

# **ACUTE TOXICITY OF RESMETHRIN, MALATHION AND METHOPRENE TO LARVAL AND JUVENILE AMERICAN LOBSTERS (HOMARUS AMERICANUS) AND ANALYSIS OF PESTICIDE LEVELS IN SURFACE WATERS AFTER SCOURGE™, ANVIL™ AND ALTOSID™ APPLICATION**

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## ACUTE TOXICITY OF RESMETHRIN, MALATHION AND METHOPRENE TO LARVAL AND JUVENILE AMERICAN LOBSTERS (*HOMARUS AMERICANUS*) AND ANALYSIS OF PESTICIDE LEVELS IN SURFACE WATERS AFTER SCOURGE<sup>TM</sup>, ANVIL<sup>TM</sup> AND ALTOSID<sup>TM</sup> APPLICATION

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**ABSTRACT** Acute toxicity and immune response, combined with temperature stress effects, were evaluated in larval and juvenile American lobsters (*Homarus americanus*) exposed to malathion, resmethrin and methoprene. These pesticides were used to control West Nile virus in New York in 1999, the same year the American lobster population collapsed in western Long Island Sound (LIS). Whereas the suite of pesticides used for mosquito control changed in subsequent years, a field study was also conducted to determine pesticide concentrations in surface waters on Long Island and in LIS after operational applications. The commercial formulations used in 2002 and 2003—Scourge, Anvil and Altosid—contain the active ingredients resmethrin, sumithrin and methoprene, respectively. Concentrations of the synergist piperonyl butoxide (PBO) were also measured as a proxy for pesticide exposure. Acute mortality in Stage I-II larval lobsters demonstrated that they are extremely sensitive to continuous resmethrin exposure. Resmethrin LC50s for larval lobsters determined under flow-through conditions varied from 0.26–0.95  $\mu\text{g L}^{-1}$  in 48- and 96-h experiments at 16°C, respectively. Increased temperature (24°C) did not significantly alter resmethrin toxicity. Malathion and methoprene were less toxic than resmethrin. The 48-h LC50 for malathion was 3.7  $\mu\text{g L}^{-1}$  and methoprene showed no toxicity at the highest (10  $\mu\text{g L}^{-1}$ ) concentration tested. Phenoloxidase activity was used as a measure of immune response for juvenile lobsters exposed to sublethal pesticide concentrations. In continuous exposures to sublethal doses of resmethrin (0.03  $\mu\text{g L}^{-1}$ ) or malathion (1  $\mu\text{g L}^{-1}$ ) for 7 d at 16 or 22°C, temperature had a significant effect on phenoloxidase activity ( $P \leq 0.006$ ) whereas pesticide exposure did not ( $P = 0.880$ ). The analytical methods developed using high performance liquid chromatography coupled to time-of-flight mass spectroscopy (LC-TOF-MS) provided high sensitivity with mass detection limits of 0.1–0.3 ng  $\text{L}^{-1}$ . Pesticide levels were often detected in the ng  $\text{L}^{-1}$  range in Long Island surface waters and western LIS (except in open waters), but rarely at concentrations found to be toxic in flow-through laboratory exposures, even immediately after spray events.

**KEY WORDS:** *Homarus americanus*, American lobster, resmethrin, malathion, methoprene, toxicity, immune response, LC-TOF-MS

### INTRODUCTION

The spread of West Nile virus focused attention on the human and ecologic risks associated with using chemical insecticides to manage the virus's mosquito vectors. Common synthetic insecticides used for this purpose are organophosphates that function by inhibiting the nervous system enzyme acetylcholinesterase (Kennedy 1991), type I pyrethroid adulticides that modulate nervous system sodium channels (Bradbury & Coats 1989), and methoprene-based larvicide formulations that function as insect growth regulators (Celestial & McKenney Jr. 1994). The organophosphate malathion, and pyrethroids sumithrin and resmethrin, were used extensively in New York City during September 1999, immediately after the first outbreak of West Nile virus in the United States. Concurrent with the outbreak, the American lobster (*Homarus americanus* H. Milne Edwards, 1837) population in Long Island Sound (LIS) began showing signs of a massive die-off that was most pronounced in western LIS, closest to the region of spraying (CTDEP 1999). Contributing to the concern about the juxtaposition of pesticide spraying and lobster mortality was the occurrence of tropical storm Floyd that delivered more than eight inches of rain to the New York metropolitan area on September 16, 1999, subsequent to a period of intensive pesticide spraying (K. Chytalo, pers. comm.). Results of attempts to determine the exact cause of lobster mortality in 1999 were inconclusive, with the high incidence of a *Paramoeba* sp. infection in the nervous tissue of

dead and dying lobsters (Russell et al. 2000) the only significant finding. This allowed for the possibility that pesticide exposure combined with other stressors (e.g., hypoxic bottom waters, elevated temperatures) may have contributed to the death of the lobsters or caused them to be more susceptible to opportunistic diseases.

Pyrethroids are highly toxic to fish with 96-h LC50s ranging from <1  $\mu\text{g L}^{-1}$  to 10  $\mu\text{g L}^{-1}$  in flow-through exposures (reviewed in Bradbury & Coats 1989 and Coats et al. 1989). Estuarine and marine crustaceans are generally more sensitive with 96-h LC50s reduced by as much as two orders of magnitude (Cripe 1994). Compared with other pyrethroids, resmethrin and structurally similar sumithrin have received little attention. Rand (2002) reviewed available data on resmethrin, most of it from unpublished reports, citing 48- to 96-h LC50s for aquatic invertebrates and fish that ranged from 0.22–15  $\mu\text{g L}^{-1}$ . Malathion, by comparison, ranked among the least toxic of three organophosphate and three pyrethroid insecticides to the mysid shrimp *Mysidopsis bahia*, with a flow-through 96-h LC50 value of 2.6  $\mu\text{g L}^{-1}$  (Cripe 1994). De Guise et al. (2004) recently reported a 96-h LC50 of 38  $\mu\text{g L}^{-1}$  for adult *H. americanus* exposed to malathion in static tests. Methoprene has the lowest reported acute toxicity of the pesticides used to mitigate West Nile virus. *Mysidopsis bahia* exposed continuously to the more potent isomer, *S*-methoprene, showed no mortality at 62  $\mu\text{g L}^{-1}$  and complete mortality at 125  $\mu\text{g L}^{-1}$  in 96-h flow-through experiments (McKenney Jr. & Celestial 1996). Sublethal effects of these pesticides, including immunosuppression, growth inhibition, developmental delays and reproductive effects, have been observed at exposure concentrations significantly less

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than those that cause mortality (McKenney Jr. & Celestial 1996, Rand 2002, De Guise et al. 2004).

One measure of immune response is phenoloxidase (PO) activity. Phenoloxidase is an important enzyme in crustacean defense and recognition that mediates the melanization reaction around foreign substances and plays a role in wound healing, cuticle pigmentation, and sclerotization (Söderhäll 1982). Several studies have reported decreased PO activity in crustaceans exposed to environmental toxicants such as PCBs (Smith & Johnston 1992), copper sulfate (Cheng & Wang 2001), harbor dredge spoils (Smith et al. 1995), and benzalkonium chloride (Cheng et al. 2003).

