining weights until day 16 when the remaining individuals were scarified due to high mortality (Table 18). There was also a reduction in fish dry weight

at 100 µg/L on days 20 and 25. Overall growth was reduced at the 100 µg/L treatment level compared to the control. The LOEC for the fatheads was 108.1 µg/L with no NOEC being determined. The reduction in larval dry weight summarized the impact of Se on the food chain, since the fish represent the highest trophic level. In a food-only experiment, selenate has been reported to reduced larval growth at 566 µg/L (USEPA 1987). Halter et al. (1980) reported a 14 day $LC_{50}$ of 600 µg/L using 30 day old larvae exposed to selenate in the water only. The lowest value reported value for a water only exposure is an LOEC of 300 µg/L after 30 days (Adams and Johnson 1981). It is clear that when fathead minnows are exposed to both water borne and food routes of Se exposure that toxicity is increased.

Se levels in the whole body of the larval minnows were generally higher at the upper treatment levels and increased during the test (Table 19 and Figure 11). Because of mortality at 400 µg/L, there were only two measurements (Table 19) taken which showed a decline in tissue levels. This was probably due the fact they these minnows were losing weight. At 200 µg/L tissues levels increased from 75.3 on day 7 to 89.0 µg/g on day 16 when all surviving fish were scarified. At 100 µg/L level whole body concentrations of Se ranged from 47.5 on day 4 to 76.0 µg/g on day 20 (Table 19 and Figure 11). Levels in the tissues rose until day 20 then declined slightly on day 25 (Figure 11), paralleling the fluctuations seen in the Se content of the rotifers, and algae (Figures 6 and 9). The concentration of Se in the fish was generally slightly

higher or the same as the concentration found at the same treatment levels in the rotifers (Tables 16 and 19. Bennett, et al. (1986) found whole body Se levels between 51.7 and 61.1 μg/g in larval minnows fed rotifers containing between 46 and 91 μg/g Se, which is in general agreement with our results.

BCFs were dependent on dose ranged from a low of 154 at 400 μg/L on day 11 to a maximum of 711 at 100 μg/L on day 20 (Table 20 and Figure 12). Values at 200 μg/L were relatively stable, only increasing from 320 on day 7 to 379 on day 16 (Figure 12). It should be noted that fish dry weight was decreasing during this period (Table 18). At 100 μg/L BCFs increased from 445 on day 7 to 711 on day 20, then dropped to 574 on day 25 (Table 20 and Figure 12). These concentrations are in general agreement with concentration factors found by other researchers (USEPA 1987).

## CONCLUSION

Our results support the work of earlier researchers (Cumbie and VanHorn 1978, Lemly 1985, Ingersoll et al. 1990, Hermanutz et al. 1992, Besser et al. 1993, Coyle et al. 1993) who found that selenium had a negative impact on aquatic biota at concentrations less then 100 μg/L, and as low as 10 μg/L in some food chain studies. Most studies that have found fish to be sensitive to Se only when exposed to long-term exposure from both the food and water. Generally the food chain studies that have detected an impact to the top trophic level at low Se concentrations, have not detected any impact to the food

organisms. In this research, selenium as selenate, was found to reduce larval fathead minnow biomass at 108.1 μg/L, and impaired both the algal and rotifer population growth rates at similar Se concentrations. The effects observed to the lower trophic levels were subtle and could have easily been undetected in a less controlled experiment.

The values observed for accumulation and bioconcentration of Se are in general agreement with what has been reported by other researchers (USEPA 1987, Besser et al. 1993). The question of whether all three test organism were in equilibrium with the exposure levels is undetermined. Selenium concentration in the test organisms fluctuated, initial by rising to a distinct peak and then declining. Fluctuations at the higher trophic levels parallelled changes in the algae suggesting that these trophic levels were in equilibrium with the exposure levels. Other researchers (Besser et al. 1993) have reported that algae reach their maximum Se uptake within 24 hours and *Daphnia* do so between 24 and 96 hours. For the two lower trophic levels that were growing exponentially it appears likely that Se concentrations in the tissues were in equilibrium with the Se they were exposed. The fluctuations in Se concentration in the algae suggest that the algal population was regulating the amount of Se taken up and that this quantity was decreasing as the population was exposed. Since the levels in the larval fathead minnows did not plateau, it difficult to determine whether the fish had reached steady-state. If the fluctuations are attributed to changes in the quantity of Se in the food supply

then it is probably that the Se concentrations in the larval minnows were at equilibrium with their environment. It should be noted that in contrast to many food chain experiments, there was a substantial increase in biomass over the duration of the experiment. Larval fathead minnows increase their weight at the 100 $\mu$g/L treatment by a factor of 10 between days 7 and 25. Overall the three-trophic level test system was well designed to evaluate the impact of Se on aquatic biota.

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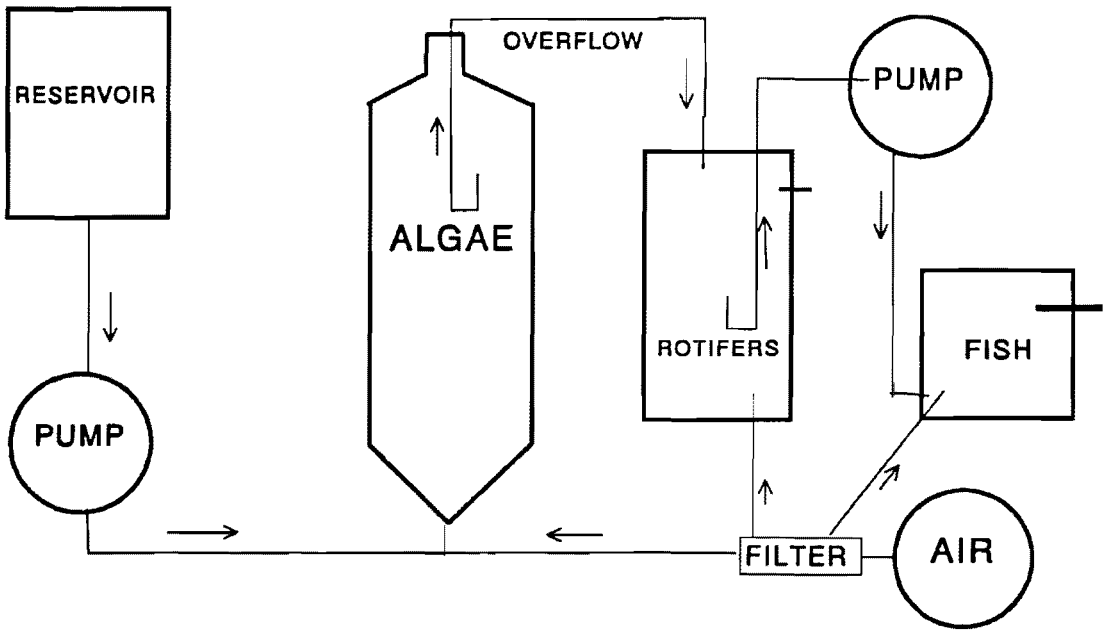


**Figure 1: Diagram of Trophic Test System**

Table 1. Temperature program for the determination of selenium using a Perkin-Elmer HGA 300 graphite furnace.

| Step | Temperature (°C) | Ramp Time (s) | Hold Time (s) | Function |
|---|---|---|---|---|
| 1 | 90 | 1 | 15 | |
| 2 | 130 | 15 | 15 | |
| 3 | 1200 | 5 | 30 | |
| 4 | 20 | 1 | 15 | Stop Flow |
| 5 | 2200 | 0 | 5 | Stop Flow, Read |
| 6 | 2650 | 1 | 10 | |
| 7 | 20 | 1 | 4 | |

Table 2. Measured Se concentrations ($\mu$g/L) in test solution reservoirs for algal screening test (n = 8).

| NOMINAL CONC. (ppb) | TOTAL | | DISSOLVED | | RATIO |
|---|---|---|---|---|---|
| | MEAN | STD ERR | MEAN | STD ERR | |
| 10 | 8.3 | 0.3 | 8.7 | 0.2 | 1.05 |
| 100 | 81.7 | 1.5 | 79.5 | 1.8 | 0.97 |
| 1000 | 1053.7 | 7.6 | 1045.2 | 12.5 | 0.99 |

Table 3. Dissolved Se concentrations ($\mu$g/L) in algal chemostats during the screening test. Means are based on 2 replicates per treatment per day, except on day 7 where the samples were pooled.

| DAY | 10 | | 100 | | 1000 | |
|---|---|---|---|---|---|---|
| | MEAN | SD | MEAN | SD | MEAN | SD |
| 0 | 9.3 | 0.9 | 84.7 | 2.6 | 1047.6 | 6.7 |
| 2 | 9.2 | 0.0 | 82.4 | 0.7 | 1012.2 | 23.4 |
| 3 | 7.9 | 0.4 | 80.0 | 1.8 | 1014.6 | 20.0 |
| 4 | 8.4 | 0.4 | 79.8 | 2.2 | 974.5 | 70.1 |
| 6 | 10.8 | - | 86.0 | - | 920.2 | - |
| 7 | -* | - | -* | - | 1028.7 | - |
| MEAN | 9.1 | | 82.6 | | 999.6 | |
| STDERR | 0.4 | | 1.1 | | 18.7 | |

* = Sample lost.

