

Report as of FY2007 for 2007ME114B: "A Novel Approach to Assessing Multiple Stressor Effects in Maine Fishes"

Publications

Project 2007ME114B has resulted in no reported publications as of FY2007.

Report Follows

**A Novel Approach to Assessing Multiple Stressor Effects
in Maine Fishes**

FINAL REPORT

**The George Mitchell Center for the Environment
Maine Water Resources and Research Institute**

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Submitted by

Adria A. Elskus^{1,2}, Frank Drummond^{2,3},
Rebecca van Beneden^{4,5} and L. Brian Perkins⁶

¹U.S. Geological Survey, Maine Field Office, University of Maine, Orono, ME 04469

²School of Biology & Ecology, University of Maine, Orono, ME 04469

³University of Maine Agricultural Experiment Station, Orono, ME 04469

⁴School of Marine Sciences, University of Maine, Orono, ME

⁵Biochemistry, Microbiology and Molecular Biology Dept, University of Maine, Orono, ME

⁶Dept of Food Science and Human Nutrition, University of Maine, Orono, ME

Abstract

Maine Atlantic salmon (*Salmo salar*) rivers experience a broad range of stressors, including acidity, aluminum (Al), and pesticides, which are present simultaneously but whose combined effects on resident salmon are unknown. Such stressor combinations are best tested using multiple concentrations and combinations; however the slow developmental rate and seasonal limitations of egg production preclude the use of Atlantic salmon early life stages as efficient toxicant screens. We conducted studies in both Atlantic salmon and in zebrafish (*Danio rerio*), a species with rapid development (5 days from fertilization to feeding). Our objectives were to (1) evaluate potential sublethal effects of current use and proposed blueberry pesticides, (2) determine whether pesticide-contaminated, acid/Al rivers pose a greater threat to salmon than pesticide-contaminated rivers alone, and (3) evaluate the potential of early life stage zebrafish as a toxicology model for early life stage Atlantic salmon. For objective one, we found the blueberry pesticides (formulation/active ingredient: VelparTM/hexazinone, ImidanTM/phosmet, CallistoTM/mesotrione, SpinTorTM/spinosad) had no significant effect on innate immunity, development rate or behavior (spontaneous swimming, prey capture) in zebrafish early life stages at environmentally realistic concentrations (0.2-3.0 ppb active ingredient) or concentrations 10-fold higher (2 - 30 ppb a.i.). For objective two, we hypothesized that combinations of acid/Al (AA) + pesticides would have stronger sub-lethal effects on early life stage salmon than either stressor alone. We found that in 5 day exposures, pesticide treatments alone (0.75-7.5 ppb a.i.) at neutral pH (6.96 ± 0.06 SEM) had no effect on the survival of salmon fry relative to untreated controls, but AA treatments with or without pesticides (254-573 ppb inorganic Al; pH 4.98 ± 0.25) significantly reduced survival. Of the four multiple-stressor (pesticide + AA) groups, three sustained mortalities significantly higher than those of the AA control. However, it is likely that the dramatic drop in pH on day 2 (pH 3.8-4.3 across all acid/Al treatments), rather than a multiple-stressor effect, drove this difference in mortality. High variability masked potential treatment effects on prey-capture; the immune assays were inconclusive. We conclude that blueberry pesticide effects on salmon fry may increase in the presence of acid/Al, suggesting a multiple stressor effect, but further studies with more replicates and longer exposures are needed. To make firm conclusions regarding objective three, prey capture and immune assays must be repeated using optimized protocols to better facilitate comparisons between zebrafish and salmon fry responses.

Problem and Research Objectives

Maine rivers and lakes feature a broad range of stressors, including acidity, aluminum (Al), endocrine-disrupting chemicals, organochlorines and pesticides. Many of these are present simultaneously. Exposure to a mixture of contaminants could have effects on fish that would not be predicted from exposure to individual stressors alone. For example, co-exposure of amphibians to the pesticide carbaryl in the presence of predator scent increased carbaryl toxicity 2-4 fold relative to carbaryl exposure alone (Relyea and Mills 2001). Of particular concern for fish in Maine's Downeast rivers (Washington County) is the combination of blueberry pesticides, acidic water and elevated levels of Al, each of which has been implicated in hindering the recovery of endangered Maine Atlantic

salmon (*Salmo salar*) (National Research Council 2004). Whether the pesticide mixtures alone, or in combination with high acidity and elevated Al levels, adversely affect fish health, is unknown. An additional concern is the need to provide input on the effects of proposed changes, including alterations in pesticide usage and plans to neutralize river acidity. For example, the Maine blueberry industry currently proposes to supplement two of the pesticides found consistently in Maine rivers, phosmet and hexazinone, with spinosad and mesotrione, respectively. Plans by NOAA-Fisheries to neutralize the acidity of Downeast rivers as a mitigation tool have been put on hold until more information is available on the potential beneficial/detrimental effects of this action (D. Kircheis, pers comm).

Atlantic salmon are developing in the presence of blueberry pesticides and acid/aluminum in some Maine rivers. The Maine Board of Pesticide Control has consistently detected blueberry pesticides, including phosmet and hexazinone, in Downeast salmon rivers (Jackson 2003). In addition, during spring and fall rains, these rivers experience dramatic fluctuations in acidity, reaching pH levels as low as 4.6. Elevated acidity induces the release of sediment-bound aluminum into the overlying water (Gensemer and Playle 1999). One result is that fish are developing in the presence of blueberry pesticides (0.2-3 ppb), highly acidic water (pH<5.6) and elevated levels of aluminum (Al>70 ppb), a multiple-stressor combination that may seriously compromise development, physiological processes and fitness. While acidic conditions toxic to fish (Lydersen et al. 2002) can be exacerbated in the presence of aluminum (Driscoll Jr. et al. 1980; Staurnes et al. 1993), how acid/Al conditions and pesticides interact to affect toxic potency is completely unknown.

There are known hazards to fish associated with the current-use pesticides (EPA 1988; Nieves-Puigdoller and McCormick 2004), but little information exists on the proposed supplements. Phosmet is a cholinesterase inhibitor that affects muscle contractions, respiration, is extremely toxic to many fish species (EPA 1988; Orme and Kegley 2004) and, like other organophosphates, may deleteriously affect fish immune function (Galloway and Handy 2003; Harford et al. 2005) and behavior (Levin et al. 2003; Swain et al. 2003). Phosmet pulses have been detected in July in Downeast rivers in every year sampled (2000, 2001, 2003) (Jackson 2003), yet phosmet continues to be used, with caution, because of its low water solubility and short half-life (hours to days). Recent work has shown, however, that even short-term exposure to pesticides that degrade rapidly in the environment can have lasting, deleterious effects to aquatic life (De Guise et al. 2004). Spinosad, the proposed supplement to phosmet, is a bacterial fermentation product. It is considered only slightly toxic to early life stage trout (0.962 ppm lowest observable effects concentration). Its effects on the nervous system of insects (hyperstimulation of muscle contractions) occur through biological receptors present in both vertebrates and invertebrates (Salgado 1998; Salgado and Saar 2004), suggesting it could also have sub-lethal, behavioral effects in fish. The current use herbicide, hexazinone, has a long half-life and can have sub-lethal effects on respiration in early life stage fish (Nieves-Puigdoller and McCormick 2004). Because of hexazinone's propensity to enter and remain in the groundwater, the herbicide mesotrione has been proposed as a supplement (D. Yarborough, Univ Maine Blueberry Extension, pers comm.). Mesotrione

is not persistent in water or soil, but its degradates are mobile and may contaminate groundwater, particularly in cold climates with low pH soils (EPA 2001), conditions which define the Downeast river watershed. Mesotrione inhibits carotenoid biosynthesis in plants but is considered virtually non-toxic to fish based on acute 96 h exposure studies (EPA 2001); however, the effects of mesotrione on fish early life stages are unknown. Based on the known toxicity of acidic water and inorganic aluminum, and the neurotoxic and immunotoxic effects of the blueberry insecticides, we expected the combination of low pH, elevated aluminum, and blueberry pesticides to delay hatch, alter immune function and affect behavior.

