MODULE 4 SPECIAL STUDIES

4.1

ESTROGENICITY OF WASTEWATER

4.1 Monitoring estrogen active compounds in wastewater effluent and determination of novel biological effects in zebrafish (*Danio rerio*)

FINAL REPORT

2006-2007

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SURFACE WATER AMBIENT TOXIC MONITORING PROGRAM
DIVISION OF ENVIRONMENTAL ASSESSMENT
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Background and Objectives:

The occurrence of pharmaceuticals and personal care products in the aquatic environment is of growing concern in the industrialized world. One class of pharmaceutically derived environmental contaminants includes the synthetic estrogens commonly found in oral contraceptives and hormone replacement therapies. Synthetic estrogens such as these mimic natural estrogens at the receptor level, but are more resistant to degradation by natural processes [1]. Because of its greater stability and higher potency in vivo, the synthetic estrogen 17α-ethinylestradiol (EE_2) may be of disproportional toxicological importance despite being found at much lower concentrations than natural steroids such as 17β-estradiol (E_2) or estrone (E_1).

POTWs handle domestic and industrial wastes in the state of Maine. Effluents from POTW's are often chemical mixtures of a variety of xenoestrogens [2-4]. Domestic wastewater treatment effluent can potentially contain significant levels of natural and synthetic hormones such as E_2, E_1 and EE_2 [2-4]. Estradiol and ethinylestradiol are the most potent of the potential estrogenic compounds found in wastewater treatment effluent, followed closely by metabolites of E_2 E_1 and estriol (E_3) [3,5]. It has been shown that conjugated forms of estrogens, such as naturally excreted metabolites, can be activated during wastewater treatment processes [6,7]. The degree of percent reactivation of conjugated estrogen metabolites is dependant upon treatment type, retention time, chemical modulation, and variable other factors in the wastewater treatment process.

Most estrogens are known to exert effects at very low concentrations, in the ng/L range. A USGS survey of more than 100 U.S. streams revealed median concentrations of 73ng/L for ethinylestradiol, 30ng/L for estradiol, 27ng/L for estrone, 800ng/l for nonylphenol and 140ng/L for bisphenol A, showing these compounds are present in the aquatic environment at sufficient concentration to exert biological effects[8]. Many of these compounds are persistent, lipophilic and tend to bioaccumulate in aquatic organisms [9]. Roach (*Rutilus rutilus*) downstream of sewage treatment plants in England were found to have a high level of intersexuality, fish having both male and female gonadal characteristics [10]. Other studies exposing fish to wastewater treatment effluent have shown that exposure to even minimal levels of estrogens in effluent result in increased plasma vitellogenin in male fish [11]. Additionally, xenoestrogens have been linked to changes in sex ratio, embryonic damage, and reduced fecundity in various vertebrate species [12]

The objectives of this study were to:

1. Determine estrogenicity of undiluted wastewater effluent from multiple publicly owned treatment works (POTWs) that discharge effluent into the Penobscot River, Maine.

2. Determine estrogen-induced, biological effects of undiluted effluent, mixing zone water, and river water from downstream transects at multiple POTWs.

Materials and Methods:

MVLN Cell Exposure and Luminescence Assay

MVLNs, MCF-7 human breast cancer cells stably transfected with luciferase reporter gene downstream of *Xenopus laevis* vitellogenin promoter, were developed by Dr. Michael Pons and graciously donated by Dr. John P. Giesy [72]. Cells were maintained in 1:1 DMEM and Ham's F-12 media with phenol red and exposed to treatments in 1:1 DMEM and Ham's F-12 media without phenol red to reduce estrogenic interference. The vitellogenin promoter region is characterized by four estrogen responsive elements. Relative vitellogenin expression was determined by measuring luminescence after cell exposure. Luciferase is produced in response to estrogen receptor agonists. To ensure that estrogen agonists activated transcription via estrogen responsive elements, the estrogen antagonist ICI-182780 was utilized as a negative control to ensure that luminescence was estrogen receptor mediated. Cells were maintained and exposed according to sterile technique. All waters were sterile filtered using 0.2µm Acrodisc syringe filters (Gelman Sciences, Ann Arbor, MI.). MVLN cells were exposed to media alone or with added fish room water as negative controls and 17□-ethinylestradiol (10 nM) as the positive control., Efluent grab samples were sterile filtered and appropriate amounts were added to the test system. Assays were carried out in 96-well polystyrene microplates for 72 hours, consistent with protocols designed by the Giesy laboratory of Michigan State University [72]. All treatments consisted of water in media at 1:5 respectively to ensure adequate nutrients for cell growth. Luminescence was determined using the Promega Steady-Glo Luciferase Assay system and measured by the Packard Fusion™ plate reader (Perkin-Elmer, Inc., Wellesley, MA.)

Adult Zebrafish Exposures:

One year old zebrafish were maintained at the University of Maine zebrafish facility with a light:dark cycle of 14:10 hours. Prior to EE_2 exposure, 20 male and 20 female fish were placed in separate 3.5 liter tanks for each exposure regime with water from the University of Maine zebrafish facility (carbon filtered and UV treated Orono, ME city water, with 7.5 mg/L dissolved oxygen and 42 mg/L hardness)

and maintained at 27.6ºC. Aqueous 17□-ethinylestradiol (CAS 57-63-6, Sigma E4876) was diluted in ethanol to produce a stock concentration of 2 mg/L and added to tanks to yield final EE_2 concentrations of 1 ng/L, 10 ng/L or 100 ng/L. Maximum ethanol levels were 0.05%, two orders of magnitude below the lowest observed effect concentration of ethanol for zebrafish (Dlugos and Rabin 2003). Although no discernable difference in transcript abundance of NER genes could be detected between 0.05% ethanol exposed and unexposed zebrafish (data not shown), control fish were exposed to 0.05% ethanol under the same conditions as 17□-ethinylestradiol exposed fish for proper vehicle control. Experimental and control fish were exposed for 7 days in static water with complete renewal once per day. During daily water renewal, fish were visually inspected for overall health. Fish were fed commercially available fish food daily, two hours prior to water renewal to minimize any adherent interactions between food and 17□-ethinylestradiol.

RNA isolation:

Total RNA was isolated from pooled samples of five livers from the same sex adult fish using phenol-free total RNA isolation procedures (RNAqueous, Ambion). Fish were anesthetized by a brief immersion in ice water and immediately euthanized by a sharp blow to the head (Beaver et al. 2000). Liver and intestinal tissues were surgically removed, after which the liver was separated from intestinal tissue. Liver samples were lysed with 500 µl cold guanidinium thiocyanate lysis/binding solution, manually homogenized and diluted with an equal volume ethanol. Samples were then bound to a glass fiber filter and washed three times with ethanol. Total RNA was eluted with 50-80 µl of 75ºC DNAse/RNAse free water (Invitrogen) and stored at -80ºC. Three to five distinct RNA samples were collected for each experimental and control exposure. RNA integrity and concentration was assessed utilizing micro-capillary electrophoresis with an Agilent 2100 bioanalyzer (Agilent). One microliter of total RNA from each sample was compared to 1 µl of RNA ladder (RNA 6000 ladder Ambion) with a known concentration of 150 ng/µl and six RNA transcripts of various sizes. RNA quality was verified by comparing corresponding 18S and 28S peaks on electropherograms for each sample tested. Only intact RNA was used for further analysis.

