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The Effect of Tetrachloroguaiacol on the Growth, Survival and Development of the Fathead Minnow (*Pimephales promelas*)

Cindy Lee Ann Woodland

A Thesis

in

The Department

of

Biology

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ABSTRACT

Fathead minnow (Pimephales promelas) larvae, within 24 hours of hatching, were exposed for 7 days to TeCG (tetrachloroguaiacol), at concentrations of 0, 25, 50, 100, 200 and 400 µg/l. No significant difference in growth or survival was found among treatments. Embryos, within 24 hours of fertilization, were exposed to TeCG at concentrations of 0 and 100 µg/l for ten days. No significant difference was found between the control and treatment groups for survival, body length or body width of the larvae, or for yolk size of the eleutheroembryos. However, a significant difference was found in the hatching success of the embryos: fewer hatched at the 100 µg/l TeCG concentration than in the control. The ecological significance of this difference found in hatching success is that fathead minnows have been known to spawn in areas close to sites that discharge BKME into receiving waters.

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TABLE OF CONTENTS

ABSTRACT..... iii

ACKNOWLEDGEMENTS..... iv

LIST OF FIGURES..... vii

GLOSSARY..... ix

INTRODUCTION..... 1

1.1. What is TeCG?..... 1
1.2. Why Study TeCG?..... 2
1.3. Mode of Toxic Action..... 4
1.4. Ecological Impact of TeCG Contamination.... 5
1.5. Pimephales promelas as a Test Species..... 6
1.6. Objectives of this Study..... 7

MATERIALS AND METHODS..... 8

2.1. Introduction..... 8
2.2. Culturing Fathead Minnows..... 8
2.3. 7-Day Growth and Survival..... 9
2.4. Hatching and Early Development..... 16
2.5. Statistical Analysis..... 23

RESULTS..... 25

3.1. 7-Day Growth and Survival..... 25
3.1.1. Mean Dry Weight..... 25
3.1.2. Lethal Bioassays..... 25
3.2. Hatching and Early Development..... 25
3.2.1. Body Length..... 25
3.2.2. Body Width..... 32
3.2.3. Yolk Absorption..... 32
3.2.4. Hatching Success Study..... 37
3.2.5. Lethal Bioassays..... 37

DISCUSSION..... 42

4.1. Growth and Mortality..... 42
4.2. Hatching and Early Development..... 45

CONCLUSION..... 51

RECOMMENDATIONS..... 52

REFERENCES..... 53

LIST OF FIGURES

Figure 1.	TeCG Molecule	2
Figure 2.	Photograph depicting the experimental apparatus, contained within a wall-mounted fumehood, that was used for all exposure experiments. Two waterbaths, each containing one replicate at each treatment level, and a waterbath containing renewal solutions, which are depicted as being aerated, are a part of the experimental apparatus.	12
Figure 3.	A diagram depicting the measurement taken to determine the body width of larval fathead minnows. (Modified diagram of fathead minnow larva from a reprint of Environment Canada, 1992).	19
Figure 4a.	A photograph depicting an eleutheroembryo with a large rotund yolk sac. (This embryo was exposed to 200 µg/l TeCG 24 hours after fertilization until hatching. At hatching, the embryo was not able to swim.)	21
Figure 4b.	An eleutheroembryo that has absorbed most of its yolk. The yolk elongate. This eleutheroembryo was not exposed to TeCG and was able to swim freely at hatching.)	21
Figure 5.	Mean dry weight of larval fathead minnows exposed to TeCG for 7 days.	26
Figure 6.	Mean proportion of larvae surviving after 7 days of exposure to TeCG.	28
Figure 7.	Mean length of larval fathead minnows exposed to TeCG for 10 days.	30
Figure 8.	Mean width of larval fathead minnows exposed to TeCG for 10 days.	33
Figure 9a.	Mean yolk area after exposure to TeCG.	35
9b.	Mean yolk width after exposure to TeCG.	35
9c.	Mean yolk length after exposure to TeCG.	35
Figure 10.	Mean proportion of embryos that hatched after exposure to TeCG.	38

Figure 11. Mean proportion of larvae surviving after 10 days exposure to TeCG. 40

GLOSSARY

Bioaccumulation: The accumulation of chemicals, from the water or food by aquatic organisms.

Bioavailability: The degree to which a chemical is potentially physically available to an organism in the surrounding environment.

BKME (bleached kraft mill effluent): A liquid waste product that is released into the aquatic environment by the pulp and paper industry as a result of the pulping and bleaching of wood.

Hatchability: The percentage of fertilized fish eggs laid that successfully hatch out of the egg membrane to enter the eleutheroembryo stage of development.

LC_{50}: The concentration of a toxicant in the surrounding environment that causes 50 percent mortality in a test population of organisms over a designated period of time (e.g. 96-h)

Lipophilicity: The degree to which a chemical has an affinity for lipids. This is a parameter that is used to estimate the ease with which a chemical will pass through biological membranes and is therefore used as a measure of toxicity. It is often estimated using the octanol water partition coefficient (see below).

LOEC/LOEL: Lowest observed effect concentration/level. This is the lowest concentration/level of a chemical that causes a significant adverse effect on the test population of organisms that is exposed to the chemical as compared with a control population.

NOEC/NOEL: No observable effect concentration/level. This is the highest concentration/level of a chemical that a test population of organisms is exposed to at which no significant effect occurs when compared with a control population.

Octanol-water partition coefficient (K_{ow}): The ratio of the solubilities of a chemical in n-octanol and water at equilibrium.

TEF (toxicity equivalency factor): An approach use to estimate the toxicity of individual compounds within a mixture of compounds in order to estimate the overall toxicity of the mixture. The TEF is calculated using the known toxicity of a reference compound that is similar in structure and mode of biological action to the individual compound that is being investigated. The TEF is a coefficient that is multiplied by the empirically derived toxicity values of the reference compound to estimate the unknown compound's toxicity.

INTRODUCTION

1.1. What is Tetrachloroguaiacol?

One of the many challenges facing Canadian society today is the estimation and abatement of the environmental risk of pulp and paper effluent. Each stage in the production of paper has its own unique type of effluent which contains different individual compounds (Oikari et al.,1985a; McLeay, 1987, Suntio et al., 1988; Berry, et al., 1991, Luthe, 1992; Carey et al., 1992). On May 20, 1992, amendments were made (Fisheries and Oceans Canada 1992b) to the Pulp and Paper Effluent Regulations (PPER) of the federal Fisheries Act (Fisheries and Oceans Canada,1992a). As a part of these amendments, environmental Effects monitoring studies (EEM) are to be conducted at all pulp and paper effluent discharge sites in the aquatic environment. These studies will assess the adequacy of these regulations in protecting fish and fish habitat. A required test included in the EEM program used to assess the toxicity of pulp and paper effluent is the 'Test of Larval Growth and Survival Using Fathead Minnows' (Environment Canada, 1992).

The purpose of this study is to determine the effects of tetrachloroguaiacol (TeCG), a compound found in pulp and paper effluent, (Leuenberger et al., 1985; Allard et al., 1988; Kovacs, 1991) on the embryo and larvae of the

fathead minnow (Pimephales promelas).

1.2. Why Study TeCG?

Tetrachloroguaiacol is a chlorophenol that is produced during the degradation of lignin with chlorine, and in the bleaching of pulp (fig.1).

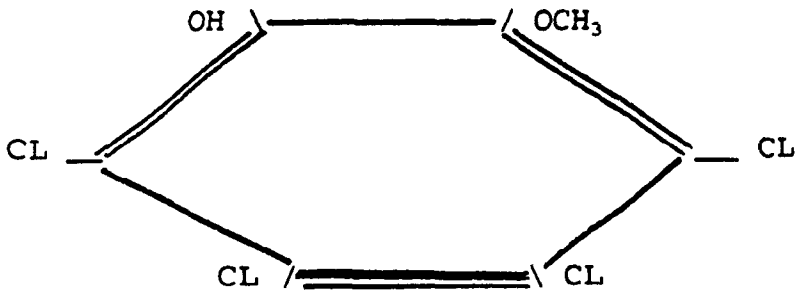


Figure 1. TeCG molecule.

When compared with other chlorophenols, pulpmill chloroguaiacols are particularly prominent in the biota, water and sediment of the receiving area of pulp mill discharges (Paasivirta et al., 1980; Carey and Hart, 1988; Rogers et al., 1989). Of the dominant chloroguaiacols found in wastewater, TeCG has the relatively high toxicity and has been found in bleached kraft mill effluent (BKME) at levels between 1-220 µg/l in a number of different studies (McLeay, 1987). Even 44 km downstream from a discharge site, the concentration of TeCG was between 4.5-20 µg/l (Hodson et al., 1992). The 96-h LC_{50} for TeCG in juvenile rainbow trout (Oncorhynchus mykiss)is 320 µg/l (Leach and

Thakore, 1975), whereas the 96-h LC_{50} for larval and adult zebra fish (Brachydanio rerio) are 300-500 µg/l and 650 µg/l respectively (Allard et al.,1988, Neilson et al., 1990). The ChV for median survival time of unfed larval zebra fish was found to be 245 µg/l of TeCG (Nielson et al., 1991), while the ChV for sea urchin (Stongylocentrotus purpuratus) fertilization was found to be 87 µg/l of TeCG (Cherr et al., 1987).