For pesticides such as pyrethroids that are of toxicologic concern at concentrations in the  $\mu\text{g L}^{-1}$  range, standard analyses need to be modified to allow detection at low  $\text{ng L}^{-1}$  levels. Analytical methods with detection limits exceeding this are inadequate for assessing the occurrence, risk or environmental fate of such compounds in surface waters. Pyrethroids have rarely been detected in surface waters after spray events because of low application rates, insensitive analytical methods and possible removal by environmental processes such as photochemical transformation and sediment sorption. Recently, Weston et al. (2004) showed that various synthetic pyrethroids can be detected in surface sediments of streams carrying pesticide run-off and, in several cases, the reported levels were of toxicologic concern.

The objectives of this study were to assess acute toxicity and immune suppression in larval and juvenile *H. americanus* exposed to malathion, resmethrin and methoprene, the pesticides used most commonly in New York to control West Nile virus, and to determine effects of temperature stress. A field campaign was also conducted to measure pesticide levels in surface waters on Long Island and in LIS after operational application of pesticides in 2002 and 2003. The analysis used methods providing detection limits at  $\text{ng L}^{-1}$  levels and below, and provides an initial data set allowing comparisons of observed surface water pesticide concentrations to those shown to harm larval and juvenile lobsters. These data should be useful in future assessments of the ecologic risks associated with pesticide application for mosquito control in coastal areas.

## MATERIALS AND METHODS

### Chemicals

Resmethrin (98% pure, a mixture containing 90% trans and 10% cis isomers), malathion (98% pure), and methoprene (98% pure, racemic mixture of R and S isomers) were obtained from Chem Service, Inc. (West Chester, Pennsylvania). Deuterated *d*-6 malathion was from CDN Isotopes (Quebec, Canada) and terbutylazine was from Crescent Chemical (Islandia, New York). All solvents were analytical grade Burdick and Jackson (VWR Scientific Products, Bridgeport, New York). All other chemicals were from Sigma Aldrich (St. Louis, Missouri).

### Experimental Animals and Dosing System

Larval and juvenile lobsters were obtained from the Lobster Rearing and Research Facility at the New England Aquarium in Boston, Massachusetts. For the larval experiments, 1- to 2-day-old animals were shipped overnight to our laboratory and acclimated upon arrival for 2–6 h to 16°C or 24°C, respectively, prior to pesticide exposure. The short acclimation time ensured the lobsters did not molt into stage III larvae prior to the conclusion of the

experiments. Compared with earlier larval stages, stage III larvae are increasingly cannibalistic (D. Fiore, pers. comm.), which would have contributed to control mortality. Most of the larvae remained at stage I throughout the exposure period based on microscopic analysis. Larvae were fed as described by Goldstein and Bartko (1999) using a 1:1 wet weight mixture of live brine shrimp nauplii (Gold Label, Argent Laboratory, Redmond, Washington) and frozen adult brine shrimp (Kordon, Royal Pet Supplies, Brentwood, New York). Experiments with juvenile lobsters were conducted with animals ranging in age from 1- to >2-y old (2.4 g to 21.2 g wet weight and 4.5 cm to 9.3 cm total length). These animals were acclimated for 7 days to 16°C or 22°C prior to pesticide exposure, and given small PVC tubes to serve as surrogate burrows. Juveniles were fed frozen adult brine shrimp every 3rd day (Goldstein & Bartko 1999).

All animals were housed in 2-L beakers and kept on a 10-h light:14-h dark photoperiod in a flow-through system at a salinity of 27 ppt. Seawater was delivered at a flow rate of  $500 \text{ mL min}^{-1}$  to the larvae and  $200 \text{ mL min}^{-1}$  to the juvenile lobsters. The experimental system consisted of a head tank delivering water by constant gravity flow to a system of 6 diluter boxes. Each box received a carrier solvent (ethanol or acetone) or a pesticide dosing solution delivered by a multichannel WPI model sp220i syringe pump (World Precision Instruments, Sarasota, Florida) at a flow rate of  $3 \mu\text{L min}^{-1}$ . The contents of each diluter box were routed to 4 or 5 replicate chambers, and the waste effluent passed through a charcoal trap before exiting the system. Either 20 larvae or a single juvenile lobster was placed in each beaker. At the end of the exposure period, larvae were sieved from the beaker, rinsed into Petri plates, and the survivors enumerated. Larvae were considered dead when there was no visible heartbeat and gentle prodding produced no movement. Surviving juveniles were removed from the chambers and kept in a frozen seawater slurry as an anesthetic until hemolymph was drawn to assay phenoloxidase activity. The molt stage was determined from the pleopods of each juvenile at the end of the exposure period (Aiken 1991).

Measured levels of the most soluble pesticide tested, malathion, in exposure chambers were found to be  $98\% \pm 28 \text{ SD}$  of nominal concentrations and demonstrated that the system was effective at supplying a known and constant dose of pesticide in a flow-through mode. Measured levels of the much more particle reactive resmethrin and methoprene were inconsistent and generally higher than nominal levels most certainly because of inadvertent sampling of fine detrital particles that accumulated in the chambers. Because the exposures to dissolved pesticides were fixed by the relative flows from the syringe pump and seawater reservoir, the aqueous exposure levels are reported here, based on nominal concentrations.

### Larval Experiments

Most of the work focused on resmethrin. Lobster larvae were exposed to nominal concentrations of resmethrin ranging from  $0.03\text{--}0.80 \mu\text{g L}^{-1}$  in a flow-through system for 48–96 h. Exposures were conducted at stressful (24°C) and nonstressful (16°C) temperatures. Larval lobsters were exposed to nominal concentrations of malathion ranging from  $0.62\text{--}50 \mu\text{g L}^{-1}$  in exposures conducted for 48 h at 16°C. Similar experiments were conducted with methoprene at concentrations ranging from  $0.12\text{--}10 \mu\text{g L}^{-1}$  for 48 h at 16°C.

### Juvenile Experiments

Juvenile lobsters were exposed continuously for 7 d in a flow-through system to either resmethrin or malathion at sublethal concentrations. Initial experiments with resmethrin indicated some organisms at the highest dose tested ( $0.10 \mu\text{g L}^{-1}$ ) showed severe locomotor impairment (animals lost ability to maintain normal posture ending up on their backs, waving their legs), therefore sublethal effects were evaluated at a lower concentration, approximately 25% of the LC50s determined from the larval exposures ( $0.03 \mu\text{g L}^{-1}$  and  $1.0 \mu\text{g L}^{-1}$  for resmethrin and malathion, respectively). Juvenile lobsters acclimated to  $24^{\circ}\text{C}$  showed significant signs of stress (reduced pigmentation and lack of responsiveness), therefore  $22^{\circ}\text{C}$  was chosen as a stressful, but nonlethal temperature, with  $16^{\circ}\text{C}$  again used as a nonstressful temperature. Hemocyte PO activity was measured at the end of the 7-d exposure to assess the immune response.

