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

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Abstract

A lobster die-off reduced the 1999 fall landings in western Long Island Sound by up to more then 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. 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. Lobsters were exposed in 20 gallon tanks, and the direct toxicity as well as sub-lethal effects on the immune system were determined. The 96 h LC_{50} for malathion upon single exposure was 38 μg/l. Malathion degraded rapidly in sea water, with 65–77% lost after 1 day and 83–96% lost after 3 days. Phagocytosis was significantly decreased 3 days after a single exposure to initial water concentrations as low as 5 ppb, when measured water concentrations were as low as 0.55 ppb. Similarly, effects on phagocytosis were observed at 1, 2 and 3 weeks after the initiation of weekly exposures. Cell counts did not differ significantly upon exposure to malathion. Malathion was not detected in muscle and hepatopancreas of exposed lobsters. Evaluation of phagocytosis is a sensitive indicator of subtle sub-lethal effects of malathion, and relatively small concentrations of malathion (6–7 times lower than the LC_{50}) can affect lobster defense mechanisms.

Keywords: Malathion; Lobster; *Homarus americanus*; immunotoxicity; LC50

1. Introduction

An unusual mortality event was observed in Long Island Sound (LIS) lobsters in the fall of 1999, resulting in a reduction of 90–99.9% of landings in western LIS and 60–80% in central and eastern LIS. The lobsters examined suffered from a *Paramoeba* sp. infection not previously described in lobsters, which mainly affected the nervous system (Mullen et al., 2003). Nevertheless, it is not known if this organism is solely responsible for the mortality event or represents a terminal opportunistic infection. The die-off corresponded in time with the application of pesticides, including malathion, for the control of mosquitoes that

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carried West Nile virus, a new emerging disease in North America at the time. Initial analyses for the presence of pesticides used for the control of West Nile virus-infected mosquitoes did not reveal detectable concentrations in randomly sampled lobsters, but these chemicals are relatively short lived and could have been metabolized. The assay results could not determine with certainty if LIS lobsters had been exposed to those pesticides. It is also possible that exposed lobsters could have suffered short- or long-term effects after the chemicals had been metabolized and were no longer detectable.

The immune system is central to health and resistance/susceptibility to pathogens in all species. Interestingly, it is also one of the most susceptible and sensitive systems to the effects of xenobiotics. It is therefore highly possible that environmental stressors (chemical or others) that would have affected the immune system of lobsters in LIS would have rendered them more susceptible to infections (for example with paramoeba) and have played a significant role in the 1999 LIS lobster die-off. Malathion immunotoxicity has been documented in several species of laboratory animals including its effects on both humoral (diminution of the antibody response upon injection with tetanus toxoid, ovalbumin and sheep red blood cells) and cellular immune responses (marked inhibition of leucocyte and macrophage migration) of mice, rats and rabbits (Banerjee et al., 1998). The effects on macrophages, were also documented, including an increased respiratory burst by peritoneal macrophages upon stimulation after in vivo exposure to malathion (Rodgers and Ellefson, 1990), using a mechanism dependent on mast cells (Rodgers et al., 1996). Malathion was also documented to affect the natural and acquired immunity of fish (Japanese medaka), including a dose-dependent reduction of the production of antibodies to sheep red blood cells and a mild decrease in the superoxide production by kidney phagocytes, which resulted in a decrease in resistance to infection by *Yersinia ruckeri*, a common bacterial fish pathogen (Beaman et al., 1999). The lethal and immunotoxic effects of malathion on lobsters have never been determined. In order to determine the possible implication of malathion 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.

2. Material and methods

Experimental exposures were performed in aerated 20 gallon tanks each containing three lobsters, with a total of nine lobsters per concentration. The three concentrations of malathion tested were 5, 21 and 46 ppb, in addition to unexposed controls. Lobsters from Maine were kept in the dark (except during sampling and water changes) in 14 °C artificial sea water (Instant Ocean, Mentor, OH) at a salinity of 24‰ and exposed to technical grade malathion (Fisher Scientific, Pittsburgh, PA) using different regimes. Standard LC_{50} experiments were performed over the course of 96 h. Acute exposure lasted 5 days, with serial sampling on day 1, 3 and 5, and consisted of a single exposure to malathion. Subacute exposure was performed over the course of 4 weeks, with weekly serial sampling, followed by a weekly water change and re-exposure to an identical concentration of malathion as the original one. All experiments started with nine lobsters per concentration, which were all serially sampled until the end of the study.