TeCG has been known to accumulate in the sediment of receiving waters (Xie, 1983, Allard et al., 1988). In one study TeCG was found to accumulate in the sediments of receiving waters of BKME; 180 µg/kg organic C in the free form and 1590 µg/kg organic C in the bound form (Allard et al., 1988). Sediment can therefore become a sink for TeCG, making it bioavailable to benthic-feeding organisms in the aquatic environment.

In one study of the Fraser River in British Columbia concentrations of TeCG 16±7 ng/l were found under high flow conditions, while under low flow conditions concentrations of TeCG were 28±11 ng/l (Carey and Hart, 1988). In another study on the Fraser river, the tissue of juvenile chinook salmon, Oncorhynchus tshawytscha, was found to have levels of TeCG between 2-111 ng/g wet weight (Rogers et al., 1989). The persistence of TeCG in the aquatic environment also depends upon oxygen levels (Remberger et al., 1986) and the presence of microbes

capable of biodegrading TeCG (Haggblom and Salkinoja-Salonen,1988). Under aerobic conditions, tetrachloroveratrole can be produced at the sediment-water interface. Under anaerobic conditions tetrachlorocatechol is produced. However, it has been found that tetrachloroveratrole, the metabolite of TeCG degradation, can have an even greater toxicity than TeCG (Neilson et al., 1984).

There have been no studies of the effects of TeCG on the early developmental stages of the fathead minnow.

Considering the reported toxicity of BKME and the myriad of compounds found in BKME, a study into the effects of TeCG on the most sensitive stages of a species of fish indigenous to the discharge sites is warranted (Oikari et al., 1985a; Oikari, 1985b; Kovacs et al., 1991; Munkittrick et al.,1991).

1.3. Mode of Toxic Action

The primary mode of toxic action of chlorophenols is believed to be the uncoupling of oxidative phosphorylation (Bostrom and Johansson, 1972; Buffa et al., 1963; Weinbach, 1958; Weinbach and Garbus, 1964; Weinbach and Garbus, 1965; Ahlers, 1988; Duxbury and Thompson, 1987). This can result in a decrease in energy available for maintenance and growth, which could ultimately reduce the survival of biota contaminated with chlorophenols (Webb

and Brett, 1973).

1.4. Ecological Impact of TeCG Contamination

Because growth rate of an organism is influenced by its metabolic rate, (Anderson and D'Apollonia, 1978), growth is one endpoint that may serve as an indicator of metabolic stress caused by chlorophenols (Bostrom and Johansson, 1972; Johansen et al., 1987; Kreuger et al., 1968; Mathers et al., 1985; Samis et al., 1991). Although growth is not always a sensitive indicator of toxicity, it does have ecological significance. Ideally, the effects of toxicants on both growth and on a more sensitive endpoint, such as mortality in early life stages, should be measured jointly in a toxicity study (Woltering, 1984).

I used hatchability, the proportion of eggs that hatch, as a quick, easy and relatively sensitive endpoint for quantifying toxic stress (Woltering, 1984).

Early development rate of fish is determined by both the rate of yolk absorption and the efficiency of yolk utilization (Heming and Buddington, 1988). Thus, altered yolk utilization is a recommended endpoint to be measured in early lifecycle stage toxicity tests where possible (McKim, 1985). One way to measure yolk utilization is to quantify yolk biomass after hatching has occurred (Leduc, 1978).

1.5. *Pimephales promelas* as a Test Species

The fathead minnow is an ideal test species for a water-borne toxicant. Being a common forage fish, any significant toxic effect on the population dynamics of the fathead minnow could result in a notable impact on higher trophic levels in the food chain.

The fathead minnow is a small fish, on average 5 cm in length, and it belongs to the largest group of freshwater fish indigenous to North America, the family Cyprinidae (Scott and Crossman, 1973). The time taken to reach sexual maturity is 4-6 months (U.S.EPA,1987) and a prolonged spawning period in indoor culture units provide yearlong availability of embryos (Benoit et al., 1982). When compared with other species of North American freshwater fish, the fathead minnow is sensitive (Rand and Petrocelli, 1985).

The fathead minnow is a standard toxicity test species. So, there is a wide data base available on them. This fish is also a benthivore and may be more vulnerable to bioaccumulation of TeCG compared to other fish species since TeCG accumulates in sediment (Hebert and Haffner,1991).

In summary, the fathead minnow provides convenience, availability, and savings in both time and money in chronic toxicity tests.

1.6. Objectives of this Study

The primary objective of this study was to investigate the effects of TeCG on the growth of larval fathead minnows. In support of the central objective, examination of the influence that TeCG has on the development of embryo and larval fathead minnows was also conducted. Development was examined from the perspective of yolk absorption and larval growth. Effects on survival, as measured by hatchability and larval survival were also determined. The hypothesis that accompanied these objectives was that, due to uncoupling of oxidative phosphorylation, TeCG would decrease larval growth and reduce the development rate of the embryo, eleutheroembryo and larval stages of the fish. Because of a decrease in available energy due to uncoupling of oxidative phosphorylation, TeCG was expected to decrease embryo hatching and larval survival.

METHODS AND MATERIALS

2.1. In January of 1991, a stock of approximately 200 fathead minnows, Pimephales promelas, was obtained from The Pulp and Paper Research Institute of Canada (Paprican) of Pointe Claire, Quebec. After acclimation, the fish were divided up into juveniles and adults, and distributed into three tanks; one held juveniles and two held adults. These glass tanks were 60 x 32 x 30 cm high and had a flow through water supply. The tanks were aerated and maintained in a 16 hr light and 8 hr dark photoperiod. Water temperature was kept within a range of 22-29°C, but generally was held between
24-26°C, within the guideline (25±°C) set by the U.S. E.P.A (1987). The extremes in temperature that occurred were due to malfunctions in maintenance of buildings water temperature. For maximum egg production. However, plumbing problems within the lab precluded the suggested range from being maintained. Twice daily, the fish were fed a diet of frozen adult brine shrimp ad libitum.

2.2. Culturing Fathead Minnows

The technique used to maximize egg production incorporated guidelines designed by the U.S. EPA for culturing fathead minnows for use in toxicity tests (U.S.EPA, 1987).

Twelve spawning tanks, measuring 40 x 20 x 25 cm high, each housed one male and two female fish and a single spawning substrate. Three community spawning tanks, measuring 60 x 30 x 32 cm high, contained three males, 6 females and three substrates.

A variety of spawning substrates were presented to the fish. Red bricks and slabs of broken concrete were assembled into cave like structures, providing the fish with a formation that they could swim through and an underside surface onto which they could deposit their eggs. An alternative substrate, grey non-toxic schedule 40 PVC plastic tubing, was also provided. The tubing had an outer diameter of 11.2 cm with an inner diameter of 10.0 cm. The tubing was cut into 10.0 cm lengths and then cut into halves. These semi-circles were then placed in a convex position on the floor of the tanks. The fish would then lay their eggs on the underside of the convex roof of the substrate. Both PVC and rock substrates were used as spawning substrates for collecting embryos for the 7-day experiments, while only PVC substrates were used for the 10-day experiments (described below).

2.3 7-Day Growth and Survival

The protocol used in this experiment was the "Biological Test Method: Test of Larval Growth and Survival Using Fathead Minnows" (Environment Canada, 1990).

Spawning substrates were inspected each morning for the presence of eggs in the culture tanks. Substrates with eggs were removed from the tanks and placed individually in white plastic hatching pails. The hatching pails were placed in flow through tanks that were held under the same conditions as the culture tanks. Each hatching pail contained eggs from a single parental group.

The hatching pails were monitored daily for the hatching of embryos, which on average took 3-5 days. When 60 eleutheroembryos or more, pooled equally from three different sets of parents, had hatched within a 24 hour period, they were collected and an experiment was begun. The experiment was designed to investigate the growth and survival of larvae over a 7-day period of exposure to TeCG.

In each replicate there were five treatment levels plus a control. Tetrachloroguaiacol was purchased from Helix Biotech Corporation of Richmond B.C. at a purity of 99+%. Solutions of the toxicant were made using 0.250 g of TeCG, 500 ml of double deionized water, 4 ml of 99% ethanol and 4 ml of NaOH (Kolokotronis, 1991). TeCG would not dissolve in the water alone, therefore ethanol and NaOH were used to dissolve it in water. The concentrations of TeCG solution used in the experiment were 25, 50, 100, 200 and 400 µg/litre. Solutions were prepared daily and nominal concentrations were calculated.