### Phenoloxidase Assay

Methods to analyze PO activity in lobster hemocytes were adapted from Söderhäll and Smith (1983) and Hernández-López et al. (1996). Hemolymph was drawn from the cardiac hemal sinus of juvenile lobsters using a 26-gauge needle, into a 1-mL syringe containing ice-cold citrate/EDTA buffer (0.45 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid and 10 mM EDTA at a pH of 4.6) at one or more volumes of hemolymph collected. The hemolymph was spun at 800 g (Marathon 26KMR centrifuge, Fisher Scientific, Hampton, New Hampshire) for 25 min at  $4^{\circ}\text{C}$  to pellet the hemocytes. After supernatant removal, the remaining pellet was washed twice, without resuspension, with sodium cacodylate buffer (10 mM sodium cacodylate and 10 mM  $\text{CaCl}_2$  at a pH of 7.0). The pellet was resuspended in cacodylate buffer and disrupted using an ultrasonic homogenizer (4710 series, Cole-Parmer Instrument Co., Vernon Hills, Illinois). The cell suspension was centrifuged at 1200 g for 30 min at  $4^{\circ}\text{C}$  to remove the cell debris, and the hemocyte lysate supernatant (HLS) was assayed for PO activity.

A microtiter plate version of the assay was conducted by adding 50  $\mu\text{L}$  of HLS to a microwell and incubating for 1 h at  $25^{\circ}\text{C}$  with either 50  $\mu\text{L}$  0.1% trypsin in cacodylate buffer (from porcine pancreas) as an elicitor, or 50  $\mu\text{L}$  cacodylate buffer alone as a control. For each sample, one control and two standard reactions were run. After the incubation, 50  $\mu\text{L}$  of L-dihydroxyphenylalanine (L-dopa,  $3 \text{ g L}^{-1}$  in Milli-Q water) were added as the substrate to every well. Phenoloxidase activity was determined by following the production of dopachrome from L-dopa at 490 nm with readings taken every minute for 15 min with a Wallac 1420 Multilabel Counter (Perkin Elmer, Wellesley, Massachusetts). After subtraction of control values, the rate change in absorbance of the linear portion of the curve was averaged for the two duplicate runs and normalized to the protein content.

Protein content of each hemocyte lysate was determined using the Pierce bicinchoninic acid protein assay microwell plate protocol. Bovine serum albumin was used as the standard. The 96-well plate was incubated at  $25^{\circ}\text{C}$  for 1 h and read at 560 nm on a Wallac 1420 Multilabel Counter (Perkin Elmer, Wellesley, Massachusetts).

### Statistical Analyses

LC50s were determined using the Probit method (EPA Probit Analysis Program) or the Trimmed Spearman-Kärber method if

the assumptions for the Probit method were not met (Hamilton et al. 1977). Juvenile PO activity data were analyzed using Minitab Statistical Software (Minitab Inc., State College, Pennsylvania). Comparisons of PO response with the control values were conducted with a 1-way analysis of variance (ANOVA) and a posthoc Dunnett test. Dixon's test was applied to detect outliers (Sokal & Rohlf 1995). The effects of temperature and pesticide exposure were compared with a 2-way ANOVA. Differences were considered significant when  $P < 0.05$ .

### Pesticide Environmental Sampling

Water samples from laboratory exposures were sampled with 10-mL volumetric glass pipets, and added to amber glass screw cap test tubes containing 1 mL of hexane and surrogate standard. For malathion analyses, samples were acidified to pH 2 with HCl to prevent base catalyzed hydrolysis during storage before analysis.

Grab samples of surface waters around Long Island were collected for pesticide analyses. During the summer of 2002, United States Geological Survey (USGS) personnel collected most of the environmental samples (United States Geological Survey). Sequential replicate samples were analyzed by the USGS laboratory in Lawrence, Kansas, and by Stony Brook University (SBU). The samples collected by the USGS included the air water interface and were collected within an hour of aerial or truck-based spraying. Samples analyzed by the USGS were further vacuum filtered through 142-mm precombusted glass fiber filters (Gelman type AE) and samples were shipped overnight on ice, unpreserved, in 1-L amber glass bottles for extraction the next day. Samples analyzed at SBU were stored in 1-L amber glass bottles with approximately 100 mL of headspace. Within 2 h of collection, 25 mL of hexane were added to each bottle and the samples were shaken. Additional samples were collected for analysis by SBU approximately 0.3 m below the water surface. All samples were stored in the refrigerator until analysis.

Pesticide spraying of coastal areas adjacent to LIS has declined significantly in New York since 1999, with no aerial spraying in Nassau County and very little along the north shore of Suffolk County. New York City, however, continues to spray, albeit on a more limited basis. Therefore, to assess potential levels of pesticides in LIS coastal waters, operational sprays in New York City and Nassau County were followed in 2003.

### Pesticide Extraction and Analysis

Three methods were used to analyze aqueous concentrations of pesticides in this study. Detailed descriptions of the methods and instrumental analysis used to analyze the field samples can be found elsewhere (Zimmerman et al. 2001, Brownawell & Ruggieri 2005). A low volume (10 mL of water) extraction method was used to analyze pesticides in laboratory exposures. Higher volume samples were analyzed at SBU (900 mL of unfiltered water) and the USGS (247 mL of filtered water). Liquid-liquid extraction (water:hexane) used with volume ratios between 10:1 and 40:1 as described by Zimmerman et al. (2001) was a common feature of all three methods. It is advantageous to use hexane as an extracting solvent because it effectively partitions the targeted analytes but is inefficient at extracting potential interferences (as compared with methylene chloride or solid phase extraction (SPE) sorbents), and it can be evaporated without significant covolatilization of target



chemicals. The high performance liquid chromatography coupled to time-of-flight mass spectroscopy (LC-TOF-MS) and gas chromatography (GC)-MS based methods used were each able to sensitively and concurrently analyze all of the target analytes with very similar sensitivities (method detection limits of 1–3 pg injected for LC-TOF-MS, and approximately 10 pg injected for GC-MS).

Gas chromatography-MS analysis of pesticides (Zimmerman et al. 2001) was conducted either on a Hewlett Packard 5890 series II Plus GC equipped with a Hewlett Packard 5970 MSD (USGS) or on a HP 5890 series II GC equipped with VG Quattro mass spectrometer (SBU). The mass spectrometers were operated with electron impact ionization in selected ion monitoring mode ( $M/Z = 173, 73, 176, 123$  and  $123$  as quantitation ions for malathion, methoprene, piperonyl butoxide (PBO), sumithrin and resmethrin, respectively). Terbutylazine was used as a surrogate standard and *d*-10-phenanthrene as an internal standard. LC-TOF-MS analyses were conducted with a Micromass LCT, equipped with a Waters 2695 HPLC and a Z-spray electrospray ionization source. The mobile phase was methanol:water with gradient elution varying from 40% to 95% methanol in 12 min and held at 95% for 4 min. The aqueous mobile phase contained 10  $\mu\text{M}$  sodium acetate, and all the pesticide analytes were analyzed as sodium adducts of parent molecules ( $M + \text{Na}^+$ ). Leucine enkephalin was added through postcolumn infusion to serve as an internal mass calibrant, to confirm analyte identification using accurate mass estimation (Benotti et al. 2003). The internal standards used included terbutylazine and *d*-6-malathion. For GC-MS analysis, the final solvent volume was adjusted to 100  $\mu\text{L}$  from which 2  $\mu\text{L}$  was injected. For LC-TOF-MS analysis, the hexane extract was brought just to dryness with a gentle stream of nitrogen and the pesticides were redissolved in 100  $\mu\text{L}$  of initial HPLC mobile phase (40:60 methanol:water), of which 10  $\mu\text{L}$  aliquots were injected.