Primer design:

Sequences for zebrafish NER genes were obtained from GenBank and Ensembl whole genome databases. cDNA sequences from multiple organisms were aligned and used to validate NER sequences in the Ensembl *Danio rerio* genomic database. Primer 3 software (http://frodo.wi.mit.edu/cgi-

bin/primer3/primer3_www.cgi) was used to design primers with appropriate quantitative RT-PCR specifications: 18-25 nucleotide length and GC content of 40-65%. NCBI's basic local alignment search tool (BLAST) was used to verify primer specificity. Amplicons from RT-PCR reactions were sequenced to ensure correct gene products. Primers used for amplification of their corresponding gene products are listed in Table 1.

Quantitative RT-PCR:

Fluorescence based quantitative RT-PCR was performed using the MX4000 Multiplex Quantitative PCR system (Stratagene). Each reaction contained SYBR green RT-PCR master mix (0.2 mM each dNTP, $MgCl_2$, Taq polymerase, 10 nM fluorescein, SYBR green dye and stabilizers, BioRad), forward and reverse primers (30 nM – 150 nM final concentration), ROX reference dye (Invitrogen), 25 ng total RNA, iScript reverse transcriptase (BioRad) and nuclease free water. cDNA synthesis was carried out at 50ºC for 10 minutes, followed by 5 minutes at 95ºC for reverse transcriptase inactivation. Forty cycles of amplification and fluorescence data collection were carried out with a two-step PCR of 10 seconds at 95ºC and 30 seconds at 55ºC. Dissociation curves were created with a 1 minute denaturation step at 95ºC, followed by a ramp of 41 cycles starting at 55ºC for 30 seconds and increasing 1 degree every cycle. Relative change in transcript abundance was normalized to 18S rRNA and calculated utilizing the $2^{-\Delta\Delta Ct}$ analysis method (Livak and Schmittgen 2001). Prior to analysis, amplification efficiency was examined using LinRegPCR software, which calculates efficiency based on raw real-time PCR data (Ramakers et al. 2003). Efficiencies for normalizing gene (18s) and all other transcripts were the same (1.8 ± 0.1). Control expression levels were normalized to a value of 1. Each RNA sample was run in triplicate with three to five samples per exposure regime. A single peak in all dissociation curves verified production of a single amplicon per primer pair.

Statistics:

Quantitative RT-PCR data were analyzed using one way analysis of variance (ANOVA). Equal variance and normality were validated on raw Ct values prior to ANOVA. Normality of error was assessed with Lillifors test. Equal variance of samples was assessed with plots of estimates versus studentized residuals and modified Levene's test. One way ANOVA was performed on $\Delta\Delta$Ct values for a given gene for all treatments. When statistically significant differences were found between treatment groups ($p<0.05$), Dunnett's test was used to determine which experimental treatments were significantly different from controls. To validate that EE_2 exposure did not alter 18S rRNA abundance, Ct values were

analyzed by one-way nested ANOVA and $p>0.8$ was used to determine no significant difference between treatments. All statistical analyses were done using SigmaStat 3.0 (SYSTAT Inc.) or SYSAT 11 software (SYSTAT Inc.).

Results and Discussion:

In vitro reporter assay based estrogenicity of wastewater

Estrogenic potential of wastewater effluent, as assessed by an in vitro reporter assay, was significantly elevated compared to control at all three test sites (Figure 1). The amount of relative estrogenic potential varied amongst sampling periods with all three treatment facility effluents having the highest reporter gene transactivation during the months of October-January. Summer months were not reported, but had a generally lower trend of estrogenicity. Peak reporter gene expression levels remained below that for the positive control of 10 nM 17□-ethinylestradiol.

This data was in contrast with *in vivo* data where estrogenic activity of wastewater was assessed by measuring transcription of hepatic vitellogenin mRNA concentrations (Figure 2). This assay also revealed an estrogenic potential for effluent from all treatment facilities. However, there was no particular temporal trend in estrogenicity of the effluents. Instead, peaks in vitellogenin expression after 7-day exposures were random depending upon sample sight. Interestingly, in February of 2007, estrogenic potential based upon reporter gene transactivation was higher in Old Town than in either Bangor or Orono. In this same sampling/exposure series, adult male zebrafish exposed to Old Town effluent also had a significantly elevated amount of hepatic vitellogenin. Together, *in vitro* and *in vivo* based assays proved to be a good combinatorial approach to determine estrogenic potential of wastewater.

Females exposed to wastewater treatment effluent (or positive controls) were not a good in vivo model to determine estrogenicity based upon vitellogenin production. In general, females have a dampened response to estrogenic upregulation of vitellogenin (Figure 3). In this set of experiments, vitellogenin levels in female zebrafish exposed to effluent in December/January were depressed indicating a novel control mechanism, or more likely confounding factors in the effluent mixture that caused a sexually dimorphic response in females. In future studies, we will only asses the vitellogenin levels of male zebrafish as an indicator of estrogen exposure.

Taken together, reporter gene analysis and whole animal studies both revealed the presence of estrogenic compounds in effluents of Old Town, Orono, and Bangor wastewater treatment facilities. Levels of estrogenic compound were not sufficient over the course of our assessments to elicit an effect greater than the equivalent of 10 nM 17□-ethinylestradiol.

In addition to vitellogenin analysis, we assayed the transcriptional response of the cytochrome P450 gene, CYP1A1. Activity of p450 function is known to be depressed upon estrogen exposure in teleosts. In controlled laboratory experiments with the semi-synthetic estrogen 17□-ethinylestradiol, we have shown that transcriptional activtion of CYP1A1 is depressed in the liver after estrogen exposure. In our assays with wastewater effluent, however, this was not the case. In both males and females CYP1A1 mRNA abundance was slightly elevated after wastewater effluent exposure (Figure 4-5). We have attributed this to one or more compounds in the effluent milleau that induces CYP1A1 expression. This corresponds to the fact that CYP1A1 has been shown to be decreased after estrogen exposure, but not in studies where estrogen and an inducer of CYP were co-administered.

