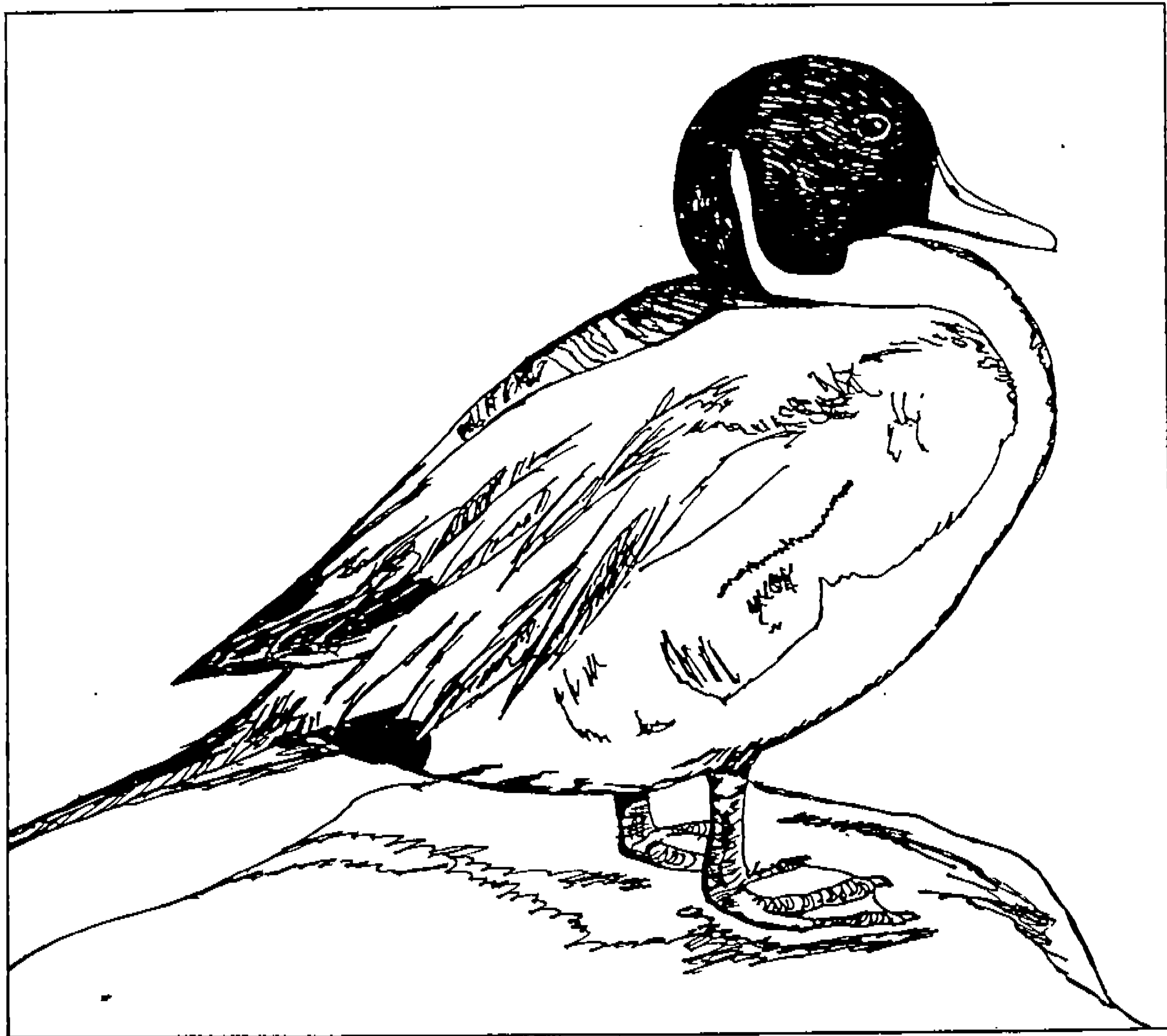
OUC 0009822

U.S. Army Eagle River Flats: Protecting Waterfowl from Ingesting White Phosphorus

Technical Report 93-1



UNITED STATES DEPARTMENT OF AGRICULTURE
DENVER WILDLIFE RESEARCH CENTER

OUC 0009823

U.S. Army Eagle River Flats: Protecting Waterfowl from Ingesting White Phosphorus

Project Coordinator: John L. Cummings

Principal Investigators: Larry Clark and John L. Cummings

Prepared for U.S. ARMY TOXIC AND HAZARDOUS MATERIALS AGENCY

Prepared by: *UNITED STATES DEPARTMENT OF AGRICULTURE*
DENVER WILDLIFE RESEARCH CENTER
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*Task A.: **Effects of Methyl Anthranilate Bead Formulations on Mallard Feeding Behavior in an Aqueous Environment.***

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FORWARD

The primary role of the Denver Wildlife Research Center in this project is to develop and evaluate methyl anthranilate as a interim remediation action to reduce waterfowl mortality caused by white phosphorus at Eagle River Flats, Fort Richardson, Alaska. This project involved close collaboration between many Federal and State agencies.

We especially thank Captain Steven Bird, U.S. Army Toxic and Hazardous Materials Agency, Aberdeen proving Grounds, Maryland for providing funding and support for this project.

We acknowledge the invaluable support of the U.S. Army 6th Infantry Division (Light). We especially single out Colonel Robert Wrentmore, Douglas Johnson, William Gossweiler and Bill Smith and personnel of the 176th Ordinance Detachment (EOD).

We acknowledge the suggestions and guidance from the Eagle River Flats Interagency Task Force, composed of representatives from the U.S. Army (Colonel Robert Wrentmore, Captain Steven Bird, Douglas Johnson, and William Gossweiler), U.S. Fish and Wildlife Service (John Nelson, Sonce DeVries and William Eldridge), Alaska Department of Fish and Game (Dan Rosenberg), Alaska Department of Environmental Conservation (Jennifer Roberts).

We greatly appreciate the assistance of CRREL personnel: Charles Racine, Marianne Welsh and Charles Collins with sediment data collection and analysis.

We appreciate the invaluable assistance provided by DWRC and ADC personnel with special thanks to Greta Schoenberger.

EXECUTIVE SUMMARIES

Task A: Effects of Methyl Anthranilate Bead Formulations on Mallard Feeding Behavior in an Aqueous Environment.

We applied two methyl anthranilate (MA) bead formulations to bottom sediment in a simulated pond setting to evaluate bird repellency to captive mallards (Anas platyrhynchos). Formulations and application rates were: DP920324B (5% MA) applied at 5.4 kg/ha and SE920326 (5% MA) applied at 5.4, 10.8, and 21.7 kg/ha. The ineffectiveness of DP920324B to reduce mallard feeding in treated pools was attributed to the pliable structure of the beads. Mallards were unable to break the beads to release the methyl anthranilate. Experiments with SE920326 at application rates of 5.4 and 10.8 kg/ha showed slight treatment effects. SE920326 applied at 21.7 kg/ha to bottom sediment was effective in reducing the time mallards spent in treated pools ($P \leq 0.05$). SE920326 applied to contaminated waterfowl feeding areas at 21.7 kg/ha could reduce feeding and mortality and warrants further testing in the field.

Task B: Preliminary evaluation of encapsulated methyl anthranilate at Eagle River Flats, Fort Richardson, Alaska.

We evaluated methyl anthranilate encapsulated in a sodium alginate bead formulation at two field sites during the spring and fall Eagle River Flats, Fort Richardson, Alaska. Encapsulated formulations of MA were able to decrease feeding activity of ducks 50-80% for up to 10 days. Based on evaluation of several formulations and their performance in the field recommendations are made or a final formulation which should have a half life of 10 days and an efficacy of at least 80% reduction of feeding activity. Sentinel studies should not be used compare relative risk of MA vs Control pens directly because even small sampling rates over a prolonged observation period place captive ducks at risk to WP poisoning. MA works by moving waterfowl away from areas of treatment, not by suppressing feeding 100%. Thus, field studies on free-ranging ducks are needed to further evaluate the efficacy of MA as a short-term remediation strategy.

Task C: Acute toxicity of methyl anthranilate to fish: Atlantic salmon, rainbow trout, channel catfish and bluegill.

Several laboratory and field studies have shown methyl anthranilate to be an effective nontoxic and nonlethal bird repellent, with application potential for protecting crops, seeds, turf and fish stocks from bird damage. Furthermore methyl anthranilate can be added to liquids for the purposes of protecting migratory birds, e.g. addition to

waste water associated with mining and to standing water pools at airports. Mammalian toxicity data are favorable. Methyl anthranilate is used as a fragrance and food flavoring and is GRAS listed by the US Food and Drug Administration. Despite the favorable outlook for methyl anthranilate's use as a safe repellent, no data exist on its environmental fate and effects. We tested the acute toxicity of methyl anthranilate in a static system for 4 species of fish. The LC_{50} at 96-h for Atlantic salmon (Salmo salar) was estimated to be 32.35 mg/L, with the no observable effect limit at 6.0 mg/L. The LC_{50} at 96-h for rainbow trout (Onchorhinus mykiss) was estimated to be 22.92 mg/L, with the no observable effect limit at 5.0 mg/L. The LC_{50} at 96-h for channel catfish (Ictalurus punctatus) was estimated to be 16.23 mg/L, with the no observable effect limit at 7.0 mg/L. The LC_{50} at 96-h for bluegill sunfish (Lepomis macrochirus) was estimated to be 9.12 mg/L, with the no observable effect limit at 7.0 mg/L.

***Task D:* Aquatic toxicity, bioaccumulation, and lifecycle effects of methyl anthranilate to daphnids.**

We evaluated the acute toxicity of five methyl anthranilate concentrations (3.1, 6.2, 11.9, 23.8 and 47.2 ppm) to daphnids. The LC_{50} of methyl anthranilate to daphnids at 24-h was estimated to be 31.3 ppm. There was no observed effect from methyl anthranilate at < 19.1 ppm and < 16.1 after a 24-h and 48-h exposure, respectively.

Executive summaries prepared by John L. Cummings and Larry Clark, U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Denver Wildlife Research Center, Denver, CO 80225.

Task A: Effects of Methyl Anthranilate Bead Formulations on Mallard Feeding Behavior in an Aqueous Environment

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ABSTRACT

We applied two methyl anthranilate (MA) bead formulations to bottom sediment in a simulated pond setting to evaluate bird repellency to captive mallards (Anas platyrhynchos). Formulations and application rates were: DP920324B (5% MA) applied at 5.4 kg/ha and SE920326 (5% MA) applied at 5.4, 10.8, and 21.7 kg/ha. The ineffectiveness of DP920324B to reduce mallard feeding in treated pools was attributed to the pliable structure of the beads. Mallards were unable to break the beads to release the methyl anthranilate. Experiments with SE920326 at application rates of 5.4 and 10.8 kg/ha showed slight treatment effects. SE920326 applied at 21.7 kg/ha to bottom sediment was effective in reducing the time mallards spent in treated pools ($P \leq 0.05$). SE920326 applied to contaminated waterfowl feeding areas at 21.7 kg/ha could reduce feeding and mortality and warrants further testing in the field.

INTRODUCTION

The U.S. Army has used Eagle River Flats (ERF) since 1945 as an impact area for artillery shells, mortar rounds, rockets, grenades, illumination flares, and Army/Air

Force Door Gunnery Exercises. In August 1981, hunters discovered large numbers of duck carcasses in ERF. Since that time, the Army and other federal and state agencies have been involved in identifying the cause of the waterfowl mortality problem. On February 8, 1990, the Army temporarily suspended firing into the ERF due to the suspected correlation between explosives and duck deaths (Quirk 1991). In July 1990, a sediment sample collected from ERF was suspected of containing white phosphorus (WP). By February 1991, it was concluded that WP in ERF was the cause of waterfowl mortality (CRREL 1991).

Waterfowl populations, especially mallards, are decreasing continent-wide (U.S. Fish and Wildlife Service and Canadian Wildlife Service 1989). ERF has been identified as an important spring (April to May) and fall (August to October) waterfowl staging area. The presence of WP in ERF could represent a hazard to feeding waterfowl (CRREL 1991). This concern has stimulated efforts to develop of an effective remediation action to reduce or eliminate waterfowl mortality caused from WP in ERF.

In response to the Army's queries about bird repellents in July 1991, the DWRC proposed to develop a repellent delivery system for testing at ERF. The objective was to deter ducks from foraging on contaminate sections of ERF. The DWRC selected methyl anthranilate (MA) for use as an active ingredient for the following reasons. MA was known to be an effective bird repellent (Kare 1961). Extensive field testing by DWRC personnel over the past 10 years showed that MA and its analogs had good potential as a repellent in a variety of circumstances (Glahn et al. 1985, Mason et al.

1985 and 1989, Cummings et al. 1991). Particularly promising was the fact that MA was not toxic to mammals. MA is GRAS listed by the FDA and is used extensively at high concentrations as a fragrance and food flavoring. We envisioned encapsulating MA into a form which could be broadcast onto the sediment and would release its contents only upon contact by foraging waterfowl. This would tend to reduce on target hazards and prolong the effective period of treatment. Our objectives in this study were to determine mallard bill pressure, develop methyl anthranilate (MA) bead formulations that were structured to rupture at less than the minimum observed bill pressure, and evaluate MA bead formulation(s) in a simulated pond setting to determine the effects on mallard behavior.

METHODS

Mallard Bill Pressure

We constructed a device (Fig. 1) to measure the applied bill pressure of a mallard. Determination of the bill pressure would allow us to develop MA bead formulations that would burst under minimum bill pressure. Six adult male mallards were randomly selected from a captive population of wild ducks. Each duck was placed in a 1.0 x 0.7 x 0.2 m holding cage. At the time of testing, individual ducks were removed individually from their cages. The displacement tab was placed in their bill about 1 cm from the tip and a pressure reading recorded. This procedure was repeated three times for each duck.

MA Experiments

We obtained two bead formulations containing 5% entrapped methyl anthranilate and ranging in size from 1 to 4 mm from PMC Specialties Group, Cincinnati, Ohio[1]. The bead shell was made of various food grade materials (alginate or gelatin). The structure strength of each bead formulation was designed to meet minimum mallard bill pressure.

We cannon-netted (Dill and Thornsberry 1950) 82 adult mallards (51 males and 31 females) on the grounds of the Denver Federal Center, Denver Colorado in February 1992. They were housed in 2 outdoor pens (8 x 4 x 2 m) with free access to food and water and quarantined for 14 days before testing.

After quarantine, 12 mallards were assigned randomly to each of 3 experiments. They included the following: Experiment 1: DP920324B (5% MA) vs. SE920326 (5% MA) both applied at 5.4 kg/ha (5 lbs/a) vs. control; Experiment 2: SE920326 (5% MA) applied at 10.8 kg/ha (10 lbs/a) vs. control; and Experiment 3: SE920326 (5% MA) applied at 21.7 kg/ha (20 lbs/a) vs. control . Within experiments, pairs of ducks were selected randomly and housed in 2 x 2 x 2 m test pens (Fig. 2) in an indoor aviary. Ducks were acclimated to their surroundings for 3 days. Each pair had free access to food and water. The floor of each pen was elevated about 20 cm and covered with

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1] Use of a company name does not imply U.S. Government endorsement of their products.

Dri-deck® matting. A circular pool 1 m in diameter and 20 cm deep was installed so that water height was the same as the floor. Mallards were able to enter the pool directly from the floor. The bottom of each pool was covered with 0.5 cm of fine sand.

We conducted each experiment between 0800 and 1600 hours for a 3-day pre-conditioning and a 3-day treatment period, except experiment 1, which included a 2-day pre-conditioning period. During each test day, a Trailmaster® motion detector affixed to each pen was used to record entries and minutes of use of the pool by mallards. In addition, a pool activity period was determined for each pair of mallards. The activity period was determined as the interval (measured in hours) between when the mallards first entered the pools until their final exit during the daily test period. The bead formulation in each experiment was designed to settle to the bottom of the pool and only release MA when broken by feeding mallards. For all experiments, the bead formulation was applied one time by hand.

Two factor repeated measure analysis of variance (ANOVA) with treatments and days as fixed effects was used to assess mallard entries and minutes of use of each pool and the activity period during the test (SAS Inst., Inc. 1988). Pairs of mallards formed the error terms. Where ANOVA results were significant ($P < 0.05$), means were separated using Duncan's Multiple Range test. Only the final day of each pre-conditioning period was used in the analyses.

RESULTS

Mallard Bill Pressure

Mallards averaged 1.77 psi bill pressure (sd = 0.65; range = 1-3). Since the minimum observed bill pressure was 1 psi, MA bead formulations were developed that would burst at 1 psi.

Experiment 1: DP920324B vs. SE920326 va. Control

*There were no significant differences among treatments in the number of entries mallards made into pools (F = 3.61; 2, 3, df; P = 0.15) or days (F = 2.92; 3, 9, df; P = 0.09), and treatment * day interaction means (F = 2.89; 6, 9, df; P = 0.07).*

Overall, entries into pools treated with DP920324B increased following the pre-conditioning period (Fig. 3). Entries into pools treated with SE920326 indicated a slight reduction on days 2 and 3 post-treatment (Fig. 4). This reduction warranted further testing of SE920326 at increased application levels, 10.8 and 21.7 kg/ha. Entries into untreated pools remained relatively constant (Fig. 5).

Experiment 2: SE920326: MA10 vs. Control

*There were no significant differences among treatments in the number of minutes ducks spent in the pools (F = 0.39, 1,4 df; P = 0.56), or days (F = 0.54, 3, 12 df; P = 0.66), and treatment * day interaction means (F = 1.50, 3, 12 df; P = 0.26). Following the pre-conditioning period, duck use of treated pools (Fig. 6) was slightly*

*reduced in comparison to untreated pools (Fig. 7). Similarly, among treatments there were no significant differences in mallard activity periods in pools (F = 1.71; 1,4 df; P = 0.26), by days (F = 0.21; 3,12 df; P = 0.88), and treatment * day interaction means or interaction terms (F = 0.87; 3,12 df; P = 0.48). Activity periods of mallards in treated and untreated pools were similar (Fig. 8,9).*

Experiment 3: SE920326: MA20 vs Control

*There were significant differences among treatments in the number of minutes ducks spent in the pools (F = 43.72; 1,4 df; P = 0.002) but no differences among days (F = 0.43; 3,12 df; P = 0.55). However, there were significant differences among the treatment * day interaction means (F = 4.21; 3,12 df; P = 0.02). Duncan's multiple range test showed that minutes spent by ducks in treated pools decreased significantly ($P \leq 0.05$) between the last pre-conditioning day and each post-treatment day (Fig. 10). The minutes spent by ducks in untreated pools remained relatively constant during the same time period ($P \geq 0.05$) (Fig. 11).*

The activity periods of mallards in treated pools decreased significantly ($P \leq 0.05$) between the last pre-conditioning day and the post-treatment days (Fig. 12). Duck activity in untreated pools remained relatively constant ($P > 0.05$) during the test period (Fig. 13).

CONCLUSIONS

The degree of repellency of MA bead formulations were influenced by bead structure and application rates. The ineffectiveness of DP920324B in Experiment 1 was due to the bead structure. Examination of the test pools following the experiment indicated that beads were pliable and would not break under duck bill pressure. In addition, some beads, were consumed intact.

MA is a chemosensory repellent acting through trigeminal senses (Mason et al. 1989). It has no aversive post-ingestional effect, thus, ducks would only avoid the pools when effective repellency levels were maintained. The avoidance of MA formulations SE920326 in Experiments 1 and 2, albeit slight, may reflect ducks differences in the degree to which chemical senses influence food consumption. Also, the low application rates may have attributed to marginal bottom coverage, precluding ducks from encountering sufficient numbers of beads during each feeding bout.

In Experiment 3, MA beads applied at 21.7 kg/ha was sufficient to cause almost complete avoidance of treated pools. Indications were that ducks encountering the higher levels of MA were likely to respond positively on subsequent treatment days. Application of MA at 21.7 kg/ha produced evidence suggesting a higher application rate causes a greater avoidance of pools than lower rates. In addition, this application rate suggests that there were residual effects. Ducks exhibited learned avoidance behavior following treatment.

RECOMMENDATIONS

MA bead formulation SE920326, applied at 21.7 kg/ha, was an effective waterfowl feeding deterrent when applied to bottom sediment. On the basis of these results, we proceeded in using a 1.5 mm gel alginate bead with 4% MA as the repellent for the spring field trials.