Although it would be preferable to conduct all toxicity studies in the endangered, focal species, Atlantic salmon, their prolonged developmental period, intensive rearing requirements, and limited seasonal availability, make them difficult to use for screening the multiple stressor conditions in Maine's waters. Hence, there is need for a rapid, biological screen in a more easily manipulated surrogate species. Zebrafish (*Danio rerio*) have a rapid development rate (fertilization to feeding in 5 days), transparent embryos, are readily obtainable in all life stages year-round, and are inexpensive to house. Moreover, because vertebrate developmental genes are highly conserved, zebrafish are widely used for studying toxicity mechanisms in vertebrates, particularly in early life stages (Spitsbergen and Kent 2003; Hill et al. 2005).

We evaluated multiple stressor combinations in early life stage Atlantic salmon and tested zebrafish as a high through-put screen to evaluate the toxicity of pesticide combinations that may be harmful to early life-stage fish. Since functional impairment in early life stages is a more sensitive measure of the response to toxicants than mortality or morphological defects (Samson et al. 2001), we evaluated two measures of fitness: behavior and immune function. Salmonid swim-up fry (Delonay et al. 1993), and zebrafish embryo-fry (Hill et al. 2005), are considered the most sensitive life stages for these fishes.

The significance of this study is twofold: an initial assessment of zebrafish as a toxicity model for salmon, and an evaluation of multiple stressors in Maine rivers. Multiple stressor approaches are rarely attempted due to the complexity of the experimental design and the numerous combination possibilities. While the effects of acid-aluminum and pesticides have been evaluated separately in fish, including Atlantic salmon, there have been no studies assessing their combined effects, despite the presence of such combinations in Maine salmon rivers.

Hypotheses

1. Combinations of acid-aluminum (AA) + pesticides have stronger sub-lethal effects on early life stage fish than either stressor alone.
2. The zebrafish is a suitable model for assessing toxicant effects in early life stage salmon.

Objectives

- Evaluate sublethal effects on fish early life stages of ImidanTM (a.i. phosmet) and VelparTM (a.i. hexazinone), two current-use blueberry pesticides, consistently detected in Maine Downeast rivers and of SpinTorTM (a.i. spinosad) and CallistoTM (a.i. mesotrione), the proposed supplemental pesticides.
- Determine if pesticides exacerbate the toxicity of episodic acid-aluminum events in Downeast rivers.
- Compare the effects of pesticides on zebrafish and Atlantic salmon using sensitive early life stages for each species.

Methodology

Materials

Pesticide formulations ImidanTM (Gowan), Velpar-LTM (DuPont), CallistoTM (Syngenta), and SpinTorTM (Dow AgroSciences) were obtained from the University of Maine Blueberry Extension Office, courtesy of Dr. Frank Drummond. Styrene-divinylbenzene (SDB-L) solid phase extraction (SPE) columns (500 mg/6 mL) were obtained from Phenomenex (Torrance, Cal.). Solvents were high purity pesticide grade from Fisher Scientific, Inc Pittsburgh, PA. Dihydrodichlorofluorescein diacetate (H₂DCFDA) was from Invitrogen (Carlsbad, Cal.), phorbol 12-myristate 13-acetate (PMA) and black 96 well plates were from Fisher Scientific, Inc. (Pittsburgh, Pa.). Pesticide standards were obtained from the U. S. Environmental Protection Agency's (USEPA) National Pesticide Standard Repository (Fort Meade, Md.). Paramecia were obtained from Carolina Biological Supply (Burlington, NC). *Ceriodaphnia dubia*, *Selenastrum capricornutum* and YTC Daphnid feed mixture were obtained from Aquatic BioSystems, Inc (Fort Collins, CO). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Source & Maintenance of Fish

Zebrafish

Zebrafish embryos (AB strain) were obtained from the University of Maine's Zebrafish Core (University of Maine, 2007). Tanks of 14 females and 12 males were spawned as needed to provide these fish.

Atlantic salmon

One thousand F1 generation of Penobscot River strain Atlantic salmon were obtained as swim-up fry, already on-feed, from the Craig Brook National Fish Hatchery (CBNFH, East Orland, ME) in mid-May 2007 and transported to the University of Maine Aquaculture Research Center (ARC). They were divided evenly among four 60 liter, aerated tanks receiving unchlorinated, flow-through, chilled well water maintained at ~ 13-14°C. Fish were fed 3 times daily until transferred into the dosing system with a combination of Cyclops EZTM and SkrettingTM feeds provided by CBNFH. To reduce

ammonia production from fish feces, urine and decaying food in the static exposure system, fish were fed only once per day during the 5 day exposure period.

Pesticide Dosing solutions

The pesticide formulations used in this study are the actual material applied by blueberry growers and contain both the active ingredient and inert substances that can have toxic effects of their own. The active ingredients (trade name in parentheses) in the pesticide formulations were as follows: hexazinone (Velpar®), a triazine-dione (3-cyclohexyl-6-(dimethylamino)-1-methyl-s-triazine-2,4-(1H,3H)-dione); phosmet (Imidan®), an organophosphate (O,O-dimethyl s-phthalimidomethyl phosphorodithioate); mesotrione (ZA1296®) (2-[4-(methylsulfonyl)-2-nitrobenzoyl]-1,3-cyclohexanedione); and spinosad (Tracer®), a combination of two macrocyclic lactones (spinosyn A + D). These pesticides are soluble in water (hexazinone, 30,000 ppm; phosmet 25 ppm; spinosad 0.495 ppm (spinosyn D) & 89.4 ppm (spinosyn A); mesotrione 160 ppm), allowing us to prepare aqueous stock solutions without the need for a solvent carrier. Dosing concentrations were based on the pesticide levels reported in the Pleasant River by the Maine Board of Pesticide Control for hexazinone and phosmet (Jackson 2003). Dosing concentrations are based on the concentration of active ingredient in the formulation (e.g. Velpar™ contains 25% hexazinone by weight; Velpar dosing concentration of 2 ppb = 2 ppb hexazinone + 6 ppb inert ingredients).

Acid-aluminum dosing solutions

For the salmon studies, pesticide, and pesticide/acid/aluminum treatments, were made up in Machias River water. Water from the Machias River was collected in May 2007 and held indoors in an aerated, 500 gallon tank and circulated through a chiller to maintain the temperature at ~ 15°C. The tank was covered in black-plastic to protect the water from light with the aim of reducing the breakdown of naturally occurring dissolved organic carbon (DOC) in the river water. River water was essential for the salmon study because DOC affects aluminum toxicity both by altering water acidity and complexing with the toxic, inorganic aluminum species. Acid-aluminum water was prepared using 6 N HCl and AlCl₃·6H₂O to achieve a target pH of 5.2 and a target inorganic (toxic) aluminum concentration of 200 ug/L, to reflect levels seen in Downeast Rivers (Jagoe and Haines 1997). Pesticides were added to all treatment buckets except the neutral control and the acid/Al control. Due to the complex interactions of acid, aluminum and DOC occurring in natural waters, there is a very narrow range of pH in which aluminum speciates into the toxic inorganic form. In the daily preparation of the acid-aluminum dosing solutions, the addition of acid was quickly buffered by these interactions. To overcome this, we adjusted the initial pH to ~ 4.5, let the solutions sit for 14 hours at 14 °C, then readjusted the pH to ~ 5.2 just prior to transfer into 17 liter headtank buckets at the start of each exposure day.

Fish Exposures & Endpoints

The exposure period, treatments and locations for these studies are presented in Tables 1 and 2.

Zebrafish exposures

The exposure period, treatments and location for these studies are presented in Tables 1 and 2. To determine whether pesticide mixtures measured in the Down East rivers of Maine have additive, synergistic, or antagonistic effects, we exposed embryos to single formulations and to mixtures (VelparTM+ImidanTM, CallistoTM + SpinTorTM), in combinations and concentrations measured in the Down East rivers of Maine for phosmet+hexazinone (0.2, 0.75, 2.0, 3.0 ppb, (Jackson 2003) and doses 10 times higher (7.5, 30 ppb). Pesticide solutions were prepared in egg water (60 micrograms of Instant OceanTM/mL nanopure water) 2 to 3 h (hours) prior to the start of each 5 day experiment. Solutions were added to each petri dish and the remaining solutions held at 28°C in the incubators with the fish on a 14-h/10-h light/dark cycle to mimic conditions under which single-application pesticide exposures 'age' in the field. To prevent the buildup of ammonia in the exposure plates, treatment water was replaced daily using the incubator-held dosing solutions. Zebrafish embryos were exposed to pesticides or egg water for 5 days in 100-mm diameter plastic petri dishes (40-50 embryos/dish) at 28 °C on a 14-h/10-h light/dark cycle from 2 to 3 h post-fertilization (Day 0) to 120 h post-fertilization (Day 4), an age where they display immunologic competence (Hermann et al. 2004). For the immune system assays, experiments were terminated on Day 4 (120 h post-fertilization). For developmental and behavioral studies, zebrafish were transferred on Day 4 from the petri dish pesticide exposures to clean egg water in 250-mL beakers suspended in flow-through tanks at 28°C. Zebrafish fry were held in the flow-through system through the end of the experiment (Day 7) with daily feedings of rotifers or dry food once per day on Days 5 to 7. Each replicate experiment used embryos from a different spawn.