Lastly, our laboratory has recently discovered a novel effect of the oral contraceptive estrogen, 17□-ethinylestradiol, in the regultion of DNA repair processes. In brief, ethinylestradiol dampens trnscription of several zebrafish hepatic nucleotide excision repair genes and hinders repair of induced DNA damage in zebrafish liver cells (data not shown). To determine if the estrogenic compounds found in wastewater effluent elicited the same effects as ethinylestradiol laboratory exposures, we assayed zebrafish livers after exposure to effluent from Bangor and OldTown. Hepatic mRNA levels of the nucleotide excision repair gene XPC were decreased after 7-day exposure to wastewater effluent (Figure 6). This is a significant finding because the nucleotide excision repair process repair DNA lesions caused by many ubiquitous carcinogens such a benzo(a)pyrene. In future studies we hope to further elucidate the effects of wastewater effluent on nucleotide excision repair processes.

Reference List

1. Jurgens,M.D. *et al.* The potential for estradiol and ethinylestradiol degradation in English rivers. *Environmental Toxicology and Chemistry* **21**, 480-488 (2002).

2. Bruchet,A., Prompsy,C., Filippi,G. & Souali,A. A broad spectrum analytical scheme for the screening of endocrine disruptors (EDs), pharmaceuticals and personal care products in wastewaters and natural waters. *Water Science and Technology* **46**, 97-104 (2002).

3. Desbrow,C., Routledge,E.J., Brighty,G.C., Sumpter,J.P. & Waldock,M. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environmental Science & Technology* **32**, 1549-1558 (1998).

4. Servos,M.R. *et al.* Distribution of estrogens, 17 beta-estradiol and estrone, in Canadian municipal wastewater treatment plants. *Science of the Total Environment* **336**, 155-170 (2005).

5. Gutendorf,B. & Westendorf,J. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. *Toxicology* **166**, 79-89 (2001).

6. Kirk,L.A., Tyler,C.R., Lye,C.M. & Sumpter,J.P. Changes in estrogenic and androgenic activities at different stages of treatment in wastewater treatment works. *Environmental Toxicology and Chemistry* **21**, 972-979 (2002).

7. Panter,G.H., Thompson,R.S., Beresford,N. & Sumpter,J.P. Transformation of a non-oestrogenic steroid metabolite to an oestrogenically active substance by minimal bacterial activity. *Chemosphere* **38**, 3579-3596 (1999).

8. Kolpin,D.W. *et al.* Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. *Environmental Science & Technology* **36**, 1202-1211 (2002).

9. Aguayo,S. *et al.* Identification of organic compounds and ecotoxicological assessment of sewage treatment plants (STP) effluents. *Science of the Total Environment* **328**, 69-81 (2004).

10. Jobling,S., Nolan,M., Tyler,C.R., Brighty,G. & Sumpter,J.P. Widespread sexual disruption in wild fish. *Environmental Science & Technology* **32**, 2498-2506 (1998).

11. Rodgers-Gray,T.P. *et al.* Long-term temporal changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. *Environmental Science & Technology* **34**, 1521-1528 (2000).

12. Pawlowski,S. *et al.* Combined *in Situ* and *in Vtiro* Assessment of the Estrogenic Activity of Sewage and Surface Water Samples. *Toxicological Sciences* **75**, 57-65 (2003).

13. Gagne,D. *et al.* Stable Luciferase Transfected Cells for Studying Steroid-Receptor Biological-Activity. *Journal of Bioluminescence and Chemiluminescence* **9**, 201-209 (1994).

14. Wjayaratne,A. *et al.* Comparative analyses of mechanistic differences among antiestrogens. *Endocrinology* **140**, 5828-5840 (1999).

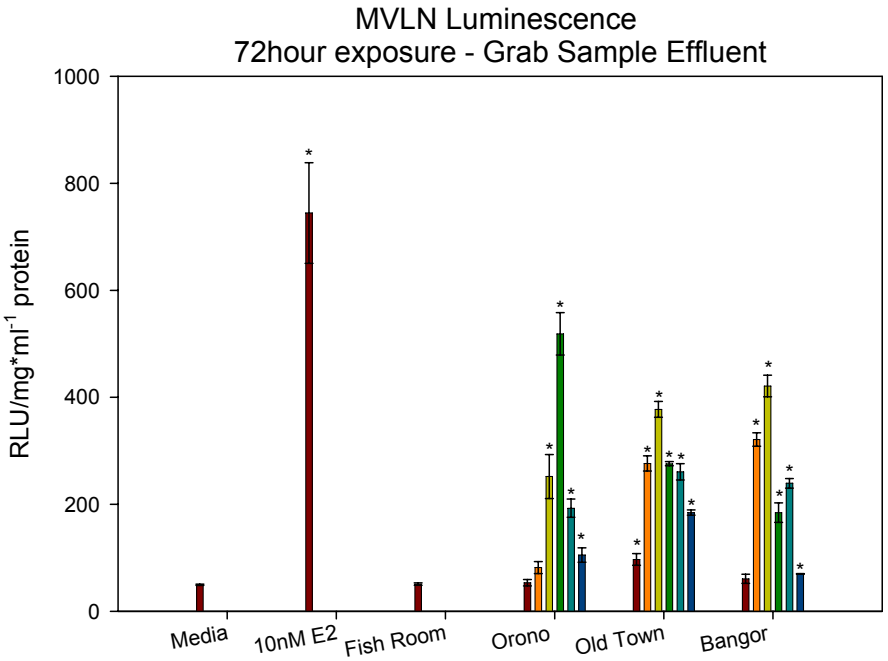

Figure 1. Reporter gene analysis showed that effluent from Orono, Old Town, and Bangor wastewater treatment facilities elicited an estrogen receptor mediated response *in vitro*. This response varied between sampling periods, with a trend in higher responses over the winter months. At no sampling period did effluent induced