ACKNOWLEDGEMENTS

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FIGURE CAPTIONS

Figure 1. *A device to measure the applied bill pressure of a mallard, February 1992, Denver, Colorado.*

Figure 2. *Test pen to evaluate methyl anthranilate formulations on mallards, February 1992, Denver, Colorado,.*

Figure 3. *Experiment 1: Mean entries by 2 pairs of mallards into pools treated with methyl anthranilate bead formulation DP920324B (5%) at 5.4 kg/ha, February 1992, Denver, Colorado.*

Figure 4. *Experiment 1: Mean entries by 2 pairs of mallards into pools treated with methyl anthranilate bead formulation SE920326 (5%) at 5.4 kg/ha, February 1992, Denver, Colorado.*

Figure 5. *Experiment 1: Mean entries by 2 pairs of mallards into untreated pools, February 1992, Denver, Colorado.*

Figure 6. *Experiment 2: Mean time spent by 3 pairs of mallards in pools treated with methyl anthranilate bead formulation SE920326 (5%) at 10.8 kg/ha, February 1992, Denver, Colorado.*

Figure 7. *Experiment 2: Mean time spent by 3 pairs of mallards in untreated pools, February 1992, Denver, Colorado.*

Figure 8. *Experiment 2: Activity period of 3 pairs of mallards following release into pools treated with methyl anthranilate bead formulation SE920326 (5%) at 10.8 kg/ha, February 1992, Denver, Colorado.*

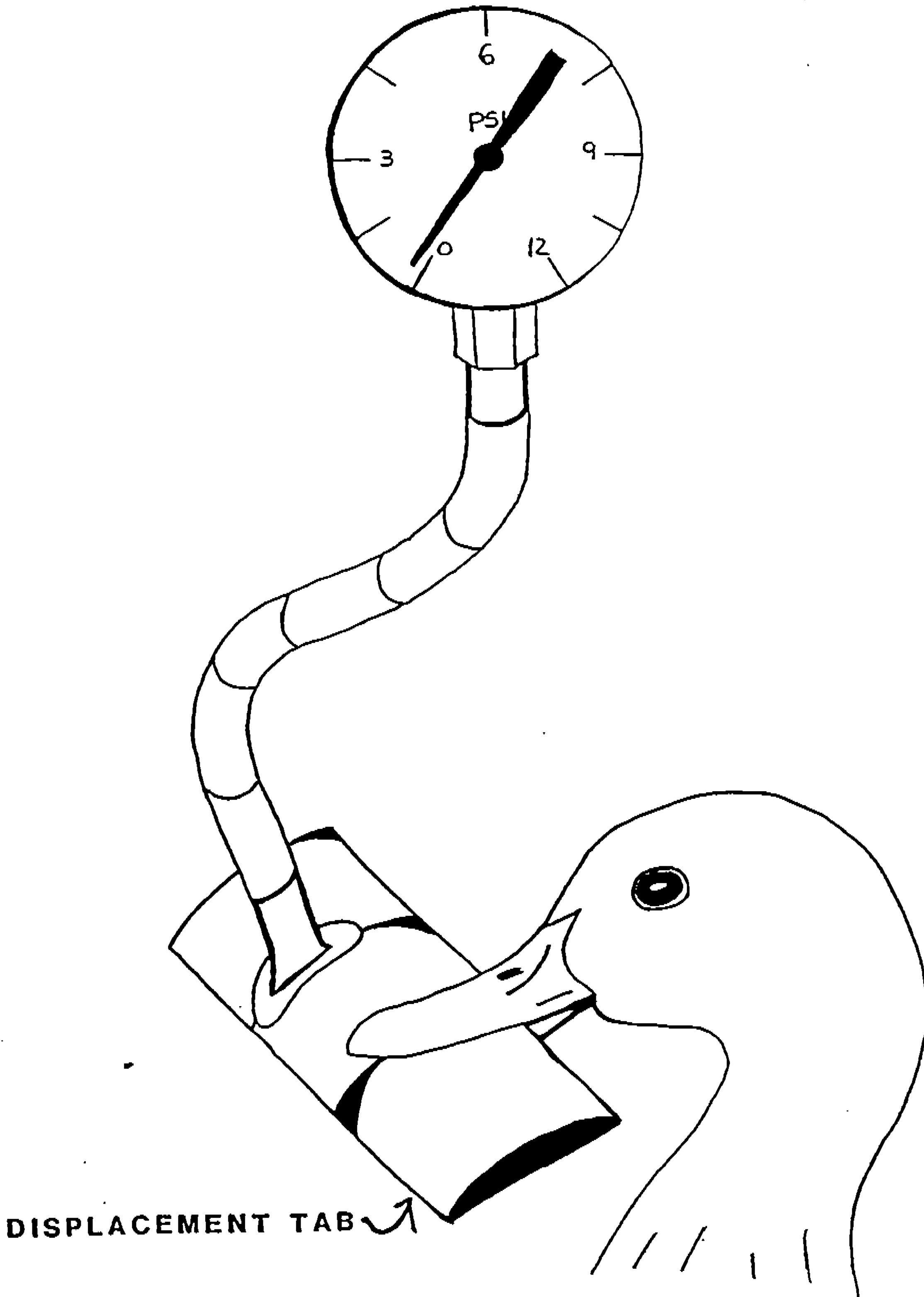
Figure 9. *Experiment 2: Activity period of 3 pairs of mallards following release into untreated pools, February 1992, Denver, Colorado.*

Figure 10. *Experiment 3: Mean time spent by 3 pairs of mallards in pools treated with methyl anthranilate bead formulation SE920326 (5%) at 21.7 kg/ha, February 1992, Denver, Colorado.*

Figure 11. *Experiment 3: Mean time spent by 3 pairs of mallards in untreated pools, February 1992, Denver, Colorado.*

Figure 12. *Experiment 3: Activity period of 3 pairs of mallards following release into pools treated with methyl anthranilate bead formulation SE920326 (5%) at 21.7 kg/ha, February 1992, Denver, Colorado.*

Figure 13. Experiment 3: Activity period of 3 pairs of mallards following release into untreated pools, February 1992, Denver, Colorado.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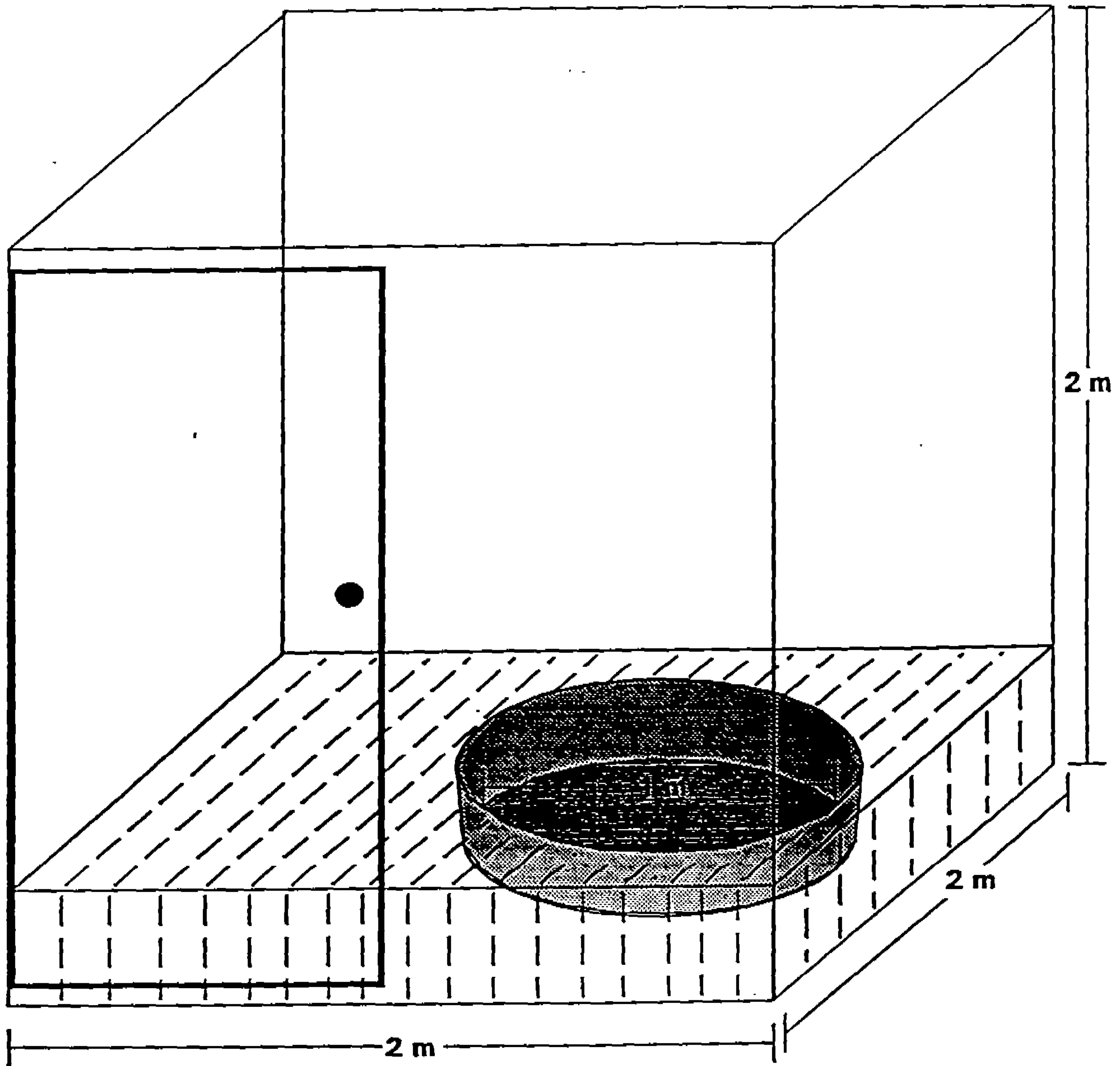
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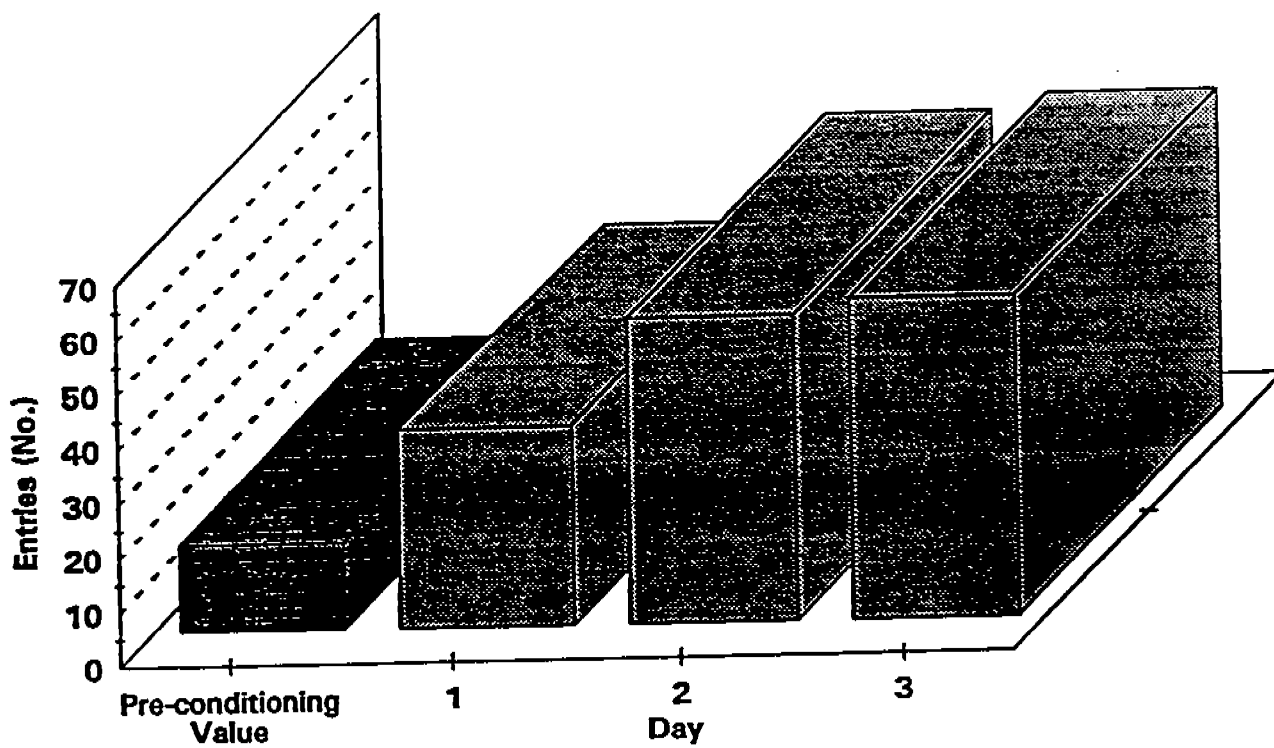
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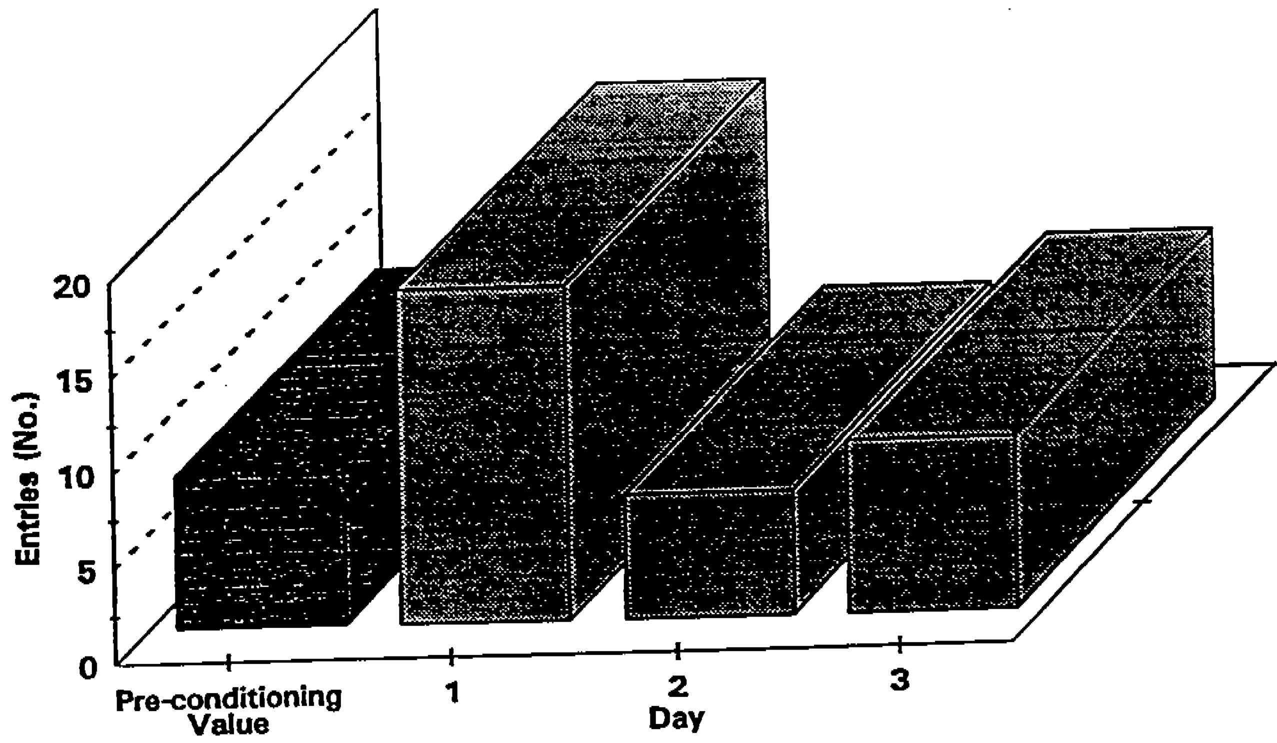
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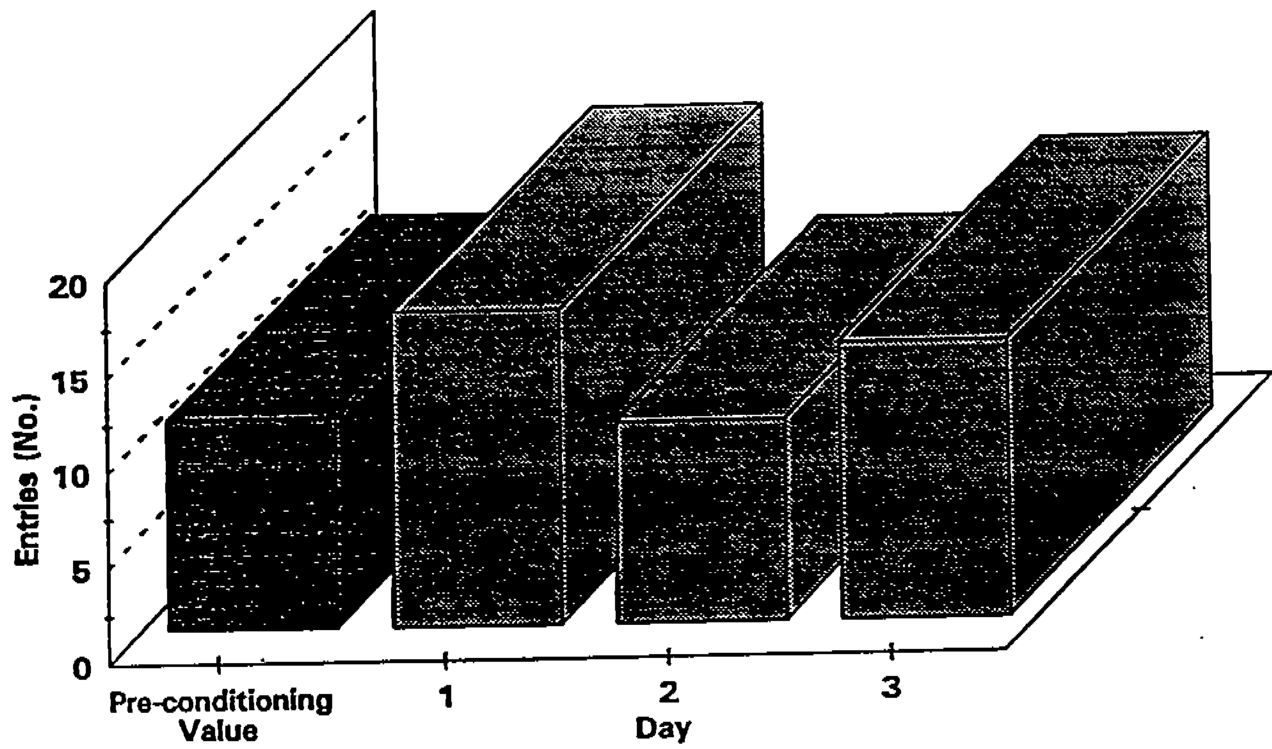
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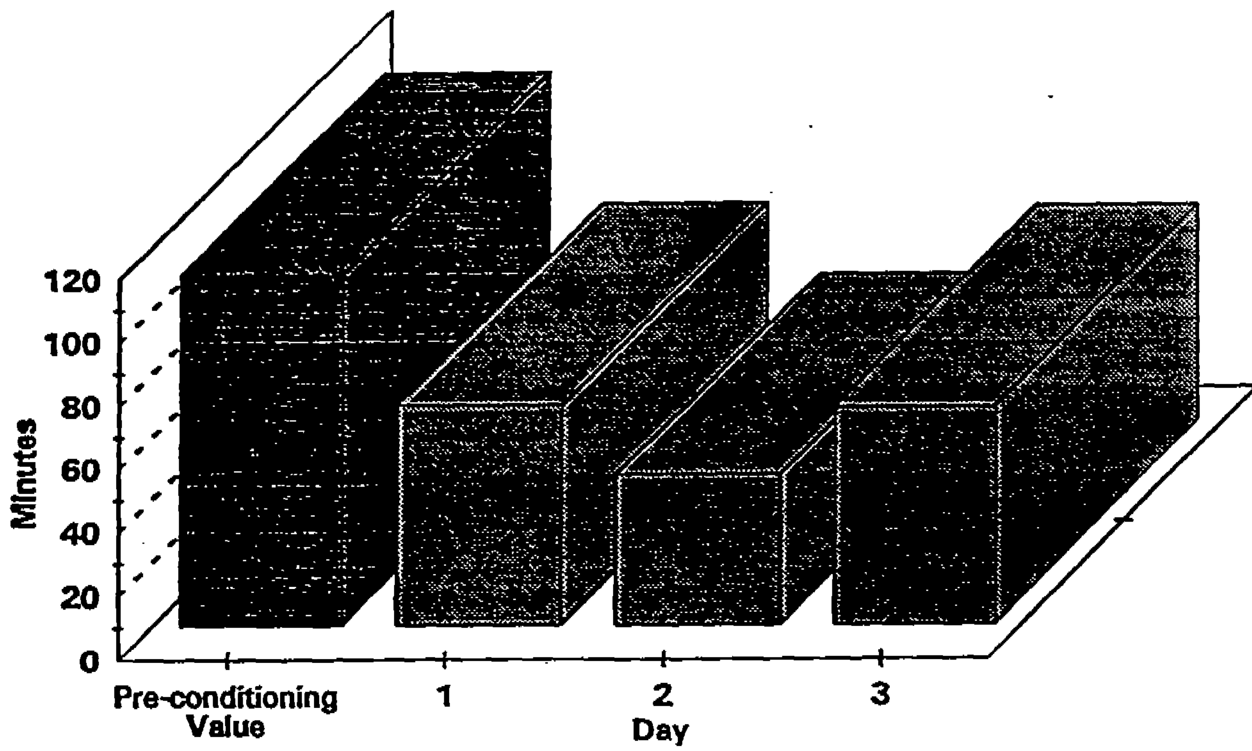
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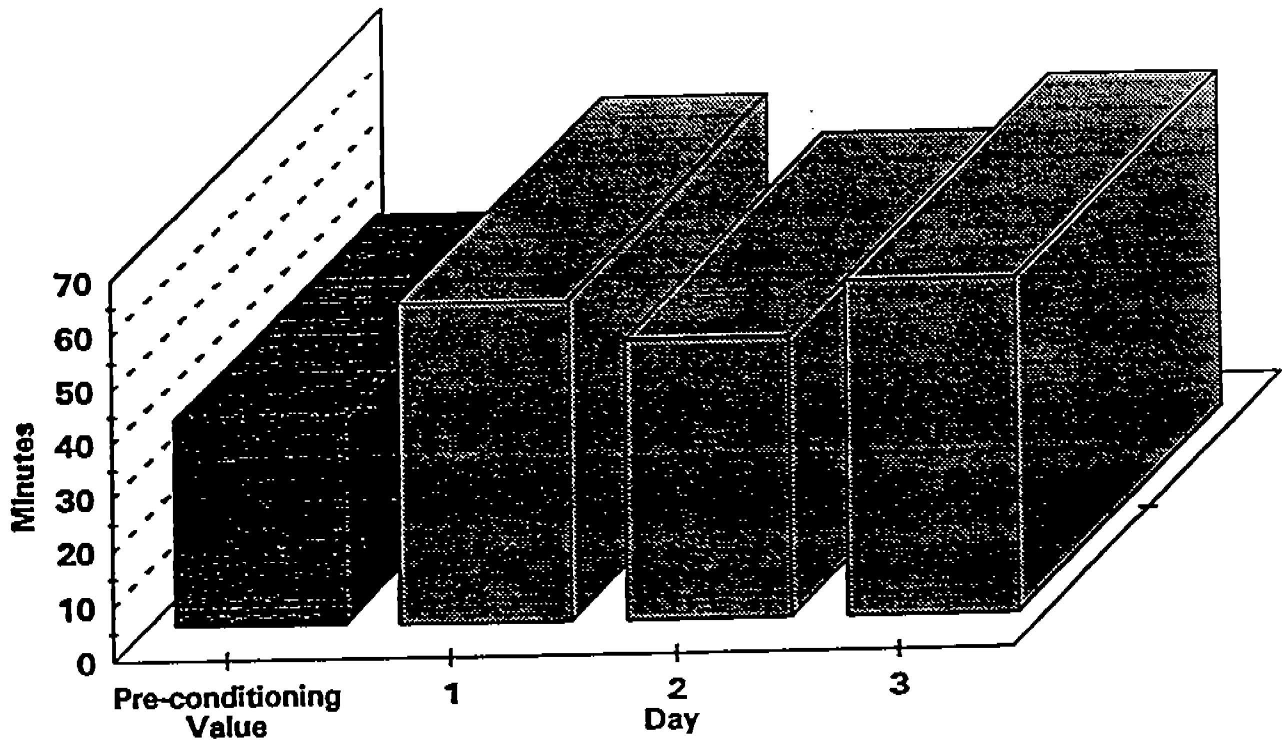
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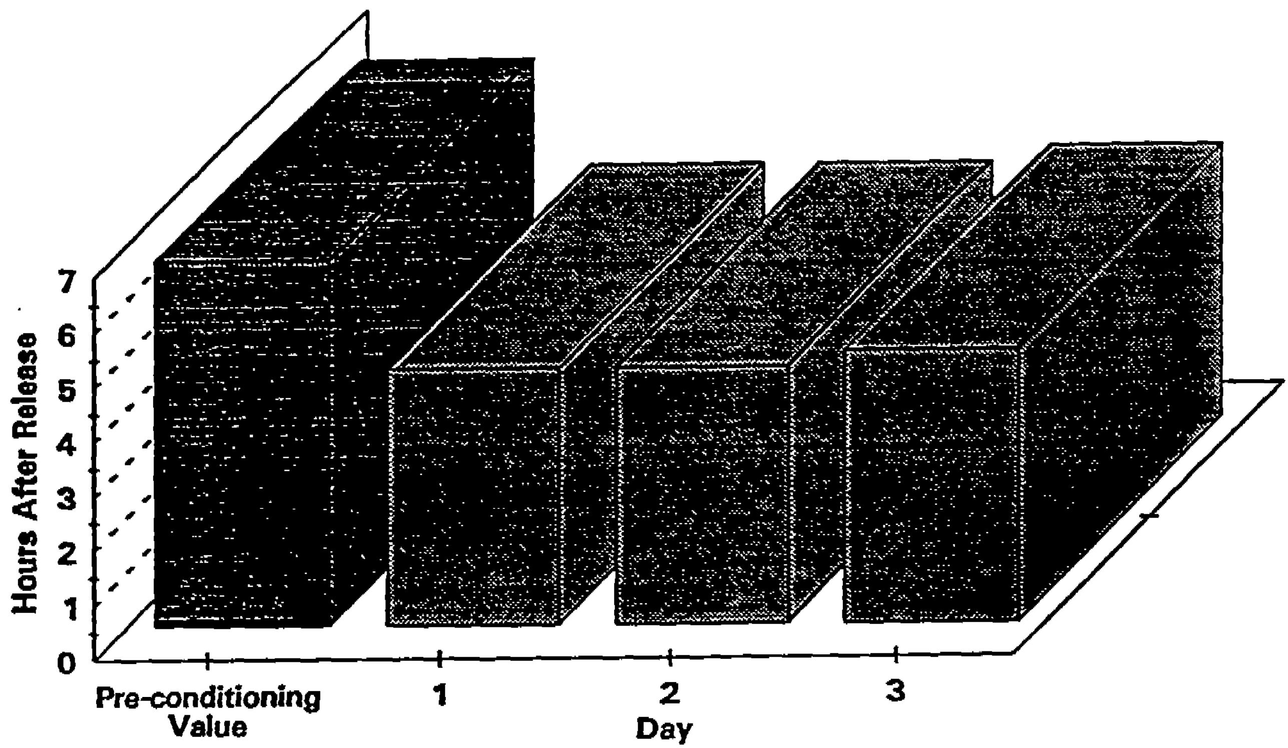
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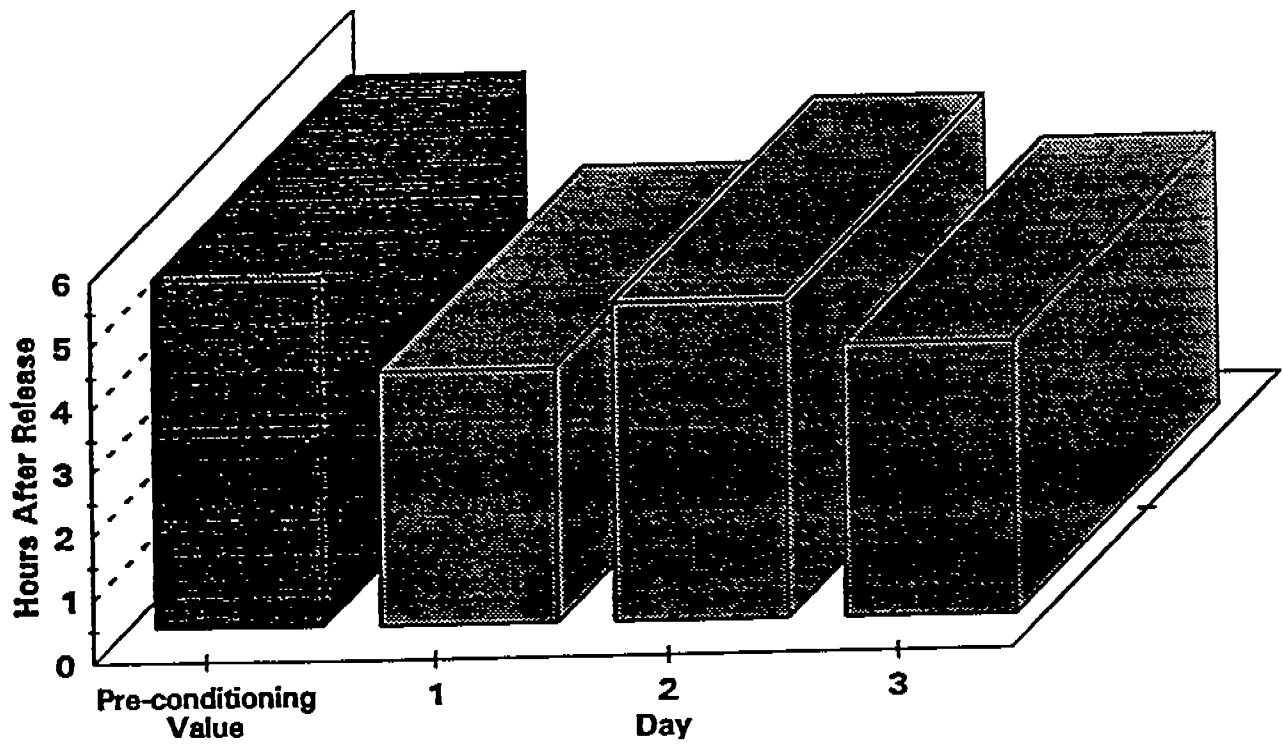
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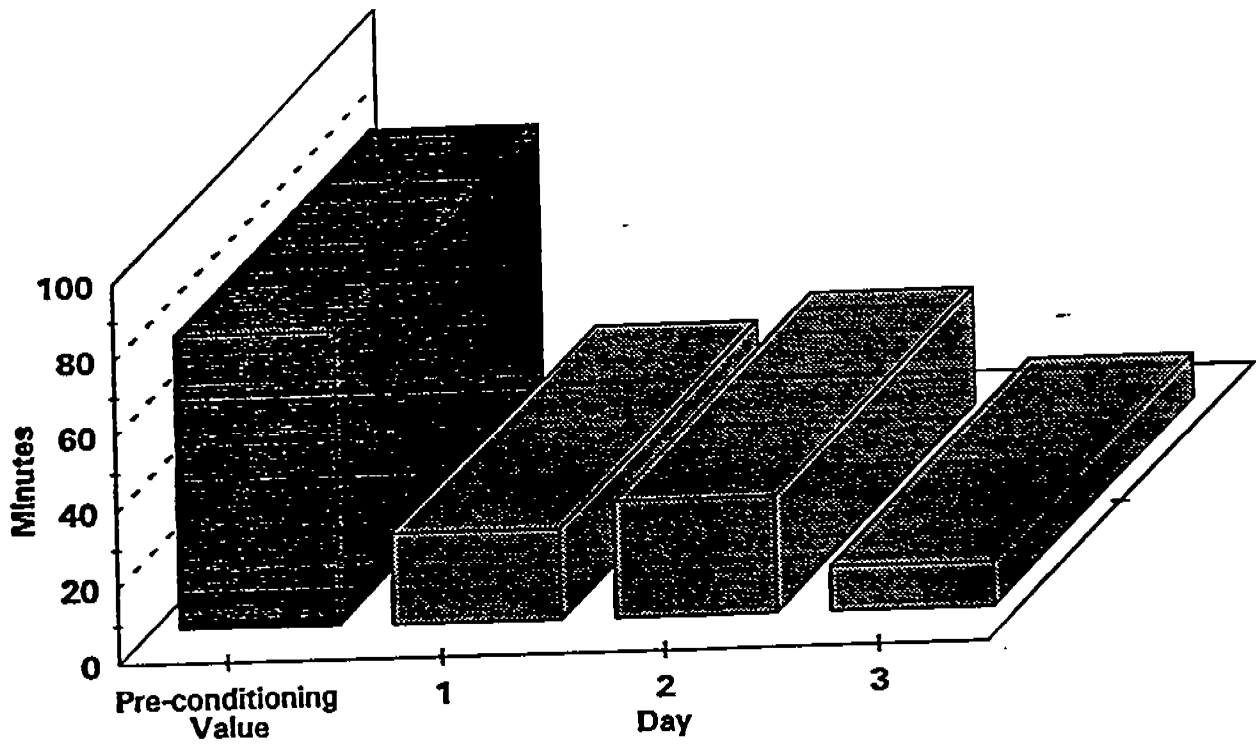
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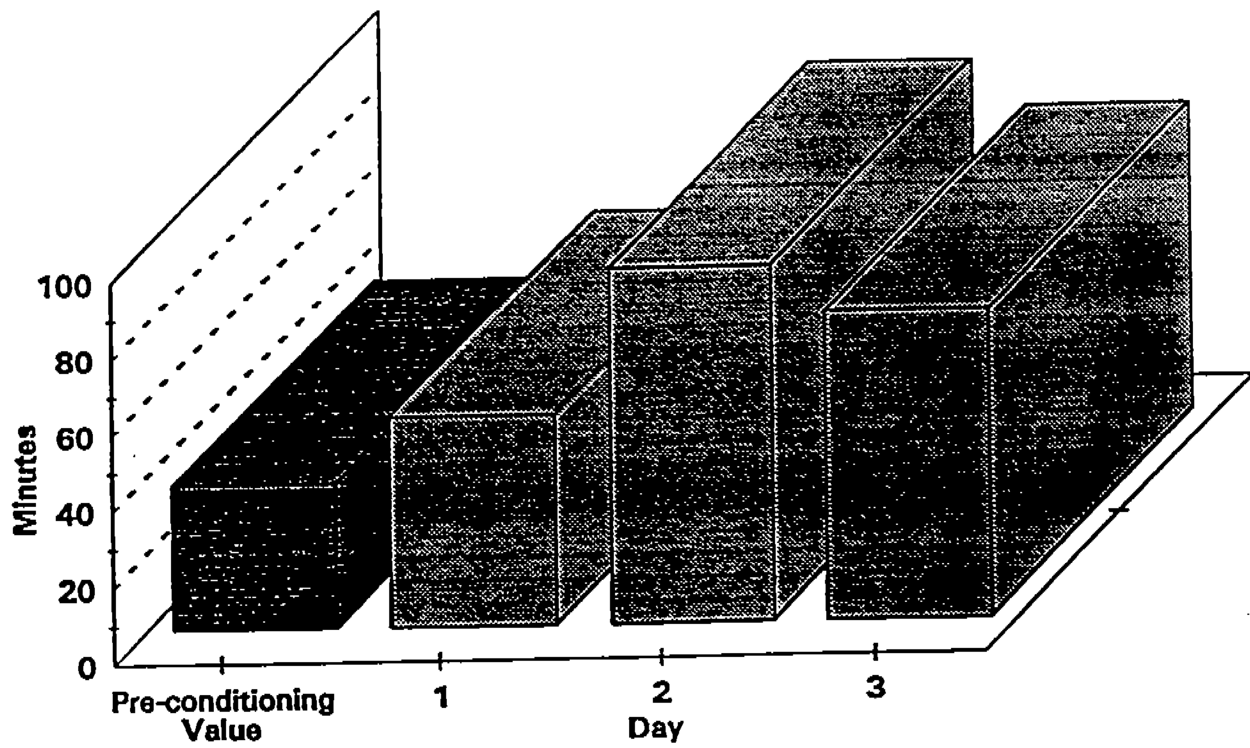