Salmon exposures

The exposure period, treatments and location for these studies are presented in Tables 1 and 2. Freshly prepared dosing solutions were allowed to equilibrate for at least 14 hours before addition to the 17 liter (5 gallon) headtank buckets (Figure 1a), each of which fed four 2 liter, gently aerated treatment buckets containing 6 salmon fry each for a total of 24 fry (n=4 buckets of 6 fry each) (Figure 1b). Treatments were conducted under these flow-through conditions in an environmental chamber on a 14/10 L/D cycle at 14°C. Pesticide treatments and pesticide-acid-aluminum treatments were prepared fresh daily; fish were fed once daily with hatchery flake food (see *Source of fish*) throughout the 5 day exposure period.



a



b

Figure 1. Flow-through exposure system for Atlantic salmon swim-up fry. Ten treatments were delivered via (a) 5 gallon headtank buckets containing 17 liters of treatment solution that each fed (b) four 2 liter exposure buckets containing 6 fry each, individually aerated, draining into 5 gallon waste buckets. The 5 gallon headtank buckets were replenished daily with freshly prepared dosing solutions.

Table 1. Exposure parameters and locations for zebrafish and salmon studies¹.

| Species | life-stages exposed | exposure duration | hatchery water | control water | treatment water ¹ |
|-----------------------|-------------------------|-------------------|----------------|---------------|------------------------------|
| Zebrafish | | | | | |
| UMaine: Hitchner Hall | embryo thru fry | 0-5 days pf | | x | x |
| Atlantic salmon | | | | | |
| CBNFH | embryo thru swim-up fry | Oct - May | x | | |
| UMaine:ARC | swim-up fry | 5 days | | x | x |

¹Treatment details are in text and in Table 2.

Table 2. Summary of treatments and endpoints for each species

| Species | Zebrafish embryo-fry | Atlantic salmon |
|------------------------|--|---|
| Life stage | embryo thru fry | fry |
| Exposure period | 5 days | 5 days |
| Treatments | Egg Water Velpar™ Imidan™ Callisto™ SpinTor™ Velpar™+Imidan™ Callisto™+SpinTor™ | River Water Velpar™ Callisto™ Velpar™+acid/AI Callisto™+acid/AI Acid/AI |
| Endpoints | Survival Days-to-hatch Innate Immunity: - <i>RBA</i> Behavior: - <i>prey capture</i> - <i>spontaneous swimming</i> | Survival N/A Innate Immunity: - <i>RBA</i> - <i>Pathogen resistance</i> Behavior: - <i>prey capture</i> |

Pesticide Analysis

To determine if nominal dosing concentrations reflect actual dosing concentrations, we are optimizing protocols for analyzing the concentration of active ingredients in our dosing solutions. While protocols for extracting phosmet and hexazinone from river water have been established, there are few protocols for the candidate pesticides, mesotrione and spinosad. We conducted preliminary experiments evaluating the utility of styrene divinylbenzene-coated silica gel (SDB-L) solid phase extraction (SPE) cartridges to capture mesotrione and spinosad from aqueous solutions of Callisto™ and SpinTor™, respectively, and confirmed and optimized the ability of this cartridge packing to capture hexazinone and phosmet from aqueous solutions of Velpar™ and Imidan™, respectively. We are currently optimizing analysis conditions for spinosad and mesotrione by high performance liquid chromatography (HPLC). Briefly, 500- to 1,000-mL pesticide dosing solutions (Velpar™, Imidan™, Callisto™, SpinTor™) were pulled through SDB-L SPE cartridges under vacuum (25 kPa), the cartridges eluted with acetonitrile (Callisto™, SpinTor™ for meotrione, spinosad) or ethyl acetate (Velpar™, Imidan™ for hexazinone, phosmet) and two fractions collected (1 mL, F1; 5 mL, F2). Eluates for Velpar™ and Imidan™ were dried over sodium sulfate (baked at 600 °C for 12 h), volume reduced to 900 µL, spiked with 100 µL of chlorpyrifos (10 ng/µL) as an

internal standard, and injected onto a Hewlett Packard 5890/5870 gas chromatograph-mass spectrometry (GC/MS) system (2 μ L, splitless), with helium as the carrier gas, under the following conditions: 275°C injection port, ramping from 80 °C – 250 °C at 20 °C/min (minute), 1.2 mL/min flow rate. Full scan mode was used to identify the quantification ions and qualifying ions for hexazinone, phosmet, chlorpyrifos (a surrogate for phosmet), and metribuzin (a surrogate for hexazinone). Eluates for Callisto™ and SpinTor™ were dried over sodium sulfate, volume reduced under high purity nitrogen to 1 mL, and injected (20 μ L) onto a Hewlett Packard High Performance Liquid Chromatograph Series 1050 fitted with a C-18 column (100 x 4.6 mm). We used a mobile phase of 75 percent acetonitrile/25 percent water, a flow rate of 1 mL/min, and monitored the analytes spinodad and mestrione at a wavelength of 271 nm. This wavelength was determined to provide the maximum signal by performing a full-UV scan of each analyte using diode-array UV detection.

Aluminum and pH analysis

Total, organic and inorganic aluminum and pH (by closed-cell) of the treatment solutions were analyzed by the University of Maine's Sawyer Environmental Chemistry Research Laboratory using protocols optimized by Ms. Tiffany Wilson and Dr. Stephen Norton. Daily monitoring of pH in each treatment was done using a calibrated pH meter.

Mortality, Time to Hatch, Developmental Abnormalities

Zebrafish embryos were monitored daily from fertilization through the end of each experiment for mortality, days to hatch, and evidence of developmental abnormalities. Salmon fry were monitored daily during the 5-day exposure period, and throughout the prey-capture and immune challenge experiments for mortality, morbidity and/or signs of infection.

Behavioral Assays

Zebrafish

We evaluated two behaviors in zebrafish, spontaneous swimming and prey capture, a well-established measure of performance fitness in fish, using the protocols described by Samson and others (Samson et al. 2001). Because larval fish quickly learn to capture prey, it is important to track individual fish to determine if there is any effect of pesticide treatment on the ability of the fish to learn. For this reason, prey capture was measured twice, on Day 5 and again on Day 7 post-fertilization. Briefly, on Day 5, fry were placed individually into one well of a 12 well microplate to which 4 mL of egg water containing 40 Paramecium was added. The number of paramecium consumed within 1 minute was observed through a microscope and recorded. After the test, individual fry were transferred to a 6 well plate, one fry per well, and assigned an identification number and maintained in an incubator at 28°C on a 14-h/10-h light/dark cycle. On Day 7, each fry was re-tested as on Day 5.

For spontaneous swimming, fry were placed individually into 100 mL of egg water in an 8-cm-diameter finger bowl placed over a 1-cm² grid. After a 2 minute acclimation period, the number of lines crossed in 30 sec (seconds) by the fry was recorded. Five

random fry were tested from each replicate for each treatment on Days 3 and 6, Days 3 and 5, and Days 4 and 7 post-fertilization.

Salmon

Prey capture was evaluated in individual fish (12 fish/treatment) as the number of prey consumed/unit time/fish using *Cereodaphnia dubia* as prey for salmon essentially as described for rainbow trout (Carvalho and Tillitt 2004). In brief, after the 5 day exposures, salmon fry were maintained for 2 days in their 2 liter buckets in clean, aerated Machias River water blended 50/50 with unchlorinated well water. After this period, the fry were placed individually into 1 liter beakers fitted with 300 micron mesh screens (to allow for water flow through the beakers) in a flow-through water table receiving unchlorinated well water and tested individually for prey capture ability. We tested the same individuals each day for three consecutive days to control for 'learning' during the trials. *C. dubia* adults were size-selected using a 300 micron screen and 30 individuals of equal size placed into each beaker. After 20 minutes, the fish were removed using a slotted spoon and the remaining *C. dubia* collected in the same sequential manner to ensure equal exposure periods. The *C. dubia* remaining were counted and prey capture assessed as number *C. dubia* consumed/20min/fish. Data are presented as percent of *C. dubia* consumed per day.