At the end of each exposure regime, lobsters were sacrificed and a gross and histopathological examination was performed to determine the presence/absence of pathological lesions, and to determine whether or not they were associated with exposure to malathion. Lobsters were euthanized using potassium chloride (Battison et al., 2000), and a complete necropsy was performed. Tissues were fixed in 10% neutral buffered formalin. Select tissues were then transferred to Dietrichs with 5% trichloroacetic acid for 48 h for partial decalcification of exoskeleton. Tissues were then trimmed and processed for paraffin embedding. Tissues were sectioned at 4 μm, routinely stained with hematoxylin and eosin, and examined by light microscopy for the presence/absence of lesions.

Muscle and hepatopancreas were also collected and frozen during the post-mortem exam to analyze for the presence of malathion. Water samples (1 l) were collected periodically using I-CHEM amber glass jars certified to meet or exceed "US EPA specifications and guidances for contaminant-free sample containers" (Nalgen Nunc International, Rochester, NY). 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. 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 quantitation at lower levels. The method detection limit (MDL) for the analysis of the malathion in water was 0.1 ppb (μg/l). The MDL for the analysis in lobster tissues was 15 ppb.

The endpoints tested include evaluation of the immune system using hemocyte counts and phagocytosis on hemolymph samples. Briefly, 2.0 ml of hemolymph was collected from the dorsal vasculature and immediately transferred to a Vacutainer tube (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 phosphate-buffered saline (PBS) and 5 μl of the bead mixture was added for every 200 μl of hemolymph. After a 1 h 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, using a forward scatter threshold to eliminate free beads from the acquisition. Phagocytosis was evaluated as the proportion of hemocytes that had phagocytized two or more beads.

Results are reported as mean ± standard deviation. A one-way analysis of variance (ANOVA) with Dunnett's test was used to compare the different experimental groups to the unexposed control group, using $P < 0.05$ for statistical significance. When the power of the analysis was below 0.80, the threshold suggested by the analysis software (Sigma Stats, Jandel Corp, San Rafael, CA), a student T-test was used to compare each mixture to the control. Regression analysis for the determination of the LC_{50} was performed using the Microsoft Excel software.

3. Results

The direct toxicity of malathion in lobster was determined through a standard 96-h LC_{50}, the calculated concentration that killed 50% of the animals. To do so, lobster mortality was recorded daily and cumulated over a 4 day exposure. The cumulative mortality was plotted against the concentrations of malathion used and a linear regression curve was determined using the Microsoft Excel software. The LC_{50} was calculated using the equation determined by the software for the regression curve. The 96-h LC_{50} was 38 μg/l (or ppb) upon single exposure (Fig. 1).

The concentration of malathion was measured in the water on day 0 (initial concentration), as well as on days 1 and 3 after a single initial exposure to evaluate its degradation. Malathion degraded rapidly in our system, with 65–77% lost after one day and 83–96% after 3 days (Fig. 2). The half-life of malathion in our system, irrespective of the initial concentration, was approximately 12 h. No malathion was detected in lobster tissues at the end of the 5 day exposure.

Phagocytosis was significantly decreased 3 days (but not 1 or 5) after a single exposure to an initial water concentration as low as 5 ppb, the lowest concentration tested (Fig. 3). The water concentration of malathion on day 3 was 0.55 ppb.