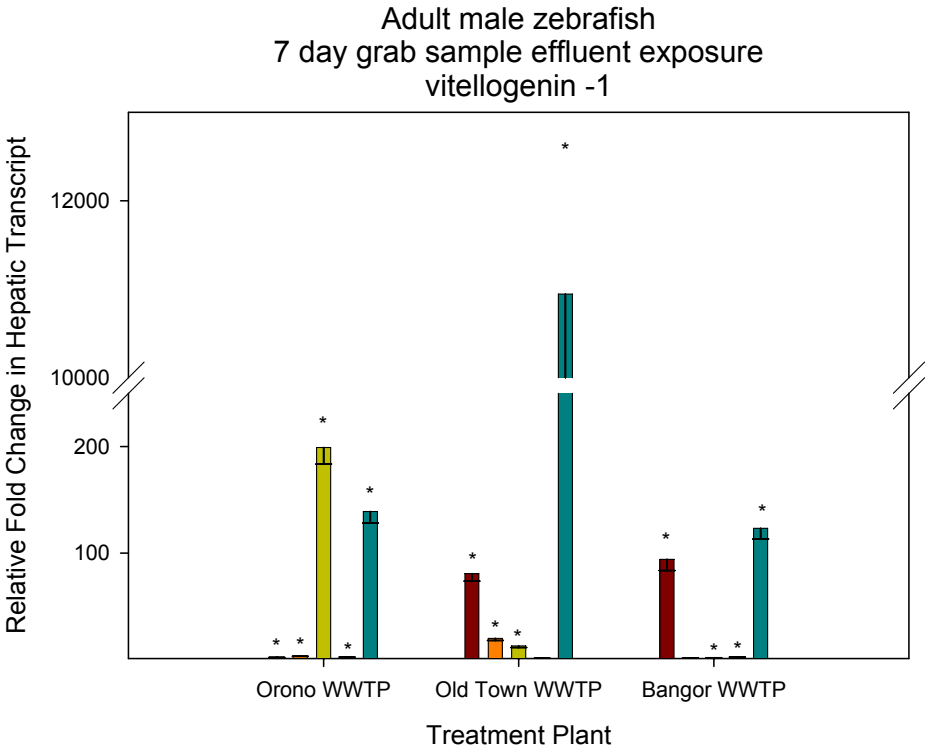

Figure 2. Adult male zebrafish exposed to wastewater effluent exhibited a mixed vitellogenic response. Only in February at the Old Town treatment facility did hepatic vitellogenin mRNA concentration rise in great enough magnitude to warrant concern. For comparison, increases in hepatic vitellogenin

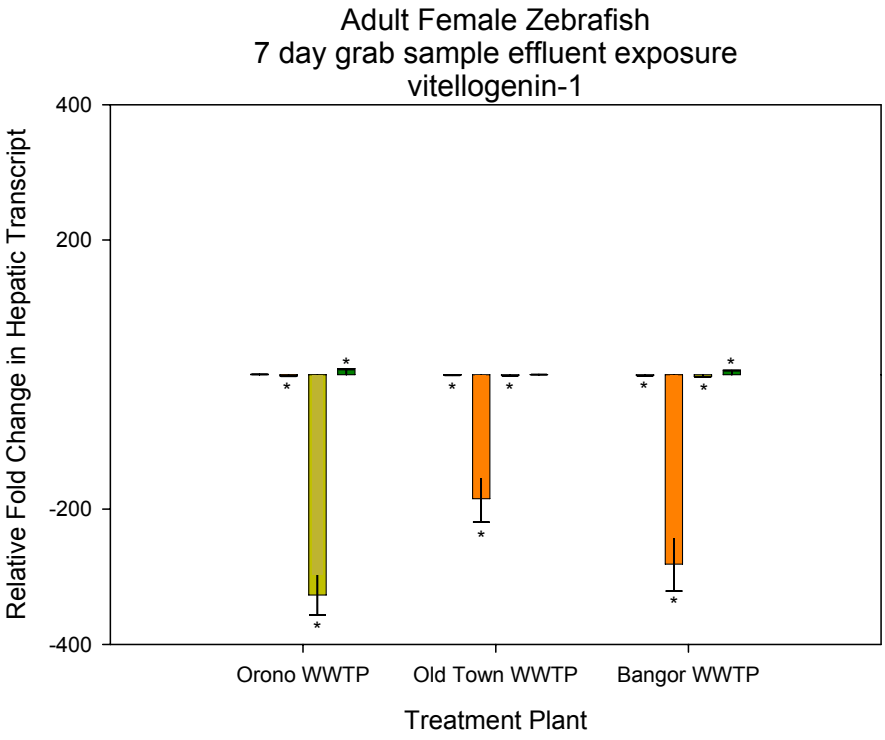

Figure 3.: Adult female zebrafish vitellogenin mRNA levels were drastically decreased after exposure to wastewater effluent at Dec. and Jan. samplings. This is a unknown phenomenon that the authors cannot explain except to attribute this effect to some other compound in the effluent. Little to no change in female

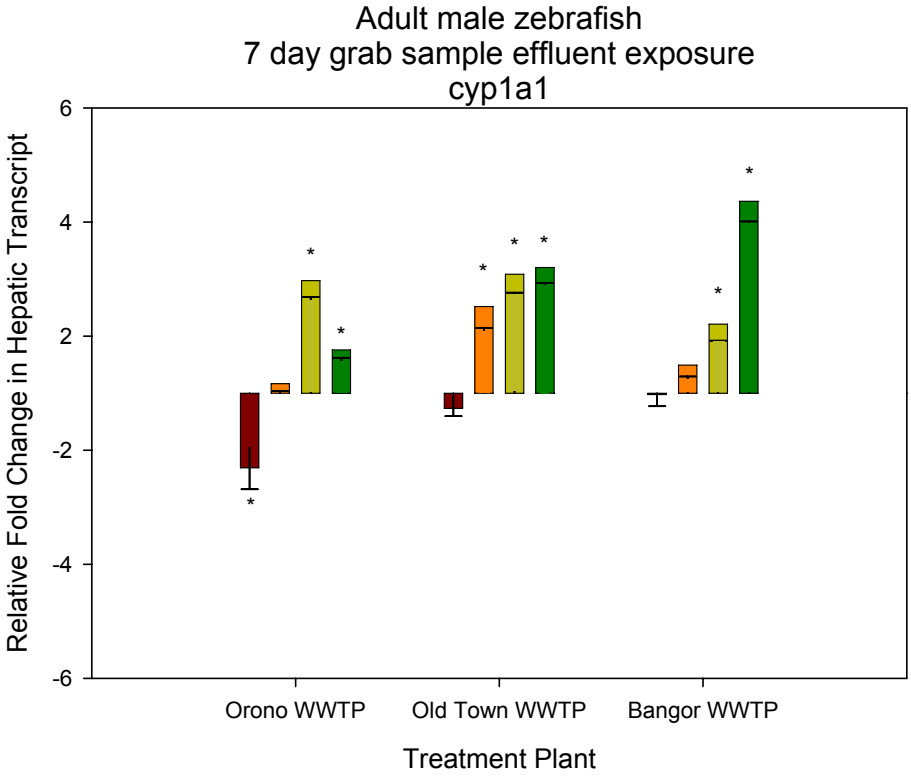

Figure 4. Adult male zebrafish are known to have reduced mRNA abundance of cyp1A1 after estrogen exposure. However, estrogen does not exhert this effect when a P450 inducer is also present in the exposure. We suggest that the increase in cyp1A1 expression is due to P450 inducers in the wastewater. This is very