Innate Immune Function

Zebrafish

For zebrafish, we evaluated respiratory burst, a simple immune system assay, as described (Hermann et al. 2004). Briefly, on Day 4 zebrafish fry were transferred from exposure dishes to black 96 well plates, one larva per well, and exposed to either substrate alone (H₂DCFDA, 6 wells) or substrate plus phorbol 12-myristate 13-acetate (PMA) (6 wells). PMA provokes the production of superoxide. In turn, superoxide oxidizes the substrate H₂DCFDA (a non-fluorescent dye) to dichlorofluorescein (DCF, a fluorescent product). In fish with a healthy immune system, PMA exposure in the presence of H₂DCFDA will provoke substantial production of DCF. PMA thus serves both as the stimulant and as a positive control to confirm the assay is working properly. Evolution of DCF was monitored for up to 3.5 h in a Perkin Elmer FusionTM fluorescence plate reader at an excitation/emission of 485nm/530nm. Response was evaluated as fold-change relative to controls (Hermann et al. 2004).

Salmon

Following the prey capture trials, salmon fry were divided into two groups and evaluated for innate immune function by respiratory burst assay or by pathogen challenge. For the respiratory burst assay we tested individual kidneys, essentially as described (Hermann et al. 2004). Briefly, kidneys were removed from salmon fry 2 days after the conclusion of the prey capture assays (7 days after the 5 day exposure period) in a laminar flow hood under sterile conditions, placed individually into sterilized 96 well black plates, H₂DCFDA in DMEM/F12 supplemented with an antibiotic/antimycotic agent added with or without PMA, and the evolution of DCF monitored as for zebrafish. Response was evaluated as fold-change relative to controls (Hermann et al. 2004).

The pathogen challenge experiment commenced 1 day after the conclusion of the prey capture assays (6 days after the 5 day exposure period). It was conducted at the University of Maine Aquatic Animal Health Laboratory (MAAHL) under the supervision of the Director, Deborah Bouchard. For this test, fry were immersed, by treatment group, in 1 liter beakers containing 500 mL of the highly infectious bacterial agent *Aeromonas salmonicida* at $> 10^5$ colony forming units/mL for 15 minutes at 11°C. *A. salmonicida* causes furunculosis in salmon (Moynier et al. 1993). Following immersion, fish were transferred to 2 liter buckets containing clean, aerated, unchlorinated well water and held for 5 days in an environmental chamber at 11°C. Fish were inspected daily for signs of infection. After 5 days, fish were removed and bagged individually in sterile Whirlpak™ bags, and stored at -80°C until evaluated (3 months later) for degree infection using a standard heterotrophic plate count method.

Statistical Analyses

Prey-capture and respiratory burst data were analyzed by one-way ANOVA. Survival analysis, using Kaplan-Meier methods and the log rank test (Bewick et al. 2004), was used to statistically compare treatment differences in salmon fry survival. All analyses were conducted using a significance level of $\alpha = 0.05$.

Disposal of pesticide and aluminum-contaminated water

Pesticides and aluminum treatment water were filtered through activated charcoal and the charcoal disposed of as hazardous waste.

Summary of Principle Findings

- Blueberry pesticide formulations (Velpar™, Imidan™, Callisto™, SpinTor™), even at doses of active ingredients 10 times higher than those measured in Maine salmon rivers, had no significant effect on early life stage (embryo-larval) zebrafish survival or development, or on the behavioral and innate immune response endpoints measured in this study.
- Pesticide combinations (e.g. Velpar™+ Imidan™) reported in some salmon rivers showed no evidence of additive, synergistic or antagonistic effects on early life-stage zebrafish survival, development, behavior or immune function, even at concentrations of active ingredients 10 fold higher than those measured in Maine salmon rivers.
- Acute exposure (5 day) to blueberry herbicide formulations (Velpar™, Callisto™) had no effect on Atlantic salmon swim-up fry survival.
- While there appear to be no effects of acute exposure (5 day) to blueberry herbicide formulations (Velpar™, Callisto™) on Atlantic salmon swim-up fry behavior (prey capture), the high variability among individuals precludes rigorous statistical analysis.

- Acute exposure to acid-aluminum significantly reduced survival of Atlantic salmon fry.
- Multiple-stressor effects of blueberry pesticides combined with acid-aluminum were not clear. While some blueberry pesticide/acid aluminum combinations (0.75 ppb Callisto™ + acid aluminum, 7.5 ppb Callisto™ + acid aluminum, 0.75 ppb Velpar™ + acid aluminum) reduced Atlantic salmon fry significantly more than acid-aluminum alone, others (7.5 ppb Velpar™ + acid aluminum) did not.
- Blueberry pesticide effects on Atlantic salmon fry prey capture and survival may increase in the presence of acid aluminum, but high variability among replicates, and the dramatic drop in pH (3.8 - 4.4) on Day 2, confound interpretation of the data.
- The current study does not conclusively support the use of early life stage zebrafish as a toxicology surrogate for early life stage Atlantic salmon; future experiments with additional common endpoints are needed.

Conclusions & Significance

The results of this pilot study indicate that acute exposure to blueberry pesticides may have little adverse effect on early life stage fish while acute exposure to acid aluminum can be highly toxic. Due to high variability among replicates, and a confounding acid event on day 2 day of the exposures, multiple stressor effects could not be rigorously evaluated. While the survival and prey capture results with salmon fry are suggestive of a possible decrease in both of these parameters when pesticides are combined with acid-aluminum, additional studies with more replicates are needed to confirm, or dispel, this possibility. Further work is also needed to tease apart the relative toxic contributions of acidity and inorganic aluminum. Additional sub-lethal, pesticide-sensitive endpoints, such as olfaction, homing, and/or reproduction, should be tested in future studies evaluating multiple stressor effects. Finally, to better mimic *in situ* conditions experienced by Atlantic salmon developing in Maine rivers, salmon should be exposed from fertilization through smolting, a 2 year period, with fish evaluated at several developmental points as the effects of early life stage exposure may not be manifested until fish reach transitional life stages (e.g. smolting, spawning).

The current study does not conclusively support the use of early life stage zebrafish as a toxicology surrogate for early life stage Atlantic salmon because the only common endpoint that could be rigorously evaluated statistically in both species was mortality. Further studies with more replicates and optimized behavioral and immune endpoints are needed to firmly evaluate zebrafish early life stages as a relevant toxicant screen and toxicology surrogate for early life stage salmonids.

Detailed Results & Discussion

This study addresses the lethal and sublethal effects on early life stage Atlantic salmon of multiple stressor conditions found in some Maine salmon rivers, assesses the effects of

blueberry pesticides on zebrafish early life stages, and provides preliminary information on the potential of zebrafish as a toxicant model for early life stage salmon.

Dosing Solution Concentrations

Pesticide standards were readily detectable by GC/MS (hexazinone and phosmet), and by HPLC (spinosad, mesotrione). Surrogates for phosmet (chlorpyrifos) and hexazinone (metribuzin) were also easily detected by GC/MS. Preliminary analyses of the pesticide dosing solutions indicate that the actual (measured) concentrations are close to, but lower than, nominal concentrations for ImidanTM and VelparTM (Table 2). Mesotrione and spinosad protocols need to be optimized before analysis of dosing solution extracts (stored for later analysis) can be completed.

Table 3. Preliminary analyses demonstrating that nominal and actual dosing solutions concentrations are similar. [ppb, parts per billion]

| Dosing Formulation | Dose | Active Ingredients | Nominal (ppb) | Actual (ppb) |
|--|-------------|---------------------------|----------------------|---------------------|
| Velpar TM plus Imidan TM | Low | hexazinone | 0.75 | 0.35 |
| | | phosmet | 0.75 | 0.21 |
| | High | hexazinone | 7.50 | 5.6 |
| | | phosmet | 7.50 | 5.0 |

ZEBRAFISH RESULTS

Innate Immune Function

We found no consistent effects of the blueberry pesticide formulations on the innate immune function of developing zebrafish. The results of initial experiments with exposure to single pesticide formulations over a wide range of doses, from environmentally relevant (0.2-3.0 ppb) to 10 times as high (7.5, 30 ppb), indicated there were no effects of any of the formulations on the respiratory burst response of embryolarval zebrafish (Figure 2). In these initial experiments, the zebrafish response was monitored for 2 h.

