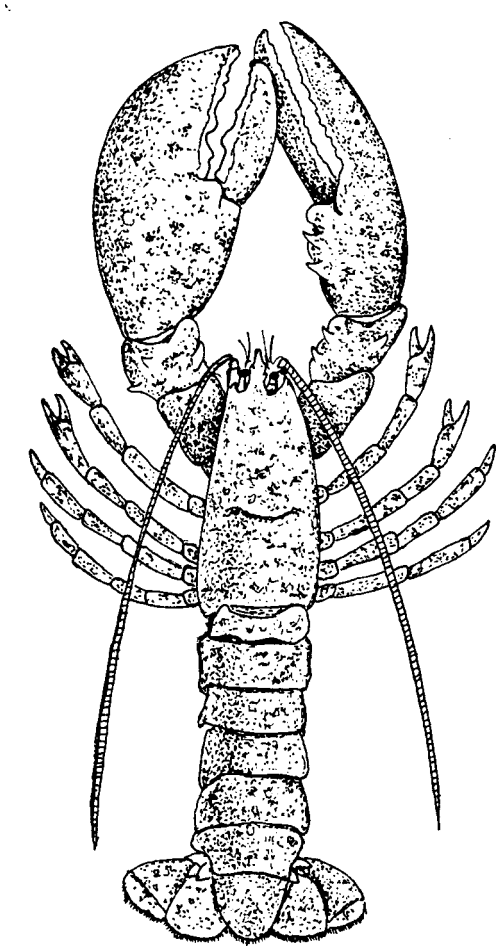
Pesticides



Effects of Pesticides on Lobster Health: Trace Level Measurements and Toxicological Assessment at Environmentally Realistic Concentrations

Anne McElroy, Bruce Brownawell, Ann Zulkosky and Joseph Ruggieri

Marine Sciences Research Center, Stony Brook University, Stony Brook, NY

Goal: Our goal is to better understand the fate and effects of pesticide use in the environment, specifically focusing on acute and chronic effects of pesticides on lobsters.

Approach:
1) Develop trace methods to analyze pesticides and their metabolites in water, sediment and possibly tissue samples;
2) measure levels occurring in the environment after applications and after significant rain events;
3) conduct toxicity studies on larval and juvenile lobsters to access acute toxicity and immune suppression after exposure to pesticides at stressful and non-stressful temperatures;
4) compare toxic levels to environmental concentrations to access risk.

Progress to date: An liquid chromatography-mass spectroscopy (LC-MS) system with electrospray ionization has been acquired and set-up, and methods developed that can measure 0.1-0.5 parts per trillion of pesticides in water samples of one liter. Using these methods, resmethrin, sumethrin, piperonyl butoxide, methoprene, and malathion can be analyzed together with comparable sensitivities. Analysis of the pesticides as sodium adducts in positive ionization mode is one of the keys to achieving high sensitivity. These methods are over 1,000 times more sensitive than those used previously to access pesticide levels in Long Island Sound, and at least ten times more sensitive than the best gas chromatography-MS based method developed to date. Sensitive methods are important both to trace inputs of highly reactive pesticides in receiving waters and to assess the risk of select pesticides that that are highly toxic. For example, there are many reports of lethal toxicity of pyrethroids to crustaceans at levels in the very low parts per trillion range.

Analytical methods have been applied to assist in the development of laboratory dosing systems and to monitor pesticide levels in surface waters following spraying events. Much of the field work was conducted collaboratively with the United States Geological Service (USGS) and the Suffolk County Department of Health. Our work to date has focused on making water measurements in shallow ponds, marshes, tidal inlets and coastal waters in the hours after spraying. In one case, following spraying on Staten Island, we followed concentrations in two water bodies before and after heavy rains that occurred the day after spraying. Most of the spraying events studied involved the pesticide formulation Scourge which consists of resmethrin and the synergist piperonyl butoxide (PPO). PPO was detected in 15 out of 19 samples at concentrations ranging from 0.7 to 15,000 parts per trillion; resmethrin was detected in only 5 of these samples at concentrations ranging from 1.7 to 980 parts per trillion. Based upon the low ratios of resmethrin to PPO in field samples relative to that of the formulations sprayed, it appears that resmethrin is being removed (probably by transformation) faster than PPO.