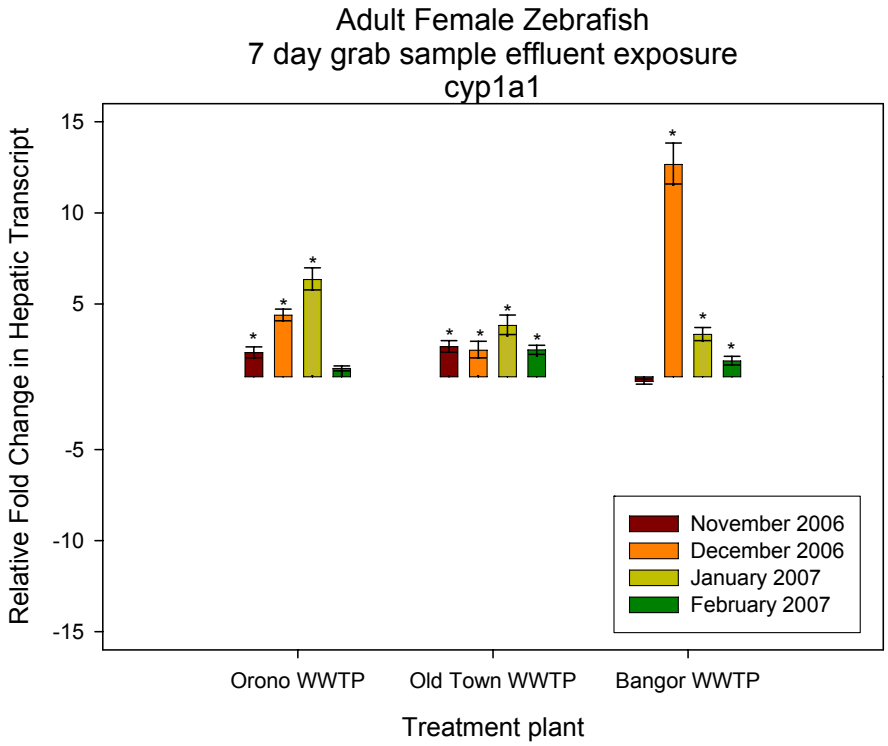

Figure 5. As in figure 4, females exhibited an increase in CYP1A1 levels after effluent exposure. Female response was generally greater than male response to these effluents.

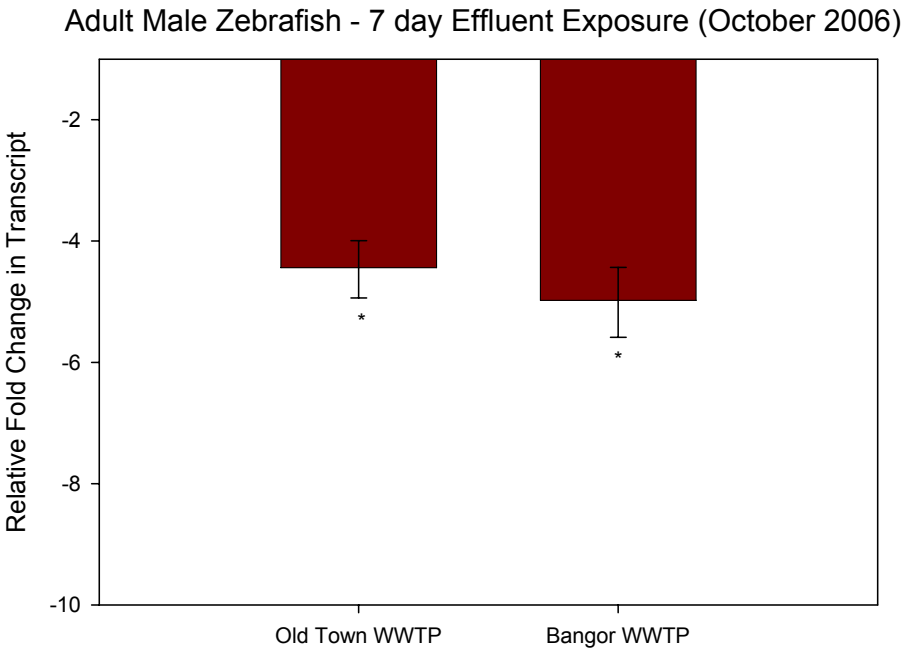

Figure 6. Male zebrafish exposed to Old Town or Bangor wastewater effluent had reduced expression of the nucleotide excision repair gene, XPC. This data corroborates our laboratories findings that 17a-ethinylestradiol dampens nucleotide excision repair. Although not a part of this project, this

4.2

EFFECTS OF BLUEBERRY PESTICIDES ON FISH

In Cooperation with the Maine Department of Environmental Protection

Pilot Study of Sublethal Effects on Fish of Pesticides Currently Used and Proposed for Use on Maine Blueberries

By Adria A. Elskus

Open-File Report 2007–1110

U.S. Department of the Interior

U.S. Geological Survey

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Conversion Factors and Abbreviations

SI to Inch/Pound

Multiply	By	To obtain
	Length	
centimeter (cm)	0.3937	inch (in.)
millimeter (mm)	0.03937	inch (in.)
	Area	
square centimeter (cm^2)	0.001076	square foot (ft^2)
square centimeter (cm^2)	0.1550	square inch (ft^2)
	Volume	
liter (L)	33.82	ounce, fluid (fl. oz)
liter (L)	2.113	pint (pt)
liter (L)	1.057	quart (qt)
liter (L)	0.2642	gallon (gal)
liter (L)	61.02	cubic inch (in^3)
	Flow rate	
milliliter per minute (mL/min)	0.0002642	gallon per minute (gal/min)
	Mass	
milligram (mg)	0.00003527	ounce, avoirdupois (oz)
	Pressure	
kilopascal (kPa)	0.009869	atmosphere, standard (atm)
kilopascal (kPa)	0.01	bar
kilopascal (kPa)	0.1450	pound-force per inch (lbf/in)
kilopascal (kPa)	20.88	pound per square foot (lb/ft^2)
kilopascal (kPa)	0.1450	pound per square inch (lb/ft^2)

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows:
°F=(1.8×°C)+32
Temperature in degrees Fahrenheit (°F) may be converted to degrees Celsius (°C) as follows:
°C=(°F-32)/1.8

Abbreviations:

ng, nanogram
nm, nanometer
mL, milliliter
µL, microliter
ppb, parts per billion
uv, ultraviolet

Pilot Study of Sublethal Effects on Fish of Pesticides Currently Used and Proposed for Use on Maine Blueberries

By Adria A. Elskus

Abstract

Blueberry pesticides have been detected consistently in some Down East Maine rivers, yet little is known about the sublethal effects of these pesticides on fish early life stages. The Maine blueberry industry is proposing to replace the insecticide Imidan™ (active ingredient phosmet) and the herbicide Velpar™ (active ingredient hexazinone), two of the pesticides found in these rivers, with candidate alternatives SpinTor ™ (active ingredient spinosad) and Callistso ™ (active ingredient mesotrione). Our objective is to evaluate potential sublethal effects of these four formulations before the industry adopts the two candidate alternatives. We exposed zebrafish (*Danio rerio*) early life stages, from fertilization through larval swim-up, to a range of pesticide concentrations and evaluated their response relative to untreated controls. In this report we provide preliminary data on immune function as well as on parameters in addition to those originally proposed: development and performance fitness. We also provide information on our progress towards optimizing chemical protocols for analyzing the concentration of active ingredient in each of our formulation dosing solutions, another new parameter we added to those originally proposed.