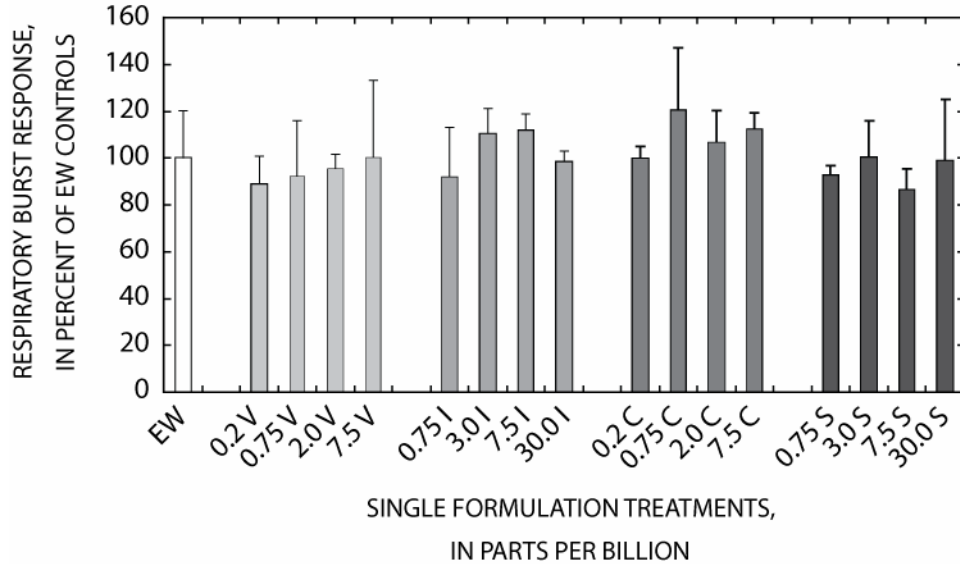


Figure 2. Preliminary data on the effects of exposure to single pesticide formulations on the innate immune system of zebrafish exposed from fertilization through swim-up fry. Bars represent means \pm SD for n=2-4 replicates of 6 fry per replicate. EW=Egg Water, V=VelparTM, I = ImidanTM, C = CallistoTM, S = SpinTorTM. Doses were 0.2, 0.75, 2.0, 3.0, 7.5, and 30 ppb. Respiratory burst was measured for 2 hours.

To improve and optimize the sensitivity of the assay, we extended the monitoring time to 3 h. However, even with the improved signal strength afforded by the longer monitoring duration, there appear to be no effects of these pesticide formulations on zebrafish respiratory burst (Figure 3).

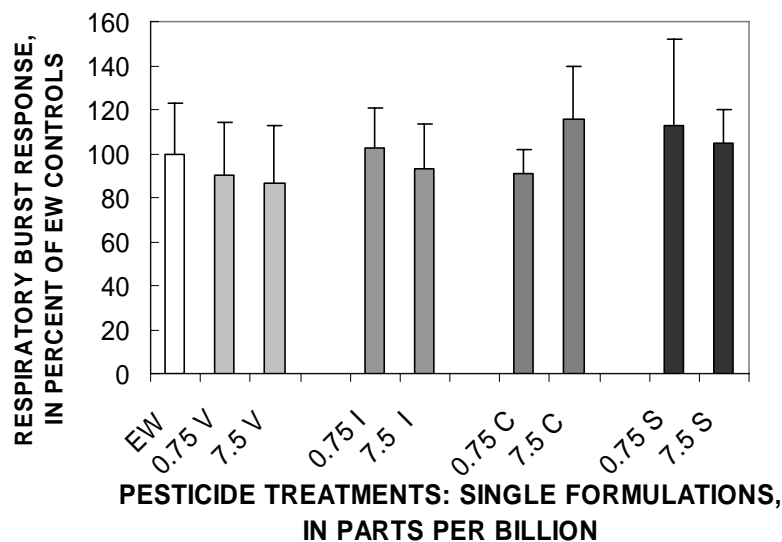


Figure 3. The effects of exposure to single pesticide formulations on the innate immune system of zebrafish embryo-fry exposed from fertilization through the swim-up larval stage. Bars represent means \pm SD for n=5 replicates of 6-12 fry per replicate. EW=Egg

Water, V=Velpar™, I = Imidan™, C = Callisto™, S = SpinTor™. Doses were 0.75 and 7.5 ppb. Respiratory burst was measured for 3 hours.

Due to ground-water contamination in the watershed, hexazinone (the active ingredient in Velpar™) is present year-round in the Pleasant River, one of the salmon rivers in Down East Maine, and thus is present in July when the insecticide Imidan™ (phosmet) is applied. To determine if pesticide mixtures have additive, synergistic, or antagonistic effects, we looked at pesticide mixtures at an average concentration found in the river (0.75 ppb) and a dose 10 fold higher (7.5 ppb). Our results indicate mixtures do not affect the respiratory burst response of developing zebrafish (Figure 4).

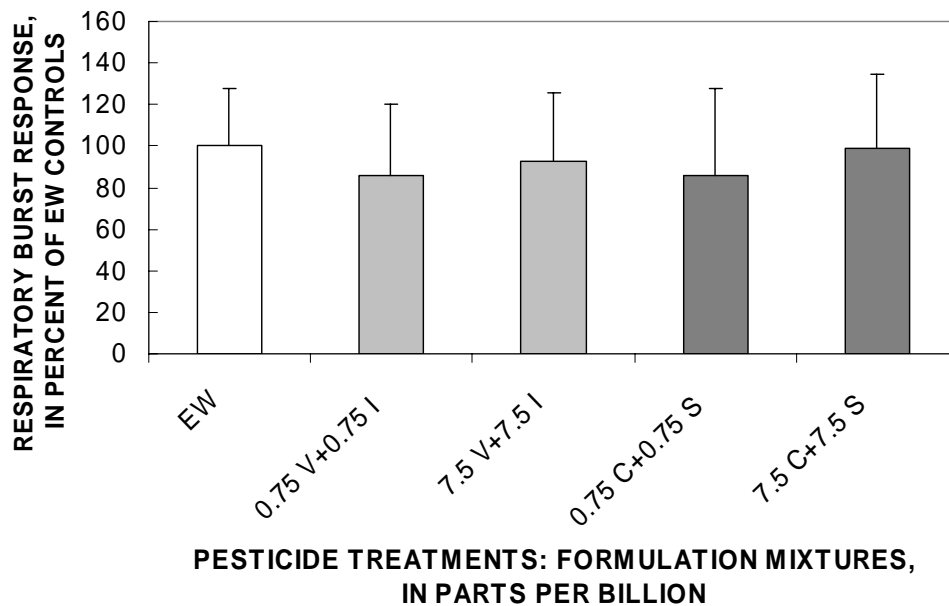


Figure 4. The effects of exposure to mixtures of pesticide formulations on the innate immune system of zebrafish embryo-fry exposed from fertilization through the swim-up larval stage. Bars represent means \pm SD for $n=5$ replicates, 6 fry per replicate. EW=Egg Water, V=Velpar™, I = Imidan™, C = Callisto™, S = SpinTor™. Doses were combinations of 0.75 ppb (low) concentrations and combinations of 7.5 ppb (high) concentrations. Respiratory burst was measured for 3 hours.

Mortality, Time to Hatch, Developmental Abnormalities

Zebrafish embryo mortality is typically 20 to 50 percent within the first 24 h post-fertilization (M. Nilan, University of Maine, oral commun, 2007). We found similar rates of mortality in our exposures, with no difference among treatments (data not shown).

We observed evidence of developmental abnormalities in both the untreated and in the pesticide treated groups. The most common abnormality was scoliosis, with fry curved into a 'C' shape; additional abnormalities observed occasionally included large head,

small or no eyes, and pericardial edema. A few individuals were moribund. All individuals displaying these abnormalities failed to hatch. There were no statistical differences among groups (Figure 5).