Table 4. Algal biomass in Se screening test (mg/ml). Means are based on 2 replicates per treatment, except on day 7 where the samples were pooled. The mean for each treatment across days does not include day 0, because these values are prior to dosing.

| DAY | 0 μg/L | | 10 μg/L | | 100 μg/L | | 1000 μg/L | | POOLED | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MEAN | SD | MEAN | SD | MEAN | SD | MEAN | SD | MEAN | STDERR |
| 0 | 0.141 | 0.011 | 0.131 | 0.008 | 0.115 | 0.013 | 0.136 | 0.000 | 0.131 | 0.004 |
| 1 | 0.132 | 0.026 | 0.124 | 0.007 | 0.123 | 0.006 | 0.130 | 0.001 | 0.127 | 0.004 |
| 2 | 0.128 | 0.016 | 0.118 | 0.015 | 0.109 | 0.001 | 0.116 | 0.001 | 0.118 | 0.004 |
| 3 | 0.132 | 0.012 | 0.118 | 0.008 | 0.099 | 0.002 | 0.109 | 0.009 | 0.114 | 0.005 |
| 4 | 0.121 | 0.002 | 0.110 | 0.009 | 0.093 | 0.015 | 0.095 | 0.012 | 0.105 | 0.005 |
| 7 | 0.110 | - | 0.112 | - | 0.106 | - | 0.100 | - | 0.007 | 0.003 |
| MEAN | 0.124 | | 0.116 | | 0.106* | | 0.110* | | 0.117 | |
| STDERR | 0.004 | | 0.002 | | 0.005 | | 0.006 | | 0.004 | |

* = Statistically different from the control at $\alpha$ = 0.05 based on ANOVA.

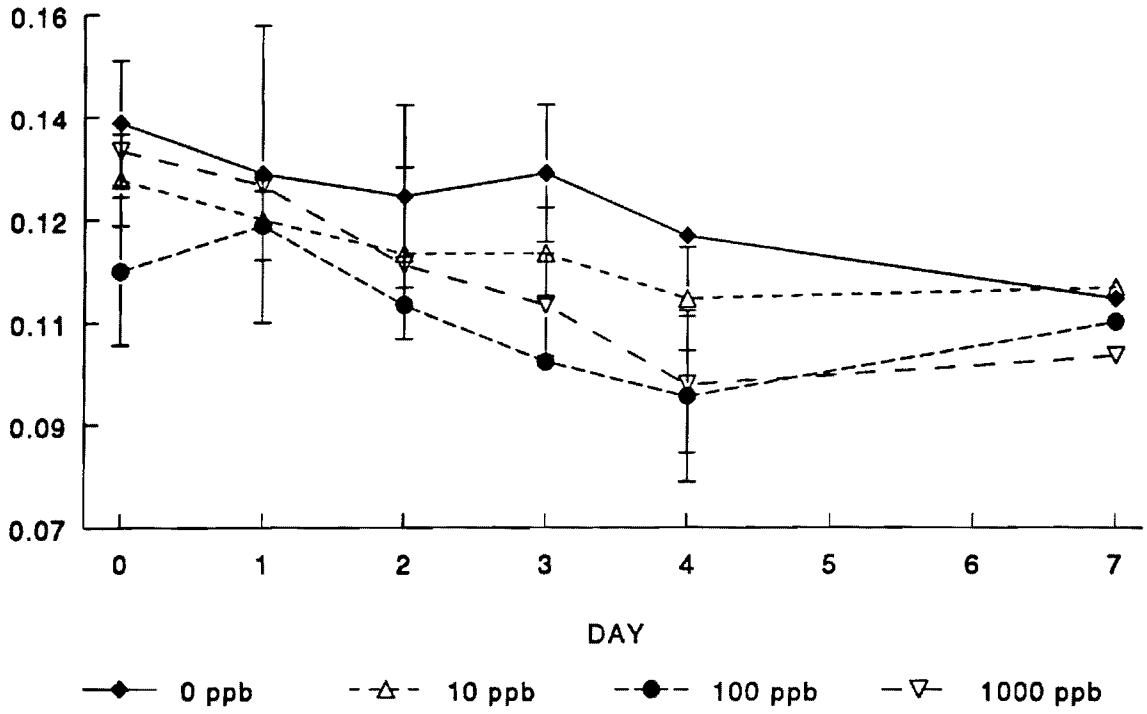


**Figure 2: Algal Screening Test**
**Mean algal biomass ± 1 sd**

Table 5. Se levels and corresponding bioconcentration factors (BCF) for the algal screening test. BCFs were calculated as the dissolved levels in the chemostats divided by the tissue concentration.

| DAY | 100 µg/L | | 1000 µg/L | |
|---|---|---|---|---|
| | MEAN | SD | MEAN | SD |
| | LOADING (ppm) | | | |
| 1 | 14.0 | 1.7 | 116.6 | 1.6 |
| 2 | 20.2 | 4.5 | 152.3 | 17.0 |
| 3 | 29.2 | 0.8 | 283.7 | 1.5 |
| 4 | 35.9 | 1.9 | 358.5 | 33.4 |
| 7 | 41.8 | - | 410.7 | - |
| | BCF | | | |
| 1 | 170 | 20 | 117 | 2 |
| 2 | 244 | 54 | 152 | 17 |
| 3 | 354 | 10 | 284 | 1 |
| 4 | 435 | 23 | 359 | 33 |
| 7 | 507 | - | 411 | - |

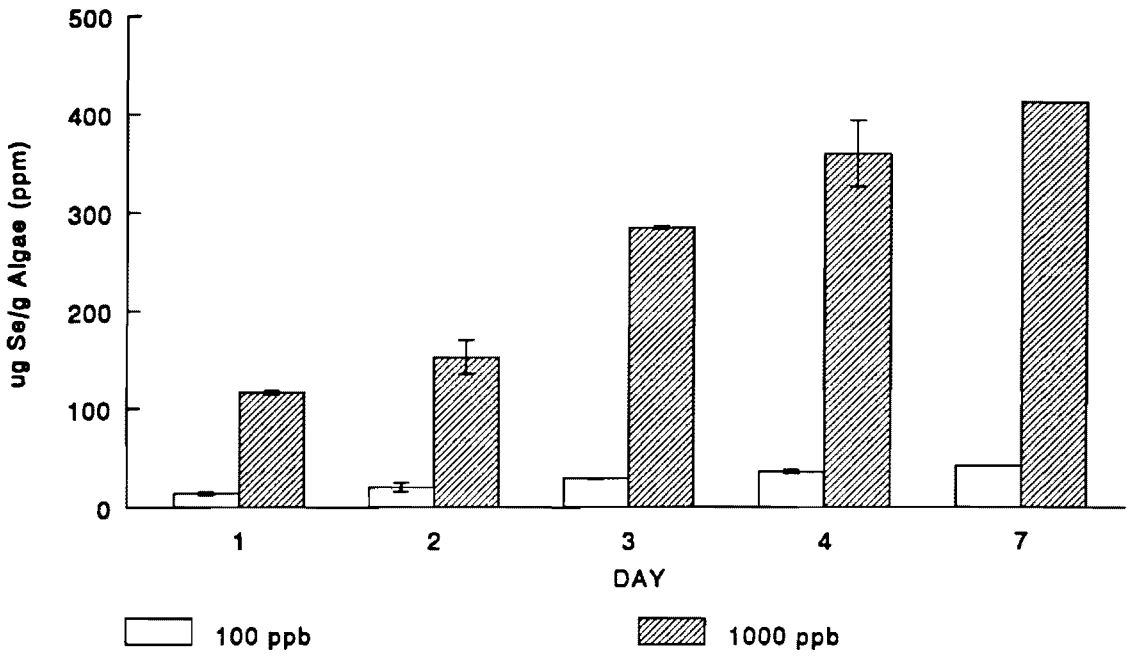


**Figure 3: Algal Screening Test**
**Se Accumulation in the algae (mean ± 1 sd)**

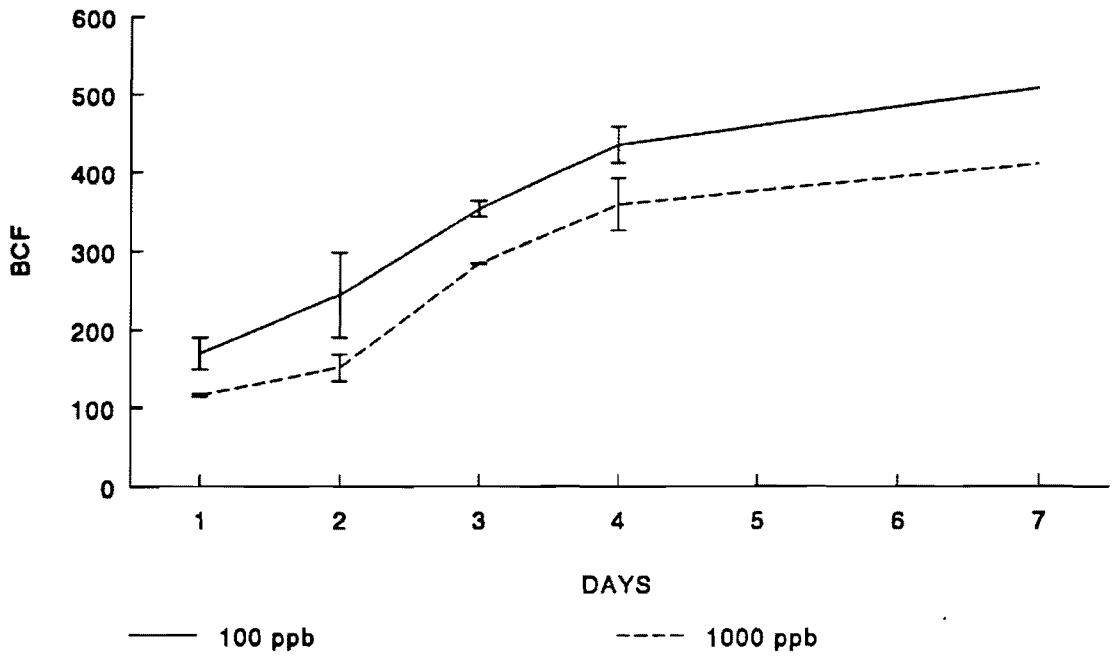


**Figure 4: Algal Screening Test**
BCF over time (mean ± 1 sd)

Table 6. Total Se concentrations (μg/L) in reservoirs over the 25-day trophic level test.