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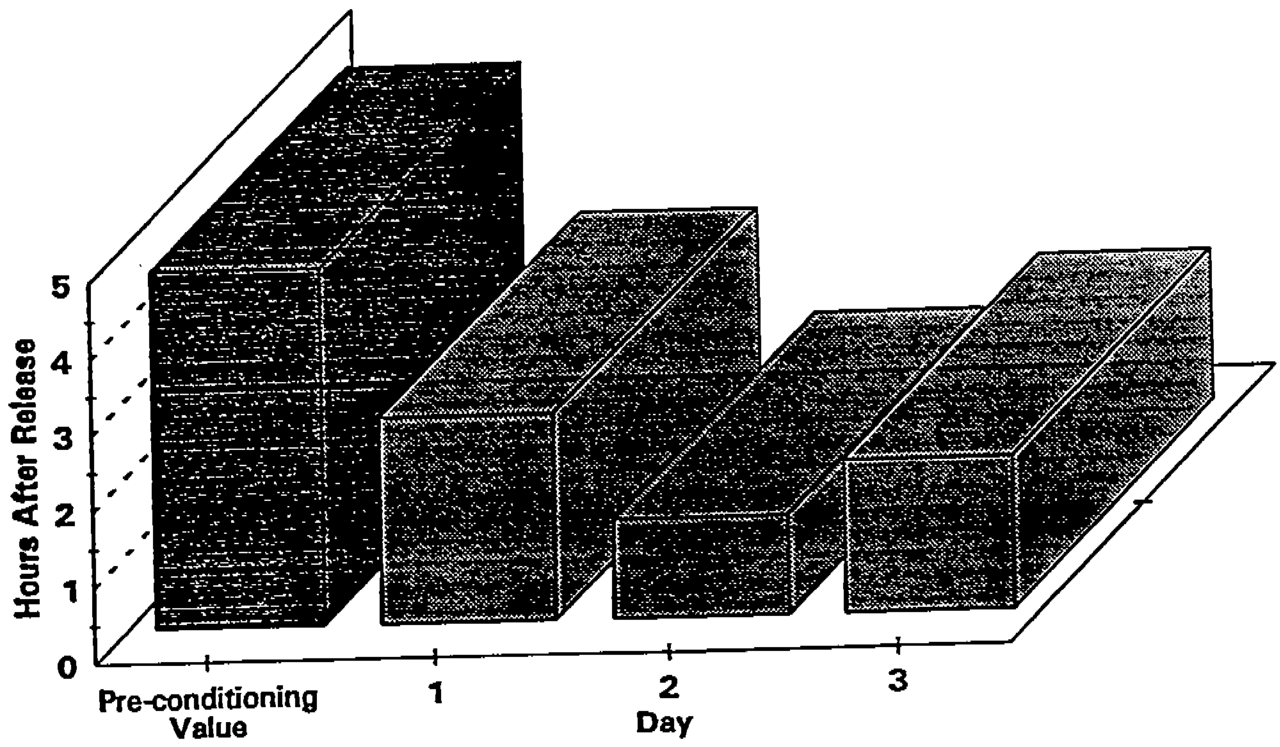
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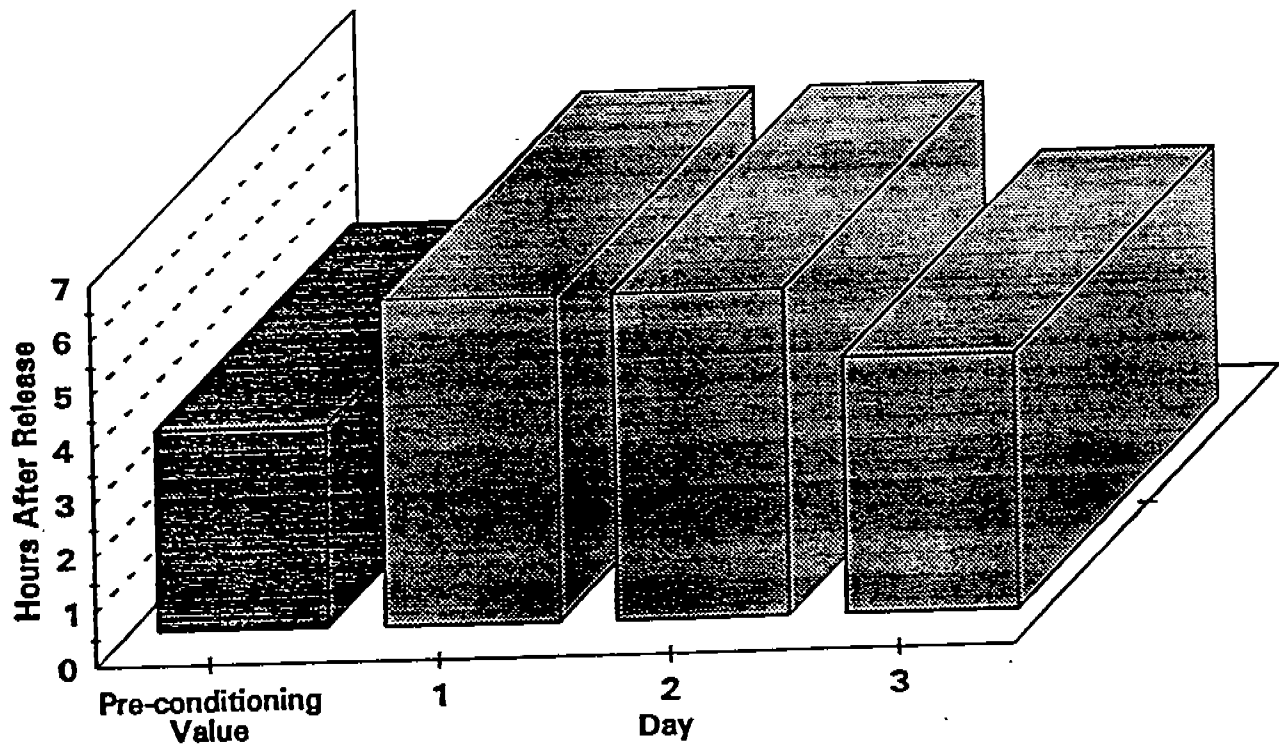

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Task B: Preliminary evaluation of encapsulated methyl anthranilate at Eagle River Flats, Fort Richardson, Alaska.

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ABSTRACT

We evaluated methyl anthranilate encapsulated in a sodium alginate capsule at two field sites during the spring and at one site during the fall at Eagle River Flats, Fort Richardson, Alaska. Encapsulated formulations of MA were able to decrease feeding activity of ducks 50-80% for up to 10 days. Further, mallard mortality was reduced 60% when ducks were continuously exposed to WP contaminated areas for up to 172. Based on evaluation of several formulations and their performance in the field recommendations are made or a final formulation which should have a half life of 10 days and an efficacy of at least 80% reduction of feeding activity. Sentinel studies should not be used to compare relative risk of MA vs Control pens directly because even small sampling rates over a prolonged observation period place captive ducks at risk to WP poisoning. MA works by moving waterfowl away from areas of treatment, not by suppressing feeding 100%. Thus, field studies on free-ranging ducks are needed to further evaluate the efficacy of MA as a short-term remediation strategy.

INTRODUCTION

The U.S. Army has used Eagle River Flats (ERF) since 1945 as an impact area for artillery shells, mortar rounds, rockets, grenades, illumination flares, and Army/Air Force Door Gunnery Exercises. In August 1981, hunters discovered large numbers of duck carcasses in ERF. Since that time, the Army and other federal and state agencies have been involved in identifying the cause of the waterfowl mortality problem. On February 8, 1990, the Army temporarily suspended firing into the ERF due to the suspected correlation between explosives and duck deaths (Quirk 1991). In July 1990, a sediment sample collected from ERF was suspected of containing white phosphorus (WP). By February 1991, it was concluded that WP in ERF was the cause of waterfowl mortality (CRREL 1991).

Waterfowl populations, especially mallards, are decreasing continent-wide (U.S. Fish and Wildlife Service and Canadian Wildlife Service 1989). ERF has been identified as an important spring (April to May) and fall (August to October) waterfowl staging area. The presence of WP in ERF could represent a hazard to feeding waterfowl (CRREL 1991). This concern has stimulated efforts to develop of an effective remediation action to reduce or eliminate waterfowl mortality caused from WP in ERF.

In response to the Army's queries about bird repellents in July 1991, the DWRC proposed to develop a repellent delivery system for testing at ERF. The objective was to deter ducks from foraging on contaminate sections of ERF. The DWRC selected Methyl Anthranilate for use as an active ingredient for the following reasons. MA was

known to be an effective bird repellent (Kare 1961). Extensive field testing by DWRC personnel over the past 10 years showed that MA and its analogs had good potential as a repellent in a variety of circumstances (Glahn et al. 1995, Mason et al. 1985 and 1989, Cummings et al 1991). Particularly promising was the fact that MA was not toxic to mammals. Indeed it is GRAS listed by the FDA and is used extensively at high concentrations as a fragrance and food flavoring. We envisioned encapsulating MA into a form which could be broadcast onto the sediment and would release its contents only upon contact by foraging waterfowl. This would tend to reduce on target hazards and prolong the effective period of treatment. Our objective in this study was to field test the most effective MA formulation resulting from pen trials in Task A.

METHODS

Spring 1992

Sediment samples were taken in areas C and B to determine the position of pens for sentinel trials. This was to done ensure that the control sites contained no WP and that the "hot" sites had some measured quantity of WP. Samples were taken using methods previously prescribed by CRREL. WP assays were carried out by CRREL using established methods.

At site B, pens were placed in proximity to one another within site of an observation blind/tower. At site C, pens were placed so as to include at least one WP sampling point. This point was used as a crude index for the level of WP ducks may

have been exposed to. We acknowledge that WP distribution is highly variable spatially and our sampling point may not reflect the integrated spatial risk foraging ducks may encounter.

Mallards were captured in Denver, CO with cannon nets (Dill and thornsberry 1950). Ducks were captured under Federal permit PRT-68014 and state permits for Colorado, 92-0060. Ducks were banded with USFWS leg bands. Ducks were transported to Fort Richardson for testing via air freight.

Upon arrival by air freight, ducks were housed in holding pens (6/pen) and quarantined for a minimum of 10 days. To prevent ducks from leaving the open test pens, primary feathers 2-9 on the right wing were clipped. Ducks were weighed with pesola spring scales and ranked according to weight. To minimize harassment, ducks were assigned to pens based upon similarities of weight. Thus the six heaviest ducks were assigned to one pen, etc. Pens were randomly assigned to treatment. At the end of field trials 2 ducks from each group were sacrificed for WP analysis. The remainder of surviving ducks were returned to DWRC.

Mallards dying during the course of the experiment were deep frozen and reserved for tissue necropsy and residue testing for WP. Any animals exposed to the WP areas were sacrificed and necropsied. One duck from each of the control (MA+ or MA-) plots was sacrificed and necropsied to verify that no WP contamination occurred. Carcasses were frozen on site and shipped to CRREL packed in dry ice and styrofoam containers via 1-day air freight delivery for necropsy.

We monitored behavior and determined the risk of mortality for foraging ducks under 4 conditions (N=3 for each treatment category for a total of 12 plots):

1. *Areas of high sediment WP*
2. *Areas of high sediment WP and presence of formulated MA*
3. *Areas with no sediment WP*
4. *Areas with no sediment WP and presence of formulated MA*

Each experimental category, hazard (presence or absence of WP) and treatment (presence or absence of MA), was replicated 3 times for a total of 12 experimental plots. Six ducks were assigned to each plot for a total of 72 experimental animals.

The day prior to a pen's first scheduled observation, encapsulated MA was broadcast spread over the surface of the sediment at a rate equivalent to 40 lb/acre. After 10 days MA pens were retreated at the same application rate.

For each site, two sets of six ducks each were taken from their holding cages, color tagged for individual identification and transferred to field pens each morning. At each site six ducks were placed in a pen treated with MA and the remaining ducks were placed in the control pen. Once the ducks were in place, ducks were observed from a tower/blind for 2 hrs. The observer alternatively carried out focal observations on randomly selected ducks within each pen for 5 minutes. For example, the observer focused observations on a single duck in a pen treated with MA, and recorded the type and duration of each activity. At the end of the 5 minute sample period the observer carried out a 5-minute focal observation on a randomly selected duck in the

other plot, i.e. control (MA absent), switching back to the first plot at the end of a 5-minute observation period, and so on. These alternating focal observations continued until 12 observation periods per plot had been obtained (approximately 2 hours). The exact overall time that ducks were in each pen were noted. This patterned observation was conducted at both the C and B sites. On the second and third days the process was duplicated for additional groups of naive ducks which were randomly assigned to the 4 treatment categories. Thus, a complete set of experimental conditions was observed each day. All 72 ducks were tested by the third day, at which point the sequence of observations was repeated.

Each pen was observed for up to 10 days post treatment. At this point the pens were retreated with an additional 20 lbs/acre of formulation and the process and sequence of observations was repeated for an additional 10 day period.

After the 2-hour observation period ducks were removed from the field pens and returned to the holding pens. Ducks were periodically observed for the next 24 hours to determine if any latent toxic effects were present. Mortality was attributed to WP poisoning if the patterned distress behavior was observed in the field or holding cages and necropsy confirmed the presence of WP in the crop or gizzard. All necropsies were carried out by CRREL personnel, who were blind to the identity of sampled ducks.

RESULTS AND DISCUSSION

Spring 1992

No detectable levels of WP were found at site B. Initial samples from site C also showed no WP levels for sampling points near the main observation tower. However, WP levels increased in area C at positions closer to the bread truck area (Fig. 1).

Foraging behavior should be observed as early as possible in the morning. Feeding activity of ducks drops off quickly. Observations past 10:00 AM are unlikely to record significant amounts of foraging activity (Fig. 2).