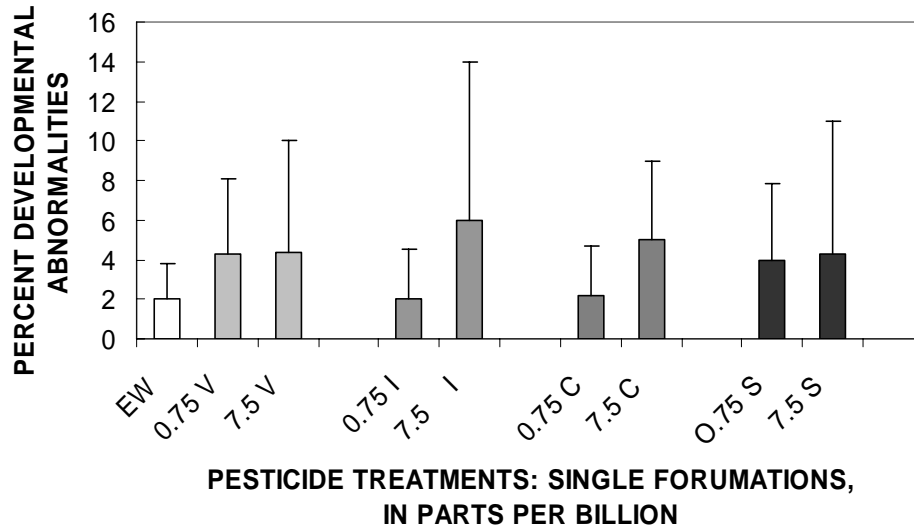


Figure 5. The effects of single pesticide exposure on the occurrence of developmental abnormalities (as percent) in zebrafish exposed from fertilization through swim-up. Bars represent mean \pm SD for n=5 fry per treatment. EW=Egg Water, V=Velpar™, I = Imidan™, C = Callisto™, S = SpinTor™. Doses were 0.75 and 7.5 ppb.

There were no significant differences in developmental rate ($P < 0.05$, two-tailed Student's t-test) as measured by the mean number of days it took the embryos to hatch (Table 3).

Table 3. Mean days to hatch for zebrafish exposed to pesticide formulations from fertilization through swim-up fry. Mean \pm SD for n=3 replicates of 50 embryos per replicate. [n, number; ppb, parts per billion; SD, Standard Deviation]

| Treatment | Dose (ppb) | Mean days to hatch | SD |
|-------------------|------------|--------------------|------|
| Egg water control | 0 | 2.90 | 0.33 |
| Velpar™ | 0.75 | 2.95 | 0.56 |
| | 7.5 | 3.08 | 0.42 |
| Imidan™ | 0.75 | 3.42 | 0.11 |
| | 7.5 | 3.18 | 0.20 |
| Callisto™ | 0.75 | 3.36 | 0.71 |
| | 7.5 | 3.18 | 0.54 |
| SpinTor™ | 0.75 | 2.89 | 0.26 |
| | 7.5 | 3.11 | 0.22 |

Prey Capture

There was no consistent effect of pesticide formulation or dose on the ability of larval zebrafish to capture prey (Figure 6). These data also indicate that the fish were as efficient at capturing prey on Day 7 as they were on Day 5, indicating no effect of pesticide formulation or dose on learning.

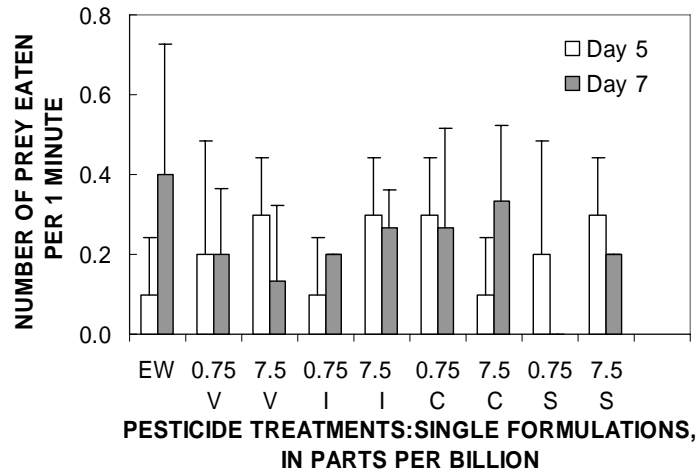


Figure 6. The effects of single pesticide exposure on prey capture by zebrafish exposed from fertilization through swim-up. Bars represent means \pm SD for 5 fry per treatment. Prey capture was measured in 2 separate experiments on Days 5 and 7 post-fertilization. EW=Egg Water, V=VelparTM, I = ImidanTM, C = CallistoTM, S = SpinTorTM. Doses were 0.75 and 7.5 ppb for Low and High, respectively.

Spontaneous Swimming

There was no consistent effect of pesticide formulation or dose on spontaneous swimming (Fig. 7). These cohorts showed little to no spontaneous movement until they reached four days post-fertilization.

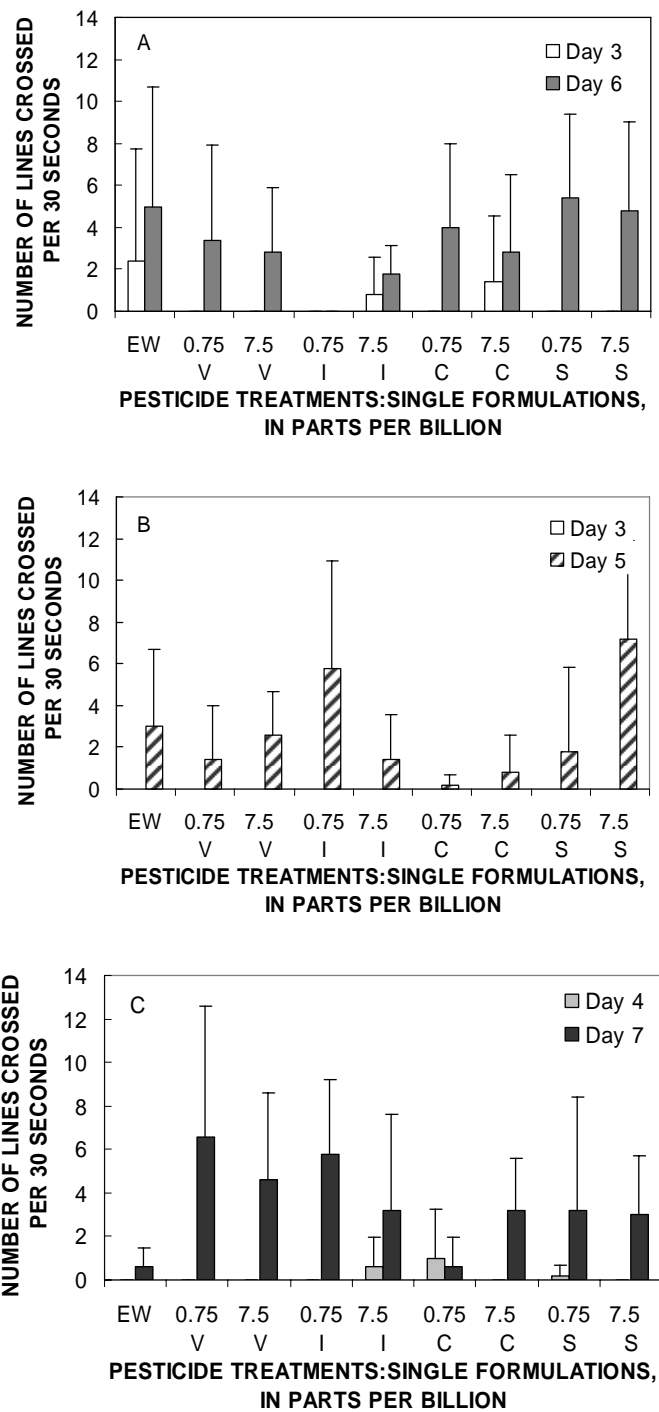


Figure 7. The effects of single pesticide exposure on spontaneous swimming in zebrafish exposed from fertilization through swim-up. Bars represent means \pm SD for 5 fry per treatment. Swimming was measured in 3 separate experiments on (A) Days 3 and 6, (B) Days 3 and 5, and (C) Days 4 and 7 post-fertilization. EW=Egg Water, V=VelparTM, I = ImidanTM, C = CallistoTM, S = SpinTorTM. Doses were 0.75 and 7.5 ppb.

SALMON RESULTS

Acid-aluminum doses

Levels of toxic, inorganic aluminum (Figure 8) ranged from 254 to 573 ppb and were thus somewhat above the target concentration of 200 ppb. However, these levels were 10 to 25 fold higher than those of the neutral treatments (19-20 ppb), producing the desired strong and significant differences among treatments.