The experiment was semistatic (fig.2). Solutions

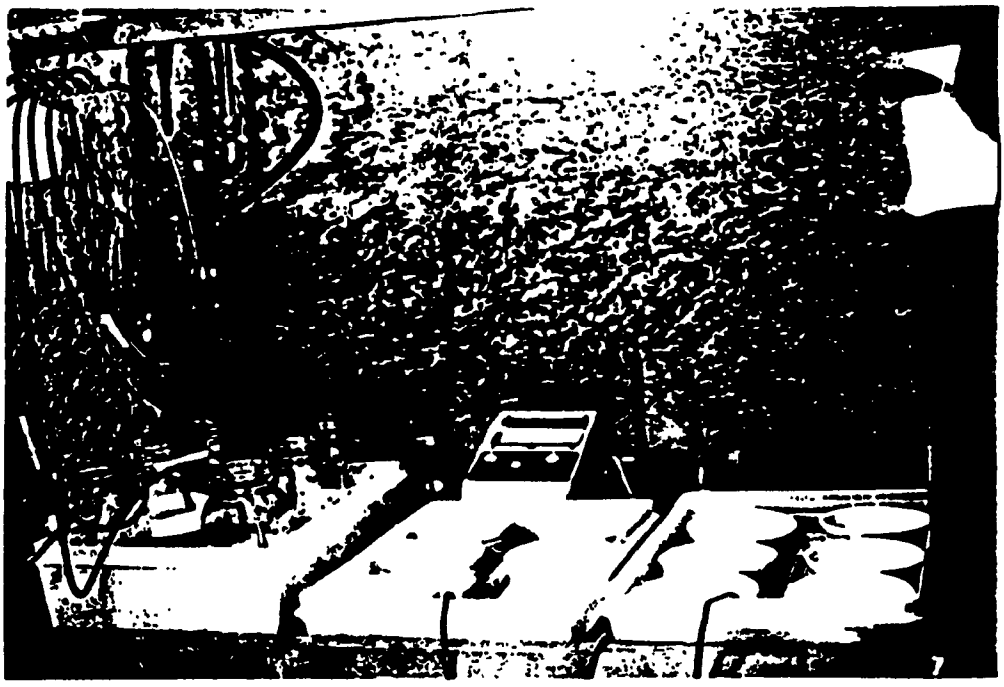
were renewed every 24 hours. To maximize oxygen concentrations prior to the initiation of an experiment, all solutions were aerated for two hours using aeration tubing, a glass pipette and aeration valve. Oxygen, pH, temperature and conductivity were measured in the aerated solutions prior to either the initiation of an experiment or the renewal of daily solutions. At the end of the 24-h period the final measurements for oxygen, pH, and temperature were taken.

The design of the experiment involved exposing groups of 10 eleutheroembryos that had hatched within 24 hours to either the control or one of the five treatment levels for a period of seven consecutive days.

In order to prevent undue temperature stress during transfer from the hatching pails to the experimental chambers, randomly chosen eleutheroembryos were placed in beakers in a water bath that was maintained at the same temperature as were the hatching pails. Three fish were randomly chosen from one hatching pail, three from another, and four from the third pail. Each hatching pail contained eggs from a different parental group.

The complete experimental apparatus was contained within a fumehood and was comprised of two water baths, each with six white porcelain bowls (10 cm diameter), containing 250 ml of solution. Immersible heaters were placed in the water bath to maintain the temperature within

Figure 2. Photograph depicting the experimental apparatus, contained within a wall-mounted fumehood, that was used for all exposure experiments. Two waterbaths, each containing one replicate at each treatment level, and a waterbath containing renewal solutions, which are depicted as being aerated, are a part of the experimental apparatus.



the desired range (23-27°C). The actual temperatures fluctuated between 23.0-26.9°C.

Groups of ten fish, pooled from three separate broods, were randomly assigned to each of the six chambers in each replicate water bath. The amount of water transferred with the

fish into the solution was 10-15 ml. The fish were then monitored for their swimming activity in order to detect any damage that may have occurred during the transfer. Damaged fish were replaced with healthy fish. Each group of ten fish was then fed 0.15 ml of a solution of live baby brine shrimp, approximately 700-800 shrimp in a given feeding which is the ration recommended in the experimental protocol. Care was taken to minimize the amount of eggs and eggshells in the brine solution. This feeding schedule was used throughout all of the larval experiments. Four hours prior to each solution renewal the fish were then fed the same amount again. Mortality was recorded at the beginning and end of each 24 hour period.

In renewing the solutions every 24 hours, the old solution and any debris on the bottom of the chamber was removed with a pipette and bulb. This debris consisted primarily of dead brine shrimp nauplii. Any dead fish were recorded and removed. Any fish killed as a result of this procedure were recorded separately from daily mortality.

Approximately 40 ml of the old solution was left in

each chamber after siphoning of the old solution and debris. The chambers were then refilled with new solutions. Once the exchange was complete the fish were fed and mortality and activity were recorded.

Two replicates were completed for the control and all treatment levels, except for the 50 µg/litre test in which only one replicate was completed, between Oct.16/91 - Oct.23/91. During this 7-day experimental period, the measurements and solution renewals were taken between 9:00 A.M.-1:00 P.M. Two more replicates at all treatment levels and the control were completed, under the same conditions as the first two replicates, between Nov.26/91-Dec.3/91. Each replicate was a group of ten fish.

The mean dry weight of the group and the proportion of mortality for each group at the end of seven days of exposure to TeCG were the endpoints measured in this experiment. Any fish that died in the groups due to handling during the experiment were subtracted from the group of ten and the mean dry weight and mortality were calculated based on the remaining fish in the group. As recommended by the protocol, mortality due to handling never exceeded 20%.

At the end of the 7-day experiment, the groups of fish in each chamber were killed with 95% ethanol and stored in vials containing 70% ethanol. The fish were not fed on the last day. After no more than two weeks of

storage in ethanol, groups of fish were dried at 100°C for 16-20 hours. Because dried fish take up water vapour readily, the boats were transferred from the oven to a desiccator before being transported to a balance weighing. Dry weights for each replicate group of fish at each treatment level were obtained using a balance that measured to 10 µg. Mean dry weights for each group were calculated.

2.4. Hatching and Early Development

During the period of May 13/92-June 13/92 I investigated sublethal effects of TeCG on development and hatching over a 10-day period. Fertilized eggs, less than 24 hours old, were placed in treatment solutions. Experiments were initiated when more than 30 eggs/replicate, pooled equally from three different broods, were available for each treatment level and the control. The eggs were assigned equally to each treatment level. Three treatment levels were used: 0, 100 and 200 µg/litre. The first replicate of the control and 100 µg/l treatments became contaminated with fungus due to a lack of sterile transfer of the eggs to each experimental chamber from the spawning substrate. Within four days of the start of the exposure to TeCG for the second replicate, embryo mortality was so high at the 200 µg/litre treatment level (90%) that only 100 µg/litre level was continued as a treatment level. The number of embryos in each replicate varied between 30-

100 since the harvest of embryos in the spawning tanks diminished dramatically during this experimental period. I decided to use the number of eggs that were available in a given harvest as long as it did not fall below 30 eggs. All measurements and solution renewals were taken between 1:00 p.m. and 5:00 p.m. daily.

A total of five replicates for the control and five replicates at the 100 µg/l treatment level were successfully completed. The three other replicates became contaminated with parasites and were deformed compared with the embryos in the other five replicates that were not contaminated with parasites. The overall design of the experiment was similar to that of the 7-day growth and survival experiments. A semistatic system was used; solutions were renewed every 24 hours, and oxygen, pH, conductivity, temperature, and mortality were measured and recorded at the beginning and end of each 24-h period. The chambers used were white porcelain bowls (15 cm diameter). To each chamber 500 ml of solution was added.

Exogenous feeding was initiated at the end of the sixth day with the addition of brine shrimp nauplii to the treatment chambers. In preliminary trials, the control embryos did not have fully opened mouths and thus could not feed exogenously before the sixth day. The same feeding regime as outlined above was employed until the final day of the test. Because there were more than ten fish in each

chamber, the amount of brine solution placed in each chamber was given in amounts proportional to the number of fish that they contained. For every ten fish approximately 0.15 ml of brine solution was administered in each feeding.

Just prior to the daily renewal of solutions, two specimens were randomly chosen from each treatment level and stored in ethanol. If the embryo was encased in the embryonic sac, the membrane was first removed with the aid of a dissecting microscope. If the embryo was damaged at all during this process it was discarded and a new specimen was obtained. The standard length and width of the fish were measured using a micrometer positioned within the ocular of another dissecting microscope that was not connected to a camera. Width was measured by aligning the '0' mark on the micrometer with the indent on the ventral side of the fish where the operculum was situated, and measuring across the lateral side of the fish to a point perpendicular on the dorsal side of the spine (fig.3).