| NOMINAL CONC. (μg/L) | MEAN | SD | STDERR | MAX | MIN | N |
|---|---|---|---|---|---|---|
| 0 | < 2 | - | - | < 2 | < 2 | 7 |
| 100 | 108.1 | 11.1 | 4.2 | 131.6 | 100.3 | 7 |
| 200 | 202.4 | 11.4 | 4.3 | 222.9 | 187.2 | 7 |
| 400 | 393.0 | 12.3 | 4.7 | 409.1 | 378.7 | 7 |

Table 7. Dissolved Se concentrations (μg/L) in algal chemostats over the 25-day trophic level test.

| NOMINAL CONC. (μg/L) | MEAN | SD | STDERR | MAX | MIN | N |
|---|---|---|---|---|---|---|
| 0 | < 2 | - | - | < 2 | < 2 | 15 |
| 100 | 105.5 | 9.9 | 2.6 | 126.9 | 86.8 | 15 |
| 200 | 204.9 | 8.4 | 2.2 | 219.5 | 188.6 | 15 |
| 400 | 397.6 | 10.9 | 2.8 | 417.8 | 377.2 | 15 |

Table 8. Dissolved Se concentrations (μg/L) in rotifer chemostats over the 25 day trophic level test.

| NOMINAL CONC. (μg/L) | MEAN | SD | STDERR | MAX | MIN | N |
|---|---|---|---|---|---|---|
| 0 | < 2 | - | - | - | - | 5 |
| 100 | 110.3 | 12.5 | 5.6 | 127.0 | 94.1 | 5 |
| 200 | 207.7 | 7.6 | 3.8 | 213.5 | 197.3 | 4 |
| 400 | 396.1 | 13.0 | 7.5 | 409.1 | 383.0 | 3 |

Table 9. Dissolved Se concentrations (μg/L) in fish chambers over the 25 day trophic level test.

| NOMINAL CONC. (μg/L) | MEAN | SD | STDERR | MAX | MIN | N |
|---|---|---|---|---|---|---|
| 0 | < 2 | 0 | 0 | - | - | 6 |
| 100 | 106.8 | 4.5 | 1.8 | 112.0 | 100.8 | 6 |
| 200 | 235.1 | 31.1 | 15.6 | 280.7 | 214.7 | 4 |
| 400 | 389.1 | 17.1 | 8.5 | 411.0 | 369.6 | 4 |

Table 10. Algal biomass (mg/ml) in Se trophic level tests, based on 2 replicates per treatment for each day. There were no significant differences at $p = 0.05$ based on ANOVA on any days.

| DAY | 0 | | 100 | | 200 | | 400 | | DAILY | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MEAN | SD | MEAN | SD | MEAN | SD | MEAN | SD | MEAN | SD |
| 0 | 0.0828 | 0.0018 | 0.0831 | 0.0004 | 0.0809 | 0.0000 | 0.0825 | 0.0009 | 0.0823 | 0.0010 |
| 2 | 0.0817 | 0.0019 | 0.0806 | 0.0059 | 0.0762 | 0.0025 | 0.0713 | 0.0062 | 0.0774 | 0.0047 |
| 4 | 0.0686 | 0.0067 | 0.0675 | 0.0098 | 0.0600 | 0.0034 | 0.0620 | 0.0078 | 0.0645 | 0.0042 |
| 6 | 0.0674 | 0.0070 | 0.0676 | 0.0113 | 0.0565 | 0.0041 | 0.0538 | 0.0016 | 0.0613 | 0.0072 |
| 7 | 0.0687 | 0.0089 | 0.0688 | 0.0127 | 0.0595 | 0.0028 | 0.0606 | 0.0099 | 0.0644 | 0.0050 |
| 11 | 0.0637 | 0.0098 | 0.0657 | 0.0103 | 0.0576 | 0.0030 | 0.0539 | 0.0060 | 0.0602 | 0.0055 |
| 14 | 0.0659 | 0.0089 | 0.0596 | 0.0004 | 0.0563 | 0.0012 | 0.0557 | 0.0082 | 0.0594 | 0.0047 |
| 17 | 0.0565 | 0.0019 | 0.0541 | 0.0004 | 0.0549 | 0.0044 | 0.0537 | 0.0012 | 0.0548 | 0.0012 |
| 20 | 0.0485 | 0.0015 | 0.0452 | 0.0004 | 0.0457 | 0.0069 | 0.0505 | 0.0022 | 0.0475 | 0.0025 |
| 24 | 0.0753 | 0.0047 | 0.0636 | 0.0027 | 0.0761 | 0.0084 | 0.0706 | 0.0124 | 0.0714 | 0.0057 |
| 25 | 0.0523 | 0.0032 | 0.0391 | 0.0022 | 0.0508 | 0.0082 | 0.0453 | 0.0118 | 0.0469 | 0.0060 |

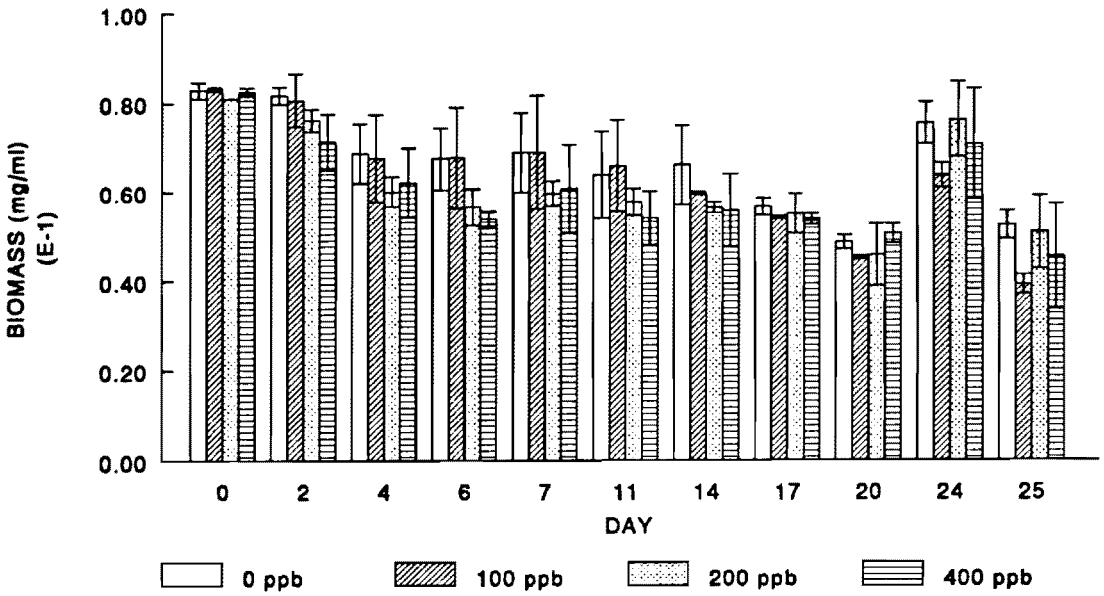


**Figure 5: Three-Trophic Level Test**
**Mean algal biomass ± 1 sd ( as dry weight)**

Table 11. Summary of algal biomass (mg/ml) for all days in Se trophic level test. Day 0 weights were not include because they are pre-exposure values.

| NOMINAL CONC. (µg/L) | MEAN | SD | STDERR | MAX | MIN | N |
|---|---|---|---|---|---|---|
| 0 | 0.0649 | 0.0109 | 0.0024 | 0.0830 | 0.0474 | 20 |
| 100 | 0.0612 | 0.0130 | 0.0029 | 0.0848 | 0.0375 | 20 |
| 200 | 0.0594 | 0.0102 | 0.0023 | 0.0821 | 0.0409 | 20 |
| 400 | 0.0577 | 0.0099 | 0.0022 | 0.0794 | 0.0369 | 20 |

Table 12. Se concentrations in algae during trophic level test. All values are $\mu$g Se/g biomass (ppm).

| DAY | 100 | | 200 | | 400 | |
|---|---|---|---|---|---|---|
| | MEAN | SD | MEAN | SD | MEAN | SD |
| 0 | 0.0 | 0.0 | 5.5 | 0.2 | 7.8 | 1.4 |
| 2 | 28.9 | 4.6 | 52.2 | 3.2 | 103.2 | 5.6 |
| 4 | 38.1 | 3.5 | 71.9 | 3.9 | 156.1 | 2.8 |
| 6 | 49.2 | 3.7 | 99.6 | 2.5 | 178.4 | 5.0 |
| 7 | 39.1 | 2.5 | 94.4 | 2.6 | 185.6 | 13.0 |
| 11 | 58.3 | 3.9 | 127.0 | 0.0 | 266.8 | 5.3 |
| 14 | 52.3 | 17.7 | 118.3 | 5.0 | 140.3 | 5.0 |
| 17 | 34.5 | 21.2 | 84.9 | 6.9 | 181.6 | 12.3 |
| 20 | 41.4 | 3.3 | 82.6 | 0.9 | 161.7 | 3.9 |
| 24 | 31.3 | 1.8 | 73.7 | 2.5 | 159.0 | 8.4 |
| 26 | 39.0 | 0.8 | 97.5 | 6.0 | 162.8 | 12.1 |