Preliminary results indicate that at environmentally realistic concentrations, these pesticides may have no significant effect on innate immunity, development rate or behavior (spontaneous swimming), however further replication is needed to confirm these initial findings. We have also observed some degree of developmental abnormalities in both pesticide-treated and control zebrafish embryos; however, additional replication is underway to determine if these groups differ significantly.

Background

Dramatic declines in Atlantic salmon (*Salmo salar*) populations in the northeastern U.S. have led to the complete loss of wild salmon in all New England states except for eight rivers in Maine where Atlantic salmon are now listed as an endangered distinct population segment (DPS) (National Research Council, 2004). Despite intensive efforts to restore Maine populations through juvenile stocking programs, adult returns continue to be well below the estimated carrying capacities of these rivers (Maine Atlantic Salmon Task Force, 1997).

The Maine Board of Pesticide Control has consistently detected blueberry pesticides, including phosmet and hexazinone, in certain DPS rivers (Jackson, 2003). With little to no data on the relative risk to salmon health posed by blueberry pesticides, restoration managers cannot rule out the possibility that these pesticides are hampering recovery and restoration efforts for endangered Maine Atlantic salmon.

Early life stages are considered the most sensitive to stressors, and because biochemical, hormonal and morphological changes that occur during early development are irreversible,

toxicant exposure during early life is almost certain to have permanent effects on populations (Lawrence and Hemingway, 2003). Indeed, developmental exposure to atrazine, a triazine related to hexazinone, produced altered immune function in adulthood (Rooney and others, 2003). Moreover, a broad range of species may be affected since pesticides, including organophosphates and triazines, deleteriously affect immune function in a wide range of invertebrate and vertebrate species (Dunier, 1996; Galloway and others, 2003).

Many pesticides exert their toxicity during metabolism and do not accumulate in biological tissues, including the pesticides being evaluated in the current study. Consequently, body burden analysis, as is done in many government-funded monitoring programs to evaluate the effect of persistent organic contaminants, will not reflect pesticide exposure or effect. Instead what is necessary is to characterize the exposure regime and define effect levels in carefully controlled exposure studies.

For this pilot study, the U.S. Geological Survey, in cooperation with the Maine Department of Environmental Protection, used zebrafish, a well-characterized aquatic toxicology model for which a sensitive assay of immune system function has recently been developed (Hermann and others, 2004), whose development is well-documented (Westerfield, 1993) and for which behavioral assays have been published (Samson and others, 2001). This paper presents the preliminary results of this study.

Objectives

The original objectives of this study were to:

1. Evaluate the immunotoxic effects on fish early life stages of two currently used blueberry pesticides, phosmet and hexazinone, consistently detected in Maine Down East rivers.
2. Evaluate the immunotoxic effects of the proposed alternative pesticides, spinosad and mesotrione.
3. Determine whether pesticide mixtures reflective of those observed for currently used pesticides in Maine Down East rivers have additive, synergistic, or antagonistic effects on immune function.
4. Provide preliminary information to the Maine Board of Pesticide Control regarding the sublethal effects of candidate pesticides on fish before they are adopted by Maine blueberry growers.

Additional objectives subsequently incorporated into this study were to:

5. Evaluate developmental and behavioral effects on fish early life stages of two currently used pesticides, phosmet and hexazinone, and their proposed alternatives, spinosad and mesotrione.
6. Develop and optimize analytical protocols for measuring aqueous concentrations of the currently used pesticides, phosmet and hexazinone, and their proposed alternatives, spinosad and mesotrione.

Materials and Methods

The materials and methods used during the course of this pilot study are presented below.

Materials

Pesticide formulations Imidan™ (Gowan), Velpar-L™ (DuPont), Callisto™ (Syngenta), and SpinTor™ (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 (H_2DCFDA) 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.).

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.

Dosing Solutions

Dosing concentrations were based on pesticide levels reported in the Pleasant River by the Maine Board of Pesticide Control for hexazinone and phosmet (Jackson, 2003). Velpar™, Imidan™, Callisto™, and SpinTor™ stock solutions were prepared in egg water and diluted to the desired concentrations. Solution concentrations were based on the concentration of active ingredient in the formulation. 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 (Velpar™+Imidan™, Callisto™ + SpinTor™), 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 as high (7.5, 30 ppb). Pesticide solutions were prepared within 2 to 3 h (hours) of the start of the experiment and then 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.

Confirmation of Pesticide Dosing Concentrations

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 at the start and conclusion of the 5-d (day) exposure periods. While protocols for extracting phosmet and hexazinone from river water have been established (L.B. Perkins, University of Maine, oral comm.), there are few protocols for the candidate pesticides, mesotrione and spinosad. With guidance from Dr. L. Brian Perkins (University of Maine) and Dr. Larry LeBlanc (University of Maine) we have 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. Dr. L. Brian Perkins (University of Maine) is working with us to optimize 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 SBD-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 μL, 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 μL) onto a Hewlett Packard High Performance Liquid Chromatograph Series 1050 fitted with a C-I8 column (100 x 4.6 mm). We used a mobile phase of 75 percent ACN, 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.

Pesticide Exposures

Zebrafish embryos were exposed to pesticides or egg water (0.6-percent Instant Ocean prepared in nanopure 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 and others, 2004). Treatment water in the petri dishes was renewed daily. 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 larvae 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.

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.

Innate Immune Function

We evaluated respiratory burst, a simple immune system assay, as described by Herman and others (2004)). Briefly, on Day 4 zebrafish larvae were transferred from exposure dishes to black 96 well plates, one larva per well, and exposed to either substrate alone (H_2DCFDA, 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 H_2DCFDA (a non-fluorescent dye) to dichlorofluorescein (DCF, a fluorescent product). In fish with a healthy immune system, PMA exposure in the presence of H_2DCFDA 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 Fusion™ fluorescence plate reader at an excitation/emission of 485nm/530nm.

Behavioral Assays

We evaluated spontaneous swimming using the protocol described by Samson and others (2001). Briefly, larvae were placed individually into 100 mL of egg water in an 8-cm-diameter finger bowl placed over a 1-cm^2 grid. After a 2-min acclimation period, the number of lines crossed in 30 sec (seconds) by the larvae was recorded. Five random larvae were tested from each replicate for each treatment on Days 4 and 7 post-fertilization. A second behavioral assay, prey capture, a well-established measure of performance fitness in fish (Samson and others, 2001) is currently underway.

Results of Preliminary Studies on the Effects of Blueberry Pesticides on Fish

A discussion of preliminary study results follows and includes a presentation of dosing solution concentrations, innate immune function, mortality, time to hatch, developmental abnormalities, and spontaneous swimming.