Toxicity assessment has been delayed by the significant time needed to develop an effective dosing system, facility and staffing issues at our marine laboratory, and limited availability of lobster larvae. We have worked on developing three different systems that have sufficient flow to maintain larval lobsters in suspension without use of aeration, that can deliver trace levels of pesticides reliably. Our third generation system has finally come on-line. It has the capability of delivering 6 different pesticides concentrations to five replicate 2 L chambers at a seawater flow rate of 0.5 L/min per chamber. With this system, measured levels of resmethrin are approximately 75-80 % of delivery calculations. This system can maintain constant temperatures

between 16 and 24 °C. A preliminary 24 hr acute test with Stage II lobster larvae indicates significant toxicity occurs near 300 parts per trillion. We anticipate that 96 hr LC50s will be significantly less.

During development of our dosing system we conducted preliminary experiments with other crustacean species to begin to assess toxicity of pyrethroids. Ten day LC50s for sediment exposures of the benthic amphipod *Leptocheirus plumulosus* were 1,000 ng/g for sumethrin and 400 ng/g for permethrin. Assuming exposure was predominately through pore waters, this roughly translates to water concentrations of 5-100 parts per trillion. We also conducted 96 hr static and flow-through acute tests with adult brine shrimp (*Artemia*). In static tests toxicity was only observed above nominal dosing concentrations of 2,000 parts per trillion. However, actually measured concentrations were much less and decayed significantly during the course of the exposure from 1,000 parts per trillion initially to 4 % of dosed levels at 24 hrs and 0.3% of dosed levels after 96 hrs highlighting the problems associated with static exposures. Measurements in flow-through systems showed toxicity at much lower levels (approximately 200 parts per trillion) after 96 hrs.

Work will continue focusing on additional field sampling for pesticides and determination of acute toxicity and immune suppression in larval and juvenile lobsters exposed to resmethrin with and without PBO, malathion, and methoprene at stressful (22-24 °C) and non-stressful (16-18 °C) temperatures.

The authors wish to thank Michael Tlusty and Denise Fiore of the Lobster Rearing Facility at the New England Aquarium, who provided organisms and technical assistance on lobster care and with the design and set-up of the dosing systems; Ken Gerold who conducted the sediment exposures with sumethrin and permethrin as part of his Intel Science Fair project; Steve Terracciano and Mike Thurman of USGS who assisted with sample collection and methods analytical methods development, and New York Sea Grant who provided financial support.

Acute Effects of Methoprene on Survival, Cuticular Morphogenesis and Shell Biosynthesis in the American Lobster, *Homarus americanus.*

Michael N. Horst[1], Anna N. Walker[1], Thomas Wilson[2], Parshall Bush[3], Ernest Chang[4], Tim Miller[5], and Robert Vogel[1]

[1]School of Medicine, Mercer University, Macon GA
[2]Colorado State University, Fort Collins, CO
[3]University of Georgia, Athens, GA
[4]Uiversity of California at Davis, Davis, CA
[5]University of Maine, Bristol, ME

The acute effects of methoprene on various life stages of the lobster are being investigated in five areas: bioaccumulation, exposure/survival studies, cytopathology/TEM studies, metabolic incorporation studies and SDS-PAGE analysis. After exposure of adult intermolt lobsters to field level concentrations of methoprene (25 ppb/ 24 h), various tissues were dissected, extracted and the concentration of methoprene in each tissue was determined by GC-MS. The highest accumulation of methoprene was observed in epithelial tissue, gonads and hepatopancreas (6.17, 5.18, and 3.97 ppm), up to 250-fold concentration of the pesticide from the surrounding seawater. Conversely, concentrations of methoprene in the stomach, connective tissue and muscle were at nearly baseline levels (0.14, 0.71, 0.16 ppm). Similar results have been obtained with other intermolt lobsters. Standard trans-S-methoprene epoxide has been analyzed by GC-MS and exhibits its own unique GC-MS profile. Analysis of S-methoprenic acid has proved unsuccessful, thus we will utilize an ELISA procedure for this component. These results indicate that adult intermolt lobsters accumulate methoprene in specific tissues "against a gradient".

Our initial metabolic studies involved exposure of intermolt juvenile lobsters to 10 ppb methoprene (18 h), injection with ^{35}S Translabel, and incubation for 24 h. We observed 90% reduction in cytosolic, mitochondrial and microsomal protein synthesis in the hepatopancreas, 50% reduction in the gills, and no effect in muscle tissue. We conclude that *in vivo*, methoprene causes tissue specific decrease in protein synthesis in juvenile lobsters.