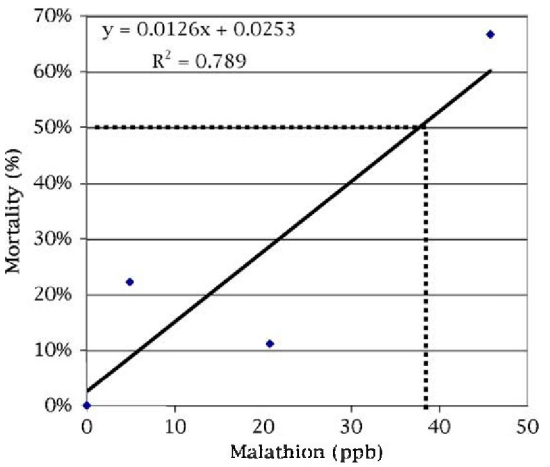

Fig. 1. The LC_{50} (dotted line) of malathion was determined in lobsters after a single exposure followed by a 4 day observation and determination of mortality. The study started with nine lobsters in each concentration group.

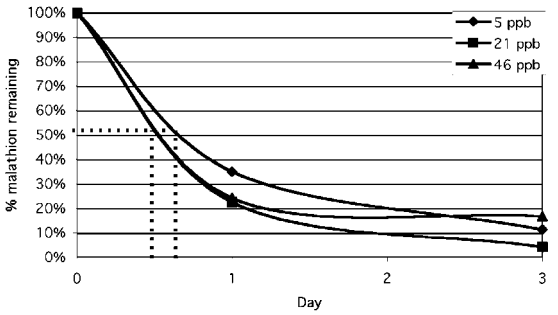

Fig. 2. Degradation of malathion in artificial sea water at 14 °C over a five day period, using three different initial concentrations. The half-life of malathion (dotted lines) was approximately half a day, irrespective of the initial concentration.

Phagocytosis was also significantly affected in the course of the sub-acute (month-long) exposure (Fig. 4). There was a significant reduction of phagocytosis one week after the initial exposure to 21 ppb, and 2 weeks after the initial exposure to 5 ppb. There was a significant reduction of phagocytosis at all concentrations tested 3 weeks after the initial exposure. On week 4, while seven animals remained in the control group, only one, three and two animals remained for the 5, 21 and 46 ppb exposure group, respectively, due to mortalities. Statistical comparison with the control group was therefore not possible for two of the three exposure groups, and the 21 ppb exposure group was not significantly different from the unexposed control group.

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

4. Discussion

While malathion is only slightly toxic to mammalian species at relatively high concentrations, its toxicity is generally much higher in aquatic species. Malathion has a wide range of toxicities in fish, extending from very highly toxic in the walleye (96-h LC_{50} of 0.06 mg/l) to highly toxic in brown trout (0.1 mg/l), rainbow trout (0.25 mg/l) and cutthroat trout (0.28 mg/l), moderately toxic in Nile tilapia (4.6 mg/l) and fathead minnow (8.6 mg/l) and slightly toxic in goldfish (10.7–11.3 mg/l), mosquitofish (0.70–12.68 mg/l) and topmouth gudgeon (14.5 mg/l) (Johnson and Finley, 1980; Kidd and James, 1991; Tietze et al., 1991; U.S. Public Health Service, 1995; Shao-nan and De-fang, 1996). The toxicity of malathion for aquatic or marine crustaceans in a 96-h bioassay ranged from 1 to 280 μg/l (Forget et al., 1998). Lobsters, with a LC_{50} of 38 ppb or 0.038 mg/l, appear to be very sensitive to the acute lethal effects of malathion compared to other aquatic species, with a LC_{50} half of that for the walleye, the most sensitive fish species, and within the relatively wide range of toxicity for aquatic and marine crustaceans. It is interesting to note that our study, as others, evaluated the toxicity of technical grade malathion, and that