## RESULTS

### Acute Toxicity of Resmethrin, Malathion and Methoprene to Larval *H. americanus*

Preliminary experiments with resmethrin indicated that regardless of dose, little dose-dependent mortality was observed in less than 24 h (data not shown). Therefore, further experiments were performed after continuous exposures of 48–96 h. Survivorship for larval *H. americanus* exposed to resmethrin is shown in Figure 1. For larvae exposed to resmethrin for 48 h at 16°C, the LC50 was 0.26  $\mu\text{g L}^{-1}$  (95% CI 0.18–0.38). After 96 h of exposure at the same temperature, control survival was lower, dropping from 88% at 48 h to 69%, as was the calculated LC50 at 0.095  $\mu\text{g L}^{-1}$  (95% CI 0.075–0.114). When the 96-h exposure was repeated at 24°C, control survival was 76% and the LC50 was calculated at 0.10  $\mu\text{g L}^{-1}$  (95% CI 0.09–0.12).

Resmethrin was significantly more toxic than malathion or methoprene to larvae. The 48-h LC50 for malathion at 16°C was calculated to be 3.7  $\mu\text{g L}^{-1}$  (95% CI 3.3–4.2, Fig. 2), whereas methoprene exposure of up to 10  $\mu\text{g L}^{-1}$  resulted in no significant mortality (Fig. 3).

### Phenoloxidase Activity in Juvenile *H. Americanus* Exposed to Resmethrin and Malathion

It was difficult to obtain adequate hemolymph volumes from juvenile lobsters of this size. In general, only 100–200  $\mu\text{L}$  of hemolymph could be obtained from all but the largest individuals.

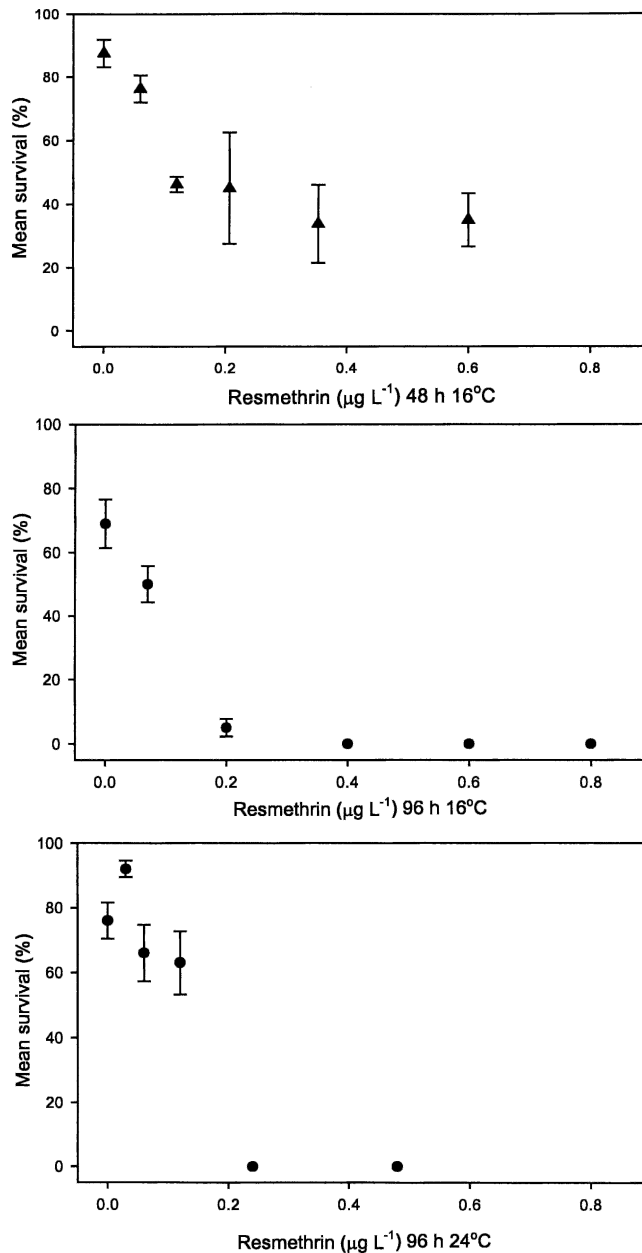


Figure 1. Acute toxicity of resmethrin to larval *H. americanus* after 48- or 96-hour flow through exposures, at non-stressful (16°C) or stressful (24°C) temperatures (mean  $\pm$  SE);  $n = 4$  replicates per treatment for 48-h exposure and  $n = 5$  for 96-h; 20 larvae per replicate.

Although PO activity was not statistically related to size in the animals studied, activity was not detectable in samples from several of the smaller organisms. No significant relationship was observed between molt stage and PO activity (data not shown).

In experiments where animals were exposed to either 0.03 or 0.10  $\mu\text{g L}^{-1}$  resmethrin at 16°C, PO activity was significantly reduced relative to controls at 0.03  $\mu\text{g L}^{-1}$  but not 0.10  $\mu\text{g L}^{-1}$  (Fig. 4). A somewhat surprising observation made during these experiments was that after 6 d of exposure, 2 of the 8 lobsters in the highest treatment group showed signs of locomotor impairment having trouble maintaining an upright posture with one flipped on its back. This inability to maintain normal posture was prevalent in

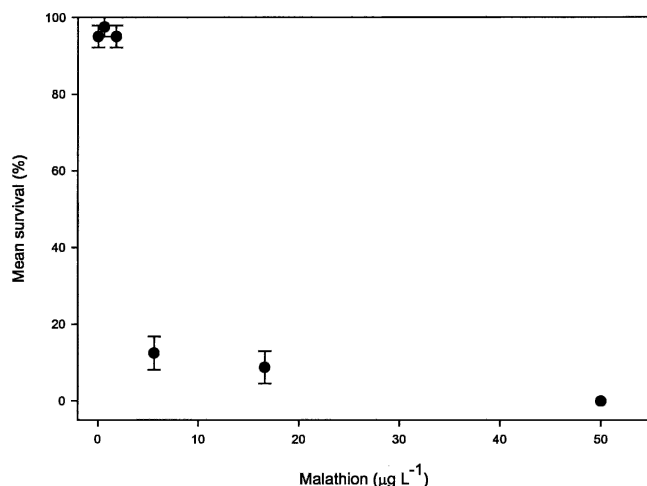


Figure 2. Acute toxicity of malathion to larval *H. americanus* after 48-h flow-through exposure at 16°C (mean ± SE); n = 4, 20 larvae per replicate.

organisms exposed to higher resmethrin concentrations in preliminary experiments at higher doses ranging from 0.10–0.50 μg L<sup>-1</sup>. Despite this, it should also be noted that no mortality of juvenile lobsters was observed at doses of 0.10 μg L<sup>-1</sup> indicating that juvenile lobsters are less sensitive than larval lobsters to acute resmethrin exposure. In two additional experiments, done as part of an effort to assess the effects of temperature, no differences were observed between juveniles exposed to 0.03 μg L<sup>-1</sup> resmethrin and control organisms (Fig. 5). Exposure to 1.0 μg L<sup>-1</sup> malathion also failed to elicit a significant PO response. Temperature, however, did affect PO activity where a 65% increase was observed at the elevated temperature ( $P < 0.006$ ).

