DEPLW-0848

SURFACE WATER AMBIENT TOXIC MONITORING PROGRAM

FINAL REPORT
EXECUTIVE SUMMARY
2006

DIVISION OF ENVIRONMENTAL ASSESSMENT
MAINE DEPARTMENT OF ENVIRONMENTAL PROTECTION
AUGUSTA, MAINE 04333

JUNE 2007

INTRODUCTION

This 2006 Surface Water Ambient Toxic (SWAT) monitoring program final report is organized into this Executive Summary (with introduction and table of contents) and 4 modules, 1) Marine & Estuarine 2) Lakes, 3) Rivers & Streams, and 4) Special Studies. The full report is available on DEP's website at http://www.maine.gov/dep/blwq/docmonitoring/swat/index.htm

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Acknowledgements
Collection of samples was conducted by the principal investigators and technical assistants listed (DEP staff unless otherwise specified) assisted by the Department of Inland Fisheries and Wildlife, and the Penobscot Indian Nation.

Chemical analyses were performed by AXYS Analytical Services, Sidney, British Columbia or other laboratories as listed in reports in individual sections.

EXECUTIVE SUMMARY

Maine's Surface Water Ambient Toxics (SWAT) monitoring program was established in 1993 (38 MRSA §420-B) to determine the nature, scope and severity of toxic contamination in the surface waters and fisheries of the State. The program must be designed to comprehensively monitor the lakes, rivers and streams and marine and estuarine waters of the State on an ongoing basis. The program must incorporate testing for suspected toxic contamination in biological tissue and sediment, may include testing of the water column and must include biomonitoring and the monitoring of the health of individual organisms that may serve as indicators of toxic contamination. This program must collect data sufficient to support assessment of the risks to human and ecological health posed by the direct and indirect discharge of toxic contaminants.

The Commissioner of the Department of Environmental Protection (DEP) must prepare a 5-year conceptual workplan that outlines monitoring approach for the following 5 years. The Commissioner must also develop annual workplans that define the work to be accomplished each year. A Technical Advisory Group (TAG), composed of 10 individuals with scientific backgrounds representing various interests and 1 legislator, is established to advise the Commissioner on the development of the 5-year framework and annual workplans.

The first 5-year framework, for the period 1994-1998, was an initial sampling of all watersheds in the state. The 5-year plans for the periods 1999-2003 and 2004-2008 were focused on problems discovered in the initial periods and were designed to confirm the initial findings and establish background conditions. Once those are established and a sufficient amount of time has elapsed, 5-10 years depending on what if any action has occurred to solve the problem, repeat sampling may be conducted to determine if the problem has been solved. The program also explores new issues as they are identified.

The SWAT program is divided into 4 modules, 1) Marine and Estuarine, 2) Lakes, 3) Rivers and Streams, and 4) Special Studies. This annual report follows the outline of the 2005 workplan recommended by the SWAT TAG in a meeting June 20, 2006. Following is a summary of key findings from the 2006 SWAT program for each module.

1. MARINE AND ESTUARINE

- Sediment monitoring occurred at one location, Mill Creek, Falmouth, in 2006. This location was selected to assess potential contamination from extensive development in upland areas, much of which is associated with Rte 1.

- Blue mussel monitoring occurred at five sites along the coast in 2006: Spruce Creek, Kittery; Back Cove, Portland; Cocktail Cove, Great Diamond Island, Portland; Mill Creek, Falmouth; and Taunton Bay, Franklin (four replicates per location).

- Lobster collections occurred at 19 stations over the southwestern half of the Maine coast in conjunction with the EPA National Coastal Assessment (NCA). 2006 marks the last of three years that DEP has sampled lobster in conjunction with NCA. When combined

with the data from the 2004 and 2005 lobster collections from other areas of the Maine coast, the 2006 data will complete geographic coverage of lobster from much of the Maine coast. DEP will continue to provide lobster data as it is reviewed and as more data arrives from the contracted laboratory. Pending review of the results, the data will be provided to the state toxicologist for use in updating public health advisories. It will also be posted on the DEP SWAT web site.

2. LAKES

- The US Fish and Wildlife Service is considering delisting the bald eagle from the Endangered Species List. A cooperative study of bald eagles with the Maine Department of Inland Fisheries and Wildlife, US Fish and Wildlife Service, Passamaquoddy Tribe, Penobscot Nation, FPL Energy, and BioDiversity Research Institute found that concentrations of mercury in nestling and adult eagles are higher than most other populations in the US and similar to those near mercury mines or other point sources. Mercury concentrations in 23-39% of Maine eagles are elevated or higher, and within the range of potential population impacts in certain hotspots. Concentrations have not diminished, and perhaps elevated, in some areas of Maine since the early 1990s.

- In 1996, mercury concentrations were found to be higher in fish and sediments from several lakes and ponds southeast (downwind) of Orrington, where there was the Holtrachem chloralkalai plant and PERC municipal waste combustor, than from the general population of Maine lakes. Samples collected from the same lakes in 2006 show that mercury levels in fish have not changed significantly since the Holtrachem plant closed in 2000.

3. RIVERS AND STREAMS

- Thirty-nine stations, primarily in the Penobscot River and North Coastal Rivers basins, were assessed for the condition of the benthic macroinvertebrate community. Results have been received to date (June 12, 2007) for twenty-three stations. Eleven of the twenty-three stations (48 %) reported failed to attain the aquatic life standards of their assigned class.

- Striped bass and bluefish exceed the Maine Center for Disease Control and Prevention's (MCDC) fish tissue action levels for mercury and PCBs in the Androscoggin, Kennebec, and Saco rivers as in past years. MCDC is leading a process with all other Atlantic coast states with significant fisheries for these fish to explore the desirability of a coast wide fish consumption advisory, since these species are coast wide migrants.

- A Cummulative Effects-driven Assessment of fish populations above and below Lincoln found little evidence of endocrine disruption, although lab data are pending. There was a small incidence of feminization of male fish below Millinockett and Lincoln in 2005

samples, but the amount (10%) is not much different from that commonly reported in the literature (5%) assumed to be background and of little consequence to the population. As in 2005, there was strong evidence for nutrient enrichment from the mills and municipal treatment plant discharges. A similar study above and below Skowhegan and the SAPPI mill on the Kennebec found no evidence of endocrine disruption, although lab samples are still pending. The nutrient enrichment measured on the Kennebec in 2004 was greatly diminished in 2006.

- A caged mussel study above and below the SAPPI bleached kraft pulp and paper mill on the Kennebec indicated nutrient enrichment, unlike the fish study. A similar study on the Penobscot above and below Lincoln, found no evidence of nutrient enrichment, unlike the fish study. As lab data are still pending, no conclusion about endocrine disruption can be made yet for either river.

4. SPECIAL STUDIES

- In a study of endocrine disruption in the Penobscot River, reporter gene analysis and whole animal studies with zebrafish both revealed the presence of estrogenic compounds in effluents of Old Town, Orono, and Bangor wastewater treatment facilities. Levels of estrogenic compound were, however, not sufficient over the course of our assessments to elicit an effect greater than the equivalent of 10 nM 17α-ethinylestradiol, the synthetic estrogen in human birth control pills.

- Preliminary laboratory studies of the potential replacement blueberry pesticides SpinTor ™ (active ingredient spinosad) and Callistso ™ (active ingredient mesotrione) indicate that at environmentally realistic concentrations, these pesticides may have no significant effect on innate immunity, development rate or behavior (spontaneous swimming) of zebrafish.. However further replication is needed to confirm these initial findings.

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MODULE 1 MARINE

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1.1 2006 Marine Sediment, Shellfish, and Lobster Tissue Analysis
(funded by DEP's oil research Surface Water Fund)

This draft report contains data on marine sediments, blue mussel (*Mytilus edulis)* tissue, and lobster (*Homerus americanus*) tissues collected in 2006. DEP is still waiting for some lobster tissue data from the contracted laboratory. Remaining lobster data will be reported as they are received and reviewed.

Mill Creek, Falmouth, was sampled for sediment in 2006. Three replicate samples were collected from Mill Creek, a tidal estuary within Casco Bay. Mill Creek was sampled to determine contaminant loading coming from extensive upland development in the area. Mill Creek was sampled on the following date:

Location	Date Sampled
Mill Creek, Falmouth	10/30/06

Sediment taken from Mill Creek, Falmouth was analyzed for: Mercury, heavy metals, PAHs, pesticides, and PCBs.

The following blue mussel sites were sampled in 2006: Spruce Creek, Kittery; Back Cove, Portland; Cocktail Cove, Great Diamond Island, Portland; Mill Creek, Falmouth; and Taunton Bay, Franklin. All samples consisted of three replicate samples. Sites were sampled on the following dates:

Location	Date Sampled
Spruce Creek, Kittery	11/02/06
Back Cove, Portland	11/29/06
Cocktail Cove, GDI, Portland	10/17/06
Mill Creek, Falmouth	10/12/06
Taunton Bay, Franklin	10/31/06

Mussel tissue from the five sites was analyzed for: Mercury, heavy metals, PAHs, pesticides, and PCBs.

Lobsters were collected as part of the National Coastal Assessment (NCA) on the southwestern half of the Maine coast in 2006. Nineteen stations were sampled over the western half of the Maine coast, and DEP dissected lobsters into hepatopancreas, muscle, and offal tissues. Whenever possible, lobster samples were composites of seven individual animals, though some samples contained fewer lobsters. EPA, as part of the NCA program, will analyze lobster muscle tissue for: Mercury, heavy metals, PAHs, pesticides, and PCBs. As part of the SWAT program, DEP analyzed the lobster muscle tissue for: Dioxins, furans, coplanar PCBs, and PBDEs. In addition, as part of the SWAT program, DEP analyzed lobster hepatopancreas for: Mercury, heavy metals, PAHs, pesticides, PCBs, dioxins/furans, coplanar PCBs, and PBDEs. Some lobster data have

been received from the contracted lab. Remaining lobster data will be presented upon their receipt, analysis, and review and are not contained in this draft report.