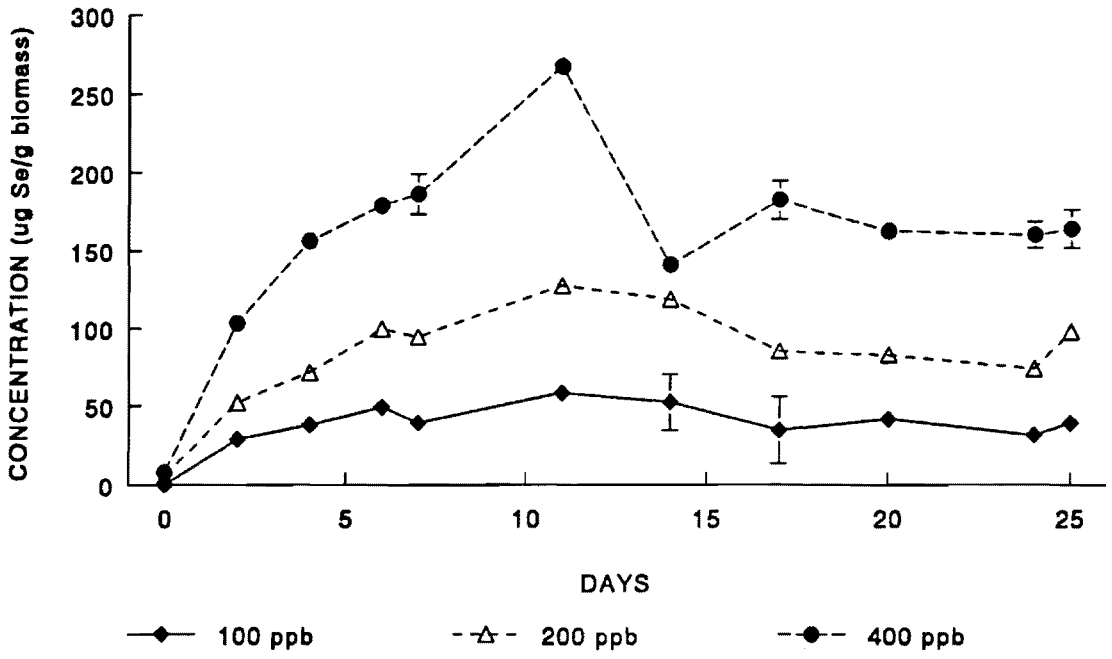


**Figure 6: Three-Trophic Level Test**
**Mean Se Concentrations (± 1 sd) in the algae**

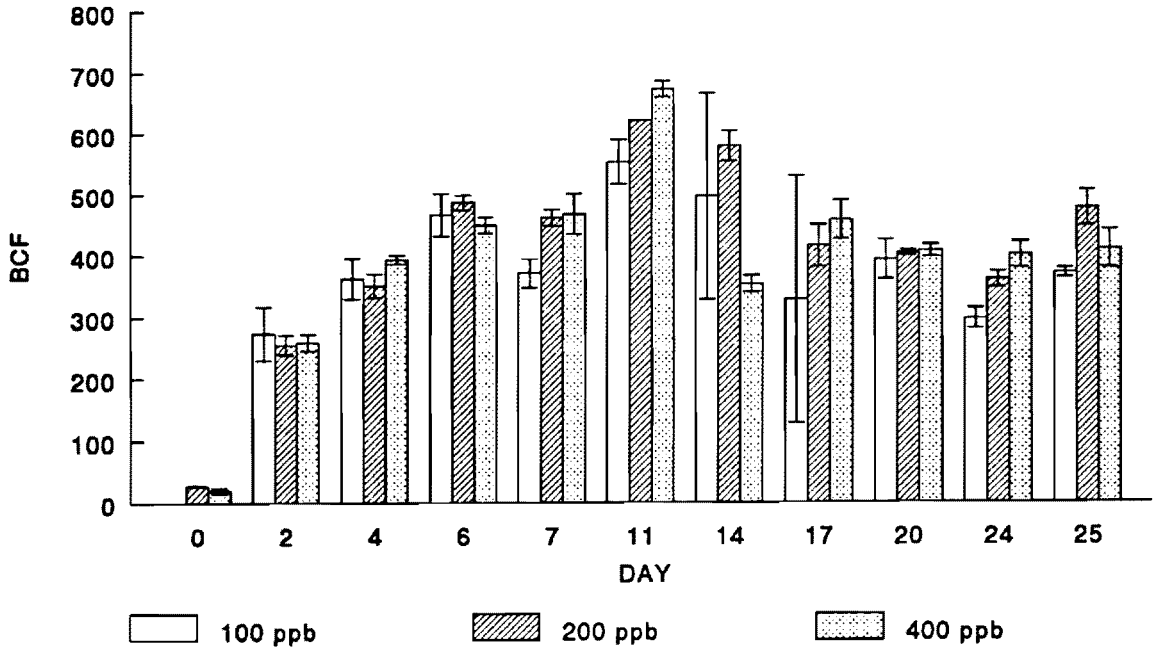


**Figure 7: Three-Trophic Level Test**
**Mean BCFs (± 1 sd) for algae**

Table 13. Bioconcentration factors (BCF) for the algae in the trophic level test. BCFs were calculated as the tissue concentration divided by the dissolved concentrations in the chemostats.

| DAY | 100 | | 200 | | 400 | | DAILY | |
|---|---|---|---|---|---|---|---|---|
| | MEAN | SD | MEAN | SD | MEAN | SD | MEAN | STDERR |
| 0 | <LOD | - | 27 | 1 | 19 | 4 | 23 | 2 |
| 2 | 274 | 44 | 255 | 16 | 259 | 14 | 263 | 10 |
| 4 | 362 | 33 | 351 | 19 | 393 | 7 | 368 | 11 |
| 6 | 466 | 35 | 486 | 12 | 449 | 13 | 467 | 10 |
| 7 | 371 | 23 | 461 | 13 | 467 | 33 | 433 | 21 |
| 11 | 552 | 37 | 620 | 0 | 671 | 13 | 615 | 21 |
| 14 | 496 | 168 | 578 | 24 | 353 | 13 | 476 | 44 |
| 17 | 328 | 201 | 415 | 34 | 457 | 31 | 400 | 45 |
| 20 | 392 | 32 | 403 | 5 | 407 | 10 | 401 | 7 |
| 24 | 297 | 17 | 360 | 12 | 400 | 21 | 352 | 20 |
| 26 | 370 | 8 | 476 | 29 | 409 | 31 | 418 | 21 |

Table 14. Rotifer biomass (mg/ml) in Se trophic level test. Based on two replicates per treatment for each day. Pooled means do not include day 0 values because they did not reflect the impact of the treatments.

| DAY | 0 | | 100 | | 200 | | 400 | |
|---|---|---|---|---|---|---|---|---|
| | MEAN | SD | MEAN | SD | MEAN | SD | MEAN | SD |
| 0 | 0.0813 | 0.0135 | 0.1056 | 0.0414 | 0.1161 | 0.0197 | 0.0909 | 0.0008 |
| 2 | 0.0330 | 0.0004 | 0.0327 | 0.0046 | 0.0347 | 0.0053 | 0.0279 | 0.0052 |
| 4 | 0.0274 | 0.0046 | 0.0244 | 0.0046 | 0.0110* | 0.0009 | 0.0030* | 0.0014 |
| 6 | 0.0251 | 0.0017 | 0.0235 | 0.0008 | 0.0037* | 0.0021 | 0.0006* | 0.0009 |
| 7 | 0.0270 | 0.0031 | 0.0251 | 0.0004 | 0.0034* | 0.0032 | 0.0017* | 0.0015 |
| 11 | 0.0208 | 0.0026 | 0.0124* | 0.0023 | 0.0006* | 0.0003 | 0.0008* | 0.0004 |
| 14 | 0.0215 | 0.0026 | 0.0183 | 0.0004 | - | - | - | - |
| 17 | 0.0197 | 0.0016 | 0.0174 | 0.0064 | - | - | - | - |
| 20 | 0.0206 | 0.0028 | 0.0140† | 0.0016 | - | - | - | - |
| 24 | 0.0204 | 0.0026 | 0.0091* | 0.0013 | - | - | - | - |
| 25 | 0.0220 | 0.0048 | 0.0082† | 0.0009 | - | - | - | - |
| POOLED | 0.0238 | 0.0047 | 0.0185* | 0.0008 | | | | |

* = Statistically different based on ANOVA at $\alpha = 0.05$.
† = Statistically different based on ANOVA at $\alpha = 0.10$.

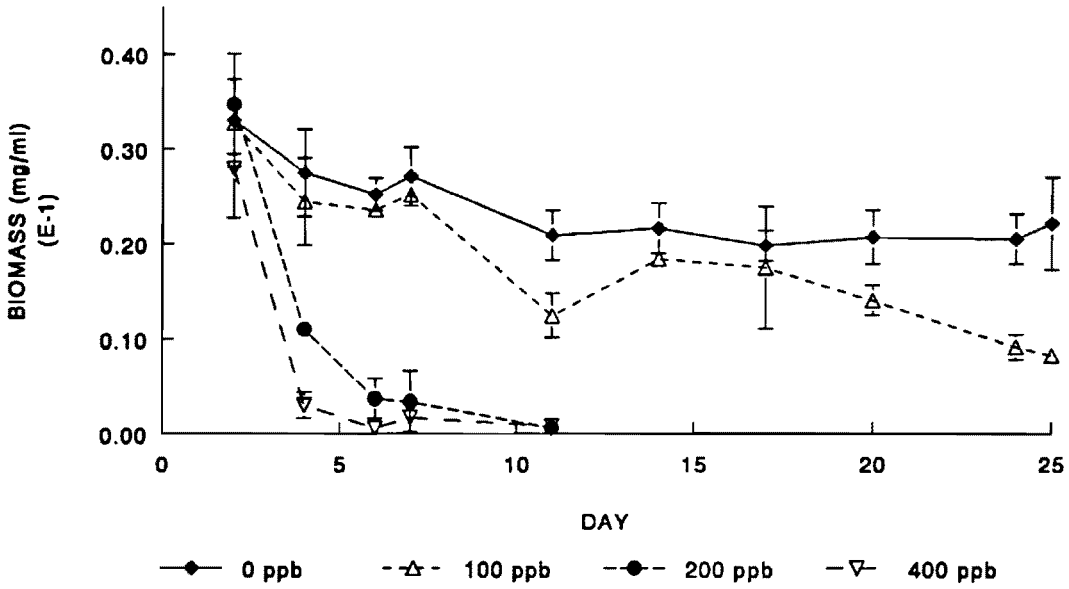


**Figure 8: Three-Trophic Level Test**
**Mean rotifer biomass ± 1 sd (as dry weight)**

Table 15. Summary of rotifer biomass for all days in Se trophic level test.