In the spring of 1992, a sodium alginate capsule with 4% MA wt/wt contained in food grade silicone oil was broadcast spread in 3 pens each at both C and B sites. MA was effective at reducing feeding activity. At site B, feeding activity in MA pens was reduced by 50% relative to controls on the first day of observation. The suppression of feeding activity was enhanced to approximately 80% of control levels for up to 7 days post treatment. By the tenth day post treatment effectiveness of the capsules began to subside (Fig. 3, bottom). Suppression of feeding activity at site C also occurred. The absolute rates of feeding activity were higher for site C. This may be attributable to the shallower depth of the pools and the difference in substrate types.

Following the initial treatment period, the pens were retreated with an additional amount of encapsulated repellent. During this period the total feeding activity at site B (even in control pens) dropped markedly. This was coincident with high tidal water

levels. Presumably, the decrease in total feeding activity in control pens reflected the difficulty the ducks had in reaching the bottom when water levels were high. As a result, the relative decrease in feeding activity at site B was only about 50% of that seen for controls. This effect was observed for a period of up to 10 days, not so much because MA was less effective, rather because the control baseline feeding rate decreased.

The water levels at Site C were more shallow than that found at Site B throughout the study period. During the second treatment period the decrease in feeding activity relative to controls was about 50%. However, absolute feeding activity in both types of pens (control and MA treated) increased during this period. This increase corresponded with an algal bloom and increased water depth. Close observations during this period indicated that a significant portion of the feeding activity was directed at the algal mat and not the substrate per se. In this event, it is unlikely that the ducks were coming into contact with MA or substrate.

In summary, the spring trials indicated that encapsulated MA could decrease feeding activity by 50-80% for up to a period of 10 days. The question remained whether this decrease in foraging probability would also result in a decreased risk of mortality.

At site C, ducks in the control pens were 1.6 times more likely to die of WP toxicosis than ducks from MA pens. The protection offered by MA would not seem great from these results. In this study the ducks were forced to reside in one location. Thus, the sampling behavior normally seen for ducks was sufficient to place all ducks

at high risk to WP poisoning. The probability of WP poisoning was more directly related to WP content of the pen than the type of treatment the pen received (Fig. 5). But it must be remembered that MA normally acts to repel birds from a resource/area. Birds normally have an opportunity to leave treated areas. Thus, we anticipate that the relative risk of poisoning in MA treated areas would decrease if the ducks were allowed to leave the area. The fall phase of the study was designed to address this question.

METHODS

Fall 1992

We attempted to test a modified encapsulated formulation in the fall field season to improve efficacy and persistence. We also attempted to decrease the relative risk of WP poisoning for MA treated pens, by offering ducks a refuge from the WP-containing/MA treated areas.

The fall field season was less than successful. Several factors contributed to the lack of success in the fall trials. Mt. Spur erupted causing an ash fall over the Anchorage, delaying the arrival of the ducks which were to be the subjects of the field test. Extremely high tides and cold and windy weather contributed to the small amount of foraging behavior observed. Finally, due to the lateness of the season and freezing over of the study area, experiments had to be terminated early. In addition, the modified formulations were larger than those tested in the spring and the walls were

substantially thicker, resulting in a larger pressure needed to break the capsule. We felt that this formulation was less likely to succeed at decreasing feeding activity.

We set out to determine whether ducks could be moved off a treated area to a "WP safe area" within a pen. This safe area was established by laying down geotextile matting atop the sediment on one half of the pen. We addressed two questions: 1) whether the matting itself had an effect on feeding activity 2) whether matting could offer a safe area which ducks could move to if they were repelled by the formulation and 3) determine morality o mallards exposed continuously to the above conditions.

The same design was used to evaluate MA formulations as outlined for the spring field trials. In addition, mortality exposure tests were conducted by placing 6 mallards each into one treated pen (MA/matting) and one control (MA/matting) pen for up to 172 hours. Supplemental food and a floating perch were placed in the pens. Mallards were removed, examined an held for 3 hours every 72 hour period.

RESULTS AND DISCUSSION

Fall 1992

To establish whether the matting had an effect we compared feeding activity between control pens and those whose sediment was covered with matting (Fig. 5). It became apparent that matting itself had a negative impact on feeding activity . It also became apparent that all activity quickly subsided. Ducks behaved normally when first introduced into the pen, but very quickly ceased all activity other than rafting together. We attribute this change in behavior to the precipitation and cold weather which

occurred on most observation days. Under such inclement weather conditions, it is advisable to curtail observation periods to 1 hr rather than the prescribed 2 hr period. While the matting did decrease the feeding activity of ducks, it did not decrease the risk of mortality. Equal numbers of ducks died in control and matted pens. The process of laying down the geotextile apparently disturbed the sediment sufficiently such that WP was over-deposited atop the matt. Three of four sediment samples collected from atop the textile after it was laid into place and allowed to stabilize contained WP. Thus, it appears that physical disturbance and strong tidal action which might result in sediment shifts may cause significant redistribution of WP. This aspect of WP movement needs to be explored more thoroughly. Any redistribution of WP would limit the success of a sediment cover as a viable strategy of protecting ducks from WP.

Although it was apparent that the initial premise that geotextile covering could provide penned ducks with a WP-free safe zone was not true, we proceeded with the movement trials to document activity levels as a function of treatment type. Pens were enlarged to accommodate equal areas of matted substrate and substrate treated with formulated MA. Control pens were also enlarged. Records were kept on the feeding activity in all halves of the pens.

Initially (1 day post treatment) the feeding activity was the same for the covered and MA treated areas (of the same pen). The activity for each treatment was lower than that seen for the controls (whose level was adjusted for equal surface area) (Fig. 6). By 3 days post treatment, the feeding activity on the MA side of pens approached

the levels seen for controls. The feeding activity for the covered areas dropped dramatically.

The rapid loss of effectiveness of the MA treatment can be attributable to a flaw in the modified formulation. Laboratory studies indicated that MA contents of individual capsules quickly leach to the environment. Within 24 hrs of being placed in an aquatic environment individual capsule are all but depleted of MA (Fig. 7). This is consistent with the field observations which showed the effectiveness of the beads vanished after 24 hrs. Two possibilities exist for the poorer performance of the modified formulation. The matrix may be more permeable to MA, but more likely is the fact that in the earlier formulation MA was dissolved in an oil. Because MA has a higher affinity for oil than water the MA is more likely to stay within the capsule so long as the oil does not permeate the alginate outer wall. Because the initial day's activity was similar to the that seen for matting, we anticipate that a reconsideration of the capsule matrix material will resolve the problem of capsule content stability.

Mortality of ducks continuously exposed to WP contaminated sediment in treated (MA/matting) or control pens was equal at 24 hrs but increased in the control pens through the conclusion of the test (Fig. 8). Subsequently, mallard mortality was reduced 60% on MA treated sites. This result indicates that ducks in treated pens showed a learned avoidance of MA treated areas after the first 24 hr period.

CONCLUSIONS AND RECOMMENDATIONS

Encapsulated formulations of MA can be effective at reducing feeding activity. Subsequent formulations are being considered which show greater promise for stability in the field. Changing the outer wall to latex will decrease permeability to MA. Reincorporating the core to an oil/MA mix will further retard leaching from the capsule. If the initial concentration of a capsule is 15% MA, and a half life of 10 days can be achieved, we estimate that the capsules will retain their effectiveness in the field for the duration of the spring or fall migratory period. Thus, only one application will be necessary each spring and each fall.

Based upon field studies where geese have been moved off MA-treated turf, we anticipate that free-ranging ducks can be moved off treated sediments. Limited field trials to test this hypothesis are planned for FY93. Based on differential feeding activity of treated and untreated areas, differential use of treated and untreated areas, probability of encountering a WP particle, and minimum toxic dose we will be able to estimate the relative risk of WP poisoning for free-ranging ducks. This model will be useful in evaluating application strategies for short-term remediation efforts.

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FIGURE CAPTIONS

Figure 1. The distribution of white phosphorous particles at the C-site study area as a function of concentration per gram of sample. The "X"'s indicate locations of observation towers. The top panel depicts individual sample points, the bottom panel depicts a contour map estimating WP distribution. Samples were analyzed by CRREL.

Figure 2. The relationship between observed feeding activity (2 hrs) and time when behavioral observations were begun. Data based on a subset of observations derived from B-site in the spring of 1992.

Figure 3. Spring 1992. Top panel. The feeding activity for 2 hr observation periods for the 2 sites (B=circles and C=inverted triangles) and 2 treatment types (MA=solid symbols and control=hollow symbols). Vertical bars are ± SE with n=3. Sampling interval were days post-treatment. Bottom panel. The relative suppression of feeding activity attributable to MA as a function of days since treatment application. A score of 0 indicates no MA effect on feeding activity relative to the control pen. Negative scores indicate the magnitude of the MA effect at inhibiting feeding activity relative to matched controls.

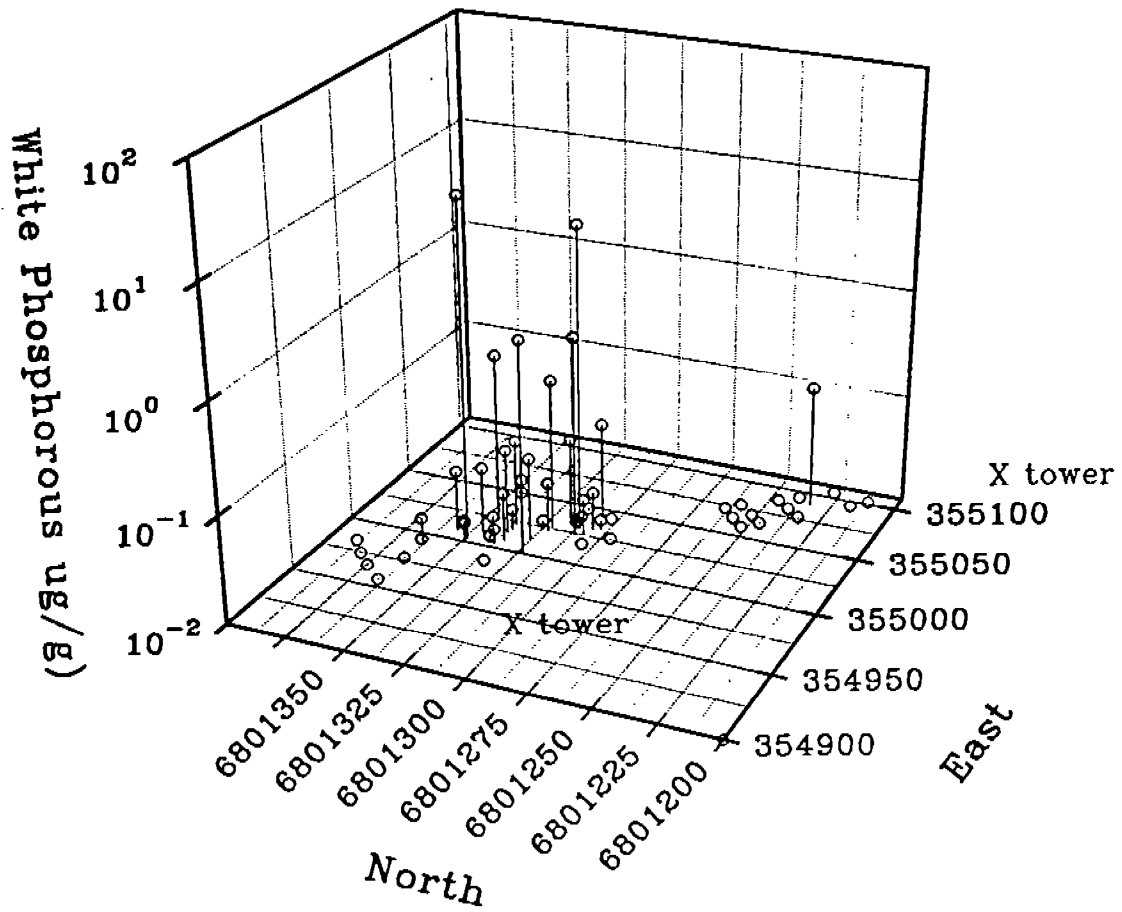
Figure 4. Spring 1992, site C. The relationship between the rank order of WP content of test pens and the rank order of mortality of test pens. Open circles are control pens, solid circles are pens treated with MA.

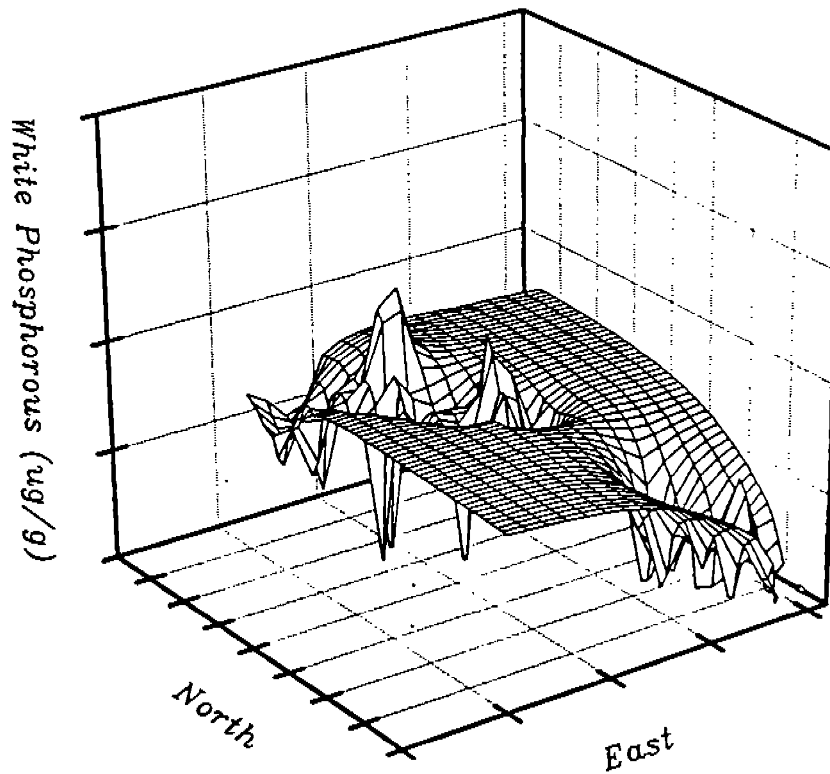
Figure 5. Fall 1992, site C. The number of feeding bouts per 30 s as a function of time during the observation period.

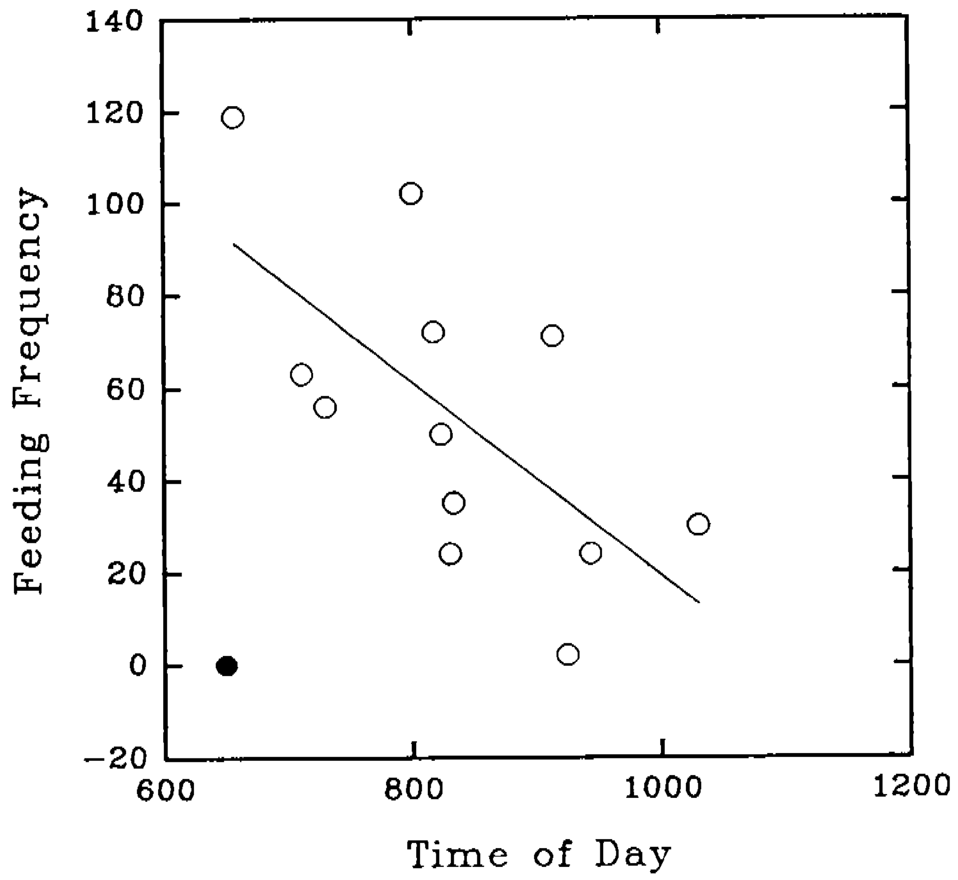
Figure 6. Fall 1992, site C. The total feeding activity during a 2 hr observation period as a function of days post-treatment with the modified MA formulation. Circles depict pens where sediments were treated with geotextile matting and MA. Solid circles depict activity on the matted half of the pen, while open circles depict activity on the MA treated half of the pen. The inverted triangle depicts activity in control pens.

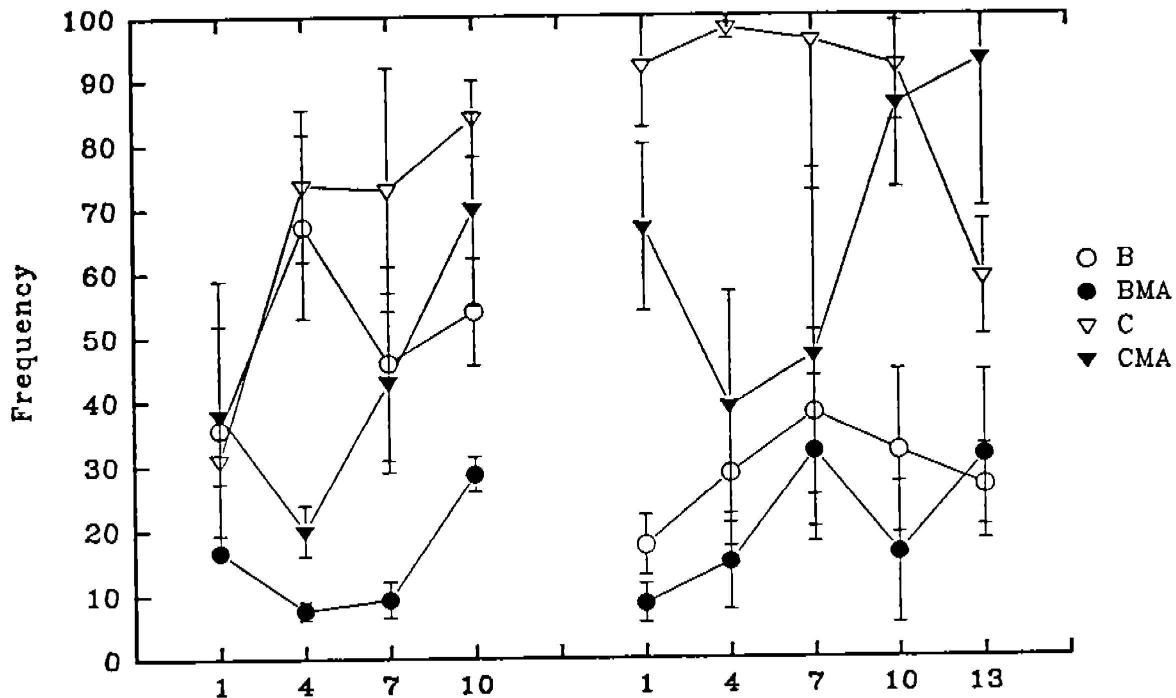
Figure 7. Fall 1992. The leaching rate of MA from modified bead formulations. The amount of MA in a water sample as a function of time.

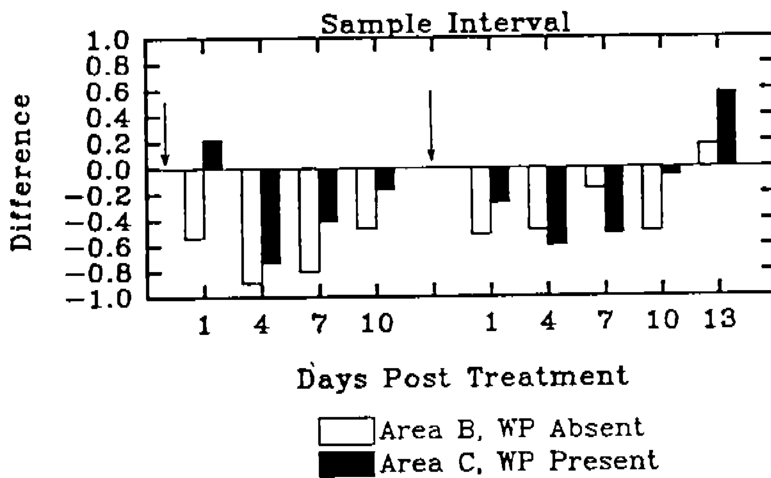
Figure 8. Mortality of ducks (6 introduced per pen) continuously exposed to WP contaminated sediment in treated (MA/matting) or control pens for approximately 100 and 172 hrs, respectively, 16-23 September 1992, Eagle River Flats.