Raw data are contained in the accompanying files.

MODULE 2 LAKES

2.1

MERCURY IN BALD EAGLES

Evaluating exposure of Maine's Bald Eagle population to Mercury: assessing impacts on productivity and spatial exposure patterns.

Submitted to:

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1.0 Executive Summary

A recent US Fish and Wildlife Service proposal to delist the Bald Eagle (*Haliaeetus leucocephalus*) from the Endangered Species List noted lasting concerns for the potential impacts of contaminants on some populations. Previous and ongoing toxicological assessments highlight specific contaminant concerns for Maine's Bald Eagle population, and warrant consideration in upcoming management decisions.

This report summarizes findings from an ongoing eagle mercury monitoring and impacts study supported by non-profit (BioDiversity Research Institute), state (Maine Dept. of Inland Fisheries and Wildlife, Maine Dept. of Environmental Protection), federal (US Fish and Wildlife Service) and industry (FPL Energy Maine Hydro) organizations. Substantial support for this project was provided by the Maine Department of Environmental Protection.

We collected and analyzed mercury concentrations in Bald Eagle nestling blood, shed adult feathers, and abandoned eggs from freshwater-based Bald Eagle nests in Maine (2001-2006) to (1) evaluate dietary mercury (Hg) exposure, (2) assess if Hg exposure might be negatively impacting eagle productivity in Maine, and (3) evaluate spatial and temporal Hg trends in Maine. The following is a summary of current findings:

- *Nestling eagle Hg exposure:* **Maine Bald Eagle nestlings and adults are exposed to elevated levels of methylmercury via the freshwater foodweb. Eagles in lacustrine habitats are particularly at risk. Blood mercury exposure levels of Maine eaglets is higher than many regional comparisons, and most similar to populations associated with significant point source pollution problems (e.g., Hg mines, dredging).**
  [Fig. 2; p. 14]

- *Adult eagle Hg exposure - feathers:* **Exposure levels in Maine's adult Bald Eagles (as indicated by shed adult feathers) is elevated in comparison to virtually all comparison populations. As found in eaglet blood, mean Hg concentrations in Maine adult eagle feathers are most comparable to levels found at a site associated with a Hg mine (Pinchi Lake, BC).**
  [Fig. 3; p. 17]

- *Hg in Eggs:* **Hg in abandoned Bald Eagle eggs from Maine study sites is elevated compared to most populations in the U.S.**
  [Table 4; p. 18]

- *Hg-Productivity Relationships: potential impacts:* **We document significant negative relationships between eagle blood Hg and 3,5, and 10- year eagle productivity (chicks fledged/occupied nest). This has not been documented in other eagle populations, suggesting Maine's eagle population may be experiencing reproductive impacts due to Hg exposure despite population growth. [Fig. 4; p. 20]**

- *Spatial Patterns:* **Eaglet blood mercury levels were significantly different among 10 Maine watersheds, but sample sizes preclude powerful analyses.**

Eaglet mercury exposure in Maine highlights geographic mercury "hot spots" that demonstrate a general agreement with Hg findings in common loons and fish. [Figs. 7-8; pps. 25-6]

- *Long-term trends:* **Mercury bioavailability as indicated by nestling blood does not appear to be markedly different in lacustrine habitats during 2001-2005 in comparison to 1991-1992. Riverine comparisons suggest that levels are likely the same or higher than 1991-1992 levels. We recommend long-term monitoring of temporal Hg trends in Maine by periodic sampling (i.e., 1—15-yr intervals) as is currently conducted in other regions. [Fig. 6; p. 23]**
- *Proportion of sampled eaglets at levels of concern:* **Our findings suggest that Maine's Bald Eagle population is within the range of negative impacts; that between 19-30% of eaglets sampled in lacustrine habitats contain blood mercury levels designated as elevated or higher (>0.70 ppm), and 4-9% of those sampled are highly elevated. [Fig. 9; p. 28]**
- *Proportions of adult feathers at levels of concern:* **Feather Hg concentrations ranging to >93 ppm indicate a substantial proportion of Maine's adult eagle population are bioaccumulating mercury; these levels are highly elevated and suggestive of impacts. [Fig. 11; p. 29]**

The full report is available as a separate file with the SWAT report at http://www.maine.gov/dep/blwq/docmonitoring/swat/index.htm

2.2

MERCURY IN EAST POND

2.2 MERCURY IN EAST POND (not funded by SWAT)

East Pond is one of several lakes in Kennebec County that has an annual nuisance algae bloom and does not meet Maine's Water Quality Standards (minimum 2 meter Secchi disk transparency), as established by the Maine Legislature (Title 38 MRSA 464-468). Maine is legally required under the federal Clean Water Act to restore water quality by assessing the cause of the impairment and implementing restoration strategies. East Pond's water quality problems are described in detail in the Maine Department of Environmental Protection (DEP) report, 'East Pond Total Maximum Daily (Annual) Load' (DEPLW2001-10). East Pond's algae bloom is largely attributed to sediment accumulation of non-point source nutrients (phosphorus), which is likely exacerbated by the presence of white perch populations. Since their introduction into East Pond, white perch have become the dominant fish biomass and play a significant role in the lake's biological equilibrium. The biological balance is influenced by the dynamic interaction between primary producers (phytoplankton or algae) and consumers, higher up the trophic scale, which include zooplankton and fish. Zooplankton consumes phytoplankton, which are responsible for the nuisance algae blooms that occur in lakes. Fish primarily consume either zooplankton or other fish depending on their size and the availability of forage. The dominant white perch population occupies a niche that exclusively targets zooplankton at early life stages and then moves on to a combination of small fish and zooplankton as they grow.

As the white perch populations increase, the lake internal mechanisms for controlling algae blooms (phytoplankton) decrease. The Biomanipulation Project in East Pond is based on work done in Europe and the mid-west to improve water clarity through the manipulation of fish populations. A number of studies demonstrate that control of algae can be achieved through the reduction of fish that target zooplankton. The project will reduce the perch populations by culling spawning adults primarily through trap-netting efforts. This reduction should allow the zooplankton populations to rebound and balance things out once more. This may take several trapping seasons before a water quality improvement becomes apparent. The current project includes the following partners: the Maine DEP (Lakes Assessment Section - Division of Environmental Assessment), the University of Maine (Department of Biological Sciences), the East Pond Lake Association and Maine Department of Inland Fisheries & Wildlife.

The project seeks to remove as many planktivorous fish (white perch, yellow perch, and black crappie) as possible. The estimated population of white perch in the lake is approximately 100,000 with a wide confidence interval. There was some thought about whether or not the white perch removed could be given to people to eat. From DEP's Fish Tissue Contamination in Maine Lakes Study in 1992-3, white perch were the species most contaminated with mercury statewide. Concentrations of mercury in smallmouth bass from East Pond in that study were quite high (mean 0.890 ug/g). Consequently, in 2006, 10 white perch were collected from East Pond and analyzed for mercury. The fish were of relatively large size (mean 249 mm, 10 in). The mean and 95th upper confidence level mercury concentration were 0.178 ug/g and 0.211 ug/g respectively, around MCDC's FTAL of 0.200 ng/g. Although concentrations were relatively low the 95th

UCL exceeded the FTAL and consequently DEP and MCDC decided not to give the fish out for human consumption. The fish are instead taken to a local farm for composting.

2.3

MERCURY IN LAKES DOWNWIND OF HOLTRACHEM

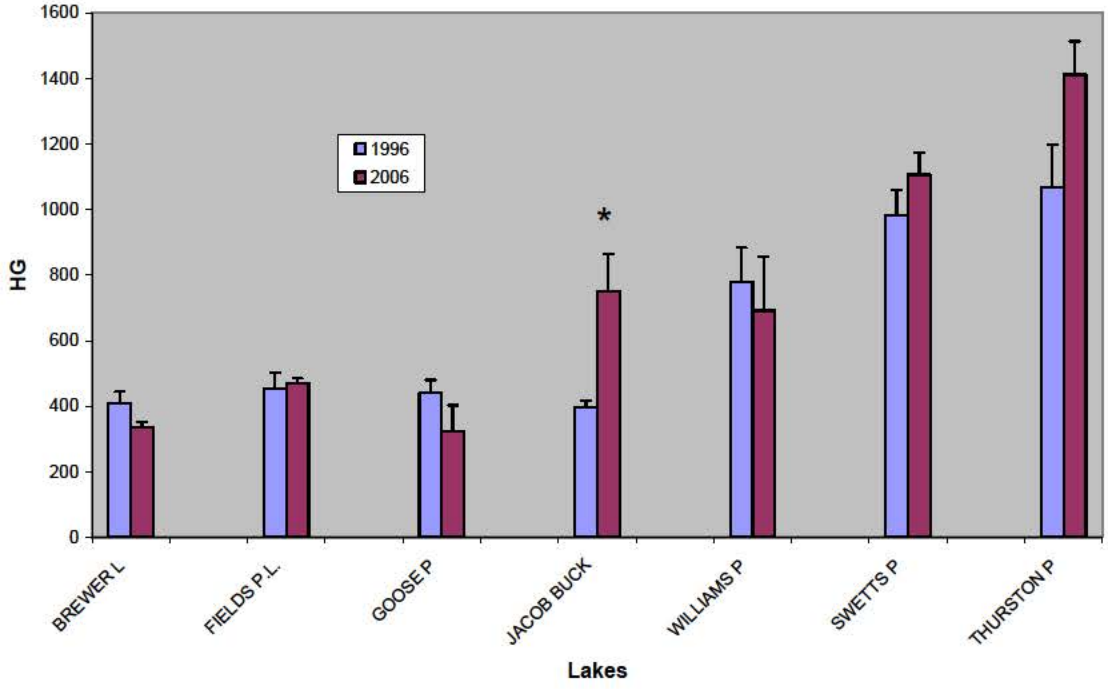
2.3 MERCURY IN LAKES DOWNWIND OF HOLTRACHEM

In 1996, fish and sediments from several lakes and ponds southeast (downwind) of Orrington were found to have higher concentrations of mercury than the general population of Maine lakes (DEP, 1997). Orrington is the location of two suspected sources of air emissions of mercury. 1) The Penobscot Energy Recovery Corporation's facility is one of waste-to-energy municipal waste combustors, which are known sources of air emissions of mercury at some level, depending on type of facility and controls. 2) The Holtrachem Manufacturing Corporation's chloralkalai facility produced chlorine using a mercury cell process until it closed in 2000. The facility had numerous releases of mercury to the air, land, and water while operating and the site is still heavily contaminated and is undergoing a hazardous waste cleanup.