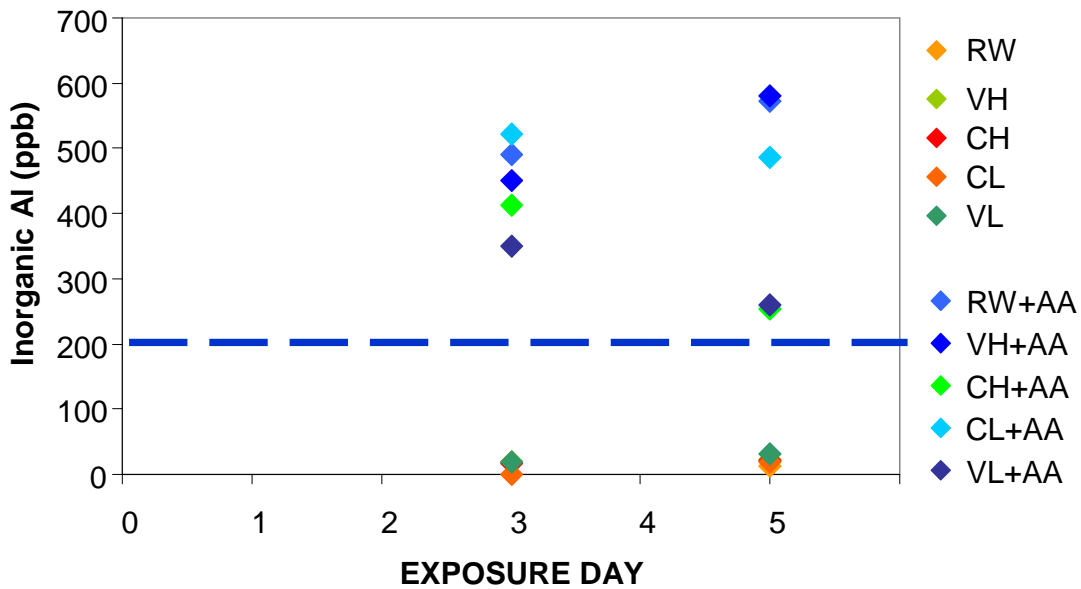


Figure 8. Concentrations of toxic, inorganic aluminum in dosing solutions measured on Days 3 and 5 of the 5 day exposure experiment with Atlantic salmon fry. RW=River Water, V=VelparTM, C = CallistoTM, L=low, H= high, AA = acid-aluminum.

Acidity (measured as pH) was significantly different between the neutral and acid-aluminum treatment groups (Figure 9). The pH of the neutral treatments averaged 6.96 ± 0.06 SEM over the 5 day exposure period; the addition of pesticides did not affect pH. Although difficult to prepare consistently each day, acid concentrations in the range of the target pH of 5.2 were achieved on 4 of the 5 treatment days. However, on Day 2, the pH in the RW+AA and VH+AA was ~ 4.4 while the pH in CH+AA, CL+AA and VL+AA fell between 3.8 and 4.0.

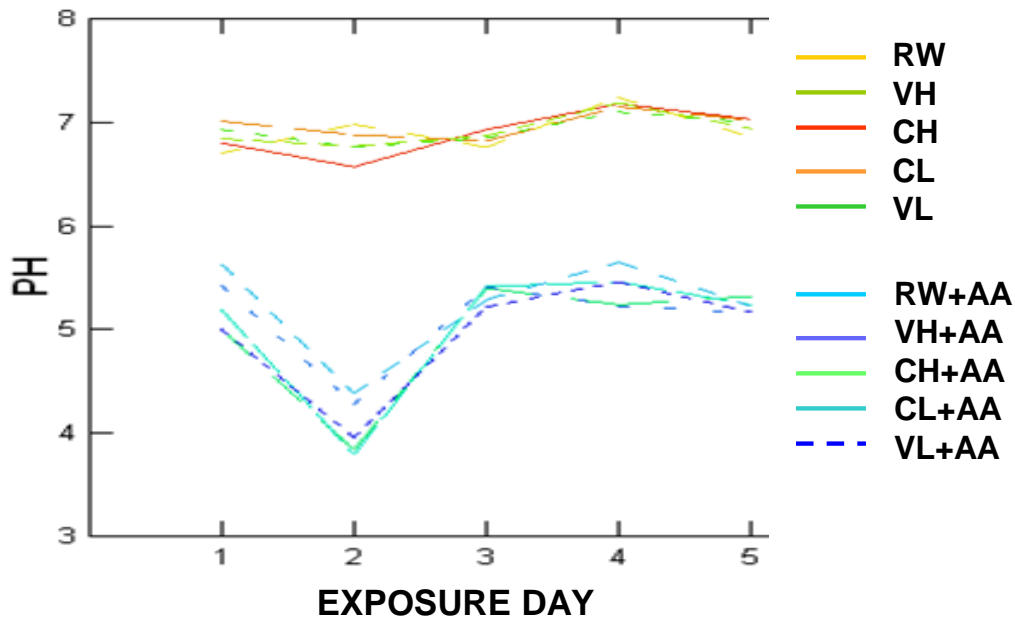


Figure 9. Concentrations of acid (as pH) measured daily in the headtank buckets during the 5 day exposure period. RW=River Water, V=VelparTM, C = CallistoTM, L=low, H=high, AA = acid-aluminum.

Mortality

Salmon fry survival was unaffected by pesticide exposure, at either dose, relative to the river water controls (Figure 10, group 1). In contrast, fry survival was significantly reduced in all treatments containing acid-aluminum relative to the neutral treatments (Figure 10, groups 2,3 versus group 1, ANOVA $p < 0.05$). Survival in the acid-aluminum treatments partitioned, statistically, into two groups; those treatments in which acidity did not fall below pH 4.4 over the 5 day period (Figure 10, group 2), and those in which acidity dropped to between 4.0 and 3.8 on Day 2 (Figure 10, group 3). The dramatic drop in pH on day 2 (Figure 9) likely had lasting effects on survival for all AA treated groups, but was particularly toxic to the 3 treatments in which pH was between 3.8 to 4 (Figure 10, group 3).

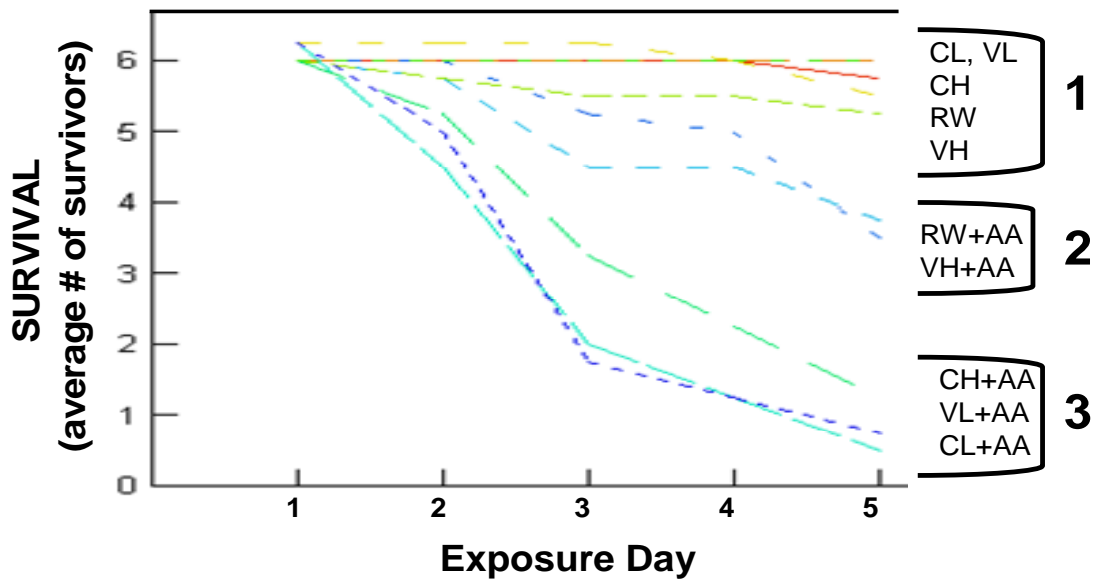


Figure 10. The effects of pesticide exposure with and without acid/aluminum on mean survival of Atlantic salmon fry exposed for 5 days in a flow-through system. Values represent the average number of survivors out of 6 fry/replicate, 4 replicates per treatment. Error bars have been removed for clarity. RW=River Water, V=Velpar™, C = Callisto™, L=low, H= high, AA = acid-aluminum. Nominal pesticide doses were 0.75 ppb and 7.5 ppb. Aluminum and acid concentrations are presented in Figures 8 and 9, respectively. Groupings with different numbers are significantly different at $p < 0.05$.