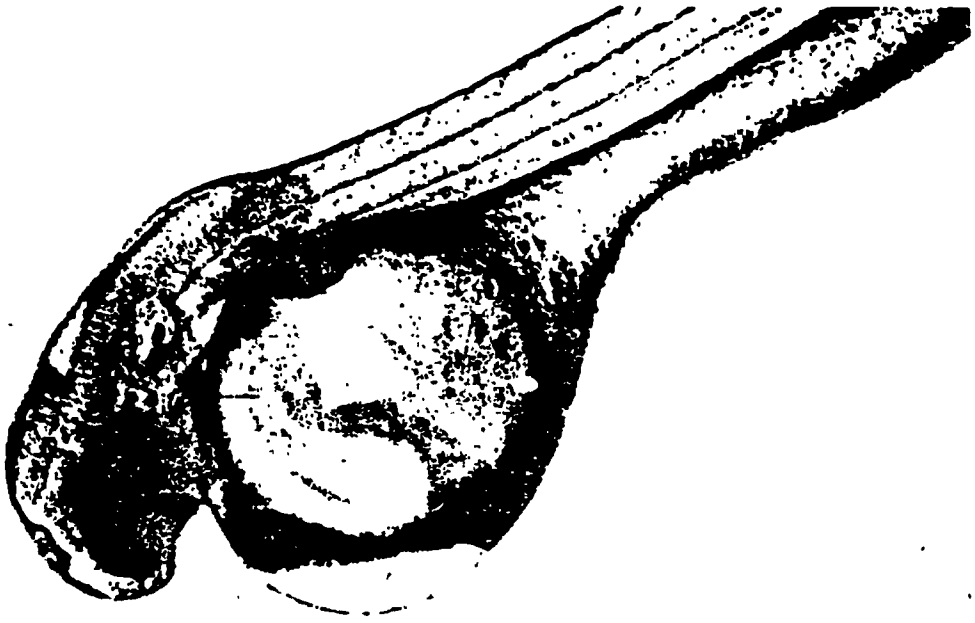
In order to obtain dimensions of the yolk sac of the fish the camera, scope and monitor were used (figs.4a,4b). A grid made up of 1 cm squares was placed on the screen of the monitor, and a rough estimate of the area occupied by the image of the yolk on the screen was estimated. Two measures of yolk area were taken and averaged for each fish. The maximum width, perpendicular to the spine and the length of the yolk were all measured

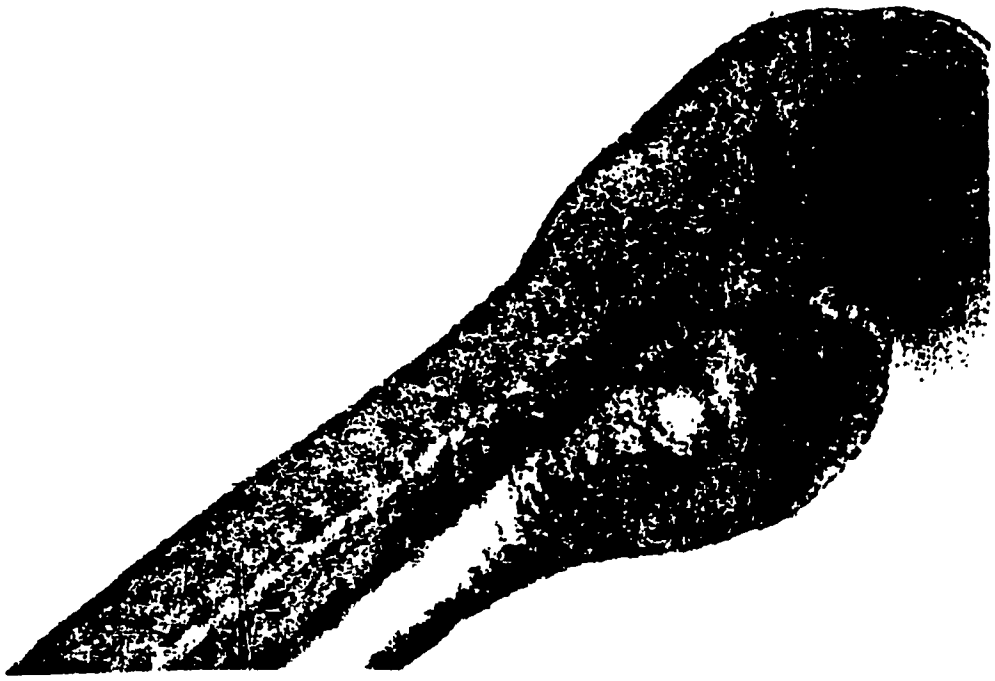
Figure 3. A diagram depicting the measurement taken to determine the body width of larval fathead minnows. (The diagram of the larva was modified from a reprint of Environment Canada, 1992).

1 mm

Figure 4a. A photograph depicting an eleutheroembryo with a large rotund yolk sac. (This embryo was exposed to 200µg/l TeCG 24 hours after fertilization until hatching. At hatching it was unable to swim.)

4b. An eleutheroembryo that has absorbed most of it's yolk. The yolk is more elongate than it is rotund. (This eleutheroembryo was not exposed to TeCG and was able to swim freely at hatching.)





using a micrometer within the ocular of the camera dissecting scope as viewed through the microscope.

The day on which embryos hatched into eleutheroembryos was recorded. The number of eleutheroembryos that hatched was recorded and used to represent proportion of embryos that hatched. The proportion of surviving fish was recorded daily.

2.5. Statistical Analysis

In the 7-day growth and survival the data was analyzed using a 1-way ANOVA to test for a significant difference in growth and survival between the treatment groups. The proportion found surviving in each replicate at each treatment level was first transformed by the arc sine square root transformation procedure and then analyzed. Tests for normality were performed using the Shapiro-Wilk's test and tests for homogeneity of variance were performed using Bartlett's test. These statistical tests are those suggested by the Environment Canada test protocol used in this experiment (Environment Canada,1990).

The survival and hatching data from the 10-day embryo-larval experiment, were transformed first to arc sine square roots and were then tested using the paired t-test. Since a significant effect of replicates was found after performing a two-way ANOVA on the data for the four yolk parameters, body length, and body width, these data

were analyzed using randomized block ANOVA to separate the replicate effects from the toxicant effects.

Tests for normality and homogeneity of variance were conducted. In those cases where normality and homogeneity were not met, appropriate transformations were performed prior to analysis.

RESULTS

3.1. 7-Day Growth and Survival

3.1.1. Mean Dry Weight

There was no significant difference in growth, as measured by mean dry weight (mg), between the control group and any of the treatment groups exposed to TeCG ($F_{5,17}$=2.66,p=.059) (fig.5). When a randomized block ANOVA was performed in order to block out any effects due to replicates, no significant difference was found between the treatment groups ($F_{5,17}$=2.30, p=.10).

3.1.2. Lethal Bioassays

In the 7-day test, larval mortality did not differ significantly among treatment groups ($F_{5,14}$=0.37,p=.895) (fig.6). Mortality in the control groups was under 20%.

3.2. Hatching and Early Development

3.2.1. Body Length

The data for body length was analyzed separately for each day that length was measured (day6-10) using a randomized blocked ANOVA to block out replicate effects. When no significant effect of replicates was found, a one-way ANOVA was performed which showed no significant difference between the control and treatment group for any day of exposure to TeCG (fig.7).

Figure 5. Mean dry weight of larval fathead minnows exposed to TeCG for 7 days (error bars represent 95% confidence intervals, n=3-4).