#### Environmental Sampling

A combination of fresh water ponds, salt marshes, tidal inlets and embayments' and marine coastal waters (off Staten Island, NY) were sampled during 2002 (Table 1). The water depth at all of these sites was shallow (depth = 1–5 m). Estuarine or salt marsh samples were obtained after three spray events in 2003 (Table 2).

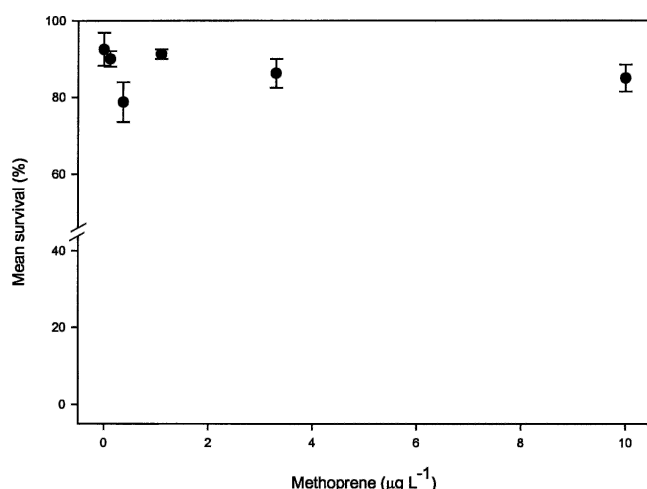


Figure 3. Acute toxicity of methoprene to larval *H. americanus* after 48-h flow-through exposure at 16°C (mean ± SE); n = 4, 20 larvae per replicate.

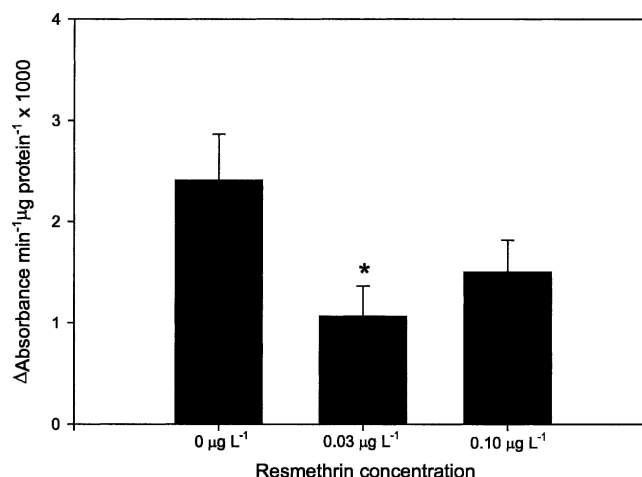


Figure 4. PO activity as a function of resmethrin exposure in juvenile *H. americanus* (mean ± SE, \*  $P < 0.05$  as compared to control response).

The geographic location of all samples is shown in Figure 6. Malathion was not sprayed in the area during the years sampled and was not detected in any sample (detection limits of 0.1–0.5 ng L<sup>-1</sup> by SBU and 5 ng L<sup>-1</sup> by USGS). Note that field measurements are reported in ng L<sup>-1</sup>, whereas toxicity results are referred to in μg L<sup>-1</sup> (Tables 1 and 2).

In 2002, measurements made on Long Island surface waters at SBU detected resmethrin, the active ingredient in Scourge, at 5 out of 10 locations sampled within an hour after spray events. Concentrations detected ranged from 1.7–980 ng L<sup>-1</sup>, but only one sample exceeded 150 ng L<sup>-1</sup>. The pyrethroid sumithrin, found in Anvil, was not detected after two spray events. Methoprene, the active ingredient in the larvicide Altosid was detected after each of two spraying events at concentrations ranging from 7.4–631 ng L<sup>-1</sup>. In contrast, the synergist PBO, included in the pyrethroid formulations Anvil and Scourge, was detected by SBU in all but two samples collected after spray events at concentrations ranging from 0.3–15,000 ng L<sup>-1</sup>. Piperonyl butoxide was still present at three locations in samples taken 3 d after a Scourge spray after a period of heavy rain (Table 1, Map numbers 11–13). Resmethrin,

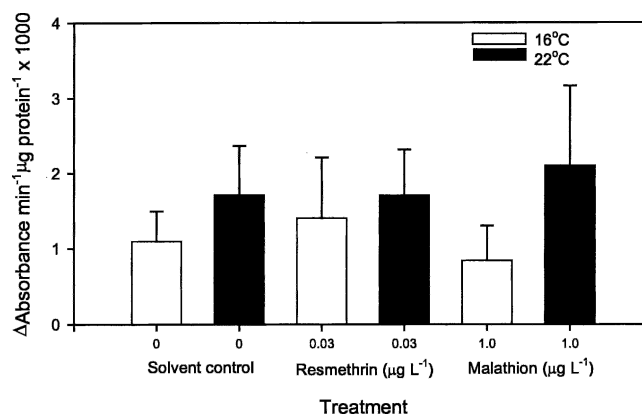


Figure 5. A comparison of the effects of temperature and pesticide exposure on phenoloxidase activity (mean ± 95% CI). The 16°C experiment was conducted separately from the 22°C experiment. The effect of temperature was significant ( $P < 0.006$ ), however, the effect of the pesticide was not ( $P = 0.880$ ).

TABLE 1.  
2002 Sampling of surface water for pesticides.