| NOMINAL CONC. (μg/L) | MEAN | SD | STDERR | MAX | MIN | N |
|---|---|---|---|---|---|---|
| 0 | 0.0238 | 0.0047 | 0.0010 | 0.0332 | 0.0185 | 20 |
| 100 | 0.0185 | 0.0080 | 0.0018 | 0.0359 | 0.0076 | 20 |
| 200 | 0.0107 | 0.0134 | 0.0042 | 0.0385 | 0.0004 | 10 |
| 400 | 0.0068 | 0.0113 | 0.0036 | 0.0316 | 0.0000 | 10 |

Table 16. Se concentrations in rotifers during trophic level test. All values are μg Se/g biomass (ppm).

| DAY | 100 | | 200 | | 400 | |
|---|---|---|---|---|---|---|
| | MEAN | SD | MEAN | SD | MEAN | SD |
| 0 | < LOD | - | < LOD | - | < LOD | - |
| 2 | < LOD | - | 28.2 | 10.1 | 45.5 | 12.6 |
| 4 | 40.0 | 4.3 | 53.5 | 9.2 | 74.8 | 7.2 |
| 6 | 46.4 | 3.7 | 74.5 | 8.7 | < LOD | - |
| 7 | 44.8 | 5.9 | 71.1 | 10.1 | < LOD | - |
| 11 | 59.7 | 4.2 | < LOD | - | < LOD | - |
| 14 | 56.9 | 3.9 | < LOD | - | < LOD | - |
| 17 | 59.4 | 0.7 | < LOD | - | < LOD | - |
| 20 | 42.6 | 4.4 | < LOD | - | < LOD | - |
| 24 | 32.9 | 4.7 | < LOD | - | < LOD | - |
| 25 | 13.1 | 2.6 | < LOD | - | < LOD | - |

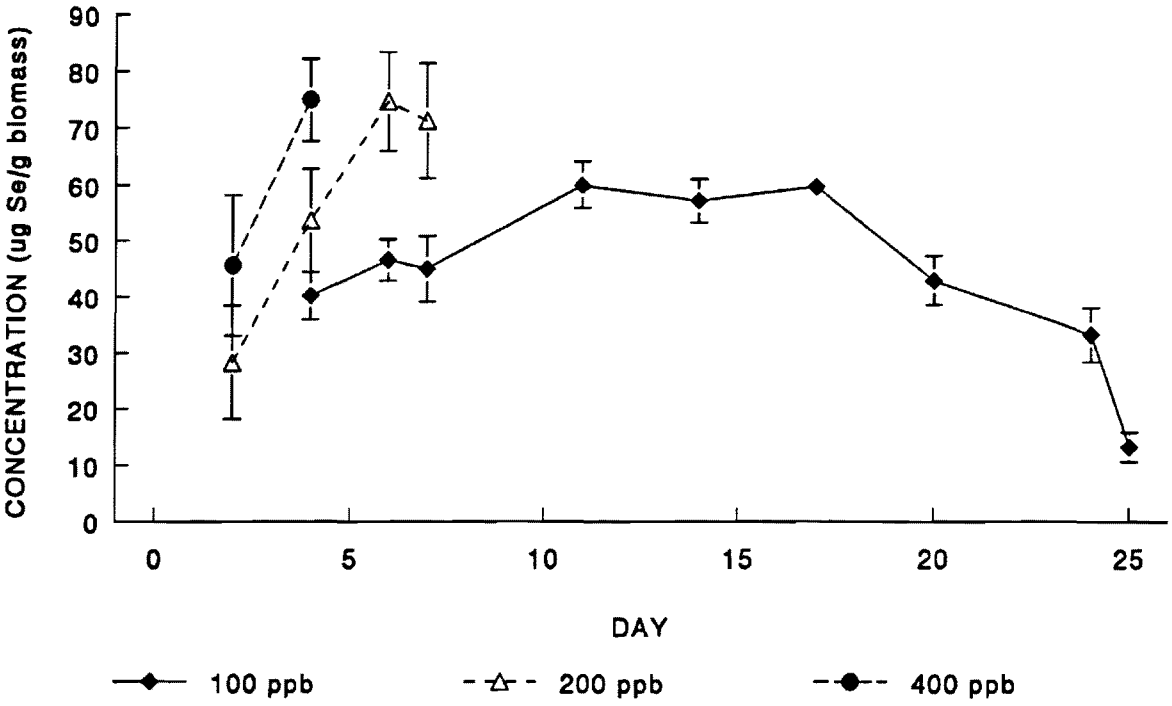


**Figure 9: Three-Trophic Level Test**
**Mean Se concentration (± 1 sd) in the rotifers**

Table 17. Bioconcentration factors (BCF) for the rotifers in the trophic level test. BCFs were calculated as the tissue concentration divided by the dissolved concentrations in the chemostats.

| DAY | 100 | | 200 | | 400 | |
|---|---|---|---|---|---|---|
| | MEAN | SD | MEAN | SD | MEAN | SD |
| 0 | < LOD | - | < LOD | - | < LOD | - |
| 2 | < LOD | - | 136 | 49 | 115 | 32 |
| 4 | 362 | 39 | 258 | 44 | 189 | 18 |
| 6 | 421 | 33 | 359 | 42 | < LOD | - |
| 7 | 406 | 53 | 342 | 49 | < LOD | - |
| 11 | 541 | 38 | < LOD | - | < LOD | - |
| 14 | 516 | 36 | < LOD | - | < LOD | - |
| 17 | 538 | 6 | < LOD | - | < LOD | - |
| 20 | 386 | 40 | < LOD | - | < LOD | - |
| 24 | 299 | 43 | < LOD | - | < LOD | - |
| 25 | 119 | 24 | < LOD | - | < LOD | - |

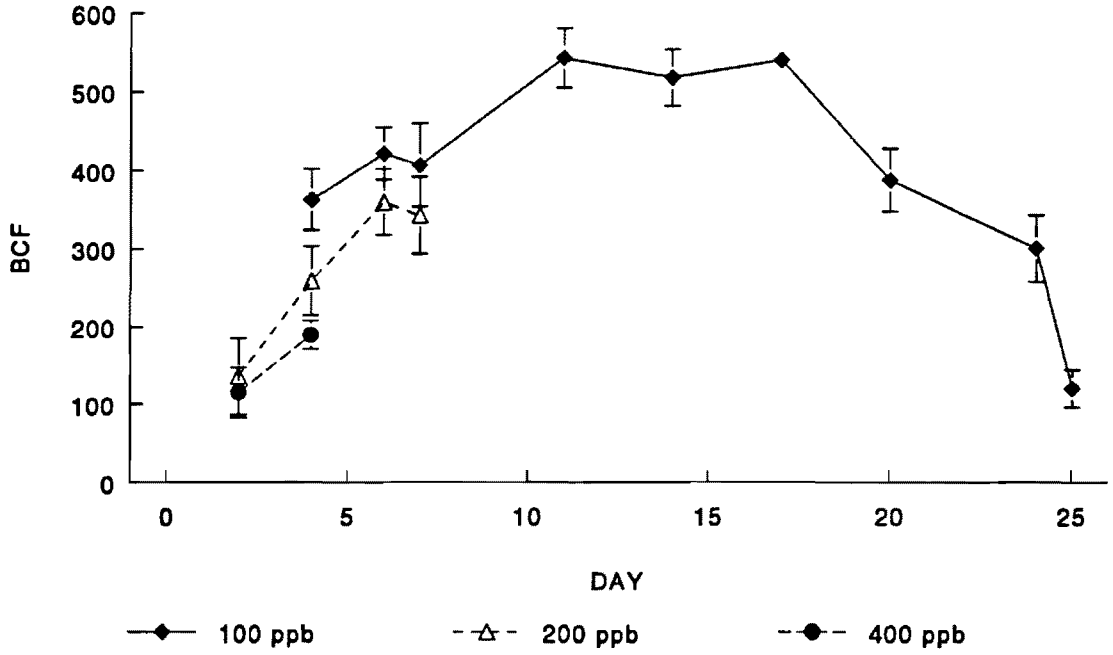


**Figure 10: Three-Trophic Level Test**
**Mean BCFs (± 1 sd) for the rotifers**

Table 18. Fathead minnow biomass (mg) in Se trophic test at the four treatment levels (μg/L). Fish were weighed on an individual basis with 15 or 16 individuals weighed for each treatment level on days 7-14, except at 200 μg/l on day 11 where 19 fish were weighed. Between 8 and 11 individuals were weighed for each treatment on days 20 and 25.

| DAY | 0 | | 100 | | 200 | | 400 | |
|---|---|---|---|---|---|---|---|---|
| | MEAN | STDERR | MEAN | STDERR | MEAN | STDERR | MEAN | STDERR |
| 7 | 0.922 | 0.063 | 0.822 | 0.050 | 0.420* | 0.028 | 0.203* | 0.015 |
| 11 | 2.019 | 0.205 | 1.739 | 0.159 | 0.389* | 0.038 | 0.178*† | 0.004 |
| 14 | 3.062 | 0.240 | 2.554 | 0.215 | 0.238* | 0.017 | - | - |
| 16 | - | - | - | - | 0.186*† | 0.024 | - | - |
| 20 | 7.559 | 0.952 | 4.635 | 0.335 | - | - | - | - |
| 25 | 15.885 | 1.815 | 7.778 | 0.889 | - | - | - | - |

* = Statistically different from control weight on that day at $\alpha = 0.05$.
† = Fish were dying rapidly so all surviving individuals were scarified on this day.