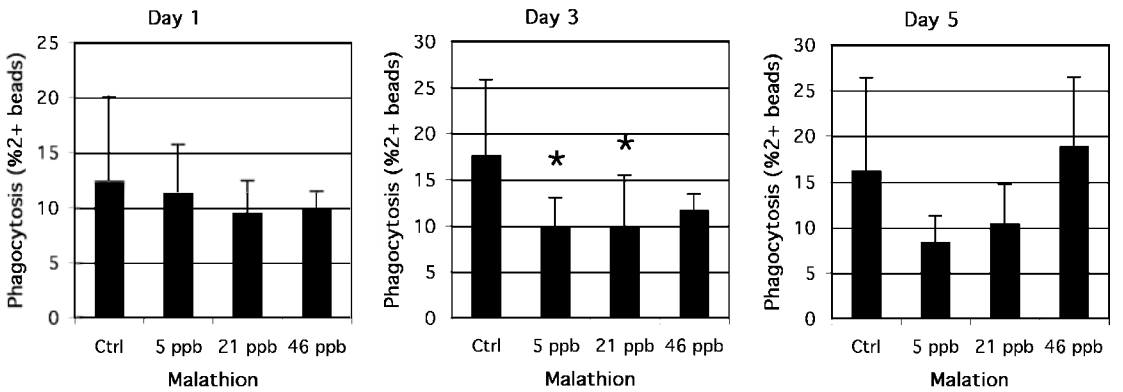

Fig. 3. Phagocytosis of lobster cells as measured 1, 3 and 5 days after a single exposure to three different concentrations of malathion. Results are reported as mean ± standard deviation (n = 9, 8, 9 and 7; 9, 7, 8 and 3; 9, 7, 6 and 2 for the control, 5, 21 and 46 ppb groups, respectively, on days 1, 3 and 5). Symbol asterisk (*) significantly different from unexposed control ($P < 0.05$).

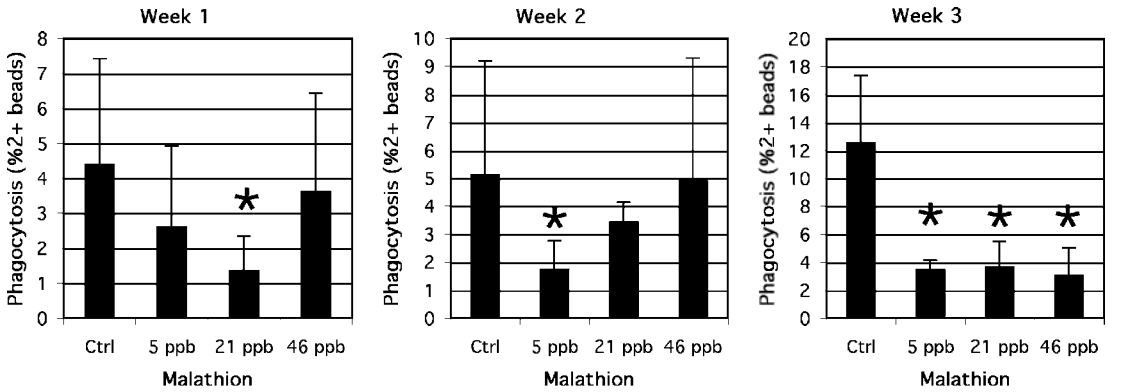

Fig. 4. Phagocytosis of lobster cells as measured on days 7, 14, 21 and 28, in each case 7 days after a weekly exposure to three different concentrations of malathion. Results for week 4 are not shown since only one, three and two animals remained for the 5, 21 and 46 ppb exposure group, respectively. Results are reported as mean ± standard deviation (n = 9, 4, 4 and 9; 9, 3, 3 and 6; 9, 2, 3 and 3 for the control, 5, 21 and 46 ppb groups, respectively, on weeks 1, 2 and 3). Symbol asterisk (*) significantly different from unexposed control ($P < 0.05$).

one study demonstrated that commercial formulations of malathion were 1.176 times more toxic than the technical material (Haider and Inbaraj, 1986).

The breakdown of malathion in our system was very rapid, with a half-life of approximately 12 h. This is in contrast with raw river water, for which the half-life is less than 1 week, and distilled water, in which malathion remained stable for 3 weeks (Howard, 1991). In general, it was determined that persistence of the pesticides decreases with increase in temperature, pH and organic content (Kaur et al., 1997). In sterile seawater, the degradation increases with increased salinity (Howard, 1991). We performed our experiments in a static/static renewal mode rather than flow through system to better mimic the sporadic environmental exposure to pesticides. Also, we chose to conduct our experiments at a temperature (14 °C) and salinity (1.024) at which lobsters were comfortable, to minimize stress and to ensure that we did not overestimate the toxicity of malathion in lobsters. Also, experiments were conducted in the dark (except for sampling and water changes) to avoid excessive breakdown of malathion through photodegradation.