Spray Date	Sample Date	Product	Map Number	Location	Coordinates	Concentration ng L <sup>-1</sup>							
						Resmethrin		Sumithrin		Piperonyl Butoxide		Methoprene	
						USB	USGS	USB	USGS	USB	USGS	USB	USGS
6/18	6/18	Altosid	1	Carmen's River	40 46.300 N 72 53.617 W	—	ND	—	ND	—	ND	—	631
7/24	7/24	Altosid	2	Heckscher State Park	40 42.250 N 73 10.233 W	ND	ND#	ND	ND#	ND	ND#	7.39	ND
7/17	7/17	Scourge	3	Shinnecock Bay 1	40 46.300 N 72 43.600 W	ND	ND#	ND	ND#	ND	ND#	ND	ND#
				Shinnecock Bay 2		ND	ND#	ND	ND#	ND	ND#	ND	ND#
7/31	7/31	Scourge	4	Mastic 1	40 44.750 N 72 50.850 W	ND	ND	ND	ND	1.4	ND	ND	ND
				Mastic 2		ND	ND	ND	ND	0.59	ND	ND	ND
8/16	8/16	Scourge	5	Lake Ronkonkoma 1	40 49.950 N 73 07.567 W	—	ND	—	ND	—	ND	—	ND
				Lake Ronkonkoma 2		—	ND	—	ND	—	ND	—	ND
8/19	8/19	Scourge	6	Gibbs Pond	40 50.683 N 73 08.383 W	146	76	ND	ND	15,000	6,910	ND	ND
8/19	8/19	Scourge	7	Spectacle Pond	40 50.150 N 73 08.056 W	1.7	21	ND	ND	11	343	ND	ND
8/26	8/26	Scourge	8	Carl's River	40 42.100 N 73 19.717 W	5.49	18	ND	ND	120	41	ND	ND
8/26	8/26	Scourge	9	Trues Creek	40 41.867 N 73 16.933 W	980	293	ND	ND	3,200	13,400	ND	ND
8/26	8/26	Scourge	10	Sampawams Creek	40 41.800 N 73 19.067 W	ND	ND	ND	ND	25	35	ND	ND
8/26	8/29	Scourge	11	Sampawams SS after rain	40 41.949 N 73 18.926 W	ND	—	ND	—	290	—	ND	—
8/26	8/29	Scourge	12	Pine Lake SS after rain	40 41.461 N 73 16.652 W	ND	—	ND	—	210	—	ND	—
8/26	8/29	Scourge	13	Argyle Park SS after rain	40 41.741 N 73 19.889 W	ND	—	ND	—	230	—	ND	—
9/10	9/10	Scourge	14	Mastic Beach 1	40 44.850 N 72 50.967 W	6.2	ND	ND	ND	68	ND	ND	ND
				Mastic Beach 2		ND	—	ND	—	46	—	ND	—
9/10	9/10	Scourge	15	Pattersquash Creek	40 45.817 N 72 51.100 W	—	ND	—	ND	—	ND	—	ND
8/28	8/28	Anvil	16	Oakwood Beach SS	40 33.090 N 74 06.683 W	ND	—	ND	—	0.70	—	ND	—
8/28	8/29			Oakwood Beach SS after rain		ND	—	ND	—	0.64	—	ND	—
8/28	8/28	Anvil	17	Wolf's Pond SS	40 30.726 N 74 11.709 W	ND	—	ND	—	0.33	—	ND	—
8/28	8/29			Wolf's Pond SS after rain		ND	—	ND	—	0.31	—	ND	—

—: Not measured, ND: Not detected (USB detection limit 0.1 to 0.5 ng L<sup>-1</sup>, USGS detection limit <5 ng L<sup>-1</sup>, except ND# where ND <200 ng L<sup>-1</sup>), SS: subsurface sample.

TABLE 2.  
2003 Sampling of coastal water after Anvil spray events.

Spray Date	Collection Date	Location	Coordinates	Concentration ng L <sup>-1</sup>	
				Sumithrin	Piperonyl Butoxide
8/13	8/14	Hutchinson River			
		Near storm drain 1	40 51.199 N 73 49.032 W	ND	1.0
		Near storm drain 2		ND	1.0
		Near pipe	40 51.091 N 73 49.037 W	ND	57
		In pipe		ND	15
8/25	9/3	Long Island Sound Transect			
		Station D-3 surface	40 59.318 N 73 24.318 W	ND	ND
		bottom		ND	ND
		Station T-1 surface	41 00.926 W 73 28.195 W	ND	ND
		bottom		ND	ND
		Station T-2 surface	40 57.601 W 73 33.572 W	ND	ND
		bottom		ND	ND
		Station T-3 surface	40 56.742 73 39.370 W	ND	ND
		bottom		ND	ND
		Station E-7	40 48.330 W 73 49.210 N	ND	0.22
	9/4	Riker's Island	40 47.759 W 73 52.475 N	ND	1.3
	9/4	Sunken Meadow	40 47.759 W 73 54.773 N	ND	6.6
9/22	9/22	Time Series at Hammonasset State Park		mean (SD) n = 3	
		Immediately after spray		1.1 (1.0)	20 (9.3)
		1 day after		ND	5.0 (4.1)
		2 days after		ND	2.5 (2.6)
		3 days after		ND	0.35 (0.03)
	10/2	10 days after		ND	0.29 (0.06)

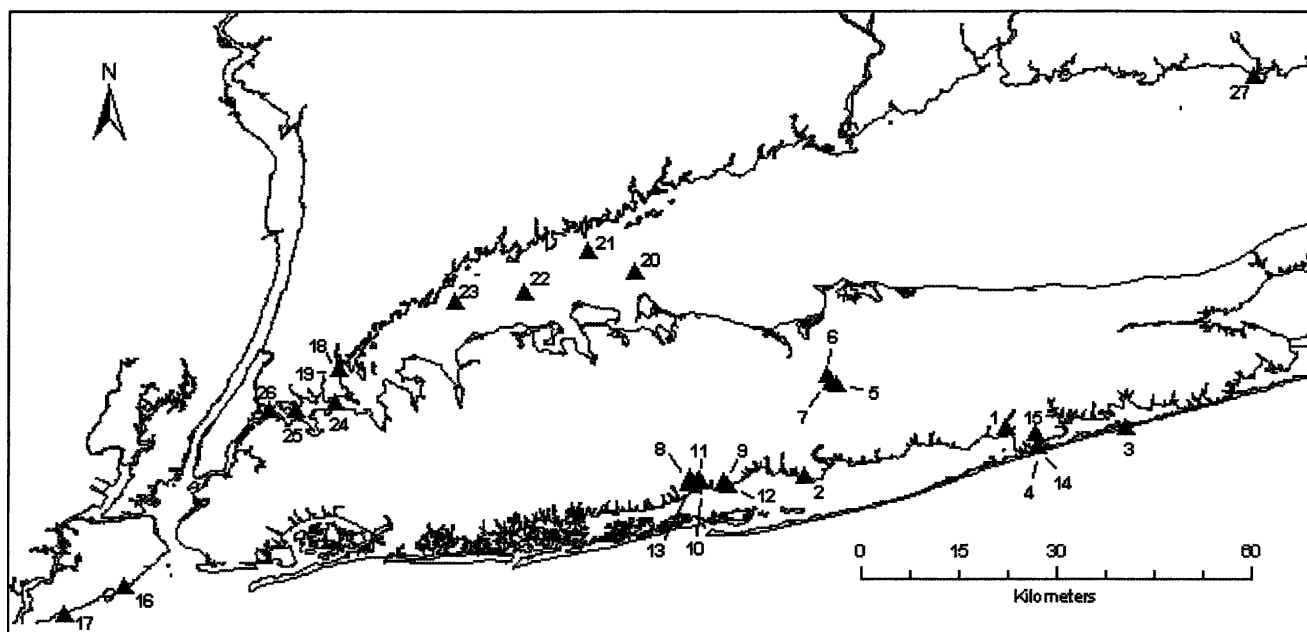


Figure 6. Field sampling locations in 2002 and 2003. See Tables 1 and 2 for coordinates and descriptions.

sumithrin, PBO and methoprene were simultaneously analyzed by the methods used. In no cases were pesticides detected other than those applied in an actual spray event, indicating that none of these compounds are persistent in the environment.