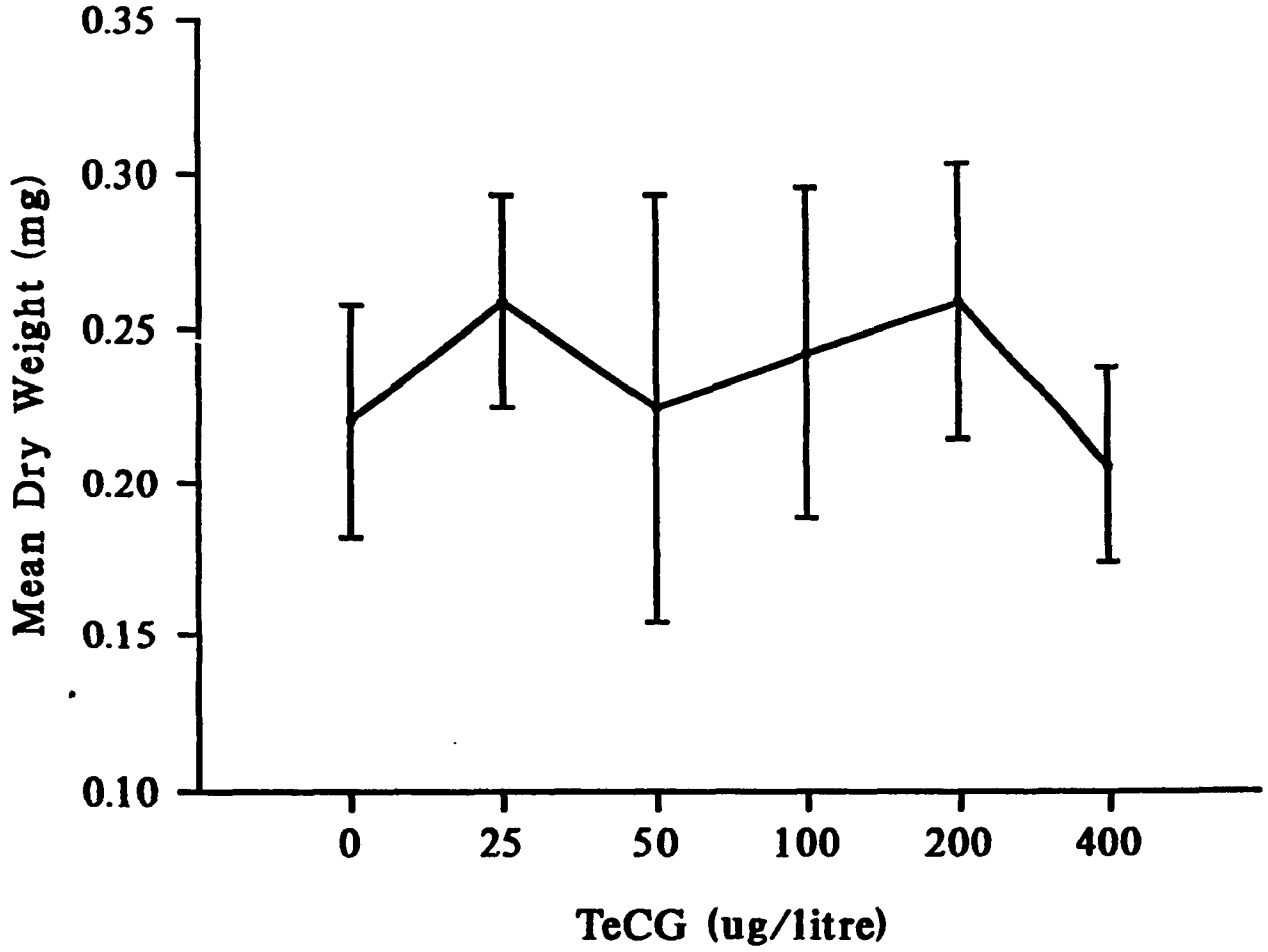

0.35
0.30
0.25
0.20
0.15
0.10
Mean Dry Weight (mg)
0
25
50
100
200
400
TeCG (ug/litre)

Figure 6. Mean proportion of larvae surviving after 7 days of exposure to TeCG. (error bars represent 95% confidence intervals, n=3-4)

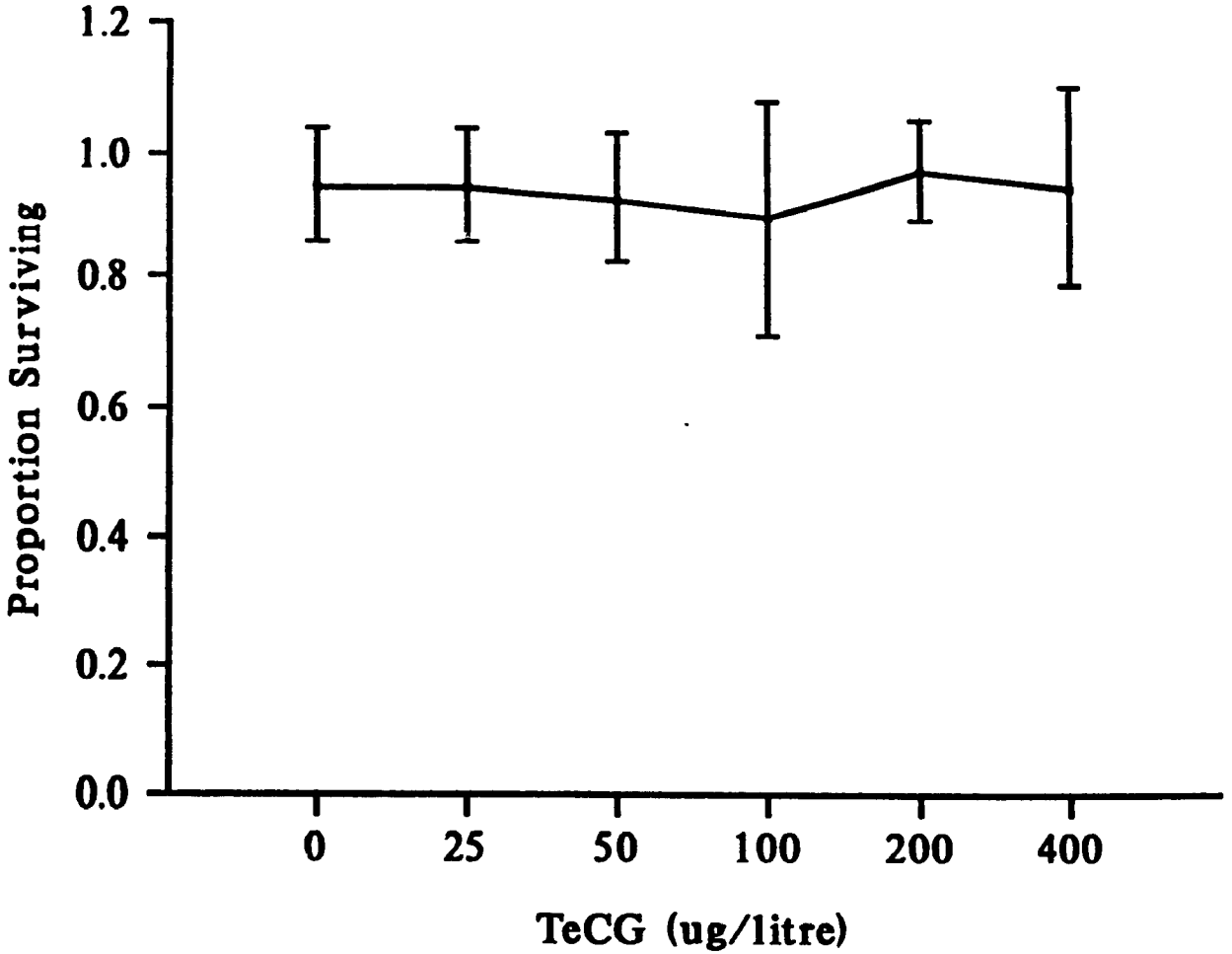
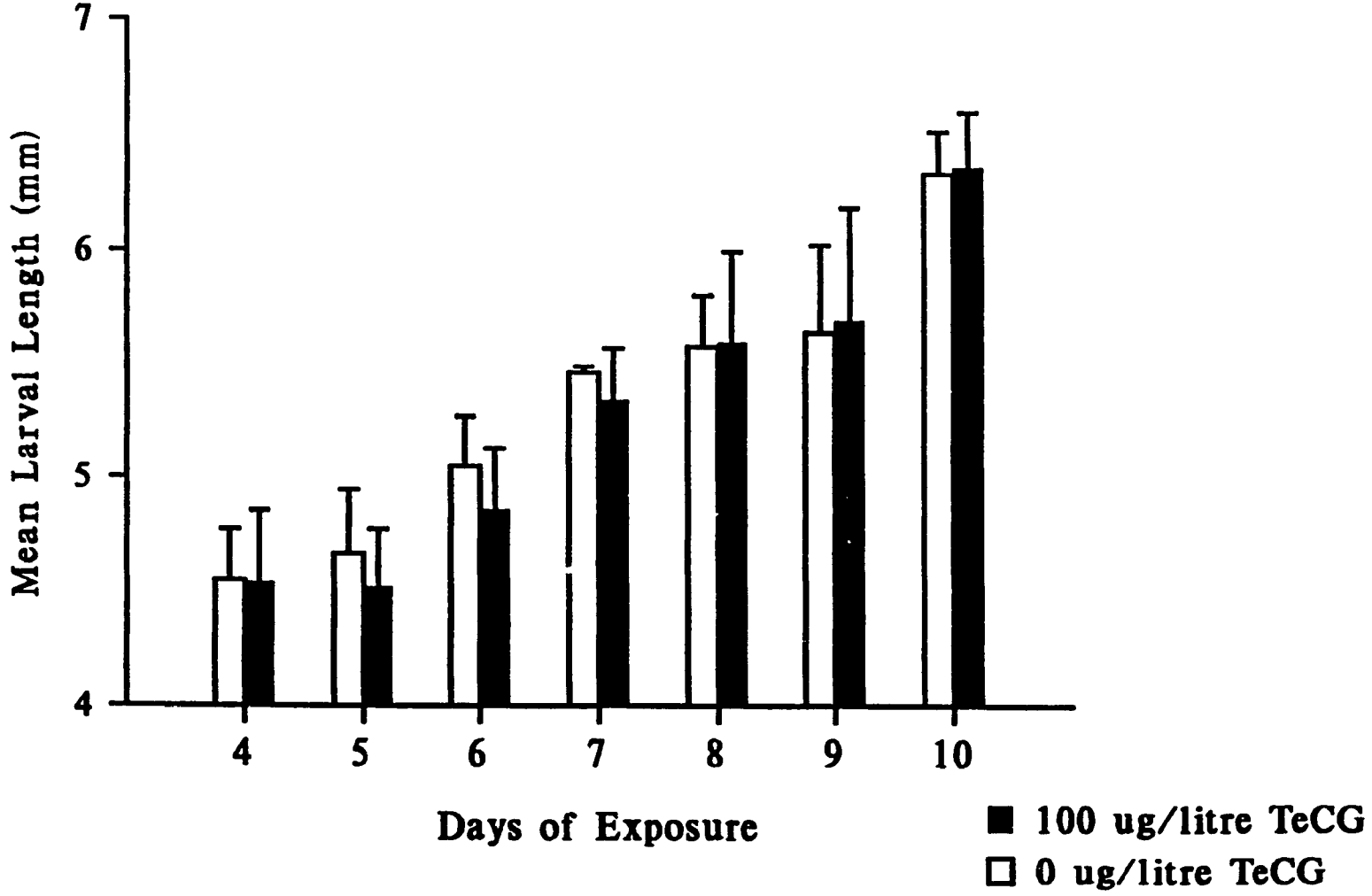