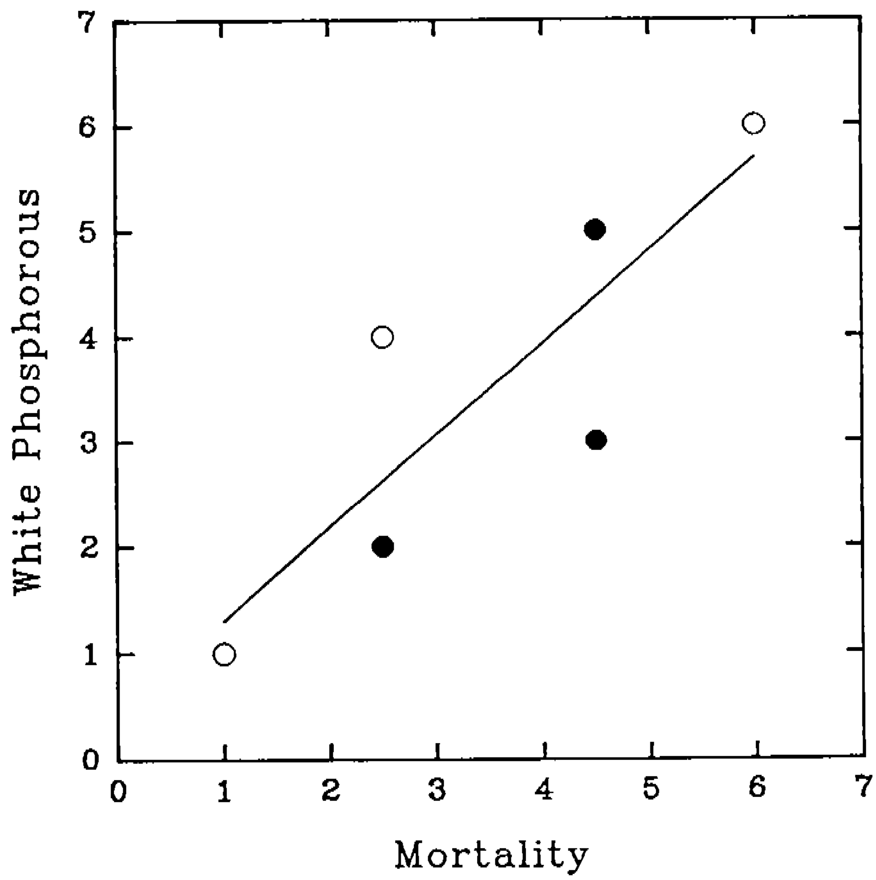

White Phosphorous ug/g)
10^2
10^1
10^0
10^{-1}
10^{-2}
X tower
X tower
355100
355050
355000
354950
354900
East
6801350
6801325
6801300
6801275
6801250
6801225
6801200
North


White Phosphorous (ug/g)
North
East

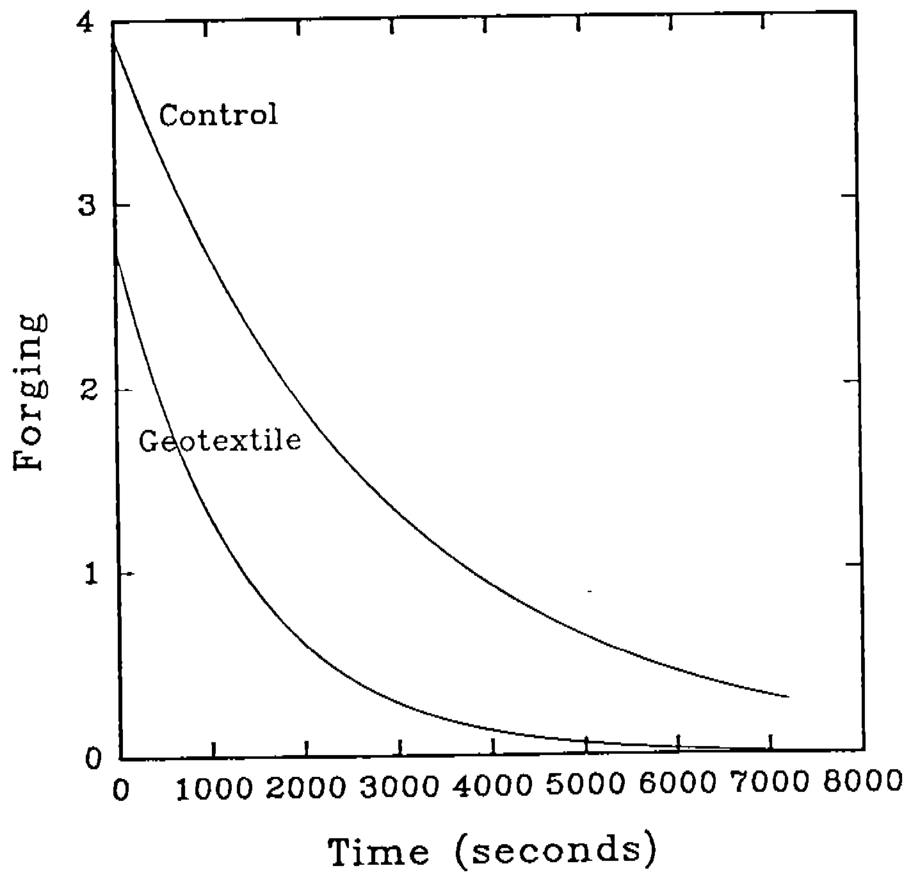

Feeding Frequency
140
120
100
80
60
40
20
0
−20
600
800
1000
1200
Time of Day

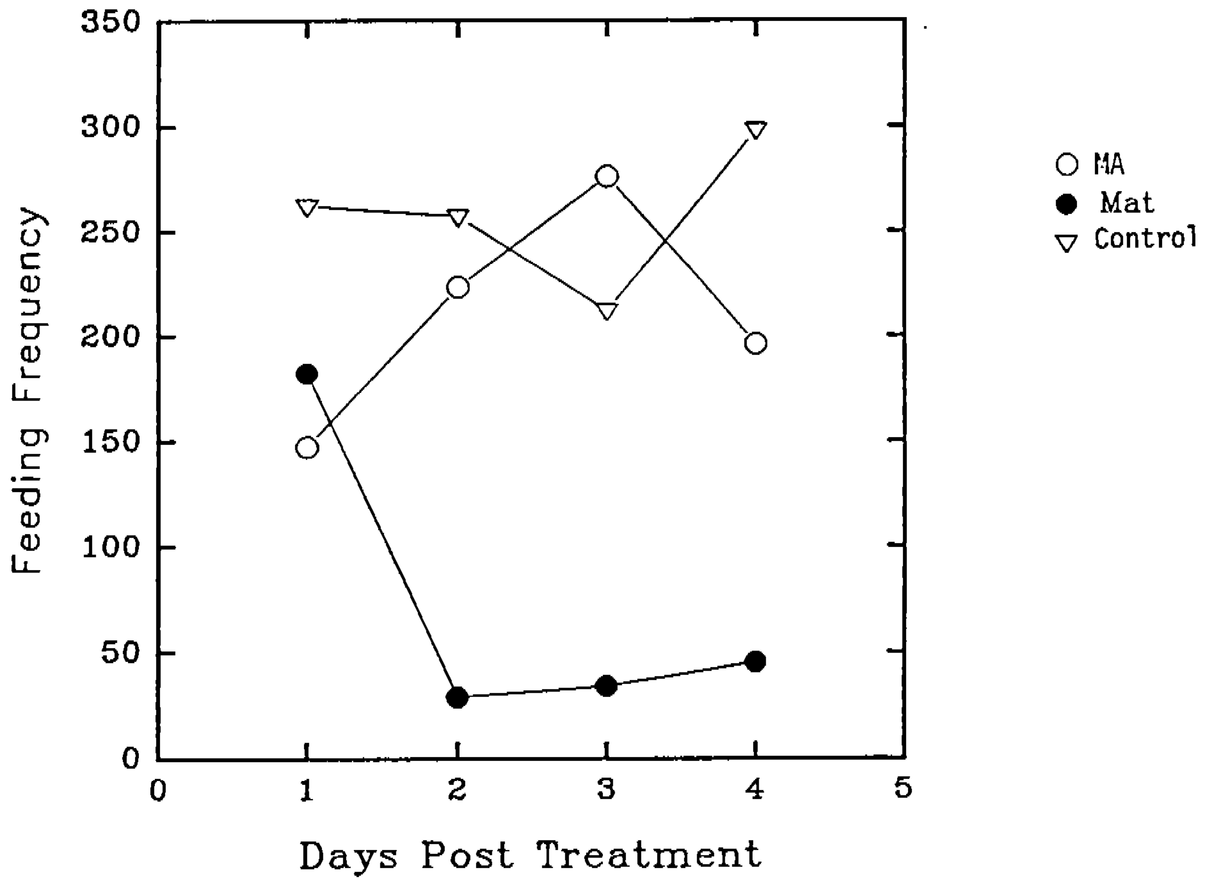

FEEDING DATA FOR EAGLE RIVER FLATS: MAY-JUNE 1992
Frequency
0
10
20
30
40
50
60
70
80
90
100
1
4
7
10
1
4
7
10
13
Sample Interval
B
BMA
C
CMA


Difference
1.0
0.8
0.6
0.4
0.2
0.0
−0.2
−0.4
−0.6
−0.8
−1.0
1
4
7
10
1
4
7
10
13
Days Post Treatment
Area B, WP Absent
Area C, WP Present

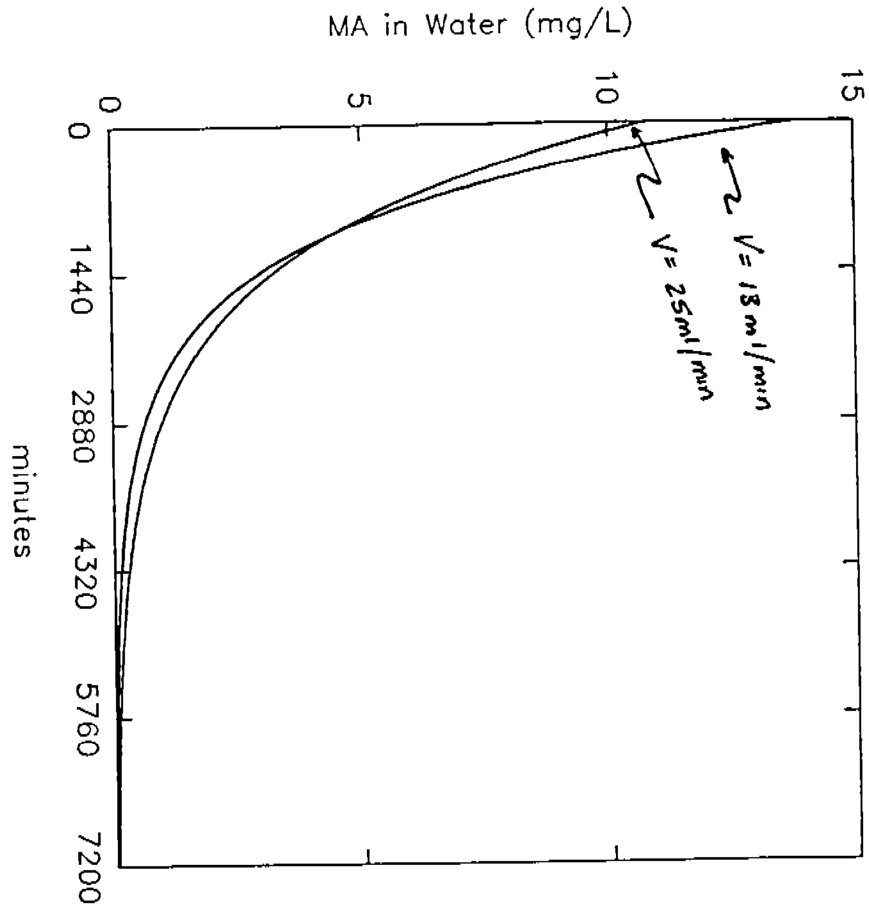


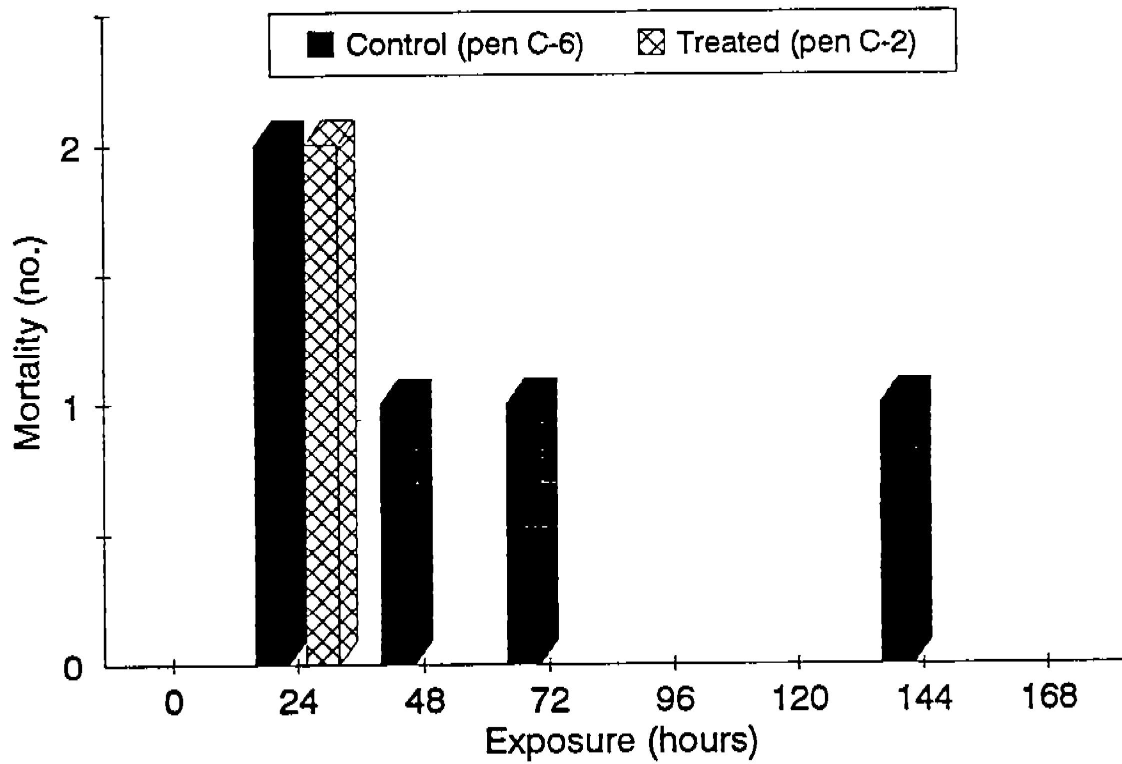
White Phosphorous
Mortality
0
1
2
3
4
5
6
7


Control
Geotextile
Forging
4
3
2
1
0
0
1000
2000
3000
4000
5000
6000
7000
8000
Time (seconds)



350
300
250
200
150
100
50
0
0
1
2
3
4
5
Feeding Frequency
Days Post Treatment
MA
Mat
Control


MA in Water (mg/L)
0
5
10
15
0
1440
2880
4320
5760
7200
minutes
V= 13 ml/min
V= 25ml/min


Control (pen C-6)
Treated (pen C-2)
Mortality (no.)
0
1
2
0
24
48
72
96
120
144
168
Exposure (hours)

Task C: Acute toxicity of methyl anthranilate to fish: Atlantic salmon, rainbow trout, channel catfish and bluegill.

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ABSTRACT

Several laboratory and field studies have shown methyl anthranilate to be an effective nontoxic and nonlethal bird repellent, with application potential for protecting crops, seeds, turf and fish stocks from bird damage. Furthermore methyl anthranilate can be added to liquids for the purposes of protecting migratory birds, e.g. addition to waste water associated with mining and to standing water pools at airports. Mammalian toxicity data are favorable. Methyl anthranilate is used as a fragrance and food flavoring and is GRAS listed by the US Food and Drug Administration. Despite the favorable outlook for methyl anthranilate's use as a safe repellent, no data exist on its environmental fate and effects. We tested the acute toxicity of methyl anthranilate in a static system for 4 species of fish. The LC_{50} at 96-h for Atlantic salmon (Salmo salar) was estimated to be 32.35 mg/L, with the no observable effect limit at 6.0 mg/L. The LC_{50} at 96-h for rainbow trout (Onchorhinus mykiss) was estimated to be 22.92 mg/L, with the no observable effect limit at 5.0 mg/L. The LC_{50} at 96-h for channel catfish (Ictalurus punctatus) was estimated to be 16.23 mg/L, with the no observable effect limit at 7.0 mg/L. The LC_{50} at 96-h for bluegill sunfish (Lepomis macrochirus) was estimated to be 9.12 mg/L, with the no observable effect limit at 7.0 mg/L.

INTRODUCTION

Methyl Anthranilate (CAS 134-20-3; 2-amino benzoic acid methyl ester) was first described as a bird repellent by Kare (1961). Since that time a series of studies have focused on its efficacy as a nonlethal avian irritant (Mason et al. 1989, Clark et

al.1991, Mason et al. 1991). In the field, methyl- and dimethyl anthranilate have proved effective at reducing bird depredations of feed at cattle feed lots (Mason et al. 1985, Glahn et al. 1989). It also has potential for use in protecting orchard crops and seeds (Avery 1992, Clark and Mason 1993). Formulated methyl anthranilate can minimize goose grazing damage to turf, and protect birds from toxicants included in granular pesticides (Cummings et al. 1991, Cummings 1992, Mason et al. 1993). Furthermore, methyl anthranilate incorporated into Concover™ dissuades gulls from using landfills (Dolbeer and Clark 1993). Methyl anthranilate can reduce water consumption of ducks, gulls and passerines (Clark et al. 1991, Dolbeer et al. 1991, Dolbeer and Clark 1992, Blante et al. 1993). Without access to fresh free-standing water at airports the risk of air collisions between aircraft and birds can potentially be reduced (Dolbeer et al. 1991., Dolbeer and Clark 1993). Decreasing the attractiveness of contaminated water (e.g., cyanide ponds associated with gold mining operations) may reduce the risk of accidental kills of birds at tailings ponds (Clark and Shah 1991, 1992, 1993).

Contributing to the attractiveness of methyl anthranilate as a nonlethal bird control agent is its use in the fragrance and flavor industries, and is GRAS listed by the US Food and Drug Administration (Furia and Bellarca 1975, USFDA 1993). Notwithstanding the favorable toxicity data for mammals (LC_{50} for mice is 3900 mg/kg), there are no data which speak to methyl anthranilate effects on the environment (Windholz 1983).

This study focuses on the acute toxicity of methyl anthranilate in static tests on fish (U.S. EPA 1993). The data herein are intended for use in the evaluation whether methyl anthranilate is suitable for broad scale environmental use as a nonlethal avian repellent.

METHODS

Test Substance

The purity of the methyl anthranilate was specified as >98% GC (Lot # 271292387, Fluka Chemical Company). All test concentrations were based on the total compound, i.e. not corrected for sample purity.

Analytical Method

A concentrated standard was prepared by dissolving 0.1 ml of methyl anthranilate with 1000 ml of doubly deionized distilled water (nominal concentration 100 mg/L). The solution was sonicated for 30 minutes followed by stirring with a magnetic glass stir bar for an additional 30-minutes. The concentrated solution was allowed to cool to room temperature and was checked to determine if any precipitate was present. A 50 mg/L working standard was prepared by diluting 100 ml of the concentrated standard to 200 ml with water. Contents were mixed thoroughly. Further working standards were prepared to nominal concentrations by quantitatively diluting concentrated standard solution with water.

In two of the four bioassays methyl anthranilate concentrations were validated using an HPLC system. The flow rate of the mobile phase was 1.0 ml/min mobile

phase, consisting of 0.5:0.5 ml, aqueous:acetonitrile. Injection volume was 130 uL at 25°C. The column was a Zorbax ODS 4.6mm x 250cm, configured with a Rainin HPXL pump (2), Rainin pressure module, Dynamax UV-M detector, Dynamax AI-2 autosampler, HP 3390A integrator and Perkins-Elmer Nelson 1020S computer integrator for archiving. The UV detector was set at 330 nm.

Approximately 3 ml of the working standard was placed in a 5 ml autosampler vial, and capped with rubber septa and cap. Samples (130 uL loop fill) were repeatedly injected (6x) to determine the HPLC system suitability for analysis. The relative standard deviation of the methyl anthranilate chromatographic peak response was not greater than 2.2% for six consecutive injections of the 50 mg/L working standard solution.

Method Validation

Four methyl anthranilate/water standard solutions were prepared ranging from 100 mg/L to 5 mg/L. Each solution was injected in quadruplicate. The simple correlation coefficient was, $r = 0.99981$ (slope = 2.93971 E-06, intercept =0), indicating that a strong linear response existed between chromatographic peak response and concentration over the range of interest.

Control water samples were treated according to the above procedure as were water samples which had fish in them for several days. No chromatographic interferences were observed.

Test Water

Dilution water used in this study was filtered by passing the water through a charcoal, sand and stone filter and was subsequently demineralized by reverse osmosis. Water was held in reservoir tanks for subsequent use throughout the aquatic facility. The test concentrations were obtained by dissolving methyl anthranilate in dilution water using the procedures described above. Stock solutions were serially diluted with dilution water to specified nominal concentrations. Prepared stock solutions and dilutions were added to the glass test tanks. Temperature in the tanks was monitored using thermocouples and data logger with constant temperature maintained via a chiller and circulating water bath.

Test Species

All test fish were held in dilution water on a 12-hour daylight photoperiod and observed for 2 weeks prior to testing. Fish culture techniques were those outlined in Brauhn et al (Brauhn and Schoettger 1975). During the holding period, fish received food once per day at a rate of 0.04g/g-fish. This feeding rate was determined to be a maintenance diet minimizing growth. Samples of fish (n=10) were weighed every few days to adjust the feed application rate. Fish in the group tank were not fed 48 hours prior to testing. Temperatures were monitored in the group holding tank and the test tanks to verify that the water temperatures were within 1.0°C prior to transferring fish to test tanks.

The 120 Atlantic salmon (Salmo salar) and the 160 rainbow trout (Oncorhynchus mykiss) used in the test were hatchery spawned and of the same size and year class. Fish were obtained from The Tunison Laboratory of Fish Nutrition of

the U.S. Fish & Wildlife Service, 3075 Gracie Road, Cortland, NY 13045. Salmon were tested at nominal concentrations of 0, 1, 6, 13, 25, and 50 mg/L. Trout were tested at nominal concentrations of 0, 1, 5, 10, 15, 20, 25 and 50 mg/L. We did not validate these concentrations with quantitative procedures, other than visually observing whether methyl anthranilate was completely dissolved. The HPLC system was unavailable at the time of testing, and the gas chromatographic method available at the time proved too slow to process all samples before microbial degradation of methyl anthranilate influenced samples (Aronov and Clark, unpublished data). Salmon were housed 5 animals to a 10 L test vessel. Twenty salmon per concentration were tested. Trout were housed 10 animals to a 10 L test vessel, with 2 test vessels per concentration.

The 280 channel catfish (Ictalarus punctatus) and the 280 bluegills (Lepomis macrochirus) used in the test were hatchery spawned and raised and were obtained from Delmarva Aquatics, P.O. Box 349, Odessa, DE 19730. All fish for each species were from the same source and year. Both catfish and bluegills were tested at nominal concentrations of 0, 5, 10, 20, 40, 50 and 100 mg/L. Concentrations were validated using the HPLC method at 0 and 96 hrs of the test. Both catfish and bluegills were housed 10 animals to a 10 L test vessel, with 4 replications per concentration.

Test Conditions

The procedures for this static bioassay were generally those suggested by the EPA.[21,23-24] The static fish bioassay was conducted in 14 liter glass vessels containing 10 liters of dilution water which was equivalent to a depth of 30 cm.

Dissolved oxygen, conductivity and pH were measured at 0 and 96-hours. Temperature of 4 test tanks within the water bath as well as room temperature was monitored every 30 minutes and recorded to a datalogger. Illuminance was maintained on a 12-hour light:dark cycle and total illuminance was monitored using a photosensor and recorded to the datalogger every 30 min. The light source was a bank of overhead florescent lights suspended over the test tanks. Total illuminance at tank level was 1.8 W/m^2.

A range finding test of 24 hours was conducted to determine the concentration range for the definitive study. The preliminary concentrations were set at 1000, 100, 10 and 1 mg/l. All fish (n=5/concentration) died within 10 seconds for each of the higher concentrations, i.e. 1000 and 100 mg/l. No fish (n=5) died at the 1 mg/l concentration.

Analyses

As a precondition to initiating toxicity tests, similarities of mass among test groups were compared using a 1-way analysis of variance. Dose-response curves and confidence intervals were generated using logit and probit procedures of the

SPSS software package (Norusis 1986). The model which had the lowest confidence interval around the LC50 value was selected as the best descriptor of mortality.

RESULTS AND DISCUSSION

Atlantic Salmon

Test conditions

The average mass of salmon was 0.3 g ±0.21 SE, with all but 4 of the 120 fish within 2.5 SD units of the mean (Fig. 1). Because these individuals were assigned to test vessels without bias, it is unlikely that they affected the outcome of testing. This is reflected in the similarity of average weights of fish across the nominal concentration groups (Fig. 1; $P>0.05$). Biological loadings across all test vessels (average = 0.152 g/L ±0.008 SE) were well below the recommended level of 0.8 g/L (Stephan 1975).

DO, pH and water temperature in the holding tank compared favorably to conditions within the test vessels at the start of the trials (Table 1). Water temperature did not deviate more than 1.5°C around the mean (15.06°C) throughout the course of the test period.