Tables 19. Se concentrations in fish whole bodies during trophic level test. All values are $\mu$g Se/g biomass (ppm)

| DAY | 100 | | 200 | | 400 | |
|---|---|---|---|---|---|---|
| | MEAN | SD | MEAN | SD | MEAN | SD |
| 7 | 47.5 | 2.2 | 75.3 | 7.6 | 73.6 | 1.1 |
| 11 | 55.9 | 13.3 | 77.4 | - | 59.9 | 7.1 |
| 14 | 70.3 | 2.3 | 82.7 | 1.8 | - | - |
| 16 | - | - | 89.0 | 9.7 | - | - |
| 20 | 76.0 | 1.3 | - | - | - | - |
| 25 | 61.4 | 4.0 | - | - | - | - |

Table 20. Bioconcentration factors (BCF) for the fish in the trophic level test. BCFs were calculated as the whole body tissue concentration divided by the dissolved concentrations in the chemostats.

| DAY | 100 | | 200 | | 400 | |
|---|---|---|---|---|---|---|
| | MEAN | SD | MEAN | SD | MEAN | SD |
| 7 | 445 | 21 | 320 | 32 | 189 | 3 |
| 11 | 523 | 124 | 329 | - | 154 | 18 |
| 14 | 658 | 22 | 352 | 8 | - | - |
| 16 | - | - | 379 | 41 | - | - |
| 20 | 711 | 12 | - | - | - | - |
| 25 | 574 | 38 | - | - | - | - |

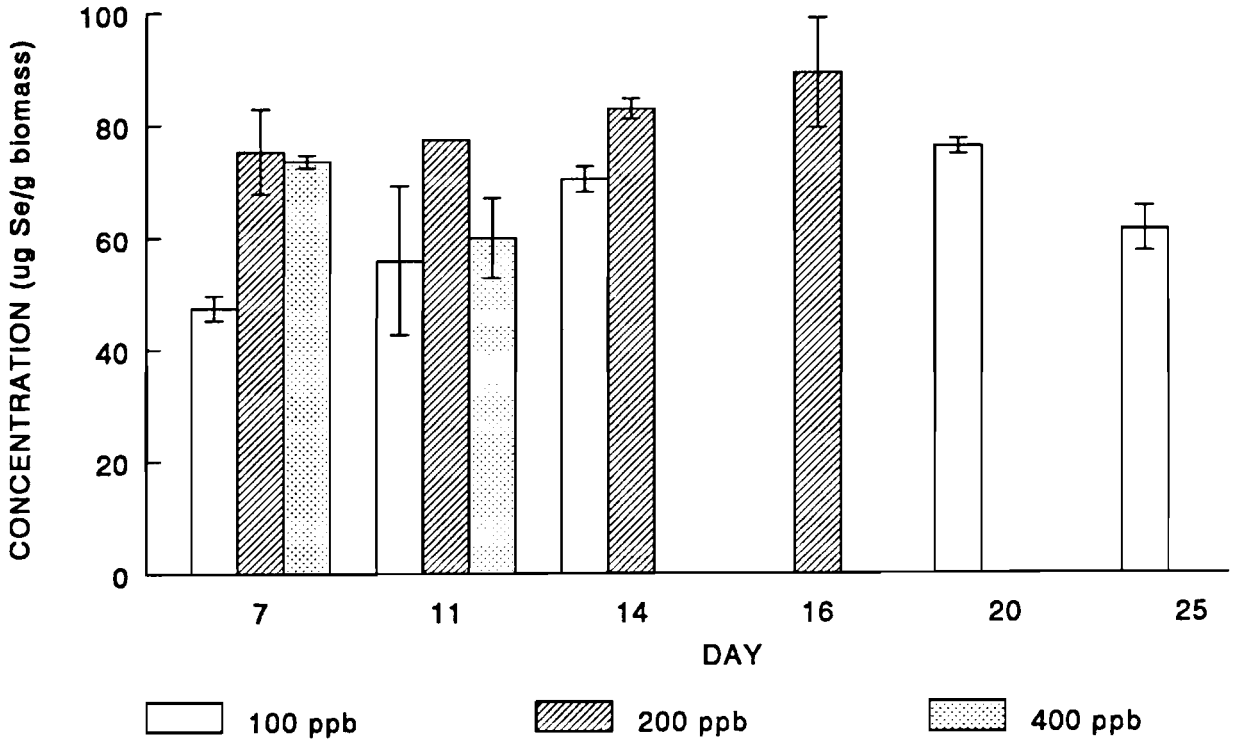


**Figure 11: Three-Trophic Level Test**
**Mean Se (± 1 sd) concentrations in the fish**

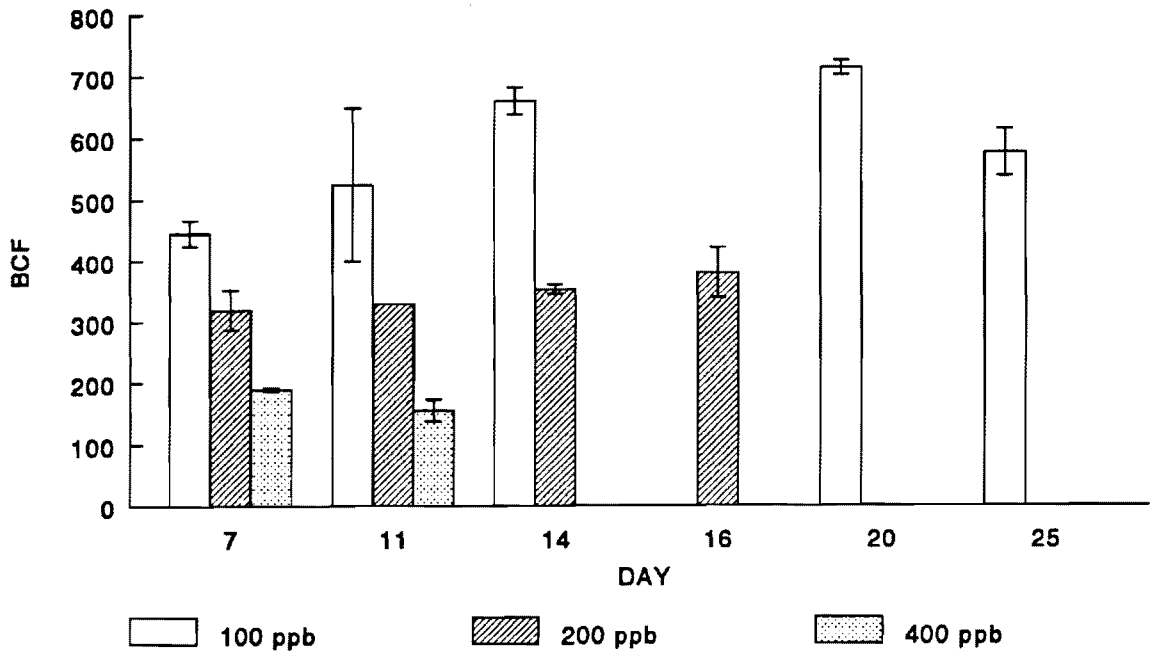


**Figure 12: Three-Trophic Level Test**
**Mean BCFs (± 1 sd) for the fish**

# CHAPTER 4: SUMMARY

## SUMMARY

This research deals with developing, testing, and validating a three-trophic level test system. The three-trophic level toxicity test is a simple linear food chain that allows one quantify the impact a chemical has on the interaction between different trophic levels. It provides an enormous advantage over single species tests, that by design limit possible conclusions about a chemical's toxicity only to the species tested.

The first part of this research focuses on developing the three-trophic level test system used to study chemical transfer up a trophic ladder and interactions between the different trophic levels. While other researchers have conducted food chain studies, the focus has generally been on the transfer of a chemical between levels and its bioaccumulation, especially at the top trophic level. While bioaccumulation is important, a chemical can also have sublethal impacts on one trophic level that are magnified up the trophic ladder. The organisms chosen for the three-trophic level test system (*Chlorella vulgaris, Brachionus calyciflorus,* and *Pimephales promelas*) are well suited for use in a laboratory food chain designed to model natural ecosystem functioning due to their rapid rates of reproduction and suitability as food for the trophic level above them. Successful development of the three-trophic level test system was made possible by relying on chemostats to culture the two lowest trophic levels. Use of chemostat technology at the two lower trophic levels made it possible to produce sufficient biomass to support the top level. Also use of

chemostats resulted in stable populations of algae and rotifers, and constant chemical concentrations, in contrast to batch culture techniques with changing populations and declining chemical concentrations. The three-trophic level test system was evaluated using copper and selenium as toxicants.

Copper and selenium are ideal chemicals to test the effectiveness of the three-trophic level test system because of their distinctly different modes of action and their influence upon of the species in the three-level food chain. Copper is more toxic to the algae at the bottom of the food chain, whereas fish at the top are the most sensitive to selenium. While Cu does bioaccumulate, direct toxicity from the water column generally is the principle mode of toxicity. Se is directly toxic to aquatic organism only at relatively high concentrations, but long term bioaccumulation of Se causes serous impairment of fish at extremely low levels.

Measurement of dry weight was found to be the most reliable parameter to estimate algal and rotifer biomass. Rotifer density after 7 days was found to be independent of the starting density and test container size. The system produced sufficient biomass at the lower trophic levels to support fathead minnow growth for at least 25 days. The fathead minnows that consume the rotifers were healthy and exhibited relatively high dry weights (0.762 ± 0.057 mg/fish after 7 days) compared to standard techniques. The algal chemostats rapidly responded to the addition of Cu and demonstrated the ability to recover from the initial exposure.