Prey Capture

Statistically, there was no effect of any treatment on the prey capture ability of Atlantic salmon fry relative to river water controls (Figure 11). However, in all cases except for VH+AA, prey capture success appeared to decrease when pesticides were added to acid-aluminum treatments (compare RW+AA versus CL+AA, CH+AA, VL+AA). However, the low number of individuals remaining in some of the treatments ($n=2$, due to high mortality likely in response to the profound drop in pH on Day 2) confounded our ability to determine if there is a multiple stressor effect or not.

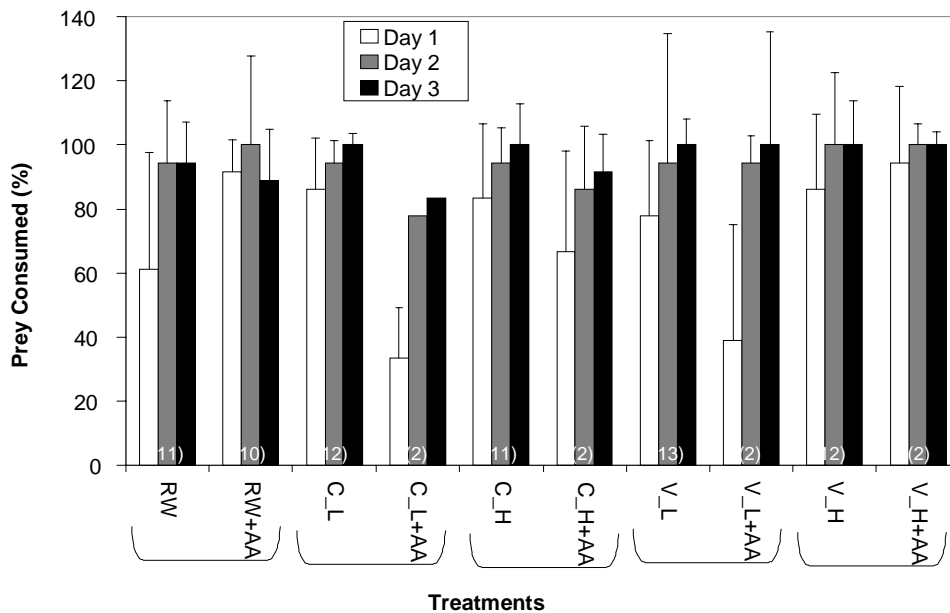


Figure 11. The effects of pesticide exposures with and without acid/aluminum on prey capture ability of Atlantic salmon fry exposed for 5 days in a flow-through system. Bars represent mean percent of prey consumed \pm SD for (n) fry per treatment. Prey capture was measured daily over three consecutive days on the same individuals. RW=River Water, V=VelparTM, C = CallistoTM, L=low, H= high, AA = acid-aluminum. Nominal pesticide doses were 0.75 ppb and 7.5 ppb. Aluminum and acid concentrations are presented in Figures 8 and 9, respectively.

Innate Immune Response

For salmon fry, both measures of innate immune response were inconclusive. Failure of the positive control (PMA stimulation of reactive oxygen production in kidneys of control fish (data not shown), suggests conditions for the kidney-based respiratory burst assay require further optimization. None of the fish exposed to the salmon pathogen, *Aeromonas salmonicida*, were successfully infected (data not shown). There are two possibilities for this. Either the immersion time (15 min) was too short to be effective at such cold temperatures (11°C), and/or the *A. salmonicida* culture had lost its toxic potency, a condition that can occur after the culture has been passaged a number of times (Dr. Ian Bricknell, MAAHL pers comm).

Discussion

It may be that inorganic aluminum, rather than low pH, is responsible for increased fry mortality in the acid-aluminum treatments in the present study. We cannot statistically tease apart the relative toxic contributions of acid and inorganic aluminum as we did not have aluminum data for Day 2 when pH levels were at their lowest and mortality spiked. However, Smith & Haines found that episodic declines to pH 4.0 did not increase mortality in swim-up Atlantic salmon fry, but that increases in inorganic aluminum (200-225 ppb) were correlated with increased mortality (Smith and Haines 1995). Interestingly, Atlantic salmon fry may be more tolerant to inorganic aluminum than smolts, at least over short exposure periods, as smolt mortality occurs at much lower concentrations of inorganic aluminum (40-50 ppb) when pH < 6 (S. McCormick, USGS, pers comm).

The findings in the current study are in keeping with those of others who reported effects of hexazinone only at concentrations over 10 times higher than ours. Exposure to 100 ppb hexazinone (21 days to the active ingredient) reduced gill Na⁺/K⁺-ATPase activity in Atlantic salmon smolts and elevated opercular movements in fry, but did not affect mortality or development in either life stage (Nieves-Puigdoller and McCormick 2004; Nieves-Puigdoller et al. 2007). In the current study, exposure to 13 to 130 fold lower doses of hexazinone (5 days to formulation) had no effect on survival or behavior of either Atlantic salmon fry or zebrafish fry. Exposure to 1.0 and 2.0 ppb atrazine, a triazine related to hexazinone, significantly reduced the olfactory response of male Atlantic parr to the female priming pheromone (Moore and Waring 1998). These doses are comparable to environmental levels of hexazinone measured in Maine salmon rivers (0.18 – 1.85 ppb, (Jackson 2003) and to the low dose used in the present study (0.75 ppb), indicating that the olfactory effects of hexazinone should be investigated in Atlantic salmon parr.

Mesotrione is considered practically non-toxic, both acutely and chronically, to fish (NYS-DEC 2002), and we found no adverse effects in Atlantic salmon fry or zebrafish embryo-larval tests. However, as for hexazinone, the olfactory effects of mesotrione should be investigated in Atlantic salmon parr.

Phosmet is highly toxic to aquatic organisms but has a short half-life (hours to days) (Turner and Mahoney 2003). However, phosmet persistence and toxicity increases as pH decreases, an important factor when studying the acidic rivers in Maine. A LOEC (lowest observed effects concentration) of 6.1 ppb (60 day exposure) significantly affected survival and growth of rainbow trout fry has been reported (Turner and Mahoney 2003), close to the high dose (7.5 ppb) used in the current study. Interestingly, 7.5 ppb phosmet (in the formulation ImidanTM) did not adversely affect mortality or behavior in the early life stages of zebrafish tested in the current study, although this may be due to the acute (5 day) nature of the exposure.

Spinosad is slightly toxic to fish and has a moderate half-life (30 – 200 days in water). The spinosad LOEC value for early life stage rainbow trout, 0.962 ppm (EPA 1997), is

100 to 1000 times higher than concentrations (0.75 and 7.5 ppb) used in the current study. At these low environmentally relevant concentrations, spinosad had no sub-lethal effects on early life stage fish in our study.

We were unable to conclusively determine whether or not zebrafish early life stages are a suitable surrogate for early life stage salmon due to loss of comparative power during the experiments. Findings for only two of the three common endpoints measured were conclusive for both for these species: survival, immune function and prey capture analyses were conclusive for zebrafish; but only survival was conclusive for salmon. This severely limited our ability to adequately compare the response of these two species to the same test conditions. As mentioned above, future experiments with additional timepoints and endpoints are needed to more robustly evaluate zebrafish as salmon surrogates.

The long-term goal of our work is to provide risk assessors, blueberry growers, and restoration managers with data to aid in making science-based decisions regarding blueberry pesticide Best Management Practices in Maine. We have little information to determine whether pesticides that are commonly in use now and pesticides that generally have fewer environmental effects and might be available in the near future (1-2 years) have detrimental effects on fish early life stages, and no information on whether combining these pesticides with other stressors, such as temperature or acidity, could produce unexpected adverse effects.

The results of this research provide the basis for more extensive studies on the sub-lethal effects of blueberry pesticides, alone and in environmentally relevant combinations, in the presence and absence of other stressors found in Downeast rivers, such as acid-aluminum. The results of such studies could influence which pesticides are used in Maine, could indicate whether Best Management Practices for currently-used and proposed for use pesticides need to be refined to further reduce potential aquatic contamination, and provide information relevant to proposed mitigation of acidic waters in salmon rivers.

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