Bioassay

Upon initial contact with methyl anthranilate mortality was swift at high concentrations. Salmon exhibited a loss of equilibrium (LOE) after 30 s at 50 mg/L. Within 1 min, in addition to the LOE, all fish became dark in color. Pumping of the operculum during this period was more rapid and exaggerated than that seen for controls. Most fish were dead within 15 min, and all fish were dead within 3 hrs. At

25 mg/L salmon showed LOE after 2 min. Within 5 min all salmon were immobile on the bottom of the vessel in darkened conditioned and exhibited rapid opercular pumping. For test concentrations of 13 mg/L the behavior and timing of toxicosis was similar to that reported for 25 mg/L. For the 6 and 1 mg/L tanks, mobility and color were similar to that of controls throughout the test period. The no observable effect limit (NOEL) was estimated to be 6.0 mg/L (nominal).

Partitioned by day, the proportion of total mortality at 24, 48 and 72 hrs was 93, 97 and 97%, respectively. After 72 hr many of the affected salmon at higher concentrations had recovered equilibrium, but were still dark and remained immobile, resting on the bottom of the test vessel. Opercular movement was still more rapid than that of controls. By 96 hr, color, mobility and opercular pumping rate returned to levels similar to that of controls. The 96 hr LC_{50} was 32.35 mg/L (Fig. 3).

Given the rapidity for the onset of mortality, the apparent cyanotic condition of the fish and the effort of affected individuals to increase ventilation rate we speculate that the short-term affects of methyl anthranilate inhibit oxygen uptake through the gills. Methyl anthranilate is highly lipophilic so it is possible that it is readily incorporated into lipid membranes of gill filaments.

Rainbow Trout

Test conditions

The average mass of trout was 0.14 g $\pm$0.03 SE, with all but 3 of the 160 fish within 2.5 SD units of the mean (Fig. 4). Because these individuals were assigned to

test vessels without bias, it is unlikely that they affected the outcome of testing. This is reflected in the similarity of average weights of fish across concentration groups (Fig. 4, $P > 0.05$). Biological loadings across all test vessels (average = 0.14 g/L ±0.03 SE) were well below the recommended level of 0.8 g/L (Stephan 1975)

Dissolved oxygen, pH and water temperature in the holding tanks compared favorably to test vessel conditions at the start of the trial (Table 1). Water temperature did not deviate more than ±1°C (average = 9.6°C) throughout the course of the 4-day test (Fig. 5).

Bioassay

Initially, mortality was swift at high concentrations. Trout exhibited a LOE after 25 s at 50 mg/L. Within 1 min all fish became dark in color. Relative to controls, opercular pumping was more rapid and exaggerated. All fish were dead within 15 min. At 25 mg/L trout showed LOE and signs of cyanosis after 40 s. Within 70 s all trout were immobile on the bottom of the vessel exhibiting rapid and exaggerated opercular pumping. All mortality recorded for this concentration occurred within the first 3 hrs of contact. For test concentrations of 20 and 15 mg/L the behavior was similar to that reported for 25 mg/L, with the exception that mortality was considerably less (i.e. 5% of the total tested; Fig. 6). For 5 and 10 mg/L only a few fish (3 @ 10 mg/L) showed any LOE. Though darker than controls, fish were considerably lighter than those exposed to higher concentrations. Mobility was similar to that of controls. At 0 and 1 mg/L all fish appeared normal, with good color and activity. Thus, the 96 hr NOEL was 5 mg/L.

Almost all mortality occurred upon initial exposure to methyl anthranilate. Partitioned by day, the proportion of total mortality at 24, 48 and 72 hrs was 97, 100 and 100%, respectively. The 96 hr LC50 was 22.91 mg/L (UCL = 24.35, LCL = 21.55).

Affected trout remained immobile and dark throughout the test. After the test, and prior to sacrificing the fish, we placed affected trout in clean water. Within 30 min all behavior and coloration returned to levels similar to controls. Thus, short term recovery was rapid once the methyl anthranilate was removed.

Catfish

Test conditions

The average mass of catfish was 0.12 ± 0.003 (SE) g, with all but 3 of the 280 catfish within 2.5 SD units of the mean (Fig. 7). These fish were assigned to test vessels without bias, therefore, we did not suspect that the results were unduly influenced by these marginally heavier fish. This was reflected in the similarity of average weights of fish across concentration groups (Fig. 7). Biological loading among the test vessels was 0.12 g/L ± 0.003 (SE). This was well below the recommended 0.8 g/L (Stephan 1975).

Water conditions between the holding tank and the test vessels compared favorably at the start of the trials (Table 1). Mean water temperature was held within 1 ^{o}C during the test period (Fig. 8).

The dissolved oxygen content in some test vessels decreased dramatically over the course of 96 hrs (Table 1; Fig. 9). Depletion of methyl anthranilate was approximately 10% of the starting concentration in each test vessel. The cause of oxygen and methyl anthranilate depletion was not immediately apparent, but oxygen depletion was not due to oxygen demand of the catfish alone. Control vessels showed only a small loss of dissolved oxygen (Fig. 9). Why then was oxygen depletion so exaggerated for some of the remaining test vessels?

In an independent set of experiments, E. Aronov and L. Clark (unpublished manuscript) showed that aerobic bacteria were capable of using methyl anthranilate alone as a nutrient source. Decomposition products appear to be sugars and amino acids. Under similar test conditions, but in the absence of fish, aerobic bacteria could deplete the extant methyl anthranilate by only 7%, yet dissolved oxygen could be depleted by as much as 76% over a 4 day period. The high variation in oxygen and methyl anthranilate depletion most likely reflects inoculation differences among the test vessels, in part due to chance, but also due to differences in timing in when dead fish were removed from the tanks. These effects were minimal at lower temperatures, such as those seen for salmon and trout (Table 1).

Bioassay

The mortality pattern for this warm water system was similar to that seen for both the cold water systems. At concentrations of 50mg/L or greater fish became immobile, showed evidence of exaggerated opercular pumping, became cyanotic and lost equilibrium within seconds. Fish did not respond to prodding. Death was

estimated to have occurred within 30 seconds. Between 20 and 49 mg/L, cyanosis, LOE, immobility and death took somewhat longer, approximately 1-2 minutes. Mortality for concentrations in the range of 7.5-20 mg/L was variable. Generally, fish died within 12 hours of introduction. Surviving fish within these concentrations showed signs of oxygen stress. They were immobile, staying near the surface gulping air and were dark in color relative to controls. Fish in vessels below 7.5 mg/L did not show any signs of stress. Activity and color were normal. Thus, the limit of no observable effect was 7.0mg/L.

Partitioned by day, the proportion of total mortality at 24, 48 and 72 hrs was 97, 90.7, and 98%, respectively. The 96 hr LC50 was 16.23 mg/L (UCL = 22.47, LCL = 11.57).

Bacterial effects on methyl anthranilate and dissolved oxygen should not alter our interpretation of methyl anthranilate's acute toxicity. Recall that most mortality occurred within minutes to hours of initial exposure. Dissolved oxygen content was high at this time. Furthermore, over the time course of the experiment, only 10% of the methyl anthranilate for any given concentration was lost. Finally, even under oxygen depleted conditions, catfish surviving initial exposure to methyl anthranilate were likely to survive the length of the static trial. Thus, our estimates for lethal concentration most likely reflect the fish's reaction to high concentrations of methyl anthranilate and its effects on oxygen transport per se. The interaction between bacteria and methyl anthranilate and its effect on available dissolved oxygen did not appear to have a large impact on mortality.

Bluegill

<u>Test conditions</u>

The average mass of bluegills was 0.62 ± 0.24 (SE) g, with all but 6 of the 280 catfish within 2.5 SD units of the mean (Fig. 11). These fish were assigned to test vessels without bias, therefore, we did not suspect that the results were unduly influenced by these marginally heavier fish. This was reflected in the similarity of average weights of fish across concentration groups (Fig. 11). Biological loading among the test vessels was 0.62 g/L ± 0.003 (SE). This was below the recommended 0.8 g/L (Stephan 1975).

Water conditions between the holding tank and the test vessels compared favorably at the start of the trials (Table 1). Mean water temperature was held within 1 °C during the test period (Fig. 12).

Oxygen depletion was observed over the course of the test (Fig. 13). As for catfish, the depletion seen for bluegills likely did not reflect oxygen demand of the fish per se. Control tanks lost between 1 and 3 mg/L of oxygen. Only vessels with methyl anthranilate content higher than 10 mg/L showed a substantial loss of oxygen. These were the vessels where some mortality was observed. In contrast to the catfish bioassay where dead fish were removed upon discovery, dead bluegills were removed once every 12 hrs. Coincidentally, the amount of methyl anthranilate lost was greater in this test (Fig. 13). Thus, we speculate that aerobic bacteria associated with fish decomposition were responsible for the depletion of dissolved oxygen and methyl anthranilate.

<u>Bioassay</u>

At concentrations of 50mg/L or greater 100% mortality was observed within 10-30 seconds. Fish would rapidly swim from the bottom to the top of the tank and die. At approximately 30-49 mg/L, mortality took several minutes. Fish would lose equilibrium, become darkened (apparently cyanotic), lie on the bottom of the tank and soon die. At concentrations of 10-29 mg/L fish were observed to remain still, positioned near the surface taking air at the surface. Mortality was variable within this range, taking from 12 to 24 hours. There was no observable effect for fish housed in tanks below 10mg/L. Fish maintained good color relative to controls, and swam freely in all layers of the tank. Thus the concentration of no observable effect was approximately 10mg/L. The 96 hr LC50 was 9.12 mg/L (UCL = 10.51, LCL = 7.98).

As for catfish, we do not believe the microbial degradation of methyl anthranilate and loss of dissolved oxygen affected the long term mortality results. At high concentrations, bluegills died within minutes, a time when dissolved oxygen content was high. Overall, 74% of the total mortality occurred within 24 hr, with 100% of the mortality occurring by 36 hr.

CONCLUSIONS

Methyl anthranilate can be acutely toxic to fish at high concentrations, ranging from 9 to 35 mg/L. We hypothesize that mortality is due to acute oxygen debt. Because methyl anthranilate is lipophilic it may bind to lipid membranes in gills, thus decreasing oxygen transport across this organ. Lower concentrations of methyl

anthranilate may hinder oxygen uptake but these concentrations did not seem to facilitate mortality, though the behavior of fish was affected. The NOEL for all species ranged from 5 to 10 mg/L.

We believe that only high concentrations of methyl anthranilate dissolved in water are of potential environmental concern. In separate experiments we have found that fish can consume up to 1000 mg/kg of methyl anthranilate incorporated into diet formulations without adverse effects (Clark and Aronov, unpublished data). Because of the high affinity of methyl anthranilate for lipids we found that partition of methyl anthranilate from food into water yielded only 10 mg/L under test conditions.

These observations will prove useful in setting guidelines for application rates of active ingredients. The impact of methyl anthranilate on fish may be reduced without affecting its bird aversive qualities by using formulations which decrease partitioning into water. While formulations may protect methyl anthranilate from microbial and photodegradation, release of unprotected methyl anthranilate into water exposes it to microbial attack. Combined with its minimal impact on vertebrates when ingested the outlook for methyl anthranilate as an environmentally safe repellent appears good.

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Table 1. Summary of water conditions in holding tanks and test vessels contained within the circulating water bath. Values are means ± SE. NM= not measured

	Holding Tank	Test Vessel at 0 hr	Test Vessel at 96 hr
Salmon	N=1	N=1	
DO (mg/L)	10.5	11.2	NM
pH	7.1	7.0	NM
Temperature (°C)	12.5	Fig. 2	Fig. 2
Trout	N=5	N=7	N=7
DO (mg/L)	10.2±0.16	9.9±0.19	7.7±0.22
pH	7.0±0.07	7.3±0.14	7.1±0.09
Temperature (°C)	12.6±0.38	Fig. 5	Fig. 5
Catfish	N=3	N=28	N=28
DO (mg/L)	6.9±0.16	7.2±0.05	4.4±0.48
pH	7.6±0.03	7.8±0.03	7.5±0.05
Temperature (°C)	22.6±0.28	Figure 8	Fig. 8
Bluegill	N=3	N=28	N=28
DO (mg/L)	6.9±0.16	7.2±0.23	2.4±0.39
pH	7.6±0.03	7.4±0.08	7.2±0.04
Temperature (°C)	22.6±0.28	Fig. 12	Fig. 12

FIGURE CAPTIONS

Figure 1. *The frequency distribution of masses recorded for salmon. Inset. Mean mass of salmon for each of the nominal concentrations tested. Vertical bars depict +SE.*

Figure 2. *Continuous temperature record for air temperature and water temperature in 4 test vessels for Atlantic salmon. Data were recorded every 30 minutes and logged to a LiCor datalogger. Horizontal dotted and dashed lines are quality control lines indicating* $\pm$ *0.5 and 1.0* ^{o}C.

Figure 3. *The 96 hr dose-response relationship for salmon. The curve for the 96 hr* LC_{50} *is a logit model of the form (Log(p/(1-p))/2+5)=intercept + BX, where B = 2.88* $\pm$ *0.593, intercept = 0.645* $\pm$*0.867, and X = concentration. The* LC_{50} *for 24, 48, and 72 hrs was 34.28, 33.31, and 32.35 mg/L, respectively.*

Figure 4. *The frequency distribution of masses recorded for trout. [inset] Mean mass of trout for each of the nominal concentrations tested. Vertical bars depict 1 SE.*

Figure 5. *Continuous temperature record for air temperature and water temperature in 4 test vessels for rainbow trout. Data were recorded every 30 minutes and logged to a LiCor datalogger. Horizontal dotted and dashed lines are quality control lines indicating* $\pm$ *0.5 and 1.0* ^{o}C.

Figure 6. *The dose-response curves for trout. No heterogeneity factor was used for the calculation of confidence limits (*P_f*=3.72, df=5, P=0.590. The mortality curve was best estimated using a logit model of the form (Log(p/(1-p))/2+5)=intercept + BX, where B = 16.681* $\pm$*3.708, intercept = -17.693* $\pm$*5.060, and X=concentration. The* LC_{50} *and confidence limits for 24, 48 and 72 hrs were 23.47 (UCL=24.87, LCL=22.11), 23.19 (UCL=24.73, LCL=21.8) and 23.19 (UCL=27.73, LCL=21.79) mg/L, respectively.*

Figure 7. *The frequency distribution of masses recorded for catfish. [inset] Mean mass of catfish for each of the nominal concentrations tested. Vertical bars depict 1 SE.*

Figure 8. *Continuous temperature record for air temperature and water temperature in 4 test vessels for Channel catfish. Data were recorded every 30 minutes and logged to a LiCor datalogger. Horizontal dotted and dashed lines are quality control lines indicating ± 0.5 and 1.0 °C.*

Figure 9. *Relationship between methyl anthranilate content in test vessels at time 0 and loss of methyl anthranilate over a 96 hr period. [inset] Methyl anthranilate content of test vessels at time 0 and the loss of dissolved oxygen over a 96 hr period. The horizontal dashed lines depict the minimum and maximum dissolved oxygen content of control test vessels.*

Figure 10. *The dose-response curves for catfish. A heterogeneity factor was needed for the calculation of confidence limits (P_f=63.67, df=22, P<0.001). The mortality curve was best estimated using a probit model of the form (Probit(p) +5 = intercept + BX, where B = 3.784 ±0.447, intercept = 0.42 ±0.565, and X=concentration. The LC_{50} and confidence limits for 24, 48 and 72 hrs were 20.08 (UCL=30.35, LCL=12.77), 17.35 (UCL=24.4, LCL=12.17) and 16.94 (UCL=23.95, LCL=11.88) mg/L, respectively.*

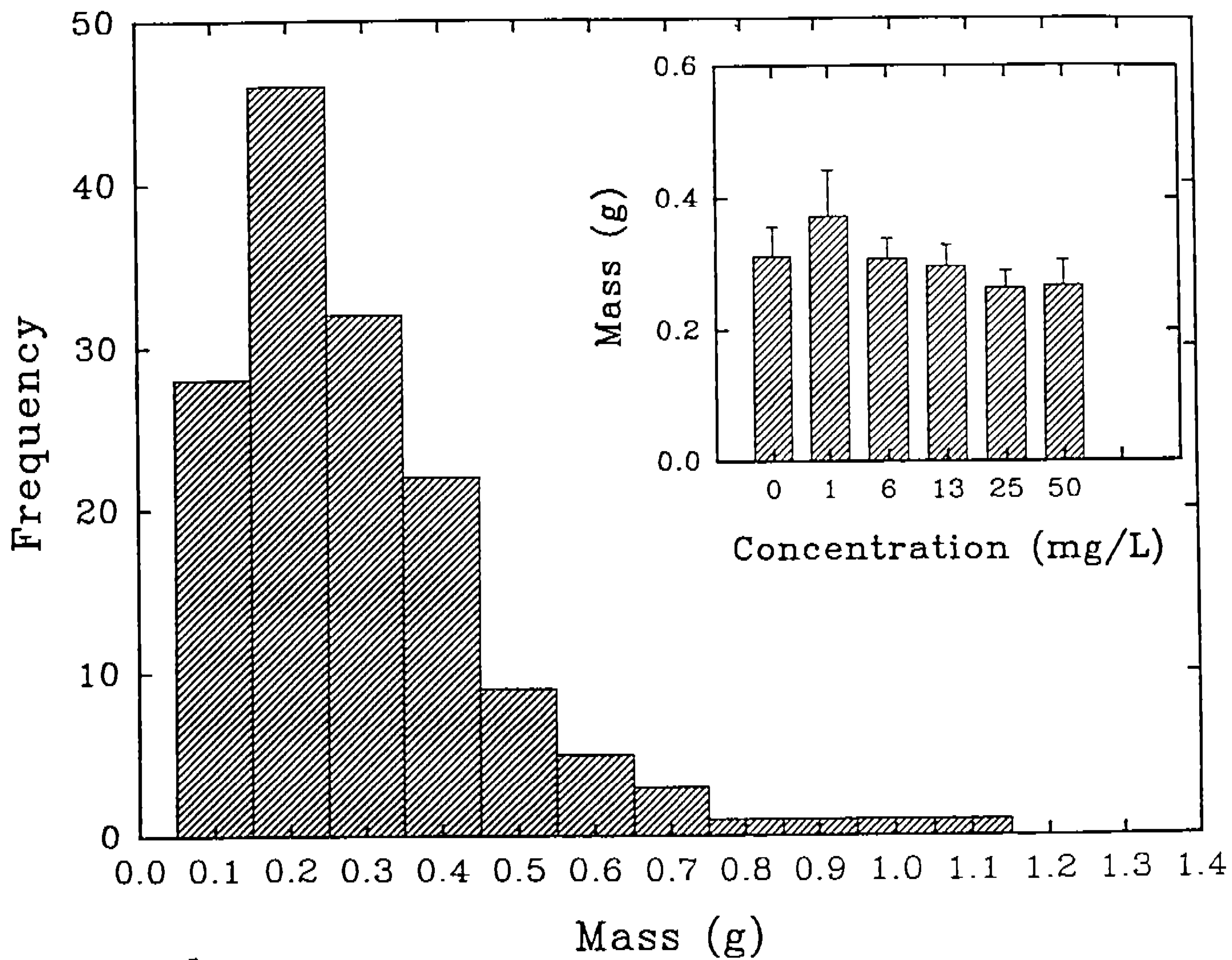
Figure 11. *The frequency distribution of masses recorded for bluegills. [inset] Mean mass of bluegill for each of the nominal concentrations tested. Vertical bars depict 1 SE.*

Figure 12. *Continuous temperature record for air temperature and water temperature in 4 test vessels for bluegill sunfish. Data were recorded every 30 minutes and logged to a LiCor datalogger. Horizontal dotted and dashed lines are quality control lines indicating ± 0.5 and 1.0 °C.*

Figure 13. *Relationship between methyl anthranilate content in test vessels at time 0 and loss of methyl anthranilate over a 96 hr period. [inset] Methyl*

anthranilate content of test vessels at time 0 and the loss of dissolved oxygen over a 96 hr period. The horizontal dashed lines depict the minimum and maximum dissolved oxygen content of control test vessels.

Figure 14. *The dose-response curves for bluegills. No heterogeneity factor was needed for the calculation of confidence limits (P_f=6.04, df=22, $\underline{P}$=<1.0). The mortality curve was best estimated using a probit model of the form (Probit(p) +5 = intercept + BX, where B = 8.019 $\pm$1.131, intercept = -2.698 $\pm$1.087, and X=concentration (Fig. 13). The LC_{50} and confidence limit for 24 hrs was 19.8 (UCL=26.24, LCL=14.16) mg/L. The LC_{50} and confidence limits for 48 and 72 hrs were the same as those for 96 hrs.*


Frequency
Mass (g)
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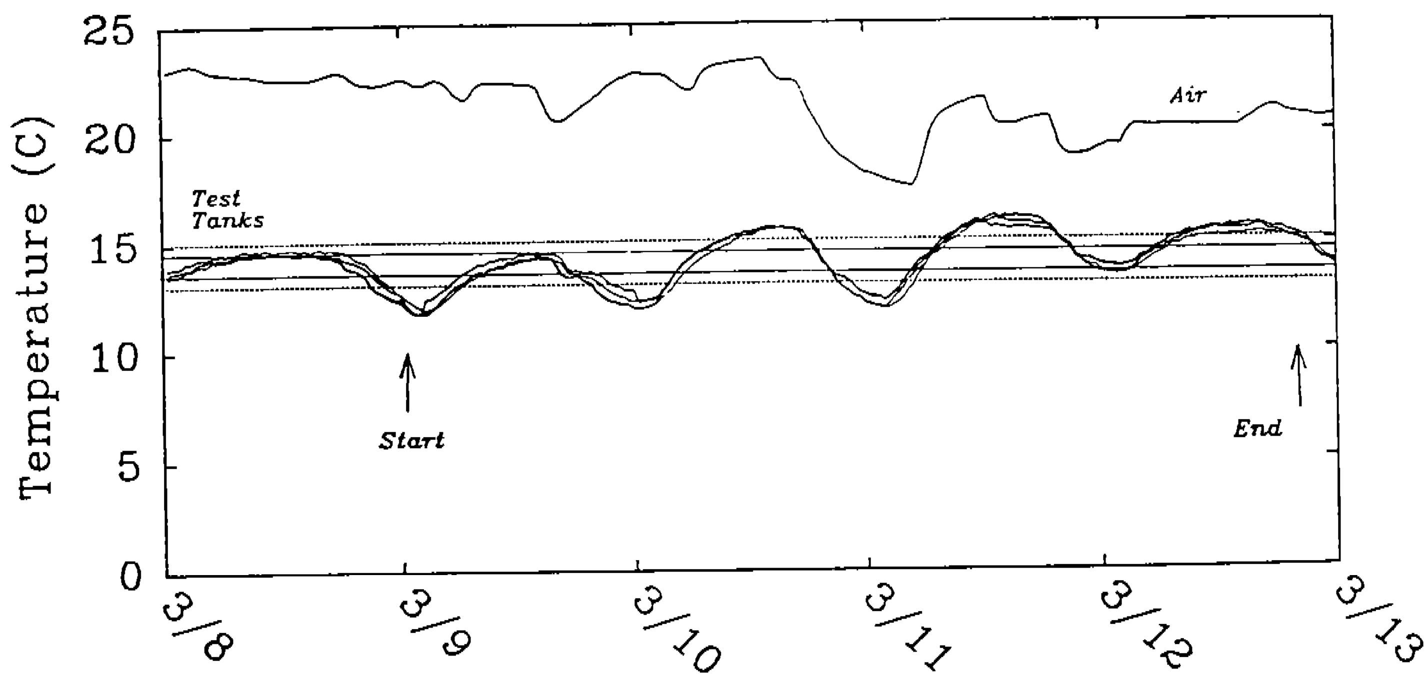
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1 1.2 1.3 1.4
Mass (g)
0.0 0.2 0.4 0.6
0 1 6 13 25 50
Concentration (mg/L)