The LOEC for Cu was 31.5 μg/L, with a corresponding NOEC of 16.2 μg/L, based on an initial decline in algal biomass in the three-trophic level test. Algal density declined initially at all treatment levels above 31.5 μg/L, but then recovered to control levels by the end of the test. The rotifer population density paralleled algal fluctuations, but was not significantly different from the control at any treatment level. Fathead minnow survival was impaired after 7 days at the 62.7 μg/L Cu level. The impaired survival at this level may be due to the reduced quality of the rotifers as food for the larval minnows. The algae bioconcentrated Cu by a factor of approximately 11,000, with more Cu at the higher treatment levels. Fathead minnows had BCFs ranging from 300 at the highest concentration to 15,000 in the control. However, it does not appear that the minnows truly concentrated Cu since the amount of Cu in the fish was approximately the same at all treatment levels including the control. Whole body concentrations in the fish ranged from 14.4 to 18.6 μg Cu/g biomass, whereas the concentration in the water ranged from 1.1 to 62.7 μg/L, over an order of magnitude.

Comparison of the trophic level test results with single species tests found the single species tests to be more sensitive due to the higher levels of dissolved Cu in these tests. The principal route of Cu exposure in the three-trophic level test was through the food while the single species tests were water borne. The three-trophic level test illustrated a number of responses to Cu that were not detected by the single species tests. First the ability of the

higher trophic levels to endure declines in the algal population can not be detected with single species tests and demonstrates the robustness that natural ecosystems also posses. In addition, the enhancement of algal growth at the 31.5 μg/L level, although unexplained, was transferred up the food chain resulting in higher rotifer densities and fish weights. These two examples demonstrate two valuable properties, although diametrically opposed, of the three-trophic level toxicity test that it shares with natural ecosystem. The transfer of a response to a chemical stressor up a trophic ladder, and the ability of an ecosystem to compensate for it, are two essential functional aspects of ecosystems not addressed in single species testing.

In the selenium experiment all three trophic levels were affected at the lowest treatment concentration of 100 μg/L. This research has significance because it demonstrated a link between sublethal effects at the lower trophic levels and reduction in biomass at the top trophic level in a relatively short term exposure. Bioconcentration in the organisms was dose dependent in algae, but dependent upon the treatment level at the other two trophic levels. The alga, *ChLorella vulgaris*, had a BCF of around 400 at all treatment levels once they stabilized. However, Se levels in the algae fluctuated, suggesting that the algal population was regulating the amount of Se within the cells. When comparing these results with literature values it is important to note that these values were obtained with a chemostat culture, compared to the more commonly used batch culture testing. The rotifer population was impaired at the two highest

treatments to the extent that the population was not able to maintain itself by day 7. At 105.5 $\mu$g/L, impairment was more subtle with reduction in the standing crop not apparent until the second half of the exposure period. Levels of Se in the rotifers paralleled the algal levels, generally being about the same or slightly higher then the concentrations in the algae.

Fathead minnows were not viable at the two highest selenium concentrations. At 397.6 $\mu$g/L the impact may be due to direct toxicity, while at 204.9 $\mu$g/l the decline in rotifer populations most likely contributed to the low fish weights and high mortality. At 105.5 $\mu$g/L fish biomass was significantly lower then the control by the end of the exposure period probably reflecting a true "trophic impact" due to the decline in the rotifer population and dietary toxicity. In the Se experiment it was demonstrated that slight sublethal changes at the lowest trophic level can be magnified up the trophic ladder.

I believe that the field of ecotoxicology needs out less emphasis on single species tests and start using a hazard evaluation scheme that relies on multi-component test systems that more accurately reflect natural ecosystem complexity. This research has demonstrated the utility of one such system using two chemicals with very different modes of action.

# CURRICULUM VITAE
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Aquatic Ecosystem Health and Management Society
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American Society of Limnology and Oceanography
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Dobbs, M.G. December 1991. Development of a Trophic Toxicity Test utilizing an alga , rotifer, and a fish. Submitted to Graduate Research development Project, Graduate Student Assembly, VPI & SU. Received February 1992. $1000.

Cherry, D.S., J.R. Bidwell, and M.G. Dobbs. October 1992. Proposed Benthic, Macroinvertebrate Study of Brandywine Creek for Sonoco Products Company, Downingtown Plant, Chester County, Pennsylvania. Received November 1992, $10,000 **(12,000 1993, 24,000 Projected for 1994).**

Cherry, D.S., Stuart R. Lynde, M.G. Dobbs. December 1993. Development of a Study Plan to Test Toxicity and Environmental Effects of Didecyldimethylammoniumchloride (DDAC) to Non-target Organisms for Biofouling Control. $108,500.

Cherry, D.S. and M.G. Dobbs. April 1994. Control of *Corbicula* and *Dreissena* by Betz Laboratories' Chemicals. $24,000.

**PUBLISHED ABSTRACTS AND PRESENTATIONS:**

Dobbs, M.G., J.L. Farris, D.S. Cherry. 1991. Acute Toxicity Testing of Copper Using Site Water for Derivation of a Site-Specific Water Quality Criterion. Twelfth Annual meeting, Society of Environmental Toxicology and Chemistry, Cincinnati, OH. (Platform Presentation).

Dobbs, M.G., D.S. Cherry, and J. Cairns, Jr. 1992. The Development of a Flow-through Algal Toxicity Test Utilizing the Green Alga *Chlorella vulgaris*. 70th Annual meeting, Virginia Academy of Science, Richmond, VA. (Platform Presentation).

Dobbs, M.G., D.S. Cherry, and J. Cairns, Jr. 1992. A Flow-through Toxicity Test Designed to Form the Base of a Three-level Trophic Toxicity Test. Thirteenth Annual meeting, Society of Environmental Toxicology and Chemistry, Seattle, WA. (Poster-discussion).

Dobbs, M.G., J.L. Farris, R. Reash, and D.S. Cherry. 1993. Derivation of a Site-Specific Acute Copper Criterion for Blaine Creek, Kentucky using Single Species Toxicity tests. 3rd International Conference, Aquatic Ecosystem Health & the Ecological Significance of Bioassay Techniques, Blacksburg, VA. (Poster Presentation).

Bidwell, J.R., D.S. Cherry, and M.G. Dobbs. 1993. An Integrated Approach Toward Determining the Impact of a Paper Mill Effluent Upon a Receiving Stream. Fourteenth Annual meeting, Society of Environmental Toxicology and Chemistry, Houston, TX. (Poster Presentation).

Cherry, D.S., J.L. Farris, M.G. Dobbs, J.R. Bidwell, and E.P. Smith. 1993. Laboratory and *In-Situ* Studies Evaluating the Potential Toxicity of a Chemical-Sanitary Water Treatment Effluent in the New River, Virginia. Fourteenth Annual meeting, Society of Environmental Toxicology and Chemistry, Houston, TX. (Poster Presentation).

Dobbs, M.G., D.S. Cherry, and J. Cairns, Jr. 1993. Development of a Three-Trophic Level Toxicity Test. Fourteenth Annual meeting, Society of Environmental Toxicology and Chemistry, Houston, TX. (Poster Presentation).

Dobbs, M.G., D.S. Cherry, and J. R. Bidwell. 1994. The Importance of a Balanced Approach when assessing the Impact of a Paper Mill Effluent upon a Receiving System. Bulletin of the North American Benthological Society, Spring 1994 11(1):179.

Dobbs, M.G., D.S. Cherry, and J. Cairns, Jr. 1994. (In review) The Toxicity and Biomagnification of Se to a Three-Trophic Level Food Chain. Fifteenth Annual meeting, Society of Environmental Toxicology and Chemistry, Denver, CO.

**PUBLICATIONS:**

Farris, J.L. D.S. Cherry, W.R. Slagle and M.G. Dobbs. 1993. Suitability of Biological Indices to Evaluate Light Inhibition Effects from Treated Pulp Mill Effluents. TAPPI Proceedings, Atlanta GA pp. 253-259.

Dobbs, M. G., J.L. Farris, D. S. Cherry, R. Reash, and J. Carins, Jr. 1994. Evaluation of the Resident Species Procedure for Developing Site-Specific Water Quality Criteria for Copper in Blaine Creek. *Environ. Tox. Chem.* 13(6):963-972.

**MANUSCRIPTS IN PREPARATION:**

Lauth, J.R., M.G. Dobbs, D.S. Cherry, and T.W. Snell. (In prep). Replacement of Brine Shrimp, *Artemia* sp., with the Freshwater Rotifer *Brachionus calyciflorus*, as a Food Source for Larval Fathead Minnows Used for Toxicity Testing.

Cherry, D.S., M.G. Dobbs, J.L. Farris, and P.R. Scheuerman. (In prep). The Impact of Road Construction on the Macroinvertebrate Community in Upper South Indian Creek and Tributaries Near Ernestville and Erwin, Tennessee: A three-year study.

Cherry, D.S., M.G. Dobbs, J.L. Farris, and P.R. Scheuerman. (In prep). Shifts in Macroinvertebrate Community Composition due to the Impact of Road Construction on Lower South Indian Creek and Tributaries near Ernestville and Erwin, Tennessee: A four-year study.

Dissertation papers in preparation:

Dobbs, M.G., D.S. Cherry, and J. Cairns, Jr. Development a Toxicity test System Designed to Evaluate the Role of Trophic Interaction has on the Toxicity of a Chemical.

Dobbs, M.G., D.S. Cherry, and J. Cairns, Jr. A Flow-though Algal Toxicity Test: Comparisons with Static Bottle Tests Using Copper as the Toxicant.

Dobbs, M.G., D.S. Cherry, and J. Cairns, Jr. Evaluation of the Possible trophic effects when a Three Level Food Chain is Exposed to Copper.

Dobbs, M.G., D.S. Cherry, and J. Cairns, Jr. Selenium Toxicity and Trophic transfer as Evaluated Using a Trophic Toxicity Test.

**TECHNICAL REPORTS (LIMITED DISTRIBUTION):**

Farris, J.L., M.G. Dobbs, and D.S. Cherry. 1991. Preliminary Artificial Stream Studies and Sampling of the James River to Evaluate Color's Effects from Virginia Fibre's Treated Effluent: Interim Report. Virginia Fibre Corporation, VA. 15 pp.