Acute toxicity studies have been initiated in all life stages of the lobster. Following exposure of stage I larvae to 25 ppb methoprene at 17 ^{o}C, we observed 100% survival of the larvae up to 48 hours. Stage II larvae were collected and individuals were exposed to methoprene (0.1, 0.5 and 10 ppb) at 18 ^{o}C for up to three days; these larvae were fed adult brine shrimp twice daily. Mortality after 24, 48 and 72 h was recorded. After 24 h exposure, no death was observed at any concentration of methoprene. After 48 and 72 h, we observed mortality in the methoprene-treated larvae, up to a maximum of 82% at 10 ppb. The estimated 72 h LC_{50} for methoprene was 2 ppb in stage II larvae. Postlarvae, juveniles and intermolt adults survive 24 h acute exposures up to 25 ppb methoprene. Following exposure of several postmolt adult lobsters to 25 ppb methoprene, we have observed death after 18 h. Since such animals were likely subjected to multiple stressors, additional studies are planned to further investigate this point.

In vitro metabolic studies with ^{35}S Translabel on postmolt adults utilized explant cultures in DMEM media supplemented with 10% fetal bovine serum and containing 1/10th the normal levels of methionine and cysteine. After 18 h incubation, we observed minimal effect of methoprene on total shell protein synthesis, but did detect a shift in the distribution of extractable proteins from the shell. The epithelial tissues from these cultures exhibited increased increased protein labeling but showed shifts of labeled precursors into buffer and urea soluble fractions. In order to study chitin synthesis, *in vitro* metabolic studies with ^{3}H D-glucosamine (GlcN) were performed as described above for ^{35}S Translabel. Methoprene reduces ^{3}H GlcN incorporation into epithelial cells and the associated microsomal fraction by 62%. Extraction of the shell fractions from this experiment indicate that methoprene decreases total ^{3}H GlcN incorporation by 17% and causes a shift in the

distribution of precursors from buffer to urea soluble. Taken together these metabolic studies indicate that even at very low levels, methoprene may cause a block in the exocytosis and/or crosslinking of ^{3}H GlcN labeled precursors into the nacent cuticle.

As part of our studies of the individual fractions solubilized from control and methoprene treated shell and epithelial cells, we have analyzed these fractions by SDS-PAGE. The gels have then been examined by total protein staining (colloidal Coommassie Blue G-250 or Sypro Ruby) and by Western blotting followed by probing with either the chitin specific lectin, ToL, or with monoclonal antibodies to specific stress proteins (HSPs). In intermolt adult lobsters, methoprene causes shifts in membrane-associated proteins of the hepatopancreas; no such changes were observed in muscle tissue. In postmolt adult epithelial cells, methoprene decreases most major cytosolic proteins and increases several, e.g., HSP-70. Decreased synthesis of tomato lectin-positive chitoproteins was observed in the cellular membrane fraction and in all fractions extracted from the shell of these samples. These results indicate that methoprene alters the synthesis, secretion and/or incorporation of chitoproteins in the postmolt lobster.

To date, our cytopathology studies have failed to show any consistent aberrations caused by low levels of methoprene (1-10 ppb); some preliminary indication of focal necrosis in the hepatopancreas has been noted but requires further investigation. Additional cytopathology and TEM studies are ongoing.

Malathion immunotoxicity in the American lobsters (*Homarus americanus*) upon experimental exposure

Sylvain De Guise[1], Jennifer Maratea[1], Christopher Perkins[2]

[1]Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT
[2]Environmental Research Institute, University of Connecticut, Storrs, CT

Introduction

A lobster die-off reduced the 1999 fall landings in western Long Island Sound by up to more than 99%. The die-off corresponded in time with the application of pesticides for the control of mosquitoes that carried west Nile virus, a new emerging disease in North America at the time. The lobsters examined suffered from a *Paramoeba* sp. infection that mainly affected the nervous system. In order to determine the possible implication of pesticide application as a direct cause or contributing factor in the die-off, we studied the effects of experimental exposure to malathion on the health of lobsters.

Material and methods

Experimental exposures were performed in aerated 20 gallon tanks each containing 3 lobsters, with a total of 9 lobsters (in 3 tanks) per dose. Lobsters were kept at 10 °C in artificial sea water and exposed to malathion using different regimes. Standard LC50 experiments were performed in the course of 96 hours. Acute exposure lasted 5 days, with sampling on day 1, 3 and 5, and consisted of either a single dose of malathion or repeated doses through daily water changes. Subacute exposure was performed over the course of 4 weeks, with weekly sampling. At the end of each study, lobsters were sacrificed and tissues sampled for the presence of gross and histological lesions, and for determination of concentrations of the chemical used in pooled muscle, hepatopancreas and hemolymph, in comparison to water concentrations.

Water and tissue samples were analyzed at the Environmental Research Institute (ERI) based upon a modified form of EPA Method 616. This EPA method is not validated for sediment and tissue from the EPA Office of Pesticide Programs (personal communication). The primary changes from the EPA method is the use of capillary column techniques in lieu of the packed column specified in the methods, and the use of GC/MS instead of a flame ionization detector. EPA method 616 is based upon older techniques and the ERI improvements to the method allow for the identification and quantification at lower levels.

The endpoints tested include evaluation of the immune system using hemocyte counts and phagocytic index on hemolymph samples. Briefly, hemolymph was collected and immediately transferred to Vacutainer tubes (Becton Dickinson, Rutherford, NJ) containing acid citrate dextrose (ACD). This proved to be the best anticoagulant for use with lobster hemolymph cells in preliminary studies in our lab. Cells were then counted using a hemocytometer and Trypan blue to determine viability. Phagocytosis was evaluated as previously described (De Guise *et al.*, 1995) with some variations. Hemocytes were incubated in their hemolymph at room temperature (20-25°C). One µm diameter fluorescent latex beads (Molecular probes, Eugene, OR) were diluted 1:10 in PBS and 5 µl of the bead mixture was added for every 200 µl of hemolymph. After a 1 hour incubation in the dark, 200 µl of each cell suspension was analyzed by flow cytometry. The fluorescence of approximately 10,000 hemocytes was evaluated with a FACScan (Becton Dickinson, Mountain View, CA) flow cytometer. Phagocytosis was evaluated as the proportion of hemocytes that had phagocytized 2 or more beads.

At the end of all studies, lobsters were sacrificed and a gross and histopathological examination performed to determine the presence/absence of pathological conditions. Tissues were fixed in Bouin's fixative for 48 hours, then in 70% ethanol for 24 hours, and further trimmed and processed for paraffin embedding. Tissues were sectioned at 4µm, routinely stained with hematoxylin and eosin, and examined by light microscopy for the presence/absence of lesions.

Results

The direct toxicity was determined through a standard 96-hour LC50, the calculated concentration that killed 50% of the animals. To do so, lobster mortality was recorded daily over a 4 day exposure. The cumulative mortality was then plotted against the concentrations of malathion used and a linear regression curve was determined using the Microsoft Excel software. The LC50 was calculated using the equation determined by the software for the regression curve. The 96 hour LC50 was 33.5 µg/L (or ppb) upon single exposure (Figure 1).

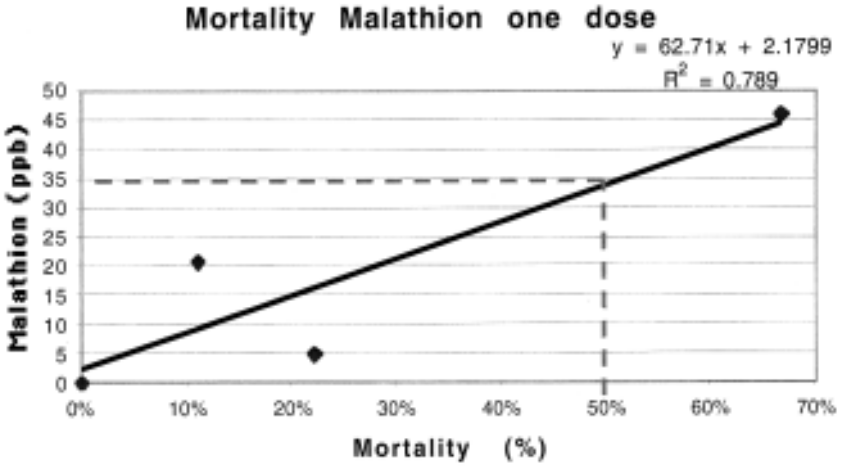


Figure 1. LC50 of malathion in lobsters.

Malathion degraded rapidly in our system, with 65-77% lost after one day and 83-96% after three days (Figure 2). No malathion was detectable in lobster tissues at the end of the 5 day exposure.

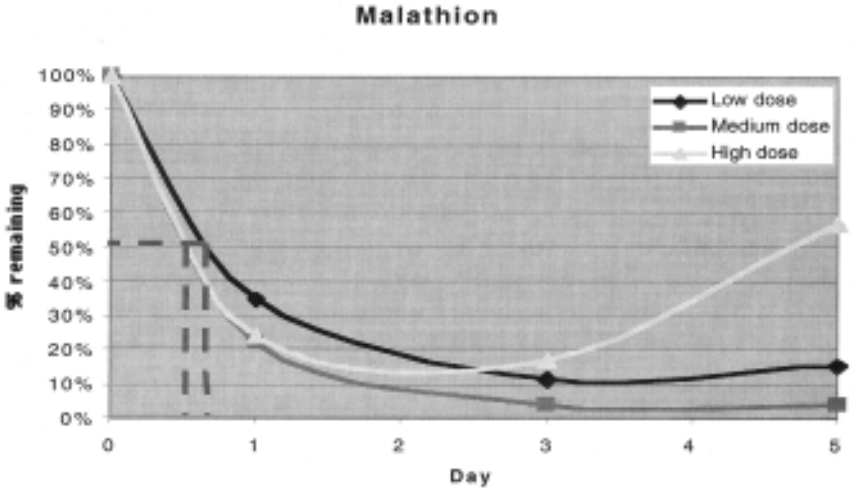


Figure 2. Concentrations of malathion in water decreased rapidly in our system.

Relatively high concentrations of malathion, upon repeated exposure, initially (day 1) increase phagocytosis, with no effects on day 3 and 5 (data not shown). Phagocytosis was significantly decreased 3 days (but not 1 or 5) after a single exposure to water concentration as low as 5 ppb (the lowest concentration tested), when water concentrations were as low as 0.55 ppb (Figure 3).

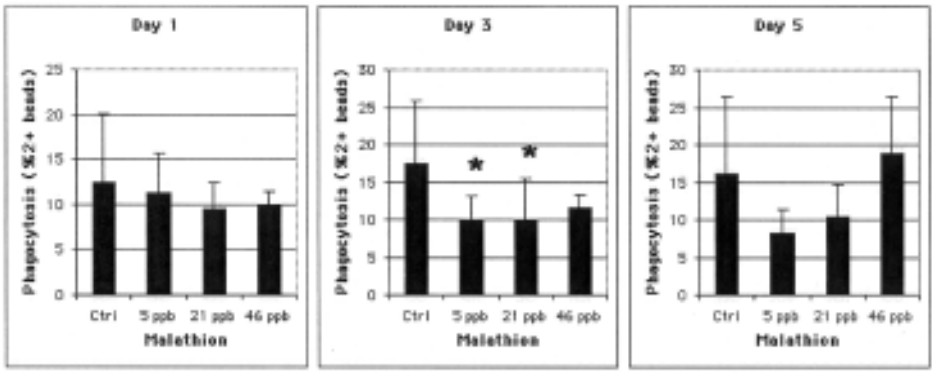


Figure 3: Phagocytosis of lobster cells after a single exposure to increasing concentrations of malathion.

Phagocytosis was also significantly affected in the course of the sub-acute (month long) exposure (Figure 4). There was a significant reduction of phagocytosis one week after the initial exposure to 21 ppb, and two weeks after the initial exposure to 5 ppb. There was a significant reduction of phagocytosis at all concentrations tested three weeks after the initial exposure.

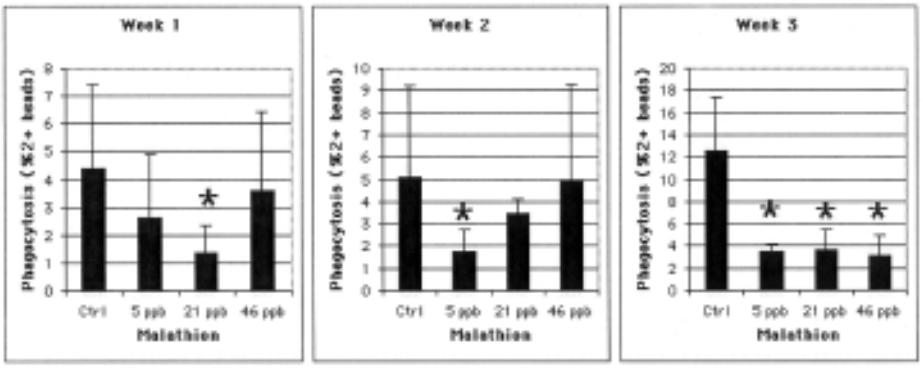


Figure 4: Phagocytosis of lobster cells after a weekly exposure to increasing concentrations of malathion.

Cell counts did not differ significantly upon exposure to malathion. Gross or histological lesions were not observed upon exposure to malathion.

Discussion

Malathion has a wide range of toxicities in fish, extending from very highly toxic in the walleye (96-hour LC50 of 0.06 mg/L) to highly toxic in brown trout (0.1 mg/L) and the cutthroat trout (0.28 mg/L), moderately toxic in fathead minnows (8.6 mg/L) and slightly toxic in goldfish (10.7 mg/L) and mosquitofish (12.68 mg/L) (Johnson and Finley 1980, Tietze *et al.*, 1991,Kidd and James, 1991, U.S. Public Health Service, 1995). Various aquatic invertebrates are extremely sensitive, with EC50 values from 1 ug/L to 1 mg/L (Menzie, 1980). Lobsters, with a LC50 of 33.5 ppb, appear to be very sensitive to the acute lethal effects of malathion compared to other aquatic species.

The very rapid breakdown of malathion in our system suggests that failure to measure malathion in water samples does not necessarily mean lack of exposure. At day 3 of our acute exposure study, the concentrations of malathion in the water were very low, yet effects on phagocytosis were demonstrated in lobsters.

Our data suggest that evaluation of phagocytosis using flow cytometry is a sensitive indicator of subtle sub-lethal effects of malathion, and that transient exposure to relatively small concentrations of malathion (6-7 times lower than the LC50) can affect lobsters' defense mechanisms, even with rapidly decreasing water concentrations. Those results are not surprising given that the immunotoxicity of malathion has been documented in several species of laboratory animals including effects on both humoral and cellular immune responses of mice, rats and rabbits (Banerjee *et al.,* 1998). Malathion was also documented to affect the natural and acquired immunity of fishes (Japanese medaka), in addition to decreasing resistance to a common pathogen (Beaman *et al.,*. 1999). Nevertheless, it is interesting to note that the initial water concentrations that resulted in immunotoxicity in lobsters (5 ppb or 5 µg/L) are 40 times lower that those which resulted in reduction in immune functions and 20 times lower than those which resulted in reduced resistance to a pathogen in the fish study (Beaman *et al.,* 1999).

In conclusion, our results suggest that lobsters are highly sensitive to both the lethal and sub-lethal toxicity of malathion in sea water. A reduction in immune functions could likely result in an increase susceptibility to infectious agents, and could have contributed to the mass mortality if exposure was sufficient.

References

Banerjee B.D., Pasha S.T., Hussain Q.Z., Koner B.C., Ray A. 1998. A comparative evaluation of immunotoxicity of malathion after subchronic exposure in experimental animals. *Indian J Exp Biol* 36(3):273-282.

Beaman J.R., Finch R., Gardner H., Hoffmann F., Rosencrance A., Zelikoff J.T. 1999. Mammalian immunoassays for predicting the toxicity of malathion in a laboratory fish model. *Toxicol Environ Health* 56: 523-542.

Johnson W.W., Finley M.T. 1980. Handbook of Acute Toxicity of Chemicals to Fish and Aquatic Invertebrates. Resource Publication 137. U.S. Department of Interior, Fish and Wildlife Service, Washington, DC.

Kidd H., James D.R., Eds. 1991. *The Agrochemicals Handbook, Third Edition*. Royal Society of Chemistry Information Services, Cambridge, UK, (as updated).

Menzie C.M. 1980. Metabolism of Pesticides. Update III. Special Scientific Report, Wildlife No. 232. U.S. Department of Interior, Fish and Wildlife Service, Washington, DC.

Tietze N.S., Hester P.G., Hallmon C.F., Olson M.A., Shaffer K.R. 1991. Acute toxicity of mosquitocidal compound to young mosquitofish, *Gambusia affinis*. *Am Mosq Control Assoc* 7: 290-293.

U.S. Public Health Service. 1995. Hazardous Substance Data Bank. Washington, DC.