25
20
15
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Air
Test Tanks
Start
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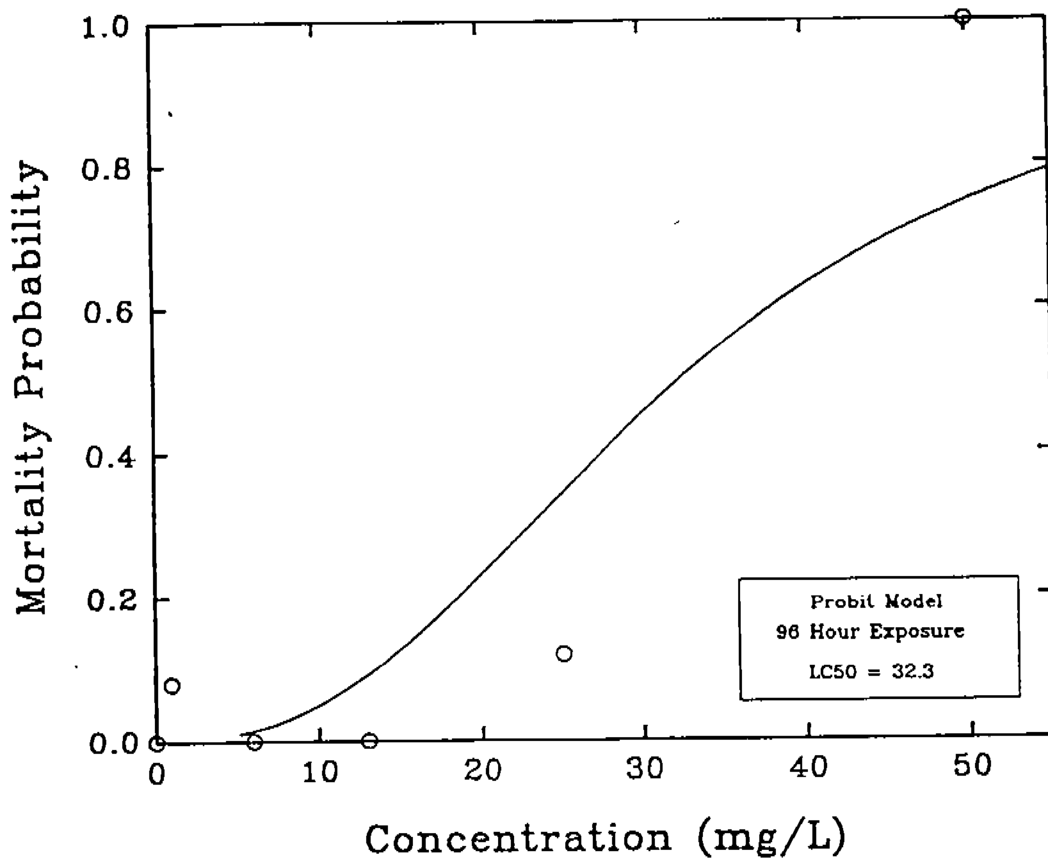
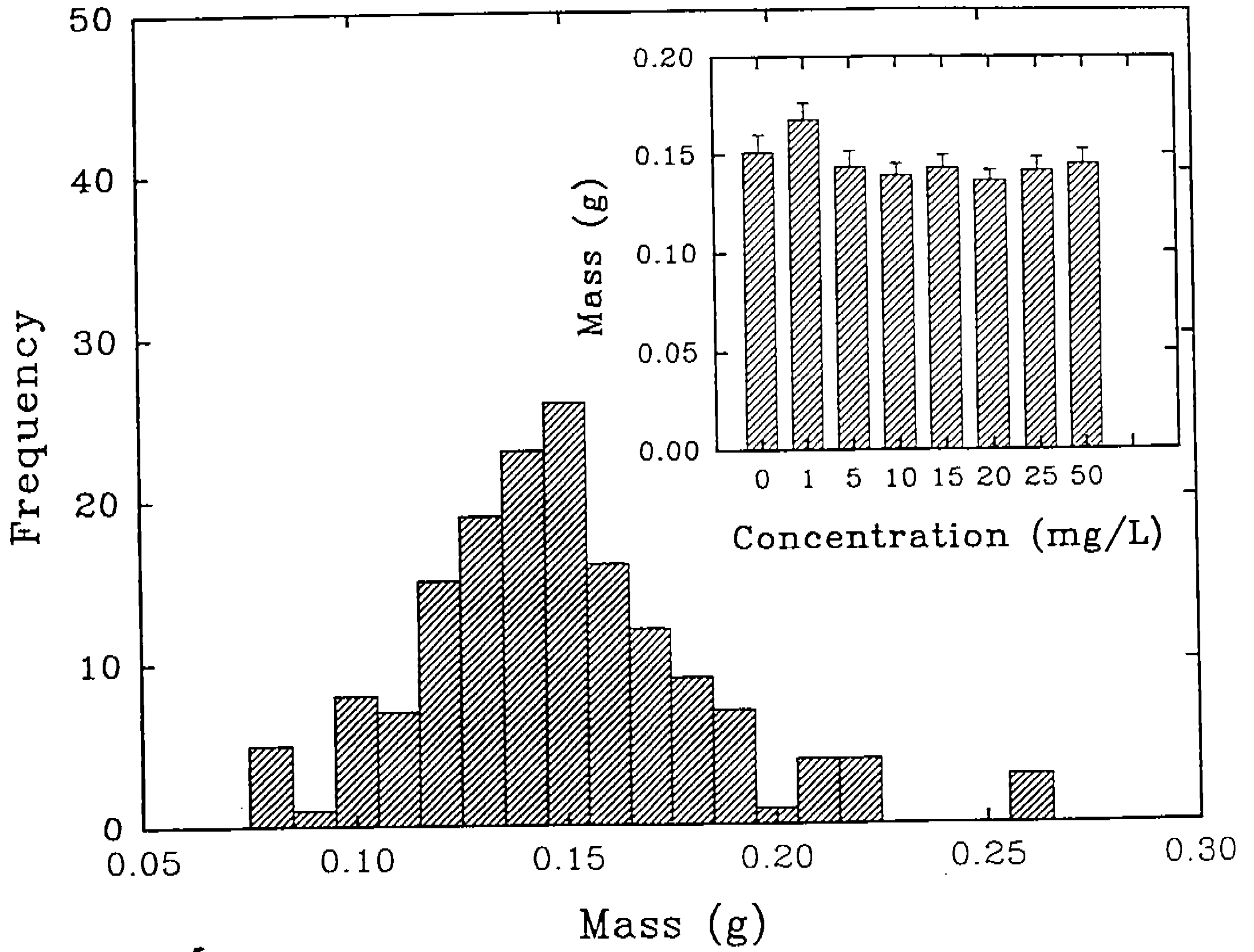
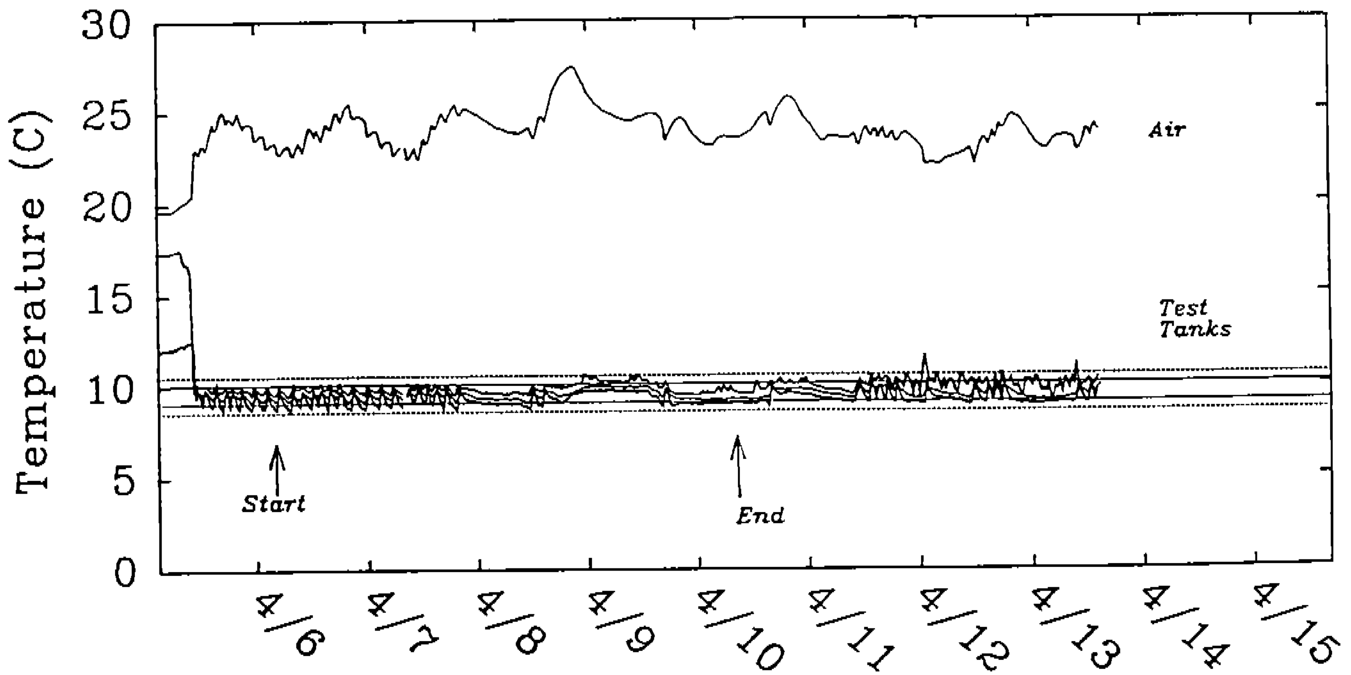
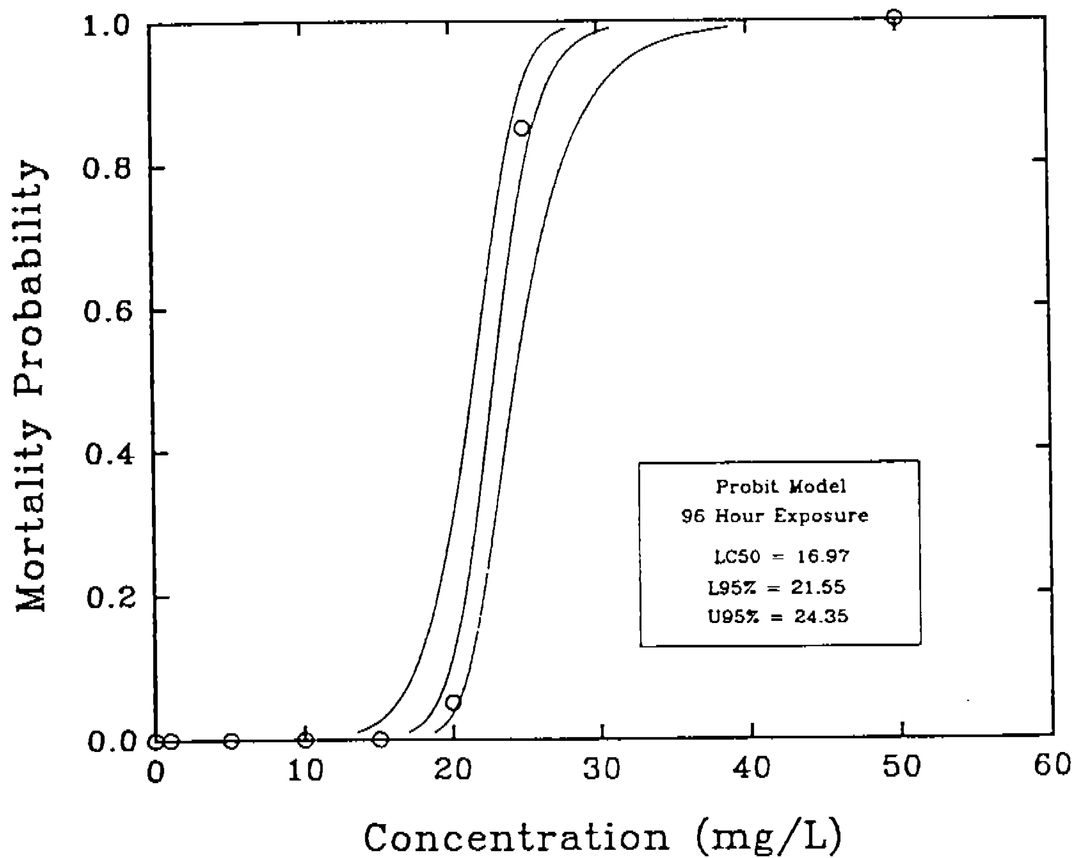


Mortality Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Concentration (mg/L)
Probit Model
96 Hour Exposure
LC50 = 32.3

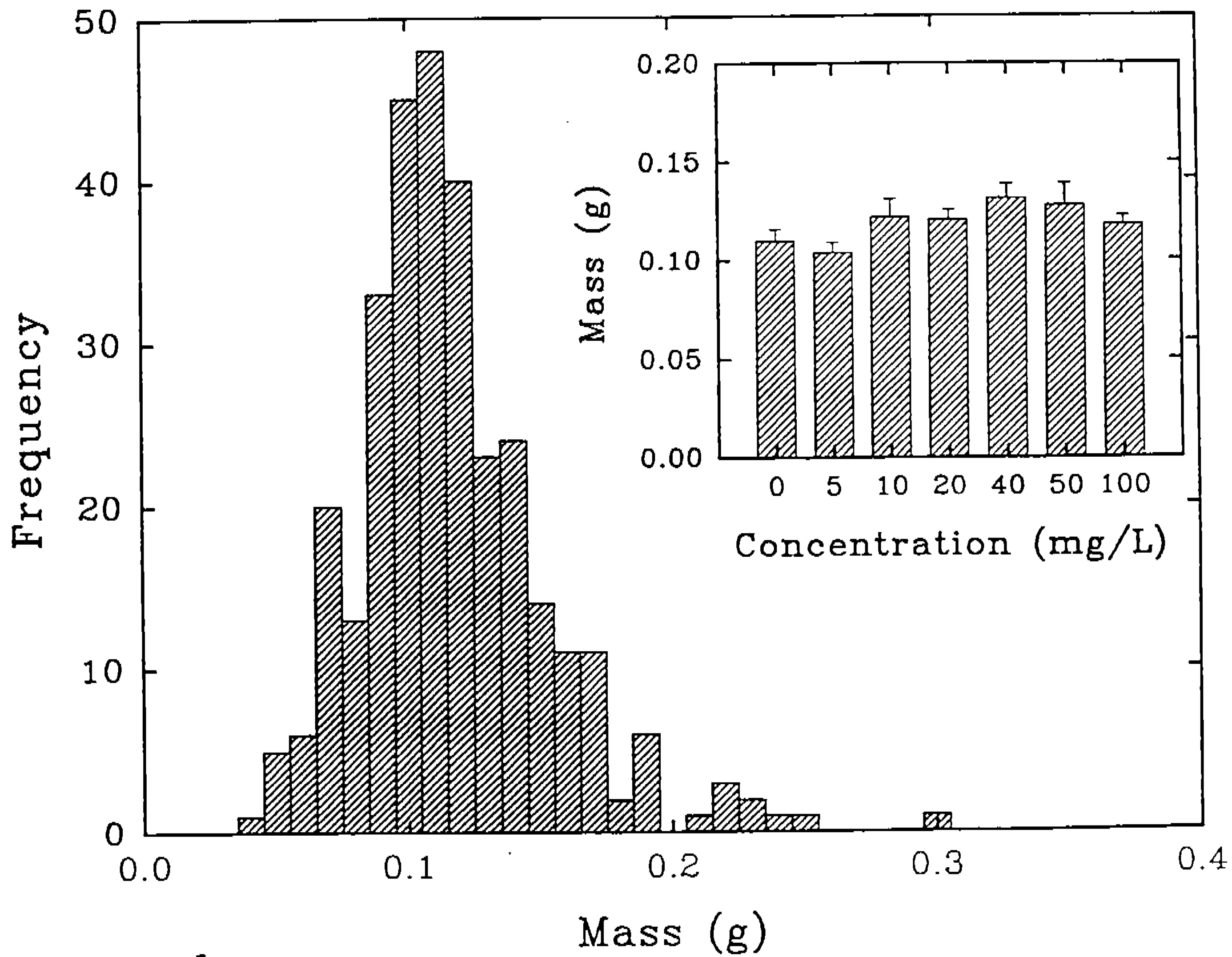

Frequency
50
40
30
20
10
0
0.05
0.10
0.15
0.20
0.25
0.30
Mass (g)
Mass (g)
0.20
0.15
0.10
0.05
0.00
0 1 5 10 15 20 25 50
Concentration (mg/L)



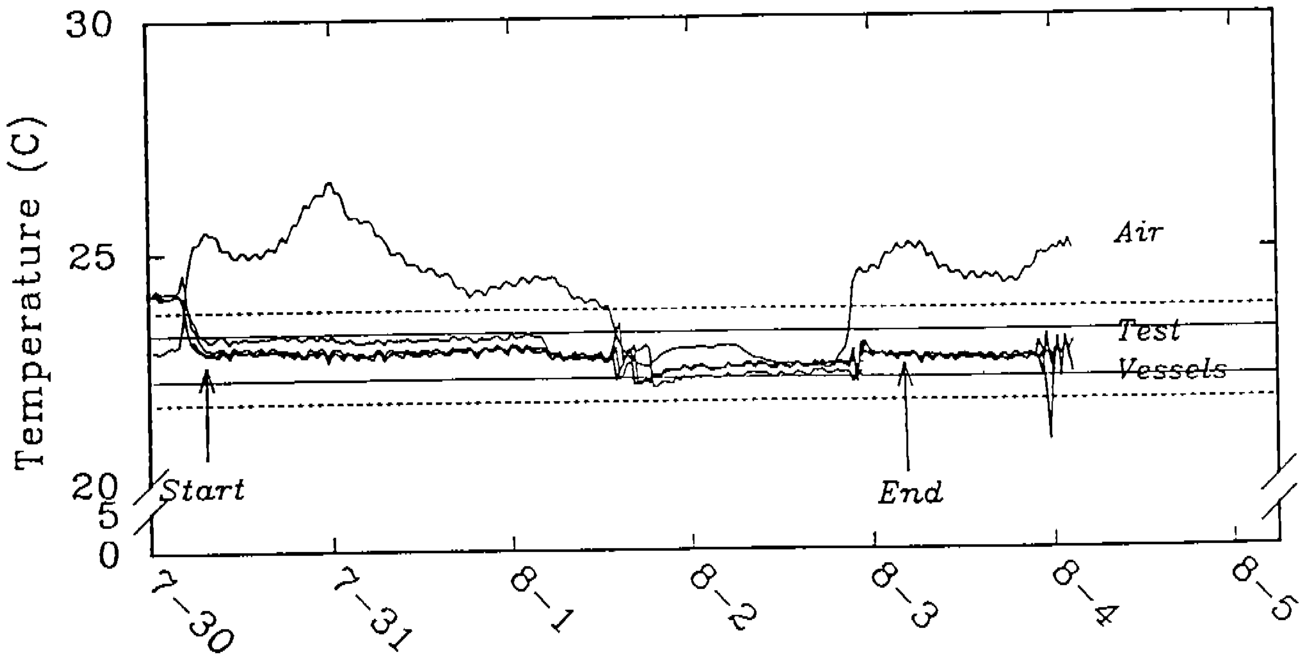
Temperature (C)
0
5
10
15
20
25
30
Air
Test Tanks
Start
End
4/6
4/7
4/8
4/9
4/10
4/11
4/12
4/13
4/14
4/15

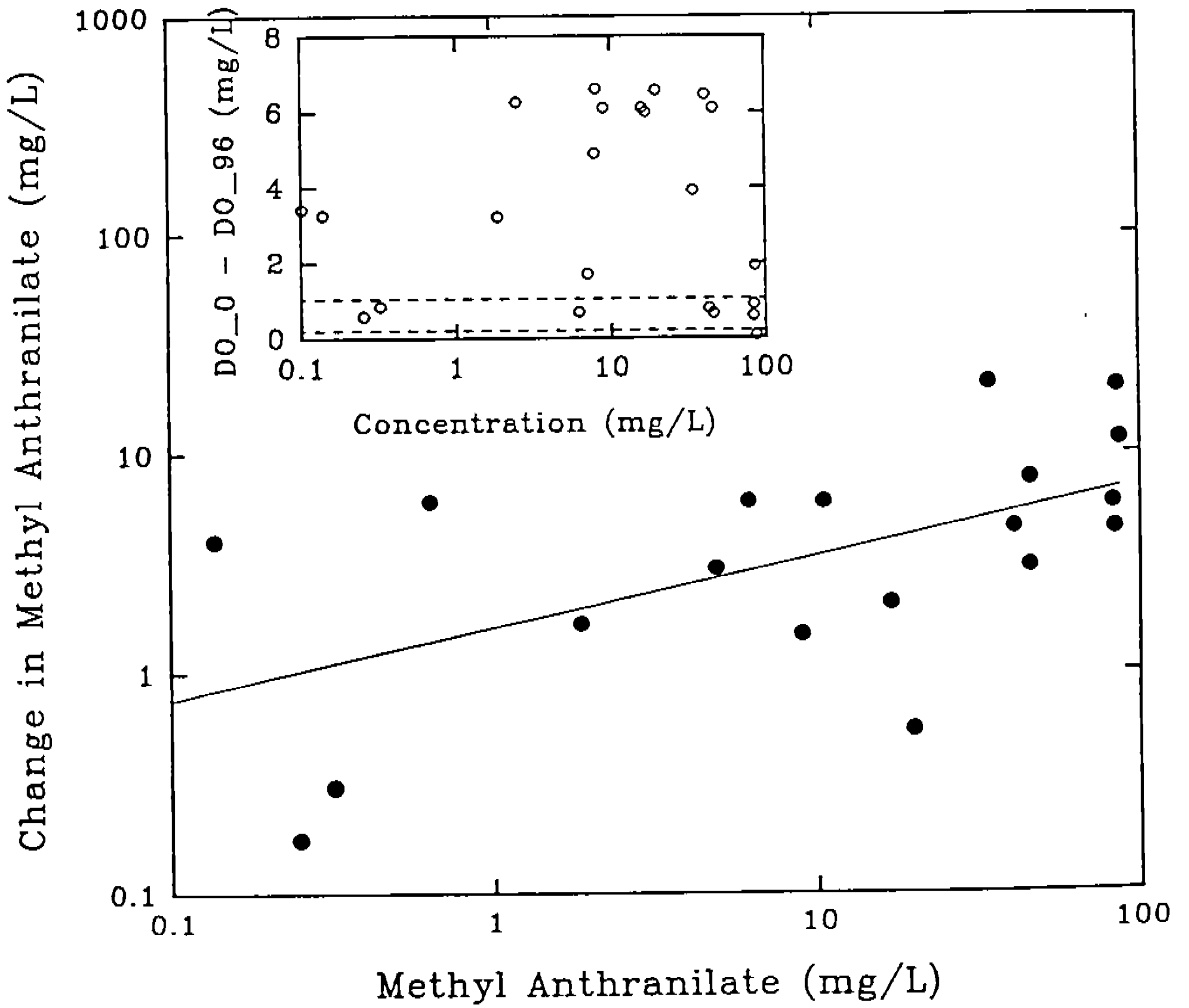


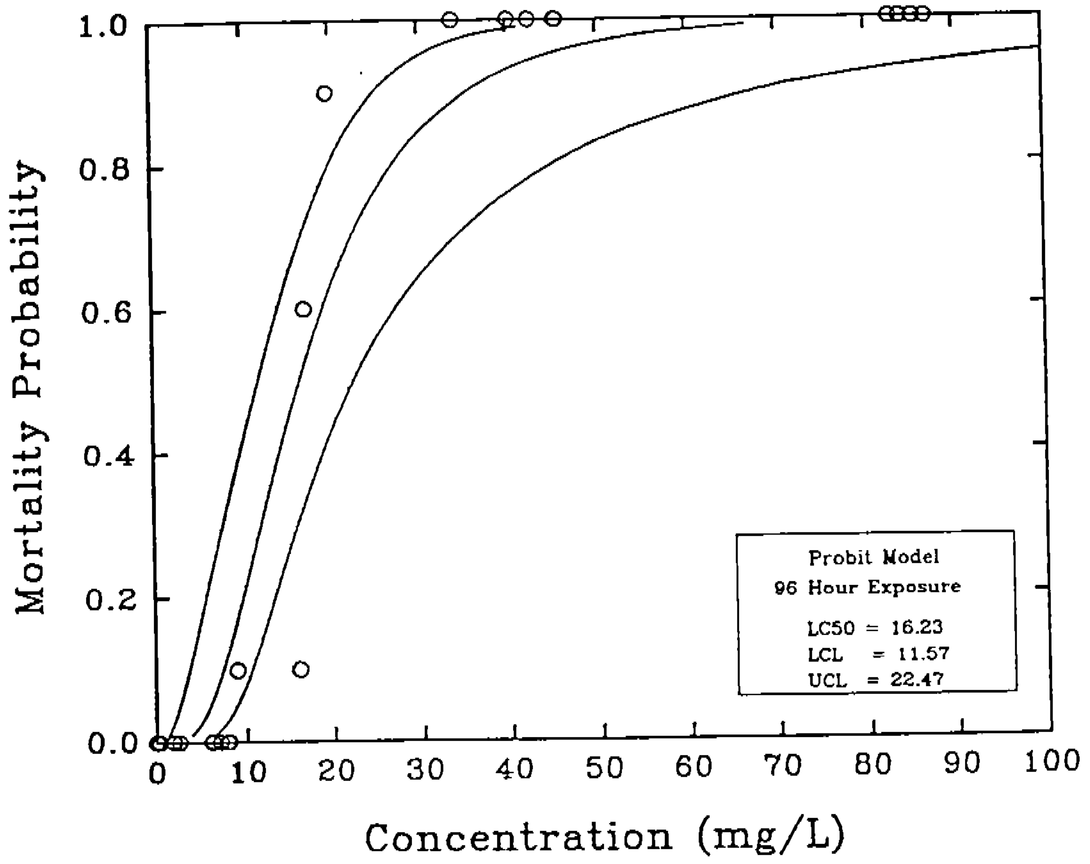
Mortality Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
Concentration (mg/L)
Probit Model
96 Hour Exposure
LC50 = 16.97
L95% = 21.55
U95% = 24.35