Farris, J.L., M. G. Dobbs, and D. S. Cherry. 1991. Acute Toxicity Testing of Copper in Blaine Creek Water for Amendment to Site-Specific Criteria. American Electric Power Service Corporation, Columbus, OH. 35 pp.

Cherry, D.S., M.G. Dobbs, A. Mikailoff, S.R. Lynde, J.R. Bidwell, M.M. Yeager, and J.C. Fisher. October 1992. Acute Toxicity and Chronic Impairment Testing of *Daphnia pulex, Ceriodaphnia dubia*, and Fathead Minnow (*Pimephales promelas*) to Robins Air Force STP - Phase II Effluent. Metacalf & Eddy, Atlanta, GA. 20 pp.

Cherry, D.S., J.L. Farris, and M.G. Dobbs. November 1992. Macroinvertebrate Community Assemblages in Upper South Indian Creek and Tributaries near Ernestville and Erwin, Tennessee - May 1992. Eastern Tennessee State University, Johnson City, TN. 40 pp.

Cherry, D.S., M.M. Yeager, M.G. Dobbs, J.R. Bidwell, J.L. Farris, and E.P. Smith. November 1992. The Influence of Effluent Temperature on the Distribution of Fishes and Other Biota in the New River near Hoechst Celanese Corporation, Virginia, Report and Proposal. 60 pp.

Cherry, D.S., J.L. Farris, and M.G. Dobbs. December 1992. Macroinvertebrate Community Assemblages in Lower South Indian Creek and Tributaries near Ernestville and Erwin, Tennessee - May 1992. Eastern Tennessee State University, Johnson City, TN. 40 pp.

Farris, J.L., M.G. Dobbs, and D.S. Cherry. 1992. Artificial Stream Studies and Sampling of the James River to Evaluate Color Effects from Virginia Fibre's Treated Effluent: Final Report. Virginia Fibre Corporation, Amherst, VA. 150 pp.

Cherry, D.S., J.R. Bidwell, and M.G. Dobbs. January 1993. Benthic Macroinvertebrate Study of East Branch, Brandywine Creek for the Sonoco Products Company, Downingtown, Chester County, Pennsylvania. 60 pp.

R.L. Shema, D.S. Cherry, M.G. Dobbs, and J.W. McIntire. January 1993. Duquesne Light Company Beaver Valley Power Station Ohio River In-situ *Corbicula* Growth Study. Duquesne Light Company, Shippingport, PA. 55 pp.

Cherry, D.S., J.L. Farris, and M.G. Dobbs. March 1993. Macroinvertebrate Community Assemblages in Upper South Indian Creek and Tributaries near Ernestville and Erwin, Tennessee - September 1992. Eastern Tennessee State University, Johnson City, TN. 40 pp.

Cherry, D.S., J.L. Farris, and M.G. Dobbs. March 1993. Macroinvertebrate Community Assemblages in Lower South Indian Creek and Tributaries near Ernestville and Erwin, Tennessee - September 1992. Eastern Tennessee State University, Johnson City, TN. 35 pp.

Cherry, D.S., J.R. Bidwell, and M.G. Dobbs. April 1993. Toxicity Tests with Betz Laboratories' Clam-trol CT-1. Betz Laboratories, Trevose, PA. 15 pp.

Cherry, D.S., J.L. Farris, and M.G. Dobbs. December 1993. Macroinvertebrate Community Assemblages in Upper South Indian Creek and Tributaries near Ernestville and Erwin, Tennessee - May 1993. Eastern Tennessee State University, Johnson City, TN. 40 pp.

Cherry, D.S., J.L. Farris, and M.G. Dobbs. December 1993. Macroinvertebrate Community Assemblages in Lower South Indian Creek and Tributaries near Ernestville and Erwin, Tennessee - May 1993. Eastern Tennessee State University, Johnson City, TN. 35 pp.

Cherry, D.S., M.G. Dobbs, and J.R. Bidwell. May 1994. Acute Flow-Through Toxicity Tests with Betz Laboratories' Clam-trol CT-2. Fathead Minnow (*Pimephales promelas*) 96-Hour Acute Bioassay. Betz Laboratories, Trevose, PA. 20 pp. (Prepared Report)

Cherry, D.S., M.G. Dobbs, and J.C. Scott. May 1994. Acute Flow-Through Toxicity Tests with Betz Laboratories' Clam-trol CT-2. Rainbow Trout (*Oncorhynchus mykiss*) 96-Hour Acute Bioassay Betz Laboratories, Trevose, PA. 20 pp. (Prepared Report)

Cherry, D.S., M.G. Dobbs, and J.C. Scott. May 1994. Acute Flow-Through Toxicity Tests with Betz Laboratories' Clam-trol CT-2. Inland Silverside (*Menidia beryllina*) 96-Hour Acute Bioassay Betz Laboratories, Trevose, PA. 20 pp. (Prepared Report)

Cherry, D.S., M.G. Dobbs, and J.C. Scott. May 1994. Acute Flow-Through Toxicity Tests with Betz Laboratories' Clam-trol CT-2. Sheepshead Minnow (*Cyprinodon variegatus*) 96-Hour Acute Bioassay Betz Laboratories, Trevose, PA. 20 pp. (Prepared Report)

Cherry, D.S., M.G. Dobbs, and J.C. Scott. May 1994. Acute Flow-Through Toxicity Tests with Betz Laboratories' Clam-trol CT-2. *Mysidopsis bahia* 96-Hour Acute Bioassay Betz Laboratories, Trevose, PA. 20 pp. (Prepared Report)

Cherry, D.S., M.G. Dobbs, M.M. Yeager, S.R. Lynde, and J.F. Scott. May 1994 Benthic Macroinvertebrate Assessment of the North Impact Area in the La Crosse River, Fort McCoy, Wisconsin. First Preliminary Draft. 8 pp.

## PROFESSIONAL ACTIVITIES

1991

Assisted in *Ceriodaphnia dubia* culturing and testing workshop. BETZ Laboratories, Trevose, PA.

Benthic macroinvertebrate sampling of South Indian Creek and tributaries to evaluate the impact of highway construction on the watershed. Statistical analysis of the data and report development. Eastern Tennessee State University, Johnson City TN.

Artificial stream and field biomonitoring studies designed to evaluate the effect of a colored paper mill effluent on James River. Virginia Fibre Corporation, Amherst VA.

Presenter VF
Benthic macroinvertebrate and fish sampling to evaluate effluent dissipation and potential toxicity of waste treatment effluent in the New River, VA. Hoechst Celanese Corporation, Celco Plant, Narrows, VA.

1992

Benthic macroinvertebrate and fish sampling to evaluate effluent dissipation and potential toxicity of waste treatment effluent in the New River, VA. Acute toxicity testing with *D. pulex* and *Isonychia bicolor*, and concurrent live box studies with *I. bicolor* and *Pimephales promelas*. Hoechst Celanese Corporation, Celco Plant, Narrows, VA.

At-large officer 1992, Biology Association of Graduate Students, VPI & SU and member of graduate student advisory committee to department chair.

Semi-annual benthic macroinvertebrate sampling of South Indian Creek and tributaries to evaluate the impact of highway construction on the watershed. Statistical analysis of the data and assisted in report development. Eastern Tennessee State University, Johnson City, TN.

Benthic macroinvertebrate sampling to evaluate the impact of a chlorinated paper mill effluent on the receiving system. Sonoco Products Company, Downingtown, PA.

1993

Analysis of waste load allocation and mixing zone for a paper mill discharge into Brandywine Creek, Downingtown, PA. Sonoco Products Company, Downingtown, PA.

Seasonal fish sampling surveys to develop a US EPA 316"a variance demonstration on heated effluent dissipation in the New River, VA. Hoechst Celanese Corporation, Celco Plant, Narrows, VA.

Benthic macroinvertebrate sampling to evaluate the impact of a chlorinated paper mill effluent on the receiving system. Sonoco Products Company, Downingtown, PA.

Reviewer of bioassay data for Quality Control/Quality Assurance between several Canadian environmental testing laboratories, Procter and Gamble Corporation, Grande Prairie, Edmonton, Alberta.

Semi-annual benthic macroinvertebrate sampling of South Indian Creek and tributaries to evaluate the impact of highway construction on the watershed. Statistical analysis of the data and assisted in report development. Eastern Tennessee State University, Johnson City, TN.

Reviewer *Environmental Toxicology and Chemistry*.

**RESEARCH INTERESTS:**

The focus of my research interests is how to relate laboratory toxicity tests with what actually happens in the freshwater ecosystems. Specific areas's of interest included metal toxicity, and the importance of metal speciation as it relates to bioavailability, and derivation of water quality criteria. I would like to continue my investigations into the subtle effects of a toxicant on testing scales larger then the individual organism.

**RESEARCH EXPERIENCE:**

Sediment toxicity testing
- *Chironomus riparius*

Freshwater acute and chronic toxicity testing (static and flow-through)
- *Ceriodaphnia dubia*
- *Daphnia magna*
- *Daphnia pulex*
- *Brachionus calyciflorus* (rotifer)
- *Pimephales promelas*
- *Lepomis macrochirus*
- *Etheostoma sp.* (darter)
- *Selenastrum capricornutum*
- *Chlorella vulgaris*
- *Gleotheca* sp. (blue-green alga)
- *Isonychia bicolor* (mayfly)
- *Stenonema* sp. (mayfly)
- *Physella* sp. (snail)
- *Eurycea bislineata* (salamander)
- *Orconectes* sp. (crayfish)
- *Corbicula fluminea*

Saltwater testing
- Oyster shell growth
- Sheepshead minnow
- Inland silverside

Field biomonitoring
- Benthic macroinvertebrate sampling and identification
- Fish sampling
- Live box studies
- Algal studies

Artificial stream systems
- Dosing and maintenance
- Algal studies

Michael G. Dobbs