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. We are currently in the process of obtaining surrogates for spinosad (spinetoram) and mesotrione (sulcotrione) from the manufacturers as they are not available from the USEPA National Pesticide Repository. These surrogates will be used to spike the dosing solutions prior to extraction through SPEs and, with internal standard spikes, will be used to correct for analyte recovery.

Preliminary analyses of the pesticide dosing solutions indicate that the actual (measured) concentrations are close to nominal concentrations for Imidan™ and Velpar™ (table 1).

Table 1. 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™ plus Imidan™	Low	hexazinone	0.75	0.35
		phosmet	0.75	0.21
	High	hexazinone	7.50	5.6
		phosmet	7.50	5.0

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 embryo-larval zebrafish (fig. 1). In these initial experiments, the zebrafish response was monitored for 2 h.

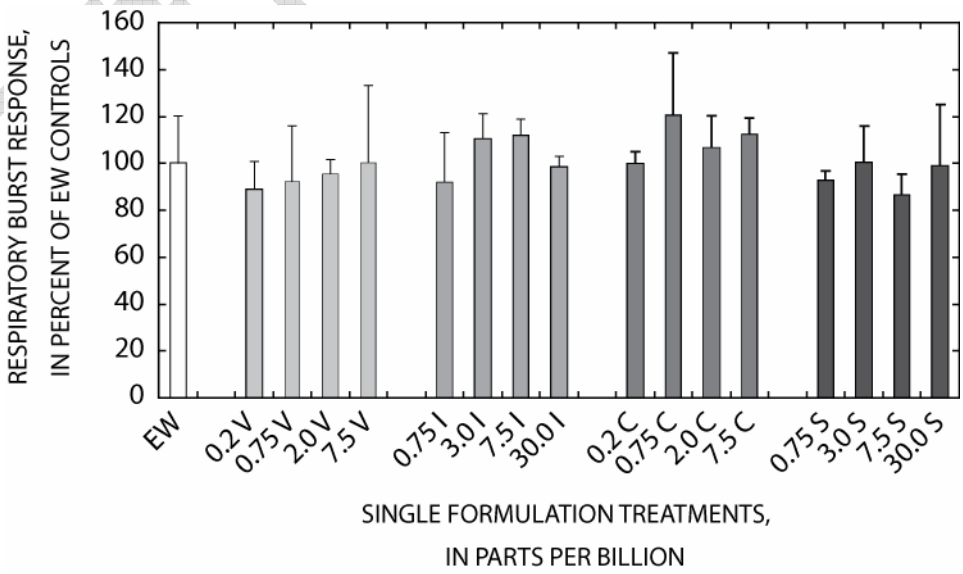

Figure 1. Preliminary data on the effects of exposure to single pesticide formulations on the innate immune system of zebrafish embryo-larvae exposed from fertilization through swim-up larvae. Bars represent means ± SD for n=2-4 replicates of 6 larvae per replicate. EW=Egg Water, V=Velpar™, I = Imidan™, C = Callisto™, S = SpinTor™. 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 3.5 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 (fig. 2).

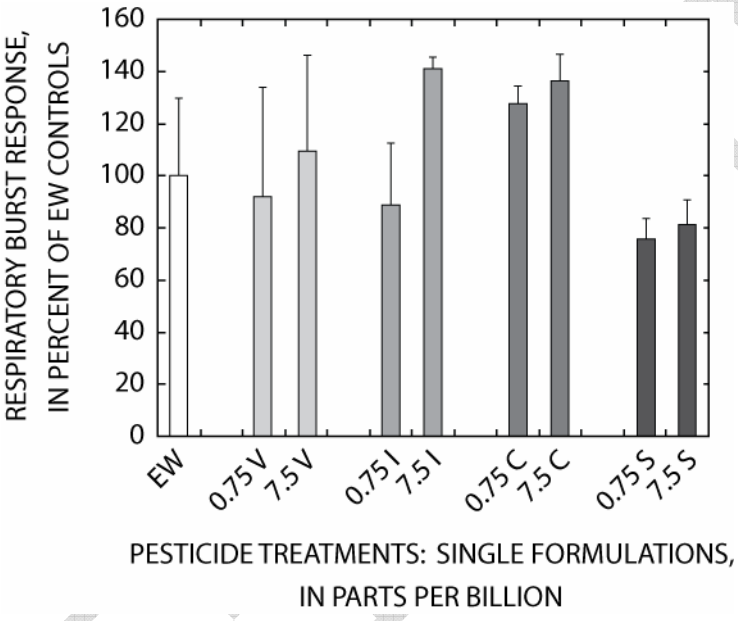

Figure 2. Preliminary data on the effects of exposure to single pesticide formulations on the innate immune system of zebrafish embryo-larvae exposed from fertilization through the swim-up larval stage. Bars represent means ± SD for n=2-4 replicates of 6 larvae per replicate. EW=Egg Water, V=Velpar™, I = Imidan™, C = Callisto™, S = SpinTor™. Doses were 0.75 and 7.5 ppb. Respiratory burst was measured for 3.5 hours.

Due to ground-water contamination in the watershed, hexazinone 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 a variety of doses. Preliminary results (for one replicate per mixture) indicate mixtures do not affect the respiratory burst response of developing zebrafish (fig. 3); however, further replication is needed before firm conclusions can be made.

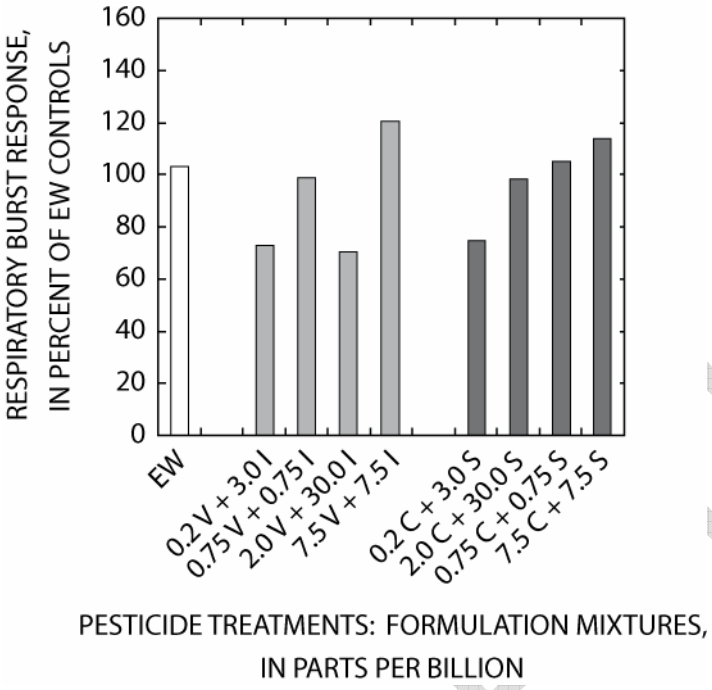

Figure 3. Preliminary data on the effects of exposure to mixtures of pesticide formulations on the innate immune system of zebrafish embryo-larvae exposed from fertilization through the swim-up larval stage. Bars represent means for one replicate, 6 larvae per replicate. EW=Egg Water, V=Velpar™, I = Imidan™, C = Callisto™, S = SpinTor™. Doses were combinations of 0.2, 0.75, 2.0, 3.0, 7.5, and 30 ppb. Respiratory burst was measured for 3.5 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 the pesticide treated groups, with very few occurrences in the controls. These abnormalities included reduced growth, small head with small or no eyes, pericardial edema, and scoliosis. A few individuals were moribund. All individuals displaying these abnormalities failed to hatch. These data will be recorded quantitatively in future exposures.