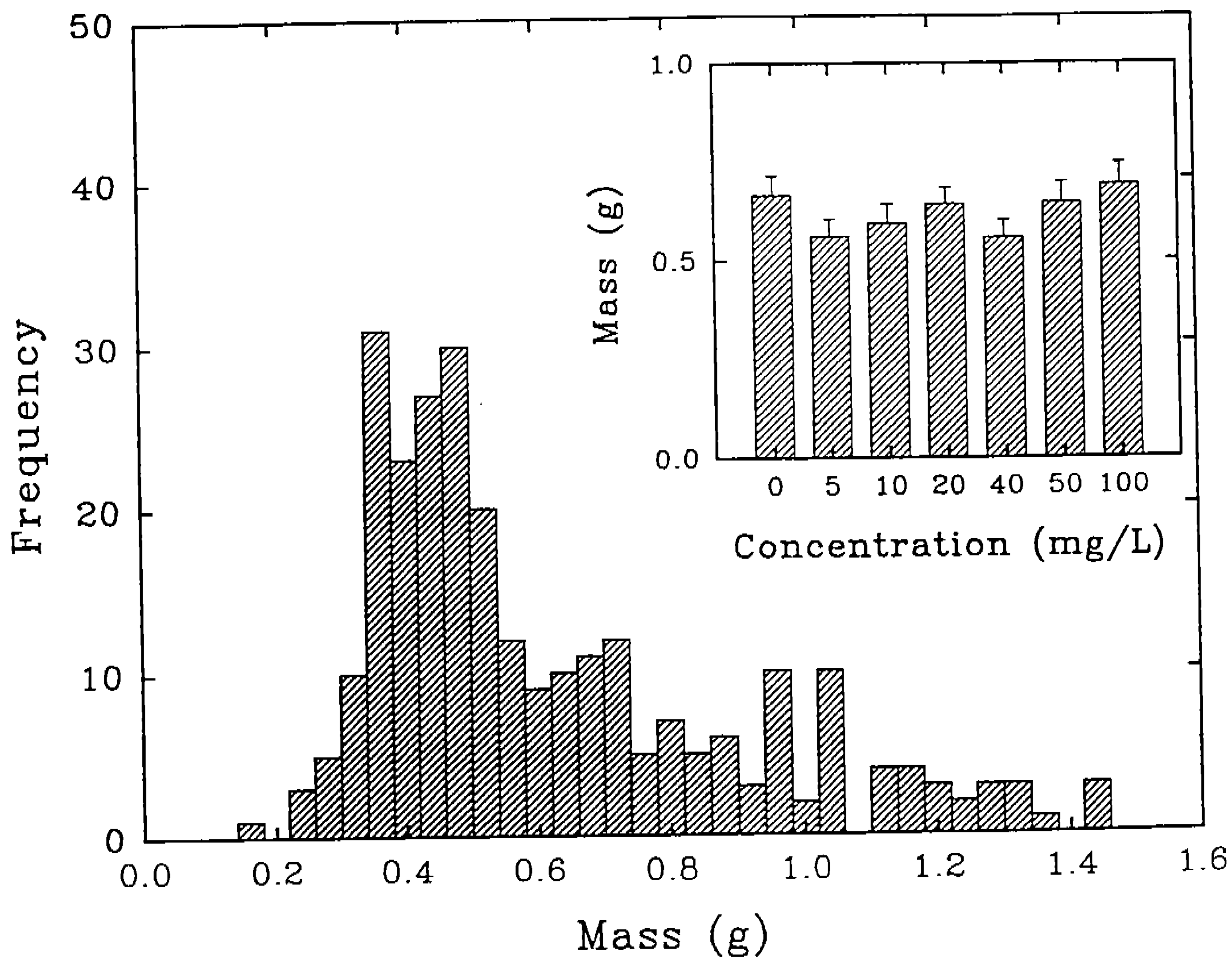


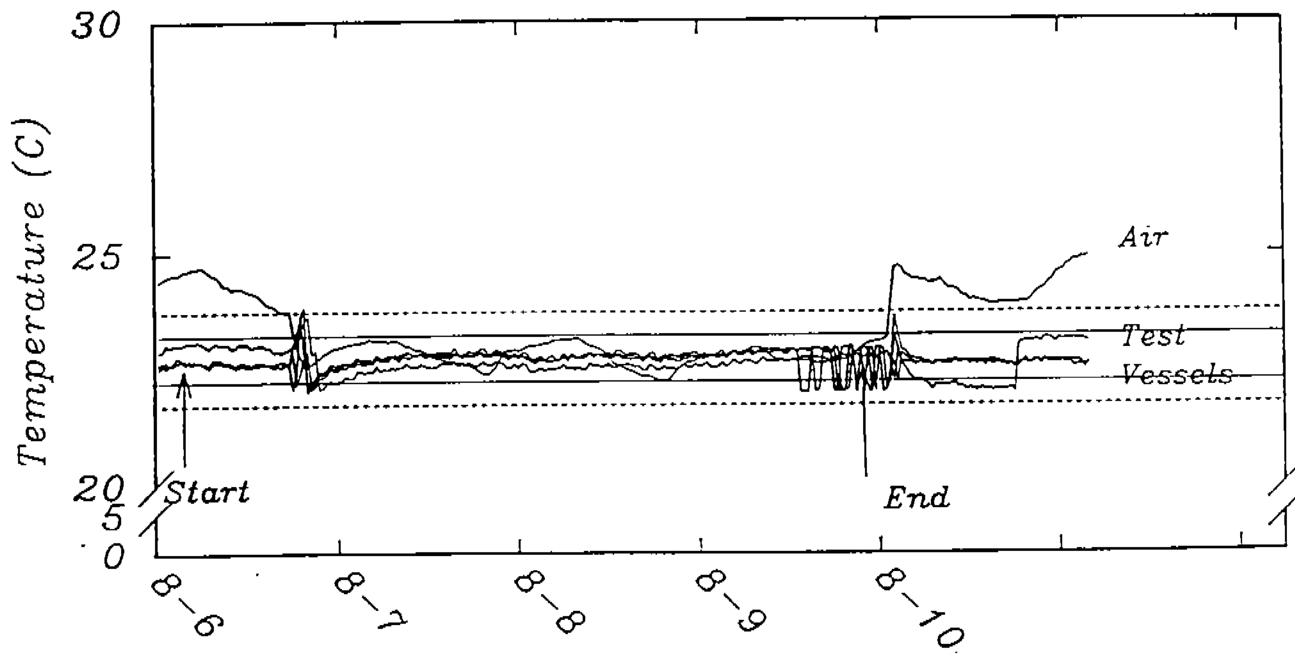
50
40
30
20
10
0
Frequency
0.0
0.1
0.2
0.3
0.4
Mass (g)
0.20
0.15
0.10
0.05
0.00
Mass (g)
0
5
10
20
40
50
100
Concentration (mg/L)

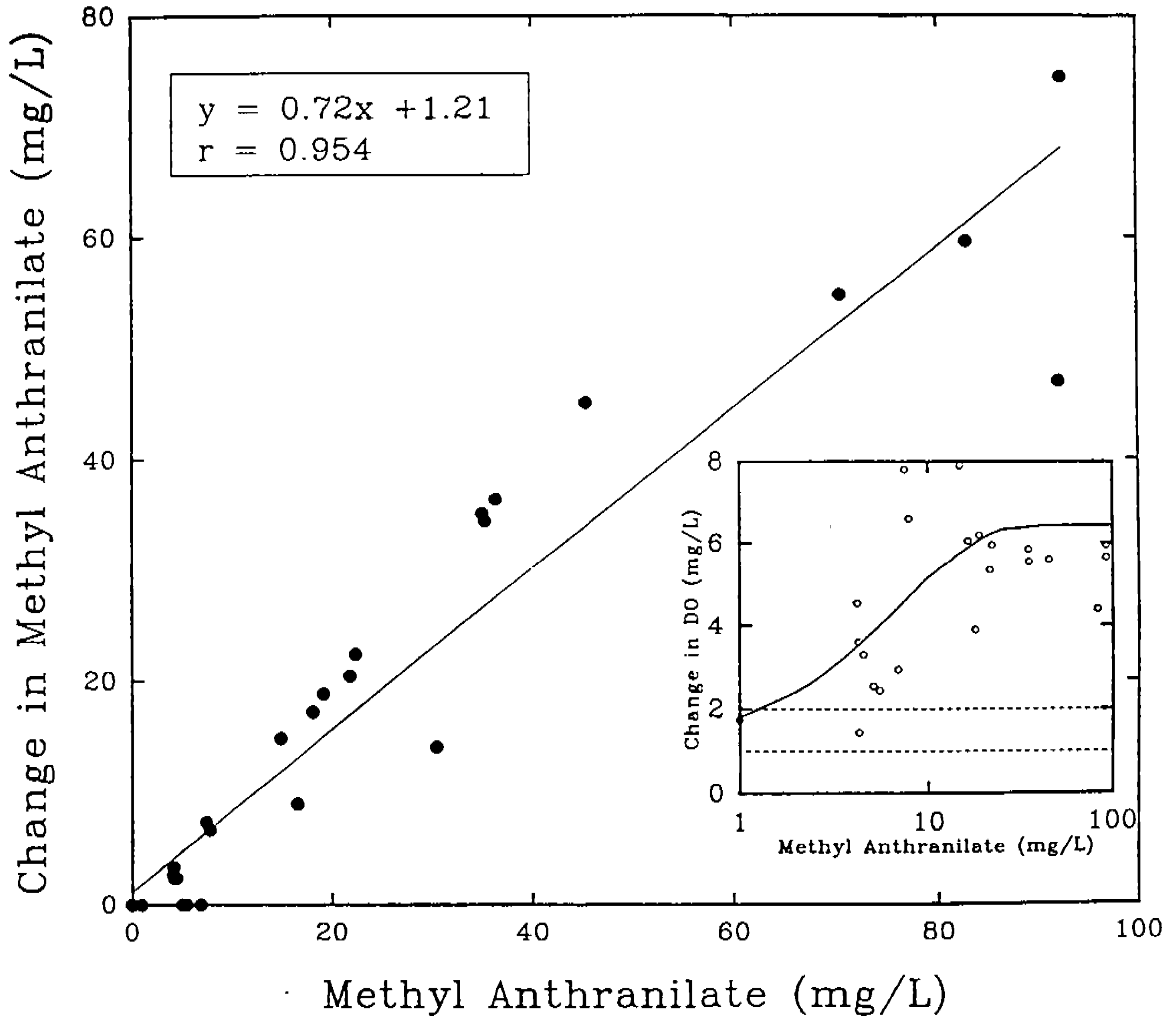

30
25
20
5
0
Temperature (C)
Air
Test
Vessels
Start
End
7-30
7-31
8-1
8-2
8-3
8-4
8-5


Change in Methyl Anthranilate (mg/L)
Methyl Anthranilate (mg/L)
1000
100
10
1
0.1
0.1
1
10
100
DO_0 - DO_96 (mg/L)
Concentration (mg/L)
8
6
4
2
0
0.1
1
10
100

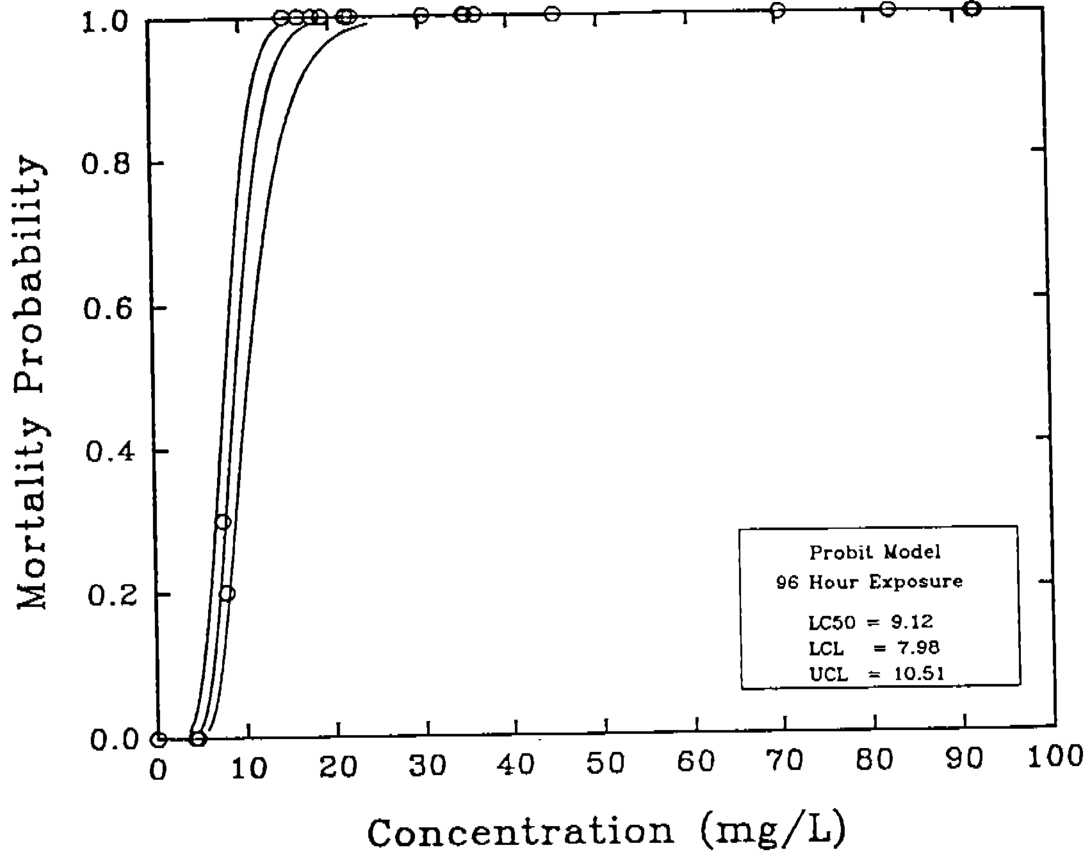

Mortality Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
80
90
100
Concentration (mg/L)
Probit Model
96 Hour Exposure
LC50 = 16.23
LCL = 11.57
UCL = 22.47


Frequency
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Mass (g)
Mass (g)
1.0
0.5
0.0
0
5
10
20
40
50
100
Concentration (mg/L)


30
25
20
5
0
Temperature (C)
Air
Test
Vessels
Start
End
8-6
8-7
8-8
8-9
8-10



y = 0.72x +1.21
r = 0.954
Change in Methyl Anthranilate (mg/L)
Methyl Anthranilate (mg/L)
0
20
40
60
80
100
Change in DO (mg/L)
Methyl Anthranilate (mg/L)
1
10
100


Mortality Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
80
90
100
Concentration (mg/L)
Probit Model
96 Hour Exposure
LC50 = 9.12
LCL = 7.98
UCL = 10.51

Task D: Aquatic toxicity, bioaccumulation, and lifecycle effects of methyl anthranilate to daphnids.

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ABSTRACT

We evaluated the acute toxicity of five methyl anthranilate concentrations (3.1, 6.2, 11.9, 23.8 and 47.2 ppm) to daphnids. The LC_{50} of methyl anthranilate to daphnids at 24-h was estimated to be 31.3 ppm. There was no observed effect from methyl anthranilate at < 19.1 ppm and < 16.1 after a 24-h and 48-h exposure, respectively.

INTRODUCTION

Several laboratory and field studies have shown methyl anthranilate to be an effective nontoxic and nonlethal bird repellent, with application potential for protecting crops, seeds, turf and fish stocks from bird damage (Mason et al. 1989, Cummings et al. 1991, 1992). Furthermore methyl anthranilate can be added to liquids for the purposes of protecting migratory birds, e.g. addition to waste water associated with mining and to standing water pools at airports (Clark and Shah 1991). Mammalian toxicity data are favorable. Methyl anthranilate is used as a fragrance and food flavoring and is GRAS listed by the U.S. Food and Drug Administration (Furia and

Bellanca 1975, U.S. FDA 1993). Despite the favorable outlook for methyl anthranilate's use as a safe repellent, no data exist on its environmental fate and effects.

This study focuses on the acute toxicity of methyl anthranilate to daphnids (Daphnia magna). The data are intended to address the Environmental Protection Agency's data requirement for registration of a biochemical repellent for us in an aquatic environment.

METHODS

Test Chemical

Chemical: Methyl anthranilate; CAS # 134-20-3; Batch/Lot 387. Carrier: ethanol (maximum concentration = 50 µL/L)

Measurement of Test Chemical: Samples for analysis were taken from each concentration at the beginning of the test, and from each test chamber at the end of the test. Samples were collected in amber bottles and transported on ice to the sponsor, who performed the chemical analyses.

Test Chemical Concentrations: A range-finding test was conducted to establish test concentrations for the definitive test. Five daphnids were exposed to each of five concentrations (3.1, 6.2, 11.9, 23.8 and 47.2 ppm) for 48 hours.

Acclimation and test Conditions for Daphnia magna

Source: In-house stock, originally obtained from EPA-Duluth

Species Verification: Brooks, J.L. (1957). "The Systematics for North American Daphnia." Mem. Conn. Acad. Arts & Sci. 13:1-180.

Temperature: 20 ± 1°C

Photoperiod: 16 hour light: 8 hour dark

Feeding Regime: Ankistrodesmus falcatus, 10^5 cells/mL, three times per week

Dilution Water: Round Valley Reservoir, NJ. Alkalinity = 39 mg/L, hardness = 44 mg/L, conductivity = 1.25 µmho

Acclimation Procedures: 2 weeks prior to test, 200 gravid females showing no signs of stress were transferred into 1-L beakers using a glass pipette inserted below the water surface. Gravid females were transferred 24 hors before test initiation. Neonates ranging from 1 to 24 hours were selected and transferred to test chambers in random order.

Test System

Start and Termination Date: April 25-27, 1992.

Temperature: 20 ± 1°C

Photoperiod: 16 hour light : 8 hour dark

Size of Test Vessel: 220-mL Anchor-Hocking plastic cups, thoroughly rinsed with distilled water.

Volume of Test Solution: 200 mL

Age of Test Animals: 1 to 24 hour neonates at start of test.

Number of Animals per Test Vessel: 5

Number of Replicate Test Vessels per Concentration: 4

Feeding Regime: Organisms were not fed during test.

Dilution Water: Round Valley Reservoir, NJ

Test Duration: 48 hours under static conditions.

Effect measured: Mortality (immobilization) and abnormal behavior

Frequency of Observations: 3, 6, 12, 24, and 48 hours.

Aeration: None; dissolved oxygen remained well within 60% and 105% of saturation.

Physical Measurements: DO, temperature, pH at beginning, 24 hours, and 48 hours in each chamber.

Reporting Results: LC_{50} and EC_{50} for 25 and 48 hors with 95% confidence intervals, calculated by trimmed Spearman-Karber analysis (Probit analysis was invalid); all observations and physical measurements were reported in tabular form.

RESULTS

Chemical tested: Methyl anthranilate

Test organism: Daphnia magna

Test administered: 40CFR 797.1300, "Daphnid Acute Toxicity Test"

Test duration: 48 hours

Responses quantified:

Dead: An animal is classified as dead if it is completely immobile and remains so when disturbed.

Affected: An animal is classified as affected if it is either dead or clearly incapable of sustained, normal swimming.

Narrative: We evaluated the acute toxicity of five methyl anthranilate concentrations (3.1, 6.2, 11.9, 23.8, and 47.2 ppm) to daphnids (Fig. 1). The LC_{50} of methyl anthranilate to daphnid at 24-h was estimated to be 32.4 ppm and at 48-h was estimated to be 31.3 ppm (Fig 2). There was no observed effect from methyl anthranilate at <19.1 ppm and <16.1 after a 24-h and 48-h exposure, respectively (Fig. 2).

REFERENCES

Brooks, J.L. 1957. "The systematics of North American Daphnia." Mem. Conn. Acad. Arts and Sci. 13:1-180.

Code of Federal Regulations. 1985. 40CFR 797.1300 - Daphnid acute toxicity test. Federal Register - Office of the Federal Register National Archives and Records Administration pp. 39333 - 39337.

Goulden, C.E. R.M. Comotto, J.A. Hendrickson, Jr., L.L. Hornig, and K.L. Johnson. 1982. Procedures and recommendations for the culture and use of daphnia in bioassay studies. Aquatic Toxicology and Hazard Assessment: Fifth Conference. ASTM STP 766. pp 139-160.

FIGURE CAPTIONS

Fig. 1. *Response of daphnids to five concentrations of methyl anthranilate in a 24- and 48-h exposure test, April 25-27, 1992, Philadelphia, PA.*

Fig. 2. *Acute toxicity (LC_{50}) of methyl anthranilate to daphnids, April 25-27, 1992, Philadelphia, PA. Confidence limits (95%) were calculated using Burlington Research's Spearman - Karber program with automatic trim calculation.*