Given that studies in other states have shown relatively rapid (3 years) declines in fish mercury levels to elimination of some local air sources of mercury, the lakes downwind of Orrington were sampled again in 2006 to determine if there have been any changes since 1996 and after Holtrachem closed in 2000. An effort was made to collect fish of the same relative size from each lake, but lengths were different between the two years for some lakes. Given that our 'Fish Tissue Contamination in Maine Lakes' study in 1993-4 documented that length was a primary determinant of mercury concentration in fish, all 1996 and 2006 mercury data were normalized to length prior for statistical analysis.

Results show that there was no lake where concentrations have significantly (Mann Whitney $p=0.05$) diminished, and only one (Jacob Buck Pond) where concentrations were significantly different, which was an increase (Figure 2.3.1). Since sample sizes for each lake were relatively small (n=3-11), these conclusions are tenuous. Consequently, data for each year were grouped to give larger samples sizes (n=49 and 31 for 1996 and 2006 respectively). There was no significant difference between the two years ($p= 0.972$).

Figure 2.3.1 Mean Mercury Concentrations (ng/g) in Fish from Lakes Downwind of Orrington



MODULE 3 RIVERS AND STREAMS

3.1.

AMBIENT BIOLOGICAL MONITORING

AMBIENT BIOLOGICAL MONITORING

As part of the SWAT program, the Biological Monitoring Unit evaluates benthic macroinvertebrate communities of Maine streams and rivers to determine if they are impaired by toxic contamination. For reasons of comparability, a small number of unimpaired reference sites is also evaluated. Benthic macroinvertebrates are animals without backbones that can be seen with the naked eye and live on the stream bottom, such as mayflies, stoneflies, caddisflies, crayfish, snails, and leeches. In 2006, we evaluated the condition of 39 sample locations, primarily in the Penobscot River and North Coastal Rivers basins.

The Biological Monitoring Unit uses a multivariate statistical model to analyze a benthic macroinvertebrate sample and predict if a waterbody is attaining the biological criteria associated with its statutory class. If a waterbody does not meet minimum state aquatic life criteria, Class C, then the model class is predicted as Non-Attainment (NA). Classes AA and A are treated the same in the model. Final decisions on aquatic life attainment of a waterbody are made accounting for factors that may allow adjustments to the model outcome. This is called the final determination.

Table 3.1.1 summarizes the results of biological monitoring activities for the 2006 SWAT Program, sorted by waterbody name. Column headings of Table 3.1.1 are described below:

- *Station* – Since waterbodies are sometimes sampled in more than one location, each sampling location is assigned a unique "Station" number.
- *Log* – Each sample event is assigned a unique "Log" number.
- *Map* – The "Map" number refers to Maps 1 through 29, which are located after the tables.
- *Location* – Some Stations are located upstream or downstream of potential sources of pollution, which are called "Issues".
- *Issue* – Issues are potential sources of pollution.
- *Statutory Class* – The state legislature has assigned a statutory class, either AA, A, B, or C, to every Maine stream and river. Class AA and A waterbodies shall support a "natural" biological community. Class B waterbodies shall not display "detrimental changes in the resident biological community". Class C waterbodies shall "maintain the structure and function of the resident biological community".
- *Final determination* – The final decision on aquatic life attainment of a waterbody. Accounts for factors that may allow adjustments to the model outcome.
- *Attains Class* – "Y" is given if the final determination is equal to or exceeds the Statutory Class. A Class B stream, for example, would receive a "Y" if its Final determination was either A or B. "N" is given if a stream does not attain its Statutory Class. A Class B stream, for example, would receive an "N" if its final determination was either C or NA. A dash ("-") is given if the sample was disturbed or provided insufficient information.
- *Probable Cause* – The probable cause column lists potential stressors to benthic macroinvertebrate communities, based on best professional judgement. In some cases, a probable cause may not be related to toxic pollution but instead to poor habitat conditions.

Data reports for each sampling event (Aquatic Life Classification Attainment Reports) are available in electronic format with the web version of this report. Supporting water chemistry data are given in Table 3.1.2. Water temperature data are given in Figure 3.1.1. For more

information about the Biological Monitoring Unit, please e-mail us at biome@maine.gov or visit our web site: http://www.state.me.us/dep/blwq/docmonitoring/biomonitoring/index.htm.

Results Summary

- Thirty-nine stations were assessed for the condition of the benthic macroinvertebrate community.
- Results have been received to date (June 12, 2007) for twenty-three stations.
- Eleven of the twenty-three stations (48 %) reported failed to attain the aquatic life standards of their assigned class.

Historical Notes

(not all of the samples listed below were collected under the SWAT Program)

- Birch Stream (Station 312) failed to attain class in 1997, 1999, 2001, 2003, 2004, and 2005.
- Dennys River (Station 297) failed to attain class in 1997, 1999, and 2003.
- East Machias River (Station 494) failed to attain class in 2001.
- Great Falls Branch (Station 504) failed to attain class in 2001.
- Millinocket Stream (Station 287) attained class in 1996.
- Mopang Stream (Station 501) attained class in 2001.
- Narraguagus River (Station 81) failed to attain class in 1984 and 1993. It attained class in 2001.
- Narraguagus River (Station 111) attained class in 1987 and 2001. It failed to attain class in 1989 and 1996.
- Narraguagus River (Station 112) attained class in 1987.
- Penjajawoc Stream (Station 314) failed to attain class in 1997, 2001, 2002, and 2003.
- Penjajawoc Stream (Station 315) attained class in 1997 and 2001. It failed to attain class in 2002 and 2003.
- Penjajawoc Stream (Station 511) failed to attain class in 2001, 2002, and 2003 .
- Penobscot River (Station 62) attained class in 1984, 1993, 1994.
- Piscataquis River (Station 83) attained class in 1984, 1985, 1989, 1996. It failed to attain class in 1990.
- Piscataquis River (Station 135) attained class in 1989, 1990, and 1996.
- Piscataquis River (Station 152) attained class in 1991, 1993, and 1995.
- St. Croix River (Station 199) attained class in 1991 and 1997.
- Shaw Brook (Station 480) failed to attain class in 2001.
- Sheepscot River (Station 74) attained class in 1987, 1989, 1990, 1992, 1995, 1996, 1998, 1999, 2000, 2001, 2002, 2003, 2004, and 2005. It failed to attain class in 1984, 1985, 1986, 1988, 1991, 1993, 1994, and 1997.
- Souadabscook Stream (Station 290) attained class in 1996.
- Souadabscook Stream (Station 291) attained class in 1996.
- Unnamed Stream 2 (Station 633) failed to attain class in 2002.
- Unnamed Stream 4 (Station 634) attained class in 2002.
- West Branch Sheepscot River (Station 268) attained class in 1995, 1996, 1997, 1998, 1999, 2001, 2002, and 2005. It failed to attain class in 2000, 2003, and 2004.

TABLE 3.1.1 - 2006 SWAT Benthic Macroinvertebrate Biomonitoring Results

Name	Town	Map	Station	Log	Location	Issue[1]	Statutory Class/ Final Determina-tion	Attains Class?	Probable Cause[1]
Birch Stream	Bangor	1	312	1549	Down-stream	Urban NPS; Airport	B / NA	N	NPS toxics; habitat
Card Brook	Ellsworth	2	814	1547		Urban NPS	B / NA	**N**	Habitat
Card Brook	Ellsworth	2	815	1548		Urban NPS	B / NA	**N**	NPS toxics; habitat
Dennys River	Meddybemps	3	297	1585		Haz Waste/ Liming	AA / A	Y	BPJ
Dennys River	Dennysville	4	741	1582			AA /		
E. Br. Penobscot River	T3R7 WELS	5	823	1589		Reference	AA /		
East Machias River	Crawford	6	494	1586			AA /		
Garland Pond Outlet	Sebec	7	817	1568			B /		
Great Falls Branch	Deblois	8	504	1579		Agric NPS	A /		
Jepson Brook	Lewiston	9	824	1592		Urban	B / NA	**N**	NPS toxics; habitat
Kenduskeag Stream	Bangor	1	829	1550		Urban NPS	C / B	Y	
Little River	Columbia Falls	10	821	1581		Reference	A / A	Y	
Little Smith Brook	Millinocket	11	819	1572			A / C	N	Habitat
Millinocket Stream	Millinocket	11	287	1571		Urban NPS	B /		
Moose Brook	Auburn	12	816	1562			B /		
Mopang Stream	T30 MD BPP	13	501	1587			AA / A	Y	
Narraguagus River	Cherryfield	14	81	1576		Agric NPS	B /		
Narraguagus River	Deblois	8	111	1577		Agric NPS	AA /		
Narraguagus River	Beddington	15	112	1578		Reference	AA /		

[1] NPS = non-point source pollution; Haz waste = hazardous waste; Agric NPS = agricultural NPS; BPJ = Best Professional Judgment

TABLE 3.2.1 - 2006 SWAT Benthic Macroinvertebrate Biomonitoring Results (cont.)