Despite evidence of abnormal development, preliminary data indicate no significant difference 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 2).

Table 2. Mean days to hatch for zebrafish exposed to pesticide formulations from fertilization through swim-up larvae. Mean ± 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

Spontaneous Swimming

Preliminary data on the effects of pesticide exposure on spontaneous swimming show no definitive trend (fig. 4). The large variability in these data reflect the small sample size (n=2). Further experiments replicating this endpoint are underway to reduce this variability. Swimming activity and the ability to capture prey are related behaviors, which together provide a measure of performance fitness. Ongoing experiments evaluating prey capture in dosed zebrafish will be related to our results with spontaneous swimming to determine if alterations in prey capture may be related to alterations in swimming activity.

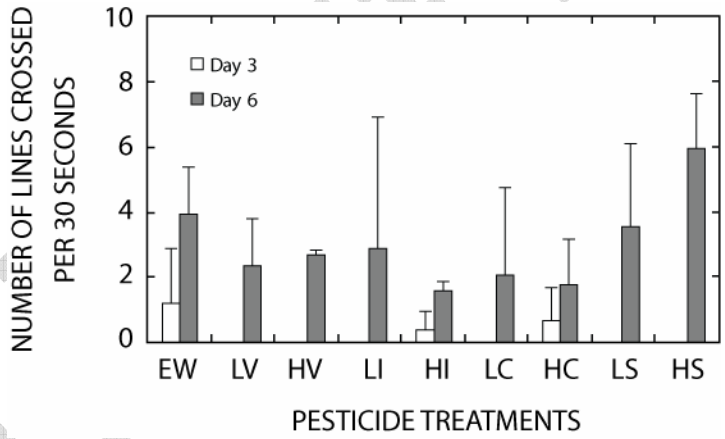

Figure 4. Preliminary data on single pesticide exposure effects on spontaneous swimming in zebrafish exposed from fertilization through swim-up. Bars represent means ± SD for n=2 replicates of 5 larvae per replicate. Swimming measured on Days 3 and 6 post-fertilization. EW=Egg Water, V=Velpar™, I = Imidan™, C = Callisto™, S = SpinTor™. Doses were 0.75 and 7.5 ppb for Low and High, respectively.

Conclusions and Additional Research

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 (University of Maine Cooperative Extension 2007). We have little to no 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.

Preliminary data from the physiological and behavioral assays indicate there are no effects of environmentally realistic, and 10 times as high, pesticide exposures, done singly or in binary mixtures, on developing zebrafish. These data are preliminary, and further replication of the assays described above is underway to assess their statistical significance.

Replication of these assays will increase the statistical power required to detect differences among treatments. These assay replications will be coupled with additional behavioral studies to assess the effects of these pesticides on the ability of larval zebrafish to capture prey, a sensitive assessment of performance fitness. We will continue to develop chemical protocols for analyzing aqueous solutions of the four pesticide formulations. These protocols will be used to measure the dosing concentrations at the start and end of the 5-d exposure periods. The results of these studies will be compared to published findings and the significance of these findings evaluated for the use of these pesticides in river systems that support developing fish.

The results of this research will provide the basis for more extensive studies on the sublethal effects of blueberry pesticides, alone and in environmentally relevant combinations, on resident fish species in the Down East rivers of Maine. 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 would provide data regarding the potential effects on resident fish of candidate pesticides before these pesticides come into use.

References Cited

Dunier, M., 1996, Water pollution and immunosuppression of freshwater fish: Italian Journal of Zoology, v. 63, p. 303-309.

Galloway, B.J., Munkittrick, K.R., Currie, S., Gray, M.A., Curry, R.A., and Wood, C.S., 2003, Examination of the responses of slimy sculpin (*Cottus cognatus*) and white sucker (*Catostomus commersoni)* collected on the Saint John River (Canada) downstream of pulp mill, paper mill, and sewage discharges: Environmental Toxicology and Chemistry, v. 22, p. 2898-2907.

Hermann, A.C., Millard, P.J., Blake, S.L., and Kim, C.H., 2004, Development of a respiratory burst assay using zebrafish kidneys and embryos: Journal of Immunological Methods, v. 292, p. 119-129.

Jackson, H.P., 2003, 2003 Drift study of two aerially applied blueberry pesticides: Augusta, Maine, Maine Board of Pesticide Control, 19 p.

Jackson, H.P., 2003, 2003 Drift study of two aerially applied blueberry pesticides: Augusta, Maine, Maine Board of Pesticide Control, 19 p.

Lawrence, A.J., and Hemingway, K.L., 2003, Effects of Pollution on Fish: Molecular Effects and Population Responses: U.K., Blackwell Publishing, 368 p.

Maine Atlantic Salmon Task Force, 1997, Atlantic Salmon conservation plan for seven Maine rivers: Maine Atlantic Salmon Task Force, March 1997, 434 p.

National Research Council, 2004, Atlantic salmon in Maine: Washington D.C., National Academies Press, 275 p.

Rooney, A.A., Matulka, R.A., and Luebke, R.W., 2003, Developmental atrazine exposure suppresses immune function in male, but not female Sprague-Dawley rats: Toxicological Sciences, v. 76, p. 366-375.

Samson, J.C., Goodridge, R., Olobatuyi, F., and Weis, J.S., 2001, Delayed effects of embryonic exposure of zebrafish (*Danio rerio*) to methylmercury (MeHg): Aquatic Toxicology, v. 51, p. 369-376.

University of Maine, 2007, Zebrafish Facility: Accessed April 10, 2007, at URL *http://www.umaine.edu/bmmb/zebrafish.htm*

University of Maine Cooperative Extension, 2007, Wild blueberry: Accessed April 10, 2007, at URL http://www.wildblueberries.maine.edu/

Westerfield, M., 1993, The zebrafish book: Eugene, Oregon, University of Oregon Press: Accessed on April 11, 2007, at URL *http://zfin.org/zf_info/zfbook/zfbk.html*