Because of its very short half-life, malathion is not expected to bioconcentrate in aquatic organisms. However, brown shrimp showed an average concentration of 869 and 959 times the ambient water concentration in two separate samples (Howard, 1991). This was not the case in lobsters from this study, for which malathion was not detected in tissues 5 days after exposure. This could be the result of complete metabolism in lobster tissues or the inability to detect malathion due to a lack of sensitivity of the analytical methods. Nevertheless, the very rapid breakdown of malathion in our system suggests that failure to measure malathion in water samples or tissues 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 exposed lobsters in which no malathion was detected in tissues.

Effects on lobster hemocyte phagocytosis were observed at relatively low concentrations, sometimes without effects at higher concentrations. Immunotoxicological effects not following a dose–response relationship have been documented before in laboratory animals. For example, Lawrence (1981) documented an increase in humoral response to sheep erythrocytes and T lymphocyte activity in mice exposed to 0.08 and 0.4 mM lead acetate for 4 weeks, while exposure to 2.0 mM did not induce significant changes, and exposure to 10.0 nM resulted in a suppression of these functions. Similarly, mitogen-induced lymphocyte proliferation in mice exposed to lead acetate for four weeks was significantly enhanced upon exposure to 0.4 and 0.08 mM for ConA and PHA, respectively, while exposure to 2.0 and 10.0 mM had no significant effect (Lawrence, 1981). In another study, exposure to

10 and 250 ppm cadmium chloride for 90 days significantly reduced mouse lymphocyte proliferation upon stimulation with PHA, Con A or LPS, while exposure to 50 ppm did not result in significant modulation of the response (Thomas et al., 1985).

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 LC_{50}) can affect lobsters defense mechanisms, even with rapidly decreasing water concentrations. These results are not surprising given that the immunotoxicity of malathion on macrophages as well as natural and acquired immunity has been documented in several species of laboratory animals (Rodgers and Ellefson, 1990; Rodgers et al., 1996; Banerjee et al., 1998) and in fish (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 reduced immune functions and 20 times lower than those which resulted in reduced resistance to a pathogen in a fish study (Beaman et al., 1999). Lobsters appear extremely sensitive to the immunotoxic effects of malathion. Various aquatic invertebrates have been documented as extremely sensitive to sublethal effects of malathion, with EC_{50} values from 1 μg/l to 1 mg/l (Menzie, 1980). Our immunotoxicity results place lobsters at the most sensitive end of the spectrum, with significant effects on the population (not an EC_{50}) at 5 μg/l. Mammalian studies comparing the relationship between immune functions and host resistance showed a good correlation between changes in the immune tests and altered host resistance, with no instances where host resistance was altered without affecting an immune test (Luster et al., 1993). We have documented immunotoxic effects in lobsters on phagocytosis, reported as the most important defense mechanism in all phyla of the animal kingdom (van Oss, 1986). Given that defective phagocyte function is an important cause of increased susceptibility to opportunistic pathogens in mammalian species (Rotrosen and Gallin, 1987), and the above referenced data in mammalian immunotoxicology (Luster et al., 1993), it is likely that malathion-induced suppression of phagocytosis in lobsters could result in a significant decrease in lobster resistance to disease.

The current study assessed the toxicity of malathion in lobsters in view of the use of malathion around Long Island Sound to control West Nile virus-infected mosquitoes and its potential role in the 1999 lobster die-off. We documented that lobsters appear to be highly sensitive to the toxic effects of malathion compared to other species for which literature was available. However, to the authors' knowledge, no data have documented the actual exposure of lobsters to malathion upon environmental application, and it is not known if the concentrations that we documented to be toxic to lobsters were ever found in their environment in the course of the die-off. It is therefore not possible to determine with any certitude the role of malathion in the 1999 lobster die-off. Our results on the toxicity of malathion to lobster can nevertheless be useful in risk assessment, and our upcoming results on the toxicity of methoprene and resmethrin will help in making informed decisions on further use of pesticides for application around Long Island Sound.

5. Conclusion

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.

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