Name	Town	Map	Station	Log	Location	Issue[1]	Statutory Class/ Final Determina-tion	Attains Class?	Probable Cause[1]
Penjajawoc Stream	Bangor	16	314	1556		Urban NPS	B / C	N	NPS toxics; habitat
Penjajawoc Stream	Bangor	16	315	1557		Urban NPS	B / C	N	NPS toxics; habitat
Penjajawoc Stream	Bangor	16	511	1555		Urban NPS	B / C	N	NPS toxics; habitat
Penobscot River	Orono	17	62	1552		Munic/Ind	B / A	Y	
Piscataquis River	Abbot	18	83	1565		Reference	A / A	Y	
Piscataquis River	Sangerville	19	135	1566		Munic/Ind	B /		
Piscataquis River	Dover-Foxcroft	20	152	1567		Munic/Ind	B /		
St. Croix River	Baring	21	199	1584		Munic/Ind	C / A	Y	
Sebec River	Milo	22	827	1569		Municipal	B /		
Seboeis Stream	Howland	23	665	1575			A / A	Y	
Shaw Brook	Hermon	24	480	1551		Urban NPS	B / NA	N	NPS toxics; habitat
Sheepscot River	North Whitefield	25	74	1539		Reference	AA / A	Y	BPJ
Souadabscook Stream	Hampden	24	290	1553		Landfill	A / A	Y	BPJ
Souadabscook Stream	Hampden	24	291	1554		Landfill	A / A	Y	BPJ
Unnamed St. 2	Topsham	26	633	1564		Urban NPS	B / A	Y	
Unnamed St. 4	Topsham	26	634	1563		Urban NPS	B /		
W. Br. Sheepscot River	China	27	268	1540		Reference	AA / B	N	
Wassataquoik Stream	T3R7 WELS	28	812	1590		Reference	AA /		
West Seboeis Stream	T4 R9	29	818	1570			A /		
Western Little River	Columbia	10	820	1580			AA / C	N	

[1] NPS = non-point source pollution; Munic/Ind = municipal/industrial; BPJ = Best Professional Judgment

TABLE 3.1.2 - 2006 SWAT Nutrients and Solids Data

Log	Waterbody	Sampling Date	DOC	NH_3-N	TKN	NO_2-NO_3-N	O-PO_4	Total P	TSS	TDS
			mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
1549	Birch Stream	8/9/2006	4.2	0.03	0.3	0.32	0.006	0.022	~1.3	360
1548	Card Brook	8/8/2006	19	0.03	0.8	0.02	0.003	0.031	5	170
1550	Kenduskeag Stream	8/9/2006	11	0.01	0.5	0.07	0.008	0.032	2	100
1571	Millinocket Stream	8/16/2006	4.4	<0.01	0.2	0.01	0.002	0.008	~1.7	21
1576	Narraguagus River	8/21/2006	7.6	0.01	0.3	0.02	0.001	0.015	~1	32
1578	Narraguagus River	8/21/2006	7.7	<0.01	0.3	<0.01	0.001	0.012	0.8	32
1555	Penjajawoc Stream	8/10/2006	12	0.01	0.7	<0.01	0.003	0.029	4	90
1556	Penjajawoc Stream	8/10/2006	11	0.01	0.7	0.02	0.004	0.025	3	150
1557	Penjajawoc Stream	8/10/2006	12	0.01	0.6	0.02	0.003	0.021	~1.6	180
1552	Penobscot River	8/9/2006	11	<0.01	0.4	0.03	0.002	0.013	~0.7	45
1565	Piscataquis River	8/15/2006	5.5	<0.01	0.2	0.02	0.001	0.019	10	24
1566	Piscataquis River	8/15/2006	5.1	0.01	0.2	0.05	0.004	0.016	~1.4	27
1567	Piscataquis River	8/15/2006	5.7	0.01	0.3	0.03	0.002	0.013	~1.4	50
1575	Seboeis Stream	8/17/2006	7	<0.01	0.3	<0.01	0.001	0.009	~0.8	<20
1551	Shaw Brook	8/9/2006	6.7	0.02	0.4	0.18	0.002	0.032	10	230
1539	Sheepscot River	8/7/2006	11	0.01	0.5	0.02	0.003	0.014	~1	50
1553	Souadabscook Stream	8/10/2006	7.8	0.03	0.6	0.01	0.007	0.032	~1	70
1554	Souadabscook Stream	8/10/2006	18	0.01	0.5	0.02	0.008	0.030	2	70
1564	Unnamed Stream 2	8/14/2006	1.9	0.03	0.2	0.89	0.003	0.015	3	150
1563	Unnamed Stream 4	8/14/2006	2.2	0.08	0.2	0.45	0.002	0.015	8	370
1563	Unnamed Stream 4 DUPLICATE	8/14/2006	2.2	0.08	0.3	0.45	0.002	0.018	8	380
1540	W. Br. Sheepscot River	8/7/2006	9.6	0.01	0.5	0.03	0.002	0.013	~1	60
1540	W. Br. Sheepscot R. DUPLICATE	8/7/2006	9.3	0.01	0.5	0.03	0.002	0.015	~1.3	60

DOC = dissolved organic carbon, NH_3-N = ammonia-nitrogen, TKN = total Kjeldahl-nitrogen, NO_2-NO_3-N = nitrite-nitrate-nitrogen, O-PO_4 = Ortho-phosphate, Total P = total phosphorus, TSS = total suspended solids, and TDS = total dissolved solids.

More detail including maps of sampling stations, temperature data, and raw macroinvertebrate data are available in the Ambient Biological Monitoring section on our website at http://www.state.me.us/dep/blwq/docmonitoring/swat/index.htm

3.2

FISH CONSUMPTION ADVISORIES

COPLANAR PCB

In 2006 the SWAT program was again integrated with the Dioxin Monitoring Program (DMP) that has been in effect since 1988. Fish samples collected at 15 DMP stations for dioxin analyses were also analyzed for coplanar PCBs in the SWAT program. All non-detects were calculated at half the detection limit. Dioxin toxic equivalents (DTEh) and coplanar PCB toxic equivalents (CTEh) were calculated using World Health Organization (1998) toxicity equivalency factors (TEFs). For comparison with the Maine Center for Disease Control and Prevention's (MCDC) (formerly Maine Bureau of Health) Fish Tissue Action Levels (FTAL) for protection of human consumers, the 95th upper confidence limits (95% UCL) were used. The 95%UCL DTEh are compared to the cancer action level, FTALc=1.5 ppt, and the 95%UCL TTEh (sum of both CTEh and DTEh) are compared to the reproductive and developmental action level, FTALr=1.8 ppt and both are compared against the potentially lower fish tissue action level (pFTAL=0.4 ppt) being considered by MCDC.

SPECIES CODES
BNT brown trout
EEL eel
LMB largemouth bass
RBT rainbow trout
SMB smallmouth bass
WHP white perch
WHS white sucker

STATION CODES
AGL Androscoggin R at Gilead
ARF Androscoggin R at Rumford
ARY Androscoggin R at Riley
AGI Androscoggin R at GIP, Auburn
ALV Androscoggin R at Livermore Falls
ALW Androscoggin Lake at Wayne
KFF Kennebec R at Shawmut, Fairfield
PBW Penobscot R at Woodville
PBL Penobscot R at S Lincoln
PBV Penobscot R at Veazie
SWP W Br Sebasticook R at Palmyra
SBN Sebasticook R at Burnham

The results show that dioxin (toxic equivalents, upper 95% confidence limit with non-detects at ½ the detection level) and coplanar PCB (toxic equivalents, upper 95% confidence limit with non-detects at ½ the detection level) both separately and combined cause many samples to exceed the pFTALs and some to exceed the FTALc and FTALr (Figures 3.2.1 and 3.2.2, Appendix 3.2.1). The contribution of each varies with station, species and (not shown) year. Concentrations of coplanar PCB are within the wide range of those seen in previous years.

As in 2004 and 2005, coplanar PCB concentrations at SEN and ALW respectively were high. But as also in 2005, coplanar PCB at SWP were much lower than those in 2004, which were unusually high. Sources of PCBs are unknown but likely include atmospheric deposition.

Figure 3.2.1. Dioxin (DTEh) and coplanar PCB (CTEh) toxic equivalents (95th UCL) in bass (and brown trout BNT, rainbow trout RBT, and white perch WHP) in the Androscoggin, Kennebec, Sebasticook,Presumpscot, and Salmon Falls rivers, 2006

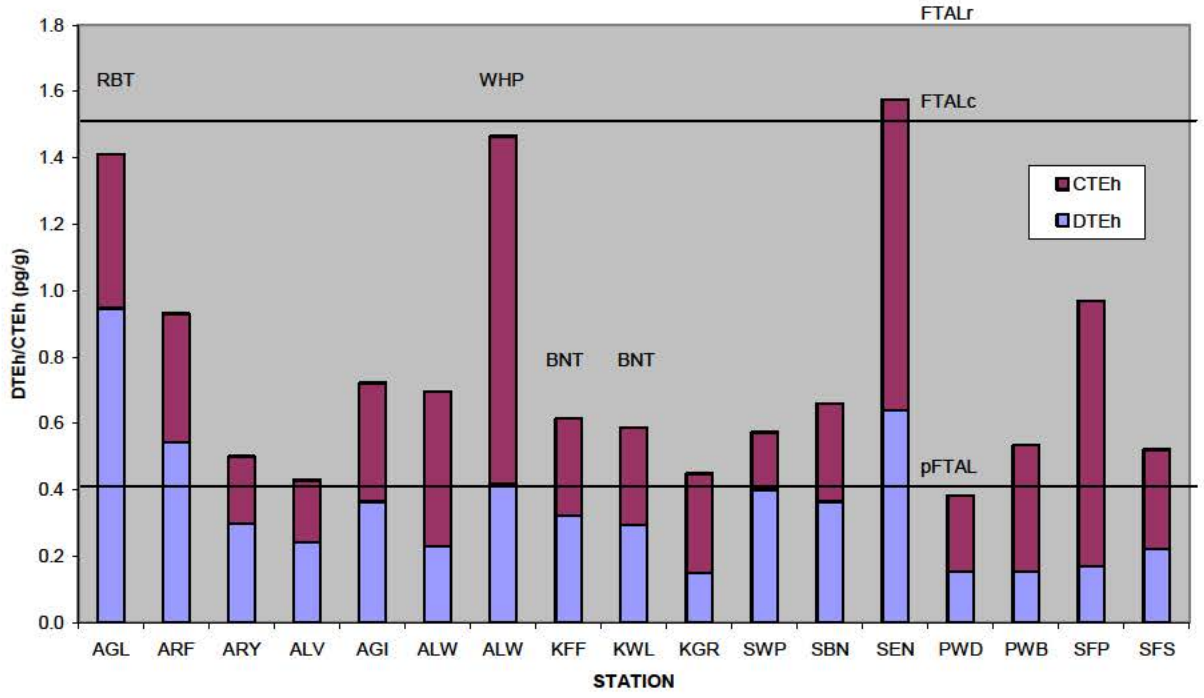
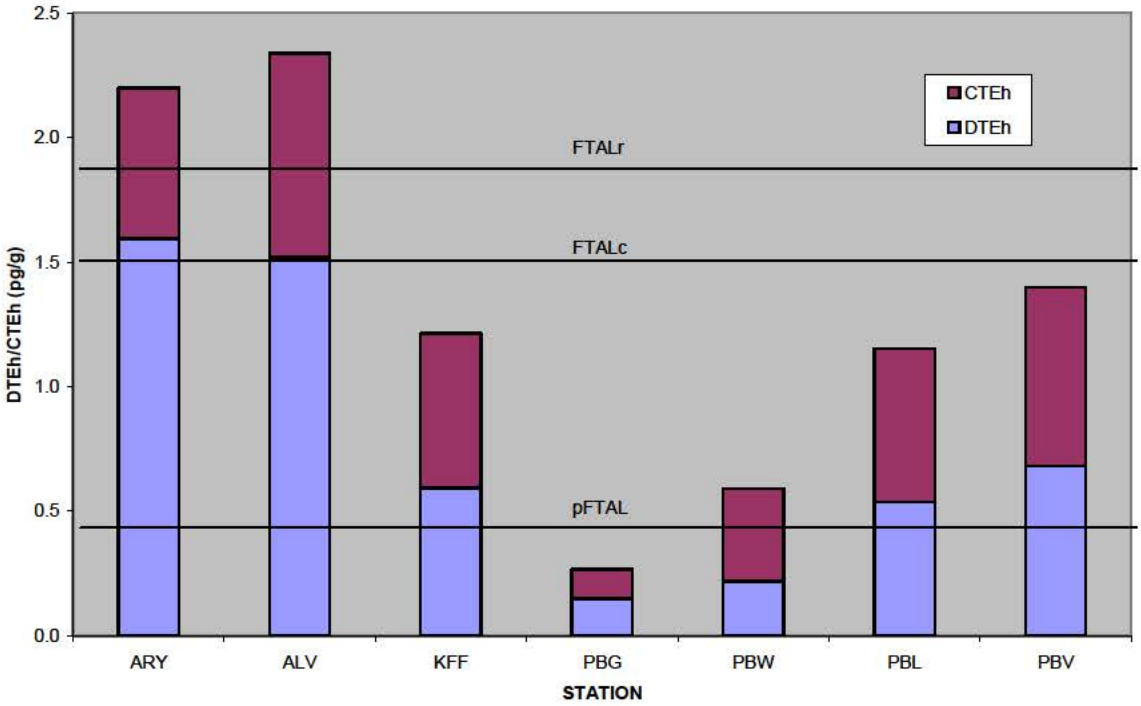


Figure 3.2.2. Dioxin (DTEh) and coplanar PCB (CTEh) toxic equivalents (95th UCL) in white suckers from the Androscoggin, Kennebec, and Penobscot rivers, 2006



DIOXIN

Dioxin concentrations in rainbow trout at Gilead, in bass from two locations on the Penobscot River, and in sludge from the Somersworth New Hampshire municipal treatment plant were measured as part of the SWAT program but are discussed in detail in the 2006 final report of Maine's Dioxin Monitoring Program (DMP) available at http://www.maine.gov/dep/blwq/docmonitoring/dioxin/index.htm . Fish from selected stations from the Androscoggin River, Kennebec River, Presumpscot River, and Salmon Falls River were funded by other sources, and are also reported in the 2006 Dioxin Monitoring Program report.

STRIPED BASS AND BLUEFISH

The current fish consumption advisory issued by the Bureau of Health for striped bass and bluefish recommends consumption of no more than 2 meals per month driven by total PCB concentrations. DEP had total PCB data from several years analyzed by various labs using some different methods. Beginning in 2003, samples have been analyzed for all 209 congeners. Data usually represent a mean of 5 individual fish.

There has been an increase in the concentration of total PCBs in striped bass and bluefish over the last three years. A significant effort is underway to revise these advisories and determine the feasibility of consistent advice from state to state along the east coast. One finding from that effort has been the need to sample striped bass from varying times to ensure different migratory stocks are being sampled. Up through 2005, striped bass were sampled during the spring fishery, but in 2006 samples were collected during the spring and fall for the Androscoggin and Kennebec rivers. We collected striped bass from the Saco River in the spring but were unable to collect any during the fall.

The upper 95th confidence level (95 UCL) mercury concentrations in striped bass in 2006 were higher than those from the other rivers and from previous years for the Androscoggin for both seasons (Table 3.2.1, Appendix 3.2.2). Concentrations for striped bass from the Kennebec River were similar to those of previous years for both seasons. Concentrations in the Kennebec striped bass were lower than found in freshwater fish from the same reach (not shown) and still exceeded the MCDC's FTAL (0.2 ppm) for most samples. There were opposite trends between spring and fall samples for the two rivers, which is interesting since the Androscoggin is a tributary to the Kennebec. Also the Androscoggin striped bass were captured not far from the confluence and freshwater fish are more contaminated in the Kennebec River. The small sample size (n=3) makes any comparisons between seasons and rivers tenuous. Spring samples from the Saco River, were similar or slightly higher than the one previous sampling, but similar to those of the adjacent Scarboro River.

Table 3.2.1 Mercury in marine fish from Maine estuaries, mg/kg 95th UCL on the mean

striped bass	season	Androscoggin	Kennebec	Penobscot	Royal	Sheepscot	Saco	Scarboro	York
Year									
1995	spring		0.35						
1997	spring		0.33						
1998	spring	0.38	0.40					0.37	
1999	spring		0.32						
2000	spring	0.22				0.22	0.18		
2001	spring			0.15					0.12
2002	spring								
2003	spring								
2004	spring	0.24	0.23	0.32	0.17				0.21
2005	spring		0.28	0.44				0.28	0.15
2006	spring	0.56	0.35				0.30		
	fall	0.91	0.34						

The upper 95th confidence level (95 UCL) PCB concentrations in striped bass in 2006 were higher than those from the other rivers (Table 3.2.2, Appendix 3.2.2). Concentrations were similar to those from recent years for the Androscoggin for the spring season but lower than in recent years in the fall. Concentrations for striped bass from the Kennebec River were similar to those of previous years in the fall but lower in the fall. Concentrations in the Kennebec striped bass were lower than in recent years but similar to those found in freshwater fish from the same reach (Table 3.2.3). Concentrations still exceeded the MCDC's FTAL (11 ppb) for all samples. There were opposite trends between spring and fall samples for the two rivers, similar to the mercury data. The small sample size (n=3) makes any comparisons between seasons and rivers tenuous. Spring samples from the Saco River, were higher than previous data but similar to the most recent sampling of the adjacent Scarboro River.

Table 3.2.2 PCBs in marine fish from Maine estuaries, ppb mean (95 ucl on the mean)

striped bass	season	Androscoggin	Kennebec	Penobscot	Royal	Sheepscot	Saco	Scarboro	York
Year									
1995	spring		23 (30)						
1997	spring		11 (14)						
1998	spring	41 (43)	16 (17)				12.2	30.3	
1999	spring		11 (12)						
2000	spring	60 (72)				24 (28)	25 (32)		
2001	spring			84					64
2002	spring	288	93.2	279		149	135		103
2003	spring								
2004	spring	201	170	211	152				147
2005	spring		193 (269)	81 (110)				82 (262)	101 (108)
2006	spring	214 (429)	85 (98)				166 (228)		
	fall	88 (126)	114 (170)						

KENNEBEC RIVER PCBs

The current advisory on the Kennebec River is no consumption of any fish from Augusta to the Chops. Limited consumption trout (5-6 meals per year) and limited consumption of bass (1-2 meals a month) from Shawmut Dam in Fairfield to Augusta. This advisory is based on a mix of contaminants. MCDC requested any fish from the river that were analyzed for dioxins and furans also be analyzed for coplanar PCBs, which was done as shown in figures 3.2.1 and 3.2.2. In addition, MCDC requested brown trout from Augusta to be analyzed for total PCBs, but none were captured. At the request of MCDC, smallmouth bass from Sidney, Augusta, and Gardiner were collected and analyzed for total PCBs to see if the trend continues downward from historically high levels responsible for the no consumption fish consumption advisory for the river below Augusta.

The results show that concentrations in smallmouth bass were perhaps slightly lower at Sidney and Augusta and similar at Gardiner to those of 2002, the most recent year sampled (Table 3.2.3). This may reflect washout of contaminated sediments since the Edwards dam was removed in 1999. Fish at Augusta have always been the most contaminated and are now showing the biggest change from previous data. Concentrations below Augusta remain elevated above the MCDC's Fish Tissue Action Level of 11 ppb.

Table 3.1.3 PCBs in smallmouth bass and brown trout from the Kennebec River
ppb mean (95 ucl on the mean or max if n=2

Smallmouth Bass

Year	Norridgewock	Skowhegan	Fairfield	Sidney	Augusta	Gardiner
1994			4.5	8.6	604	
1997		3.7 (4.5)	4.0 (4.9)	6.1 (7.2)	342 (357)	
1999					263 (323)	179 (227)
2000				32 (42)		
2002	1.6		1.7	19.5	111	47.5
2006				7.5 (10)	83 (142)	51 (75)

SALMON FALLS RIVER PCBS

The advisory on the Salmon Falls (based on data from S. Berwick) has been based on a few data (PCBs and dioxins). In 2006 largemouth bass were collected from the river in Spaulding Pond and the Somersworth impoundment in South Berwick and analyzed for total PCBs. Although historically there were discharges above and into Spaulding Pond, there are none known currently. The Berwick Sewer District discharge hosts the effluent from Prime Tanning Company and discharges above the South Berwick station. Results show that concentrations in Spaulding Pond are similar to that found upstream in Northeast Pond in 2002 (Table 3.2.4). Concentrations at South Berwick are slightly higher than the upstream stations but lower than in previous years at South Berwick, although the species sampled among the years are different. Concentrations in all samples exceed MCDC's FTAL (11ppb).

Table 3.2.4. PCBs in largemouth bass (LMB), smallmouth Bass (SMB), chain pickerel (CHP), and white perch from the Salmon Falls River, ppb mean (95 ucl on the mean)

Year	Acton	Northeast P	Spaulding P	Berwick	S. Berwick	
1997	5 (6)				75	SMB
					47 (53)	CHP
2000					83 (100)	SMB
2002		23.4	WHP	110		SMB
2006			25.5 (49)		33.2 (44)	LMB

3.3

CUMMULATIVE EFFECTS DRIVEN ASSESSMENT OF FISH POPULATIONS

CUMMULATIVE EFFECTS ASSESSMENT OF FISH POPULATIONS

Introduction

The US Clean Water Act (CWA) and Maine statutes set an ultimate goal that point source discharges be eliminated where appropriate and an interim goal that all waters be 'fishable/swimmable'. Maine Water Quality Standards further require that all freshwaters be 'suitable for the designated uses of …fishing and ….as habitat for fish and other aquatic life' and be 'of sufficient quality to support …indigenous species of fish'. EPA and DEP interpret 'fishing' to mean that not only do fish have to be present, but also healthy and safe to eat in unlimited quantities. And in order to provide 'habitat… to support a species', water quality must ensure that the population is sustainable, by allowing adequate survival, growth, and reproduction.

In the past, most SWAT studies of fish have focused on measuring the effects of persistent, toxic, and bioaccumulative (PBT) contaminants on human consumers, i.e. assessment of attainment of the designated use 'fishing', with some consideration of impacts to wildlife consumers as well. Direct effects on fish populations have been measured or estimated by other DEP programs able to detect only relatively severe impacts on survival, growth, and reproduction. Several studies (Adams et al, 1992; Kavlock et al, 1996; Munkittrick et al, 1998; Rolland et al, 1997) have measured other more subtle effects on development, immune system function, and reproduction not normally seen in more typical stressor-based testing regimes historically used by DEP. These more subtle effects may be a result of long term or cumulative exposure to relatively low levels of contaminants. These responses to pollutant challenge are often within the same magnitude as natural variation and therefore difficult to measure with the methods that are currently used. Many new techniques, such as a cumulative effects-driven assessment (CEA) of fish populations have been developed to measure some of these effects.

A CEA measures indicators of survival, growth, and reproduction. Age structure and mean age are measured as indicators of survival. Measures of energy expenditure and storage are used as indicators of growth and reproduction. Energy expenditure measures include size and size at age as indicators of growth while gonadosomatic index (GSI), fecundity, and egg size as indicators of reproductive potential. Energy storage measures include condition factor (K) as an indicator of growth and liversomatic index (LSI) and lipid storage as indicators of both growth and reproductive potential (Munkittrick et al, 2000). Response patterns of all indicators provide an integrative assessment of overall performance that may reflect different types of stresses, such as exploitation, food limitation, recruitment failure, niche shift, metabolic disruption (Munkittrick et al, 2000). Levels of circulating sex steroids are also often used as biomarkers of reproductive potential, which, along with survival, is considered an index of potential population trends.

With the assistance of Environment Canada (EC), DEP has conducted CEAs of fish populations on the St John River in 1999-2001 that have indicated probable impacts to fish populations and identified a previously unknown source. In 2000 similar studies of the North Branch of Presque Isle Stream and Prestile Stream, where high concentrations of DDT, a known endocrine disruptor, have been previously found, indicated a potential population level effects as indicated by a significant reduction in gonad size in both streams compared to two reference streams with much lower DDT levels in fish.

For Maine's major industrial rivers, the initial plan was to study what was considered the worst case first, and if no negative impacts were measured not to study the other rivers. The Androscoggin River was chosen to study first among the large industrial rivers because it had more (3) large pulp and paper mills for its size than the other major rivers and has historically had the poorest water quality. CEAs of white sucker populations in the Gulf Island Pond on the Androscoggin River from 2001-2003 did not show the evidence of endocrine disruption and metabolic redistribution found in a preliminary study in 1994. This result is possibly due to the change in bleaching technology from free chlorine to chlorine dioxide and improved waste treatment in the 3 upstream bleached kraft pulp and paper mills in the intervening years. Nor was there any evidence of endocrine disruption at any location below any of the mills in the rest of the river. There was evidence of increased eutrophication that correlated with increased nutrient levels downstream of the mills and host municipalities (DEP, 2004).

Studies of caged mussels in 2003 on the Androscoggin River showed no negative impacts on growth rate or induction of vitellin, a reproductive protein biomarker of endocrine disruption. This result is consistent with the CEA of fish populations in the river from 2001-2003. Studies of caged mussels in 2003 on the Kennebec River, however, did show induction of vitellin below a bleached kraft pulp and paper mill, evidence of endocrine disruption. Therefore, in 2004, a CEA was conducted on white suckers above and below the SAPPI Somerset bleached kraft pulp and paper mill on the Kennebec River. The results indicated possible endocrine disruption of survival, growth, and reproduction, as mean age, length in males, and levels of circulating sex steroids were reduced below the mill. Yet the results were not conclusive since MFOs, an indicator of exposure to bleached kraft mill effluent and other xenobiotics, and LSI in females were reduced below the mill while GSI and K actually increased, indicating a shift in energy storage and utilization and/or nutrient enrichment. This study was repeated in 2006.

A caged mussel study in 2004 did not show induction of vitellin seen in 2003, but the stations were different between the two years due to other priorities. The study was repeated in 2005 and showed induction of vitellin, below the mill as in 2003, although at station 5 rather than stations 3 and 4, for both males and females. In addition, lipid peroxidation, an indicator of toxicity, was elevated at all three stations below the mill. Growth in length and/or weight was increased at all stations below the mill. This study was repeated in 2006 but the data are not yet available from the lab.

Since there is some evidence of endocrine disruption below the bleached kraft mill on the Kennebec River, in 2005 a CEA was conducted above and below the Lincoln Paper and Tissue bleached kraft mill on the Penobscot River. Fish samples were collected in conjunction with the dioxin above/below (A/B) test, which allowed sampling effort and use of fish for both studies. The Environmental Effects Monitoring (EEM) program in Canada require all pulp and paper mills to conduct CEA of two species for each mill. Our 2005 Penobscot River CEA included two species as well, smallmouth bass and white sucker. Also a caged mussel study was initiated for the Penobscot for the A/B dioxin test and measurement of vitellin, but heavy fall rains and subsequent flooding prevented retrieval of the mussels.

Many studies have also documented effects of heavy metals, PAHs, sewage, and pulp and paper mill waste on fish immune systems (Voccia et al,1994; Holliday et al, 1998; Secombes et al, 1992; Ahmad et al, 1998). We have measured the spleen somatic index (SSI) and kidney somatic index (KSI) from white suckers from the Androscoggin River from 2002-2003, the Kennebec River in 2004, and Penobscot River in 2005 as rough indicators of immune system effects. There were significant decreases in SSI below the 2 most upstream mills on the Androscoggin for one or both sexes in 2002

and 2003, indicating potential immune system stress. Similarly, SSI was decreased below the SAPPI Somerset bleached kraft mill on the Kennebec River in 2004 not inconsistent with the possible decreased immune system capacity found by Hannum in head kidneys (SWAT, 2004), although the mechanism is unclear since head kidney size (KSI) in our study was no different between sites above and below the mills for either sex on either river. Both SSI and KSI were measured on both species from the Penobscot River in 2005.

Methods

In September 2006, white suckers were collected from the Kennebec River at Norridgewock (KNW) and Fairfield (KFF) above and below the discharges from the City of Skowhegan and the SAPPI bleached kraft pulp and paper mill. Similar sampling was conducted on the Penobscot River at 4 stations. The stations were 1) above Millinockett on the West Branch of the Penobscot River in the Nesowadnehunk deadwater (PBN), 2) above East Millinockett on the East Branch of the Penobscot River above Grindstone (PBG) 3) below Millinockett and East Millinockett (with 2 municipal and 2 pulp and paper mill discharges) and the confluence with the East and West branches of the river mill (PBW) at Woodland, above the Mattaceunk dam and above the Lincoln Paper and Tissue mill and 4) downstream of the Lincoln mill at South Lincoln (PBL), approximately 3 miles below the mill at the historic sampling site for the Dioxin Monitoring Program. For each of the stations, 20 males (except PBG where no males were collected) and 20 females of each species were collected during fall recrudescence. Previous studies have determined that a sample size of 20 is sufficient to reduce the variance enough to detect a difference of 20-30% in the variables measured between stations.

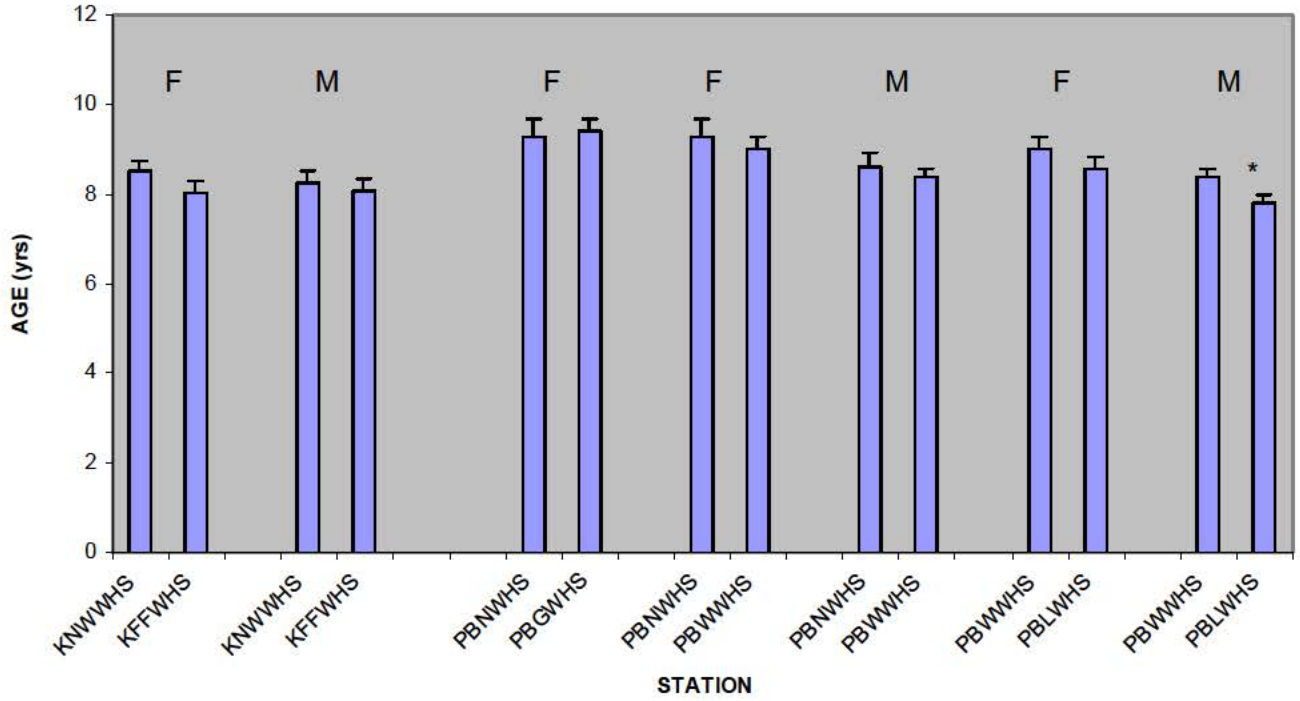
Fish were collected by gill net. Blood samples were collected from live fish immobilized in a foam cradle, into heparinized Vacutainers and placed on ice for transport to the lab the same day. The fish were then killed with a blow to the head. The operculum was collected for aging. Livers were dissected out and weighed, for calculation of LSI, and then frozen in liquid nitrogen. Gonads were dissected out and weighed for calculation of GSI and a small sample ~1 cm square was taken and placed in 10% buffered formalin for storage. Head kidney in suckers and spleen in both species were dissected out and weighed for calculation of KSI and SSI respectively.

Later the same day in the lab, the samples were placed in proper storage to await analyses. Plasma was collected from the blood samples after centrifugation in the lab and then frozen at –20C for radioimmunoassay (RIA) analysis for circulating sex steroids (testosterone T, 11 ketotestosterone 11-KT, and estradiol E2) following the method of McMaster et al (1992) and F following the method of Jardine (1996). Liver samples were stored at –80 C for MFO (CYP1A) analysis as outlined by Munkittrick et al (1992). Gonad samples remained in formalin for further analyses. Histological samples of gonads were prepared and examined for the presence of testis-ova as outlined in Gray and Metcalf (1997) or analysis of gonadal staging (McMaster, 2001). All laboratory analyses were performed by at Environment Canada's National Water Research Institute in Burlington, Ontario, Canada. Samples for aging were stored at –20C until prepared and read in the DEP lab in Augusta, Maine.

Results

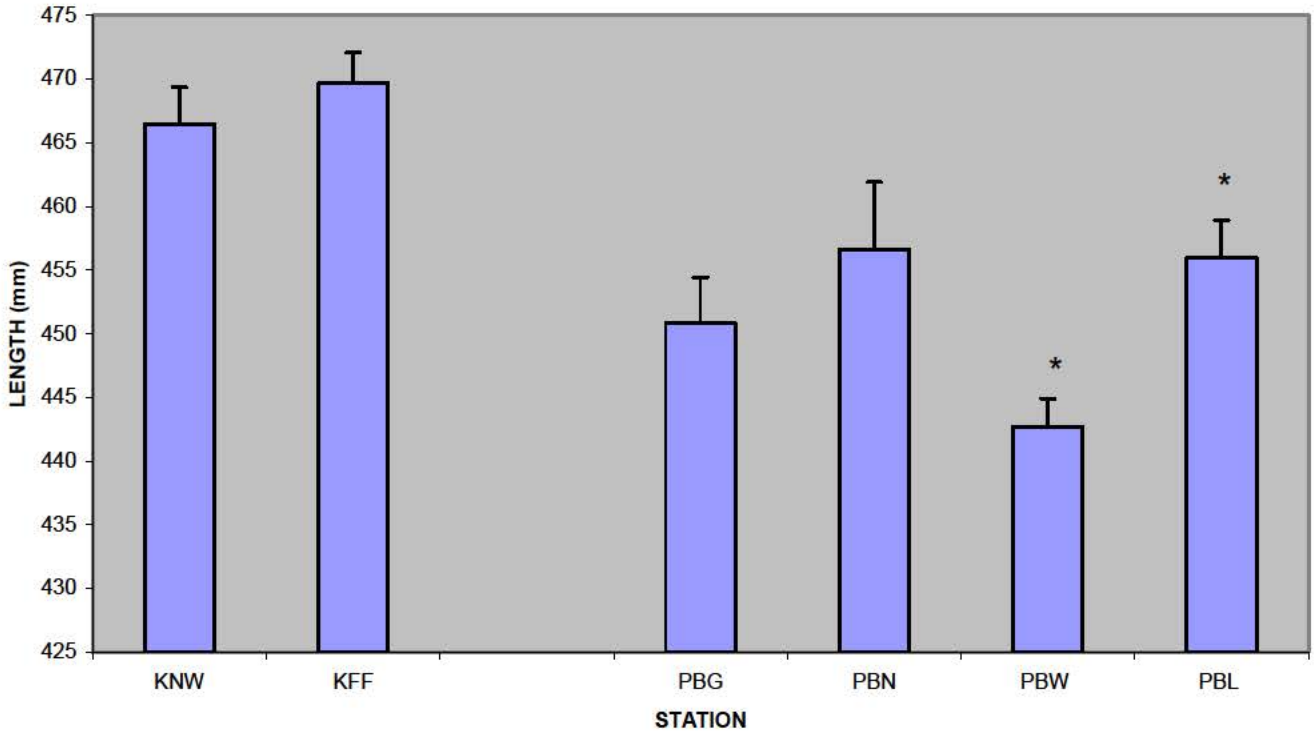
There was no difference in mean age, an indicator of survival, above and below the mill on the Kennebec River, unlike in 2004 when mean age was lower below the mill for both sexes (Figure 3.3.1). Nor was there any difference in mean age among any of the 3 stations above Lincoln on the Penobscot River. Mean age was significantly reduced in male white suckers from the Penobscot River below the mill at PBL compared to above the mill at PBW, unlike in 2005. Munkittrick (2000) gives as two possible reasons for reduced survival, 1) exploitation and 2) metabolic redistribution. Few people angle recreationally for white suckers and there is no one known to be netting white suckers for lobster bait in this reach, so exploitation is considered to be nil.

Figure 3.3.1. MEAN AGE OF MALE (M) AND FEMALE (F) WHITE SUCKERS SAMPLED FROM THE KENNEBEC AND PENOBSCOT RIVERS, 2006



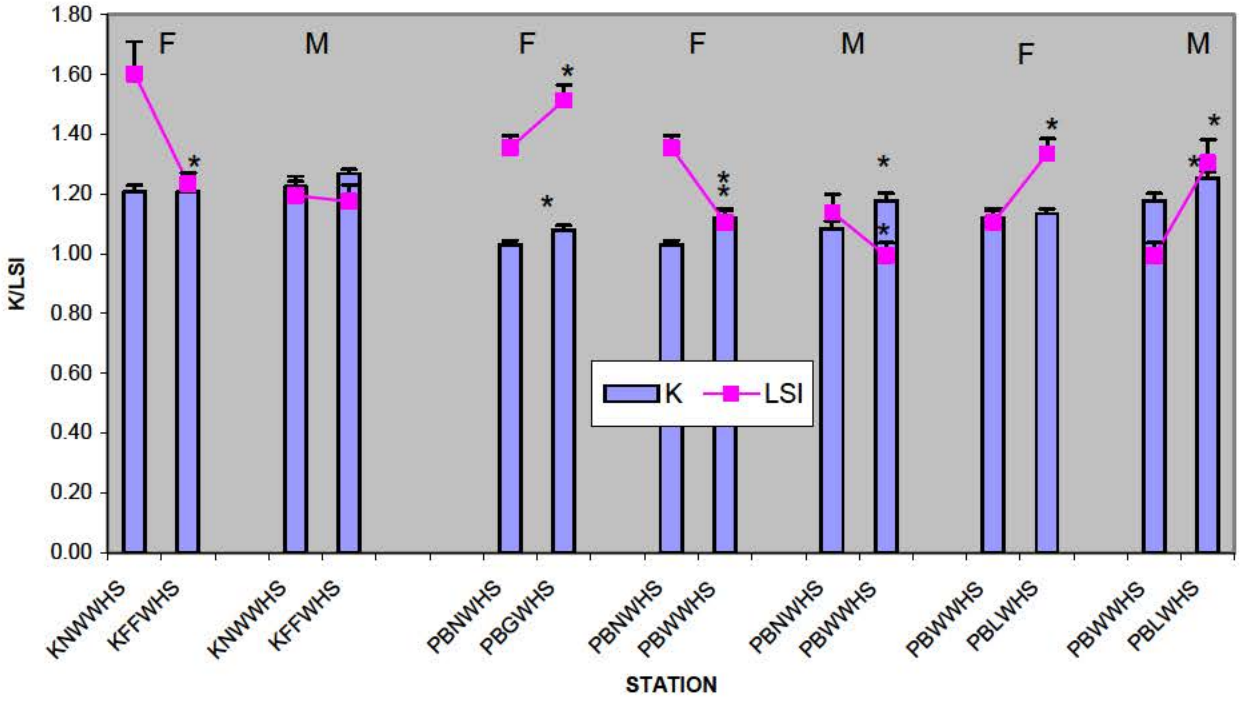
Indicators of growth include measures of energy expenditure (size and size at age) and measures of energy storage (condition factor K and liversomatic index LSI). Mean size as measured by length was biased in the 2005 study since the white suckers were also used for dioxin analysis which required that the fish be selected for a relatively uniform length. Therefore, in 2006 all fish captured were measured for length. Sample sizes were variable (PBN n=38, PBG n=28, PBW n=137, PBL n=100). Mean length was lower at PBW than any of the other stations (Figure 3.3.2). PBW is downstream of Millinockett where there are 2 municipal treatment plants and 2 pulp and paper mills. Increased length combined with decreased age below Lincoln implies increased growth, probably due to nutrient enrichment as was the case in 2005 for bass although not for suckers. There was no difference in length for white suckers on the Kennebec above and below the mill, unlike in 2004 when males were smaller below the mill.

Figure 3.3.2. MEAN LENGTHS OF WHITE SUCKERS FROM THE KENNEBEC AND PENOBSCOT RIVERS, 2006



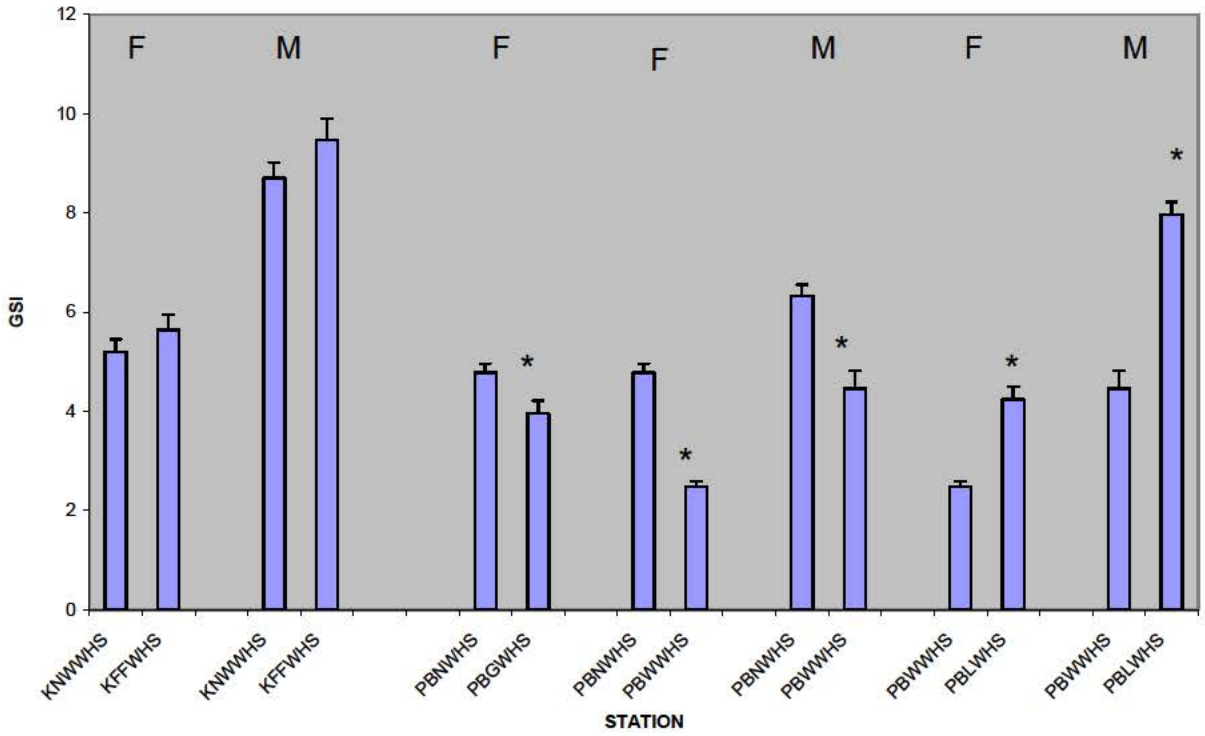
Unlike in 2004, there was no increase in K for either sex below the SAPPI mill at KFF in 2006 (Figure 3.3.3). LSI was similar to that of 2005 with a decrease in females at KFF and no change in males. Both metrics, then, indicate a loss or no increase in energy storage, unlike 2004. In the Penobscot both K and LSI increased below the Lincoln mill at PBL in both sexes, except for K in females, as was the case in 2005, indicating continued energy storage.

Figure 3.3.3. MEAN K AND LSI OF MALE (M) AND FEMALE (F) WHITE SUCKERS SAMPLED FROM THE KENNEBEC AND PENOBSCOT RIVERS, 2006



Indicators of reproduction also include measures of energy expenditure (gonadosomatic index (GSI), fecundity, and egg size) and measures of energy storage (LSI and lipid storage). Unlike in 2004, there was no increase in GSI at KFF below the SAPPI mill on the Kennebec River (Figure 3.3.4). For the Penobscot River, GSI increased at PBL below the Lincoln mill in both sexes as in 2005. Interestingly, suckers at PBW had lower GSI than the two stations upstream of Millinocket, PBN and PBG.

Figure 3.3.4. MEAN GSI OF MALE (M) AND FEMALE (F) WHITE SUCKERS FROM THE KENNEBEC AND PENOBSCOT RIVERS, 2006



Data for gonad samples of white suckers from the Kennebec River in 2004 and the Penobscot River in 2005 have been recently received and are first reported here. Testes were examined for intersex, i.e.the inclusion of ova in the testes (ovo-testis). Ovaries were examined for gonadal staging, i.e. developmental stage of oocyte, and were classified as primary (pre-vitellogenic), endovitellogenic (endogenous vitellogenic), or vitellogenic.

There were no males reported to have intersex for the Kennebec River fish. Results of the gonadal staging show no difference in mean size or percent of primary, endovitellogenic, or vitellogenic oocytes (Figures 3.3.5 and 3.3.6). Results show that for the Penobscot River there were 2 intersex males (out of 20) below Millinockett at PBW and 2 intersex males (out of 20) below Lincoln at PBL. This 10% incidence is not unusually high compared to reports from the literature, but the significance is not certain at this time. Gondal staging did show some differences above and below the mill. In white suckers, vitellogenic oocytes were significantly larger below the mill (Figure 3.3.5) and there was advanced development as evidenced by a shift from percent primary to vitellogenic oocytes there as well (Figure3.3.6). For smallmouth bass vitellogenic oocytes were also larger below the mill but there was no advanced development.

Figure 3.3.5. Mean size of primary (P), endovitellogenic (E) and vitellogenic (V) oocytes in female white suckers from the Kennebec River and the Penobscot River, 2006