Generally good agreement was seen between samples collected at the same time and location and then analyzed at either the USGS or SBU. Pesticide levels reported by the two laboratories using different methods were generally within a factor of three of each other. At locations where more than one sample was collected and analyzed by the same method, very good agreement was reached, with values being within a factor of two of each other. There were no reported detections of pesticides by the USGS laboratory that did not correspond to positive detections by SBU using more sensitive methods.

Measurements made in the East River and the Narrows of far western LIS in September 2003 10 d after spraying failed to detect sumithrin, although PBO was found at concentrations ranging from 0.2–7 ng L<sup>-1</sup> (Table 2). The highest concentrations of PBO were found in the samples collected from the stations farthest west and closest to the areas of known spraying. No methoprene (actively used in storm drains surrounding western LIS in 2003) was measured in LIS. Furthermore, no pesticides were detected in open surface waters of western LIS. After a truck spray of Anvil at Hammonasset State Park (Table 2), sumithrin was detected in a shallow creek in the salt marsh immediately after the spray event at a concentration of 1.1 ng L<sup>-1</sup>, whereas PBO was found at concentrations 20 times higher. The next day, sumithrin was no longer detectable, but PBO at concentrations in excess of 2 ng L<sup>-1</sup> was measured 2 d later, with detectable concentrations still present 10 d after spraying. Similarly, no sumithrin was detected in samples taken along the shore of the Hutchinson River (a tidal embayment in far western LIS) the day after aerial spraying of nearby Pelham Bay Park. PBO was detected in the same samples and was much higher in samples taken near tiled pipes that appeared to be storm drains emanating from the park.

## DISCUSSION

American lobster larvae seem to be extremely sensitive to resmethrin with LC50s ranging from 0.10 µg L<sup>-1</sup> after 96-h exposures to 0.26 µg L<sup>-1</sup> after 48 h. Few other studies have investigated resmethrin's effect on aquatic invertebrates; however, Rand (2002) summarized a laboratory study that also conducted flow-through exposures with resmethrin and reported a similar 48-h LC50 of 0.22 µg L<sup>-1</sup> for the freshwater crustacean *Daphnia magna*. Burrige et al. (2000) conducted acute toxicity tests with larval and postlarval lobsters with another pyrethroid, cypermethrin, and found 48-h LC50s between 0.06 and 0.18 µg L<sup>-1</sup> using static-renewal exposures. Because LC50s tend to be reduced when using flow-through systems, we would expect the toxicity of cypermethrin to be greater than that reported here for resmethrin.

Raising the exposure temperature from 16°C to 24°C failed to significantly alter the LC50 for resmethrin; however, increased toxicity caused by the elevated temperature alone may have masked any additional pesticide effects. Lobsters have a wide temperature tolerance (–1–30.5°C) but generally inhabit areas that range from 5°C to 20°C (Lawton & Lavalli 1995). Several authors have shown that stage II lobster larvae were more sensitive to elevated temperatures than any other life stage (Gruffydd et al. 1975, Sastry & Vargo 1977). There has been little work, however, on the effect of temperature on pesticide toxicity. In their review of pyrethroid toxicity to aquatic organisms, Coats et al. (1989) reported several studies showing an inverse relationship between temperature and pyrethroid toxicity in both insects and trout. He attributed this to a combination of potential temperature-dependent interactions of pyrethroids on nervous tissue, or the influence of toxicokinetic parameters enhancing concentrations at the site of action at colder temperatures.

In this study, larval lobsters were more than 20 times less sensitive to malathion than to resmethrin with malathion's 48-h LC50 calculated at 3.7 µg L<sup>-1</sup>. In contrast, De Guise et al. (2004) reported malathion 96-h LC50s for adult *H. americanus* derived



from single-dose static tests to be 10 times higher ( $38 \mu\text{g L}^{-1}$ ). The acute toxicity of malathion and a number of pyrethroid pesticides to estuarine crustacea was reviewed by Cripe (1994). Results from flow-through tests with juvenile *Mysidopsis bahia* were similar to those reported here ( $2.6 \mu\text{g L}^{-1}$ ), whereas 96-h LC50s derived from static tests with this and other estuarine crustacean species were generally higher ranging from  $5\text{--}83 \mu\text{g L}^{-1}$ . The toxicity of various pyrethroids (cypermethrin, fenvalerate and permethrin) reported for similar species was generally 10–100 times greater than that reported for malathion, agreeing well with data reported here for resmethrin. In a field study, Conte and Parker (1975) reported that the water concentrations of  $2\text{--}3.2 \mu\text{g L}^{-1}$  of malathion in surface water immediately after spraying most likely resulted in mass mortality of two shrimp species in marshes near Galveston Bay.

The juvenile hormone mimic methoprene found in Altosid was not found to be toxic to larval lobsters at concentrations as high as  $10 \mu\text{g L}^{-1}$  after 48 h of exposure. These results are not surprising given methoprene's mode of action as a molting inhibitor. In the short-term studies measuring acute lethality, we had not really expected to see much toxicity with methoprene. It should be noted that the chemical formulation tested was a racemic mixture of *S* and *R* isomers, and previous work has shown the *S* isomers to be significantly more toxic than the *R* isomers (Celestial & McKenney Jr. 1994). However, even if the *R* isomers contributed little to observed toxicity, the racemic mixture would be expected to be approximately half as toxic as *S*-methoprene. In contrast to the results reported here, Walker et al. (2005) estimated static 72-h LC50s for *S*-methoprene for stage II lobsters to be only  $2 \mu\text{g L}^{-1}$ . Results of other studies with crustaceans are in agreement with data reported here, indicating that methoprene has comparatively lower acute toxicity to aquatic crustaceans. Celestial & McKenney Jr. (1994) found elevated toxicity in mud crabs (*Rhithropanopeus harrisi*) exposed for up to 4 wk to *S*-methoprene at concentrations  $\geq 100 \mu\text{g L}^{-1}$  in static renewal tests. Studies by this group found similar results under flow through conditions with mysid shrimp (McKenney Jr. & Celestial 1996). Finally, Wirth et al. (2001) reported no acute toxicity after 96 h to shrimp (*Palaemonetes pugio*) exposed to methoprene concentrations (no information was given on the isomer distribution) as high as  $1,000 \mu\text{g L}^{-1}$ .

Mortality of larval lobsters in the control treatments ranged from 5% to 31%. Other investigators have reported similar outcomes in working with this species. Sprague and McLeese (1968) reported control mortalities nearing or exceeding 50% in 1- to 3-wk exposures. Wells and Sprague (1976) reported 21% overall control mortality in 96-h exposures and 51% in 30-d exposures. Burrige and Haya (1997) and Fiore and Tlustý (2005) both identified cannibalism as a source of control mortality when working with larval lobsters. It is possible, however, that this behavior differentially impacts the experimental treatments. Burrige and Haya (1997) noted that the larvae in controls and lower concentration treatments in exposures to pyrethroids remained active throughout the assay, whereas larval activity decreased at the highest concentrations tested. They also observed partially eaten larvae carcasses in some control treatments, but not in treatments with higher pyrethroid concentrations. Given that LC50s are calculated as a function of control mortality, this would serve to artificially increase the LC50 and underestimate a compound's toxicity.