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Proportion Surviving
0
25
50
100
200
400
TeCG (ug/litre)

Figure 7. Mean length (mm) of larval fathead minnows exposed to TeCG for 10 days. (error bars represent 95% confidence intervals, n= 8-60)


7
6
5
4
Mean Larval Length (mm)
4
5
6
7
8
9
10
Days of Exposure
100 ug/litre TeCG
0 ug/litre TeCG

3.2.2 Body Width

Body width was measured across the widest point of the body, perpendicular to the spine (fig.3). The data were analyzed separately for each day of exposure to TeCG using randomized block ANOVAs. No significant difference was found for body width between control and treatment groups on any day, except for day 10 (fig.8). On day 10 a significant difference was found for body width ($F_{1,77}$=8.24,p=.005). However, the effect of replicates on body width was also significant ($F_{3,77}$=21.69,p<.001). The interaction of replicates and TeCG concentration was also significant ($F_{3,77}$=6.81,p<.001). Thus, it cannot be concluded with confidence that the difference in body width was due to the effect of exposure to TeCG.

3.2.3. Yolk Absorption

A randomized block ANOVA was performed for each day and for each of the four yolk parameters measured (yolk area, yolk length, and maximum yolk width) to test for the main effects of concentration of TeCG and replicates on the yolk parameters. Figures 9a-9c present this data. A significant difference between the control group and the treatment group (100 µg/litre) was found for yolk length on day 3 of exposure ($F_{1,9}$=8.44,p=0.017). A significant replicate effect was also found ($F_{4,9}$=11.91,p=0.001). However, no significant interaction was found to occur

Figure 8. Mean width (mm) of larval fathead minnows exposed to TeCG for 10 days (error bars represent 95% confidence intervals, n=8-60).

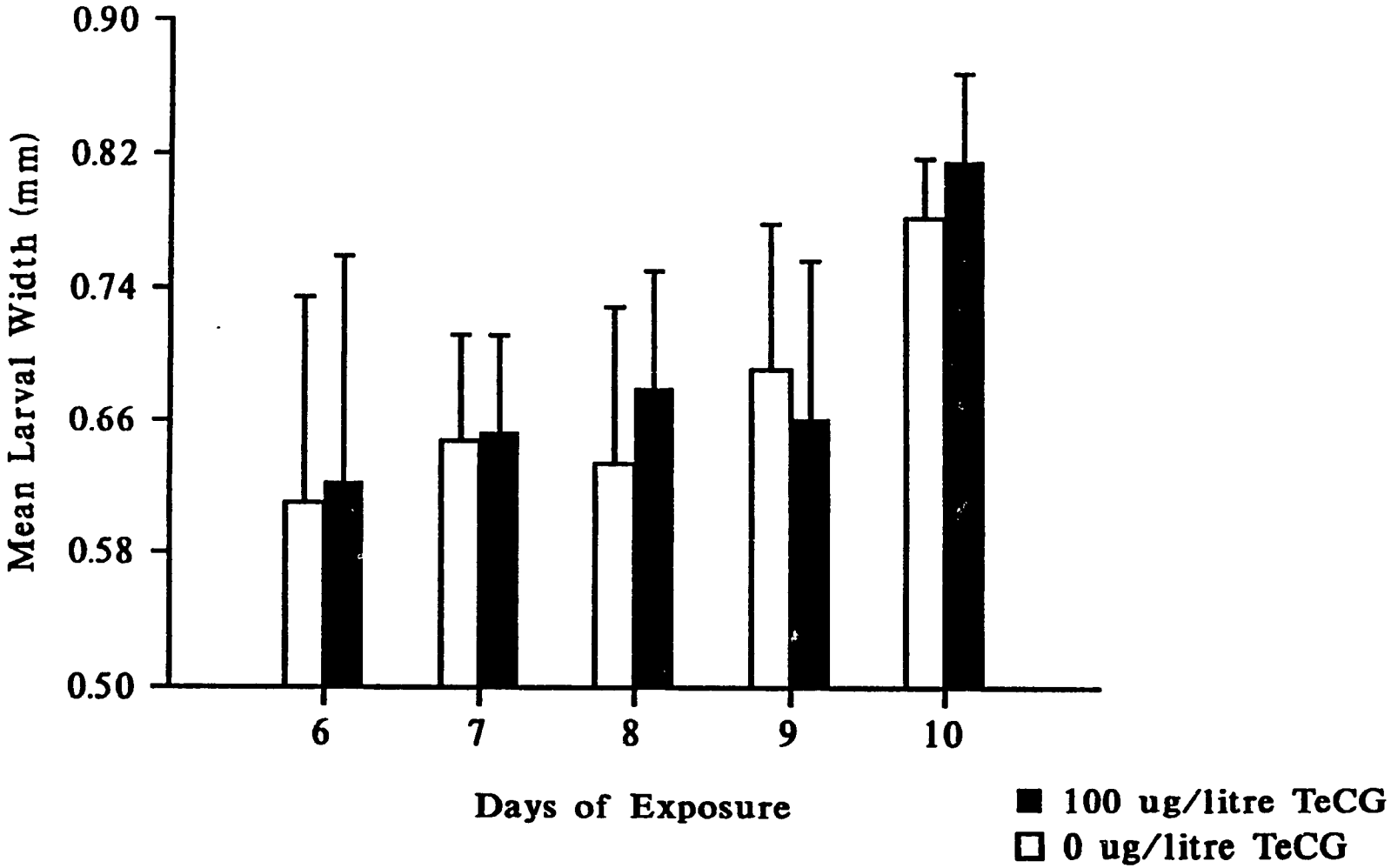
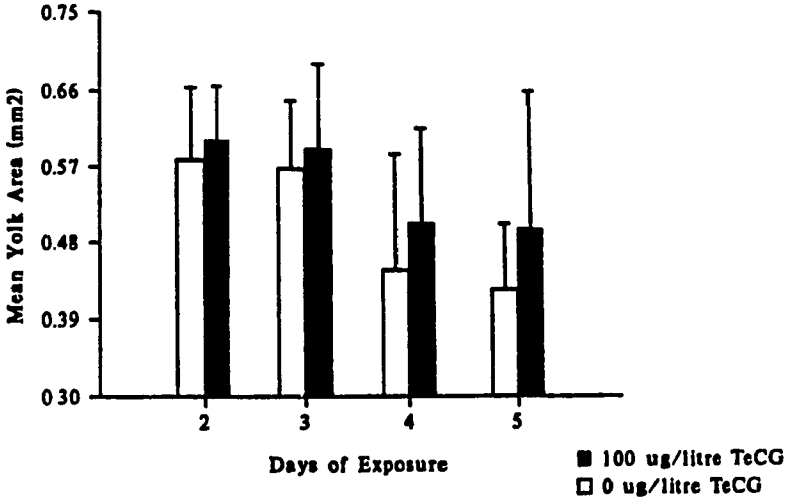
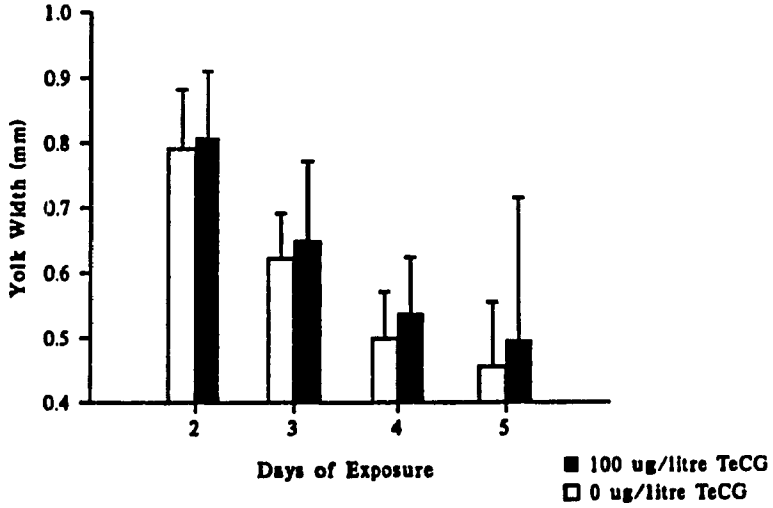

0.90
0.82
0.74
0.66
0.58
0.50
Mean Larval Width (mm)
6
7
8
9
10
Days of Exposure
100 ug/litre TeCG
0 ug/litre TeCG

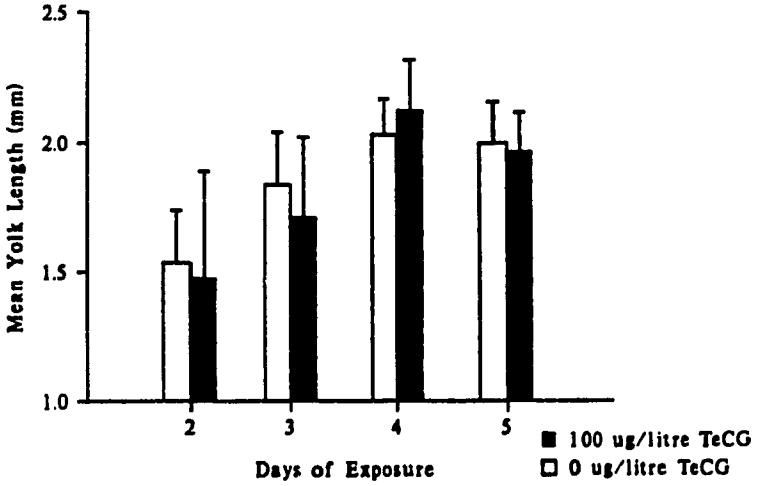
Figure 9a. Mean yolk area (mm^2) of embryos after exposure to TeCG. (error bars represent 95% confidence intervals, n=7-10).

9b. Mean yolk width (mm) after exposure to TeCG. (error bars represent 95% confidence intervals, n=7-10)

9c. Mean Yolk length (mm) after exposure to TeCG. (error bars represent 95% confidence intervals, n=7-10).


0.75
0.66
0.57
0.48
0.39
0 30
Mean Yolk Area (mm2)
2
3
4
5
Days of Exposure
100 ug/litre TeCG
0 ug/litre TeCG


1.0
0.9
0.8
0.7
0.6
0.5
0.4
Yolk Width (mm)
2
3
4
5
Days of Exposure
100 ug/litre TeCG
0 ug/litre TeCG


2.5
2.0
1.5
1.0
Mean Yolk Length (mm)
2
3
4
5
Days of Exposure
100 ug/litre TeCG
0 ug/litre TeCG

between replicates and concentration of TeCG ($F_{4,9}$=2.27,p=.14).

No significant difference was found between the control and treatment groups on any other day for any of the four yolk parameters measured.

3.2.4 Hatching Success Study

The proportion of hatching that occurred over ten days of exposure to TeCG was significantly lower in the treatment group (100 µg/litre) than in the control group ($t_{0.05(1)4}$=2.23,p=.05) (fig.10).

3.2.5 Lethal Bioassays

When larvae were exposed to TeCG for 10 days, beginning 24 hours after fertilization of the egg, no significant difference in mortality was found between the control and treatment groups ($F_{1,4}$=1.01,p=.37). Figure 11 illustrates these data; mortality was slightly higher in the treatment group when compared with the control group. However, looking at the confidence limits for each bar, it is evident that there is much greater variation found in the treatment group as compared with the control group. When group variances were tested there was no significant difference (F_1=2.37,p=.13).

Figure 10. Mean proportion of embryos that hatched after exposure to TeCG (error bars represent 95% confidence intervals, n=5).

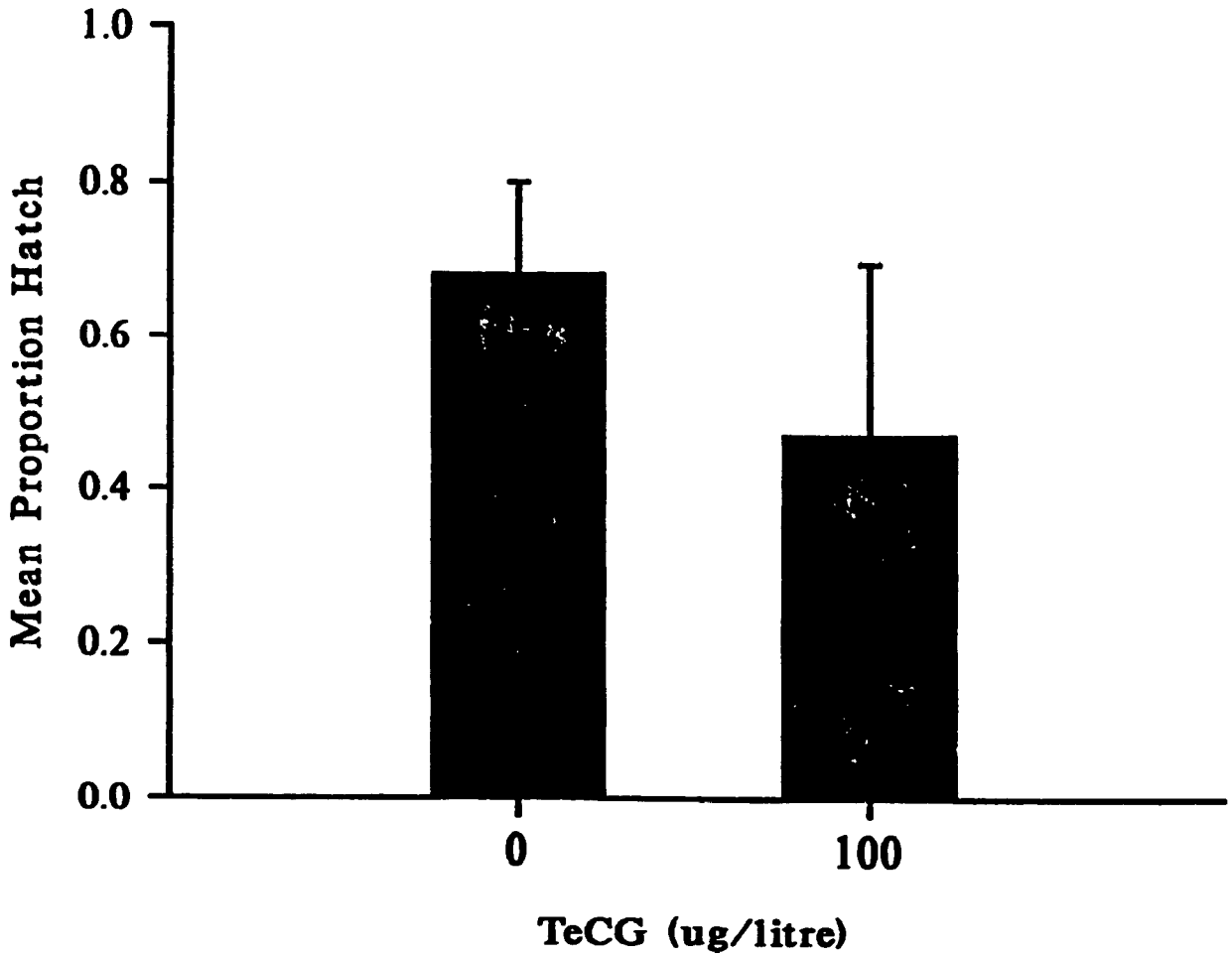

1.0
0.8
0.6
0.4
0.2
0.0
Mean Proportion Hatch
0
100
TeCG (ug/litre)

Figure 11. Mean proportion of larvae surviving after 10 days of exposure to TeCG (error bars represent 95% confidence intervals, n=5)

Proportion Surviving
0.7
0.8
0.9
1.0
1.1
TeCG (ug/litre)
0
100

DISCUSSION

4.1. Growth and Mortality

The results of the 7-day growth study showed no significant difference between the control group of fish and any of the five treatment groups of fish (25, 50, 100, 200, 400 µg/l TeCG), (fig.5). In the 10-day test, which included the embryo period, and the first few days of the larval period, body length and body width were measured each day for the last five days as an indicator of larval growth. No significant difference was found between the control group and the treatment group (100 µg/l) for length or width on any of the days that these parameters were measured (figs.7,8).

To estimate the toxicity of TeCG toxicity, equivalency factors (TEFs) have been used (Kovacs et al., 1991). TEFs are used in general to assess the risk of complex mixtures of compounds, such as BKME (Kutz et al., 1990) to aquatic biota.

In estimating the TEF for a given compound it is assumed that the compound's mode of toxic action is the same as that of the reference compound. To date, little research has been conducted on the toxic mode of action of TeCG so that any assertion made with regard to the toxic mode of action of TeCG is speculative. Thus, any use of TEFs to

estimate the toxicity of TeCG is also speculative.

In establishing a TEF for TeCG, the known toxicity of a reference chlorophenol, pentachlorophenol (PCP), is used. PCP is assigned a relative toxicity value of 1.0, while the relative toxicity of any chlorophenol, including guaiacols, with 4 chlorine atoms is assigned a value of 0.48. Thus, TeCG and tetrachlorophenol are assumed to have the same toxicities. The chronic value, ChV, the geometric mean of the NOEC, the concentration of the toxicant at which there is no observable effect in a chronic toxicity test, and the LOEC, the lowest concentration of the toxicant at which there is and observable effect, of PCP for dry weight of fathead minnow larvae in a 7-day growth test was found to be 253 µg/l (Pickering, 1988). Thus the TEF-derived ChV for TeCG based on these data is 527 µg/l. The results of my study are consistent with this estimate in that no significant decrease in growth was found below the highest concentration used (400 µg/l).

In studies that have investigated the effects of PCP on the embryo and larval stages of the fathead minnow over a 32-day period of exposure, the ChV for wet weight ranged from 40-172 µg/l (Martel and Kovacs, 1991;). Based on the results of their studies, the TEF-derived ChV for TeCG on larval growth ranges from 83-358 µg/l. Thus, it seems a 32-day test may be more sensitive for measuring the

effects of TeCG on the growth of fathead minnow larvae than is the 7-day test.

No significant difference in larval survival was found between the control group and treatment group in the 7-day test or in the 10-day test (fig.7,8). In a study that looked at the effects of TeCG on the median survival time of unfed larvae of zebrafish (Brachydanio rerio) the ChV was 250 µg/l (Neilson et al.,1991) In a comparative study that looked at the relative effects of TeCG on the median survival time of unfed larvae of zebrafish and steelhead trout (Salmo gairdneri) the ChVs were 330 µg/l and 570 µg/l respectively (Neilson et al., 1990). The highest concentration used in my study was 400 µg/l in the 7-day experiments. Because the larvae in my study were fed, starting on the day of hatch, a comparison of the sensitivity of each of these three species of fish to TeCG can not be made.

In a study that used the same 7-day test for growth and survival of fathead minnows that was used in my study, the ChV for larval survival was 362 µg/l (Pickering, 1988). Thus, the TEF-derived ChV for TeCG is 754 µg/l and no significant effect of TeCG on larval survival is expected to be found at concentrations below this value. My results are consistent with this estimate since no significant effect of TeCG on larval survival was found at levels between 0-400 µg/l.

When compared with the other chlorophenols, pulpmill chloroguaiacols are particularly abundant in biota, water and sediment of receiving areas of pulp mill discharges (Carey and Hart, 1988; Rogers et al., 1989). Of the dominant chloroguaiacols found in wastewater, TeCG has been found in BKME at levels between 1-220 µg/l in a number of different studies (McLeay, 1987). In a recent study, concentrations of TeCG 44 km downstream from a discharge site ranged between 4.5-20 µg/l (Hodson et al., 1992). Rogers et al., (1986) found that TeCG bioaccumulated in the tissue of juvenile chinook salmon, Oncorhynchus tshawytscha, and ranged from 2-111µg/kg wet weight (Rogers et al., 1986). Thus, the concentrations of TeCG that were used in this study are representative of those found in receiving waters and could result in TeCG bioaccumulation in aquatic biota.

4.2 Hatching and Early Development

Hatching success was significantly lower in the treatment group (100 µg/l) than in the control group (0 µg/l) (fig.9).

In a study that examined the effects of PCP on the hatchability of fathead minnows, ChVs ranged from 85-327 µg/l (Spehar et al., 1985). Thus, the TEF-derived ChV of TeCG for hatchability ranges from 177-681 µg/l. In another study in which the effects of PCP on the embryo and larval

stages of the fathead minnow were investigated, hatchability per se was not determined, however, viable hatch was measured (Holcombe et al.,1982). It was found that the LOEC for viable hatch was 223 µg/l PCP, giving a TEF-derived LOEC of 465 µg/l for TeCG. Viable hatch is argued to be more sensitive as a toxicity indicator than hatchability (von Westernhagen, 1988). However, based on the TEF-calculated estimate of the TeCG LOEC for viable hatch, my results suggest that hatchability is more sensitive than viable hatch since the TeCG LOEC for hatchability was 100 µg/l.

Dominguez and Chapman (1984) found a significant effect of PCP on hatchability in steelhead trout at 34 µg/l, making the TEF-derived ChV for TeCG for hatchability in steelhead trout 71 µg/l. Based on the above ChV estimates for TeCG for hatchability, steelhead trout are expected to be more sensitive to the effects of TeCG on hatchability than are fathead minnows.

I found no significant difference between the control and treatment groups for any of the yolk parameters, (area, maximum width, width perpendicular to the spine, and length), on any given day of exposure, except for yolk length on the third day post-fertilization (figs.9a-9c). On the third day the yolk length was shorter in the treatment group than what it was in the control group. Because chlorophenols may simultaneously prevent the

breakdown and synthesis of ATP (Weinbach and Garbus, 1964), I expected the yolk to be greater in the treatment group than in the control group. As embryogenesis progresses, the yolk changes from a large round ball to a long flattened yolk. Thus, I believe that my result for day 3 suggests that yolk absorption in the treatment group may have diminished in comparison with the control group since the yolk in the treatment group was more rotund and less elongate. If the energy budget is reduced by the presence of TeCG, then there is less energy available for transforming the yolk into new tissues. There is also less energy available for basal metabolism, growth and movement.

No data has been published on the effects of TeCG on yolk absorption for fish. However, one study that investigated the effects of PCP on the bioenergetics of steelhead trout found that the rate of yolk uptake decreased significantly and the rate of yolk catabolism increased significantly at 40 µg/l (Chapman and Shumway, 1978). Thus, overall yolk utilization efficiency was diminished. The corresponding TEF-derived value for TeCG is 83 µg/l. In another study, in which rainbow trout (Salmo gairdneri) embryos were also exposed to 40 µg/l of PCP a significant decrease in yolk absorption and weight at hatch was found (Hodson and Blunt, 1981). Again, the TEF-estimate of TeCG causing a significant effect on yolk absorption in rainbow trout is 83 µg/l.

There is no strong evidence, as indicated by my results, that the rate of yolk absorption was diminished due to TeCG exposure.

In order to diminish the effects of confounding variables on any of the different endpoints investigated during this study, paired t-tests and randomized blocked ANOVAs were performed on all data.

Depressed and elevated temperatures affect the bioenergetics and growth of largemouth bass (Micropterus salmoides), with highest growth occurring at 25°C and the lowest growth occurring at 18°C (Niimi and Beamish, 1974). However, these results may have occurred, in part, due to a higher rate of feeding at 25°C than at 18°C. When the feeding level was fixed, growth rate was highest at 18°C.

In another study, a significant difference in larval growth of fathead minnows was found between groups exposed to temperatures of 21.5°C, 25.9°C and 27.3°C after 45 days of treatment (Brungs 1971a). The control group (21.5°C) and the 27.3°C group had similar growth rates. Growth rate was highest at 25.9°C. No significant effect of temperature on hatchability was found within a range of 23.5-30°C.

The effect of toxicants that act on cellular enzymes involved in energy metabolism may be intensified by an increase temperature (Cairns et al., 1975). However, an increase in temperature may not always cause an increase in

toxicity since the rates of detoxification and excretion may also increase with an increase in temperature (Cairns et al., 1975).

Oxygen concentrations in water are also affected by temperature and thus, diminished oxygen concentrations can also be a confounding factor (Cairns et al.,1975). Toxicity of the test chemical may be affected by a change in dissolved oxygen which in turn is affected by changes in temperature.

Oxygen concentrations in my experiments never dropped below 40% of saturation (3.3 mg/l), as recommended by the design protocol used in my study. However, I question the safety level in preventing confounding effects due to oxygen depletion on larval growth that this criterion provides for in this type of experiment. In a literature review conducted by Sprague (1971) he states that growth of fish that are fed unrestricted diets is particularly sensitive to depressed oxygen levels (below 90% saturation). In my study, portions of brine shrimp were always present after feeding periods. I interpret this as being an unrestricted diet. In another study, Brungs (1971b) found the larval fathead minnows had significantly reduced growth at oxygen levels below 7.9 mg/l (90% air saturation).

Brungs (1971b) found that survival was also reduced at 4.0 mg/l (37% air saturation) but no effect on hatching

was found even for oxygen levels as low as 2.0 mg/l (17% air saturation). In another study, the hatchability of embryos of northern pike (Esox lucius) was not affected by depleted oxygen levels until it dropped below 33% saturation (Davis, 1978).

In summary, I believe that growth, yolk absorption and survival of the fish in all groups in my study may have been affected by depressed oxygen levels but that hatchability was not so affected (eg. Brungs, 1971b, Davis, 1978).

CONCLUSION

A significant effect of TeCG on hatchability was found at 100 µg/l. No effect of TeCG was found on larval survival, larval growth or yolk absorption. If hatchability is a more sensitive indicator of toxicity than larval growth or survival, then based on procedures used by Environment Canada to set water quality criteria, the recommended ChV for TeCG in BKME should be set at 50 µg/l.

RECOMMENDATIONS

The 7-day growth and survival experiment should be repeated under conditions of higher oxygen levels and a restricted diet so that excess amounts of brine shrimp in the experimental chambers will not contribute to oxygen depletion. The experiments should be run with higher concentrations of TeCG (0-800 µg/l) and exposure levels should be determined by gas chromatography to determine the extent to which TeCG volatilizes with aeration.

The fact that a significant effect of TeCG was found on hatchability and not on larval growth or survival suggests that further testing to determine the relative sensitivity of hatchability as a toxicity indicator for regulatory purposes be conducted. In addition, consideration should be given to decreasing the amount of time between fertilization and initial toxicant exposure in the present 7-day growth and survival test in order to ensure that the most sensitive stages of the embryonic period be included in the toxicity test.

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