Analysis of PO activity in pesticide-exposed juveniles indicated that neither resmethrin nor malathion significantly influenced the activity of this enzyme at sublethal levels. De Guise et

al. (2004) reported that exposure to single or repeated doses of as low as  $5 \mu\text{g L}^{-1}$  of malathion resulted in transitory reduction in phagocytic ability in adult lobster hemocytes. These effects were observed at doses of only 15% of the LC50 values determined under similar conditions. Although the exposure dose of malathion used in these experiments,  $1 \mu\text{g L}^{-1}$ , was less than used by De Guise et al. (2004), it was a greater proportion of the LC50 determined in continuous exposures with larvae. It should be noted that De Guise et al. (2004) only reported sustained reduction in phagocytosis after 3 wk of repeated exposures to this dose. A more prolonged period of exposure may be necessary to detect a functional immune response in this species. Alternatively, PO activity in juvenile organisms may be too low to be responsive. Several of the smaller organisms used in this study had undetectable levels of PO activity. It would be interesting to compare levels observed in juveniles to that found in older/larger juveniles or adult organisms.

Several studies have reported decreased PO activity in crustaceans exposed to other environmental toxicants. Exposure of the common grass shrimp, *Crangon crangon*, to  $50 \mu\text{g L}^{-1}$  of PCB15 reduced PO activity by a third as compared with control values, however, this study also reported mortality at this concentration that seemed to be approaching the 96-h LC50 (Smith & Johnston 1992). Another study by this group reported a reduction in PO activity in *C. crangon* exposed to harbor dredge spoils compared with reference animals exposed to clean sand, although the sample size was too small to support meaningful statistical analyses (Smith et al. 1995). Studies on the freshwater prawn, *Macrobrachium rosenbergii*, found a significant decrease in PO activity in as little as 48 h of sublethal exposure to  $300 \mu\text{g L}^{-1}$  benzalkonium chloride (Cheng et al. 2003) and  $100 \mu\text{g L}^{-1}$  copper sulfate (Cheng & Wang 2001). Both of these studies noted that PO activity continued to decrease with increasing exposure time to each compound at all concentrations tested, suggesting decreased PO activity as a possible explanation for an observed increase in susceptibility of the prawn to a bacterial pathogen (Cheng & Wang 2001, Cheng et al. 2003).

The combined effect of elevated temperature and exposure to either resmethrin or malathion also failed to produce a detectable change in PO activity, although elevated temperature itself caused a small but statistically significant increase in PO activity. Many other studies indicate that temperature can strongly influence immune response. Paterson and Stewart (1974) reported phagocytosis of sheep red blood cells by lobster hemocytes to occur more quickly at  $10^\circ\text{C}$  to  $15^\circ\text{C}$  than at  $4^\circ\text{C}$ . Steenbergen et al. (1978) found phagocytosis in lobster was reduced at temperatures above  $22^\circ\text{C}$  as compared with  $20^\circ\text{C}$ . Recently Dove et al. (2005) reported a large reduction in phagocytic activity and increased hemocyte count in adult lobster slowly acclimated to elevated temperatures ( $23^\circ\text{C}$  as compared with  $16^\circ\text{C}$ ). Chisholm and Smith (1994) surveyed seasonal variation in antibacterial activity and total hemocyte count in the shore crab *Carcinus maenas*, and found significant ( $>5$ -fold) depression in the ability of hemocytes to kill a marine bacterium at the low and high end of the temperature range for that year. Total hemocyte counts did not show this pattern. A more recent study by Hauton et al. (1997) did not show any seasonal trends in either parameter in the *C. maenas*, but did show a correlation between bacterial abundance in the water column and PO activity.

A more complete evaluation of the combined effects of temperature and pesticide exposure would benefit from additional measures of immune response. It is possible that some other mea-

sure of immune response, such as phagocytosis, may prove to be a more sensitive measure of immune suppression in young animals.

#### Field Measurements of Pesticides

Pesticides were detected at approximately 50% of the locations sampled directly after spraying. Detection of the pesticides' active ingredients was restricted to samples that were under aerial spray events and thus targeted for pesticide exposure. It was much more common to detect the synergist PBO even in surface waters that were removed (by spraying buffers, e.g.,) from areas of targeted spraying. The ratio of PBO to pyrethroid in surface water samples was always higher than that in the formulations of Scourge (resmethrin:PBO = 3:1) or Anvil (sumithrin:PBO = 1:1). These results indicate that there is either differential transport of active ingredient and synergist to surface waters or differential preservation in surface waters. Differences in transport to surface waters may occur either in the air after spraying or, in some cases, by tidal flooding or run-off from precipitation. Differences in preservation may occur as a result of the propensity of these two pyrethroids to be lost preferentially compared with the more water soluble PBO. It is believed that pyrethroids are lost rapidly from surface waters because of various processes including photochemical transformation and sorption to particles and sediments (Clark et al. 1989, Rand 2002).

In comparing the results of samples measured either as replicates or by both laboratories, it is important to recognize that results for replicate samples are from sequential samples, not duplicate splits from a larger sample. Recent studies (Brownawell & Ruggieri 2005) have demonstrated that pesticide concentrations from samples collected just after spraying at the surface and containing the air-water interface are significantly higher than concentrations from samples collected from just below the interface. Therefore, it is likely that pesticide distributions at the interface are somewhat patchy and poorly mixed, and this likely contributes to some variability in results between replicate samples.

The concentrations of methoprene, resmethrin and sumithrin measured in surface waters were usually lower, and often much lower than the levels found in this study to be acutely toxic to larval and juvenile lobsters. Of the samples analyzed, only 2 of them (Gibbs Pond and Trues Creek; Table 1, Map numbers 6 & 9) had concentrations of resmethrin approaching or exceeding those that were found to be toxic in this study. A simple hazard quotient (HQ) calculated as the ratio of resmethrin concentrations observed in the field to the lowest observable effects levels determined in the laboratory would indicate concern ( $HQ > 1$ ) only at these two sites. Those two samples were collected in a manner that included the air-water interface so much lower levels would be expected to mix lower into the water column. Furthermore, detectable levels of measured pyrethroids were found only in enclosed or semi-enclosed bodies directly under the path of aerial pesticide application, and not in nearshore coastal waters where lobsters would be found.

The results from this study suggest that pesticide levels in surface waters after spraying are low, compared with toxicologically significant exposures, under a range of typical applications. However, it remains difficult to assess the possible levels of pesticides that might have been delivered to western LIS after tropical storm Floyd in 1999, caused by the large number of applications in the watershed preceding the storm, the unusually large amount of rain deposited that might have facilitated enhanced run-off, and the rapid mixing of LIS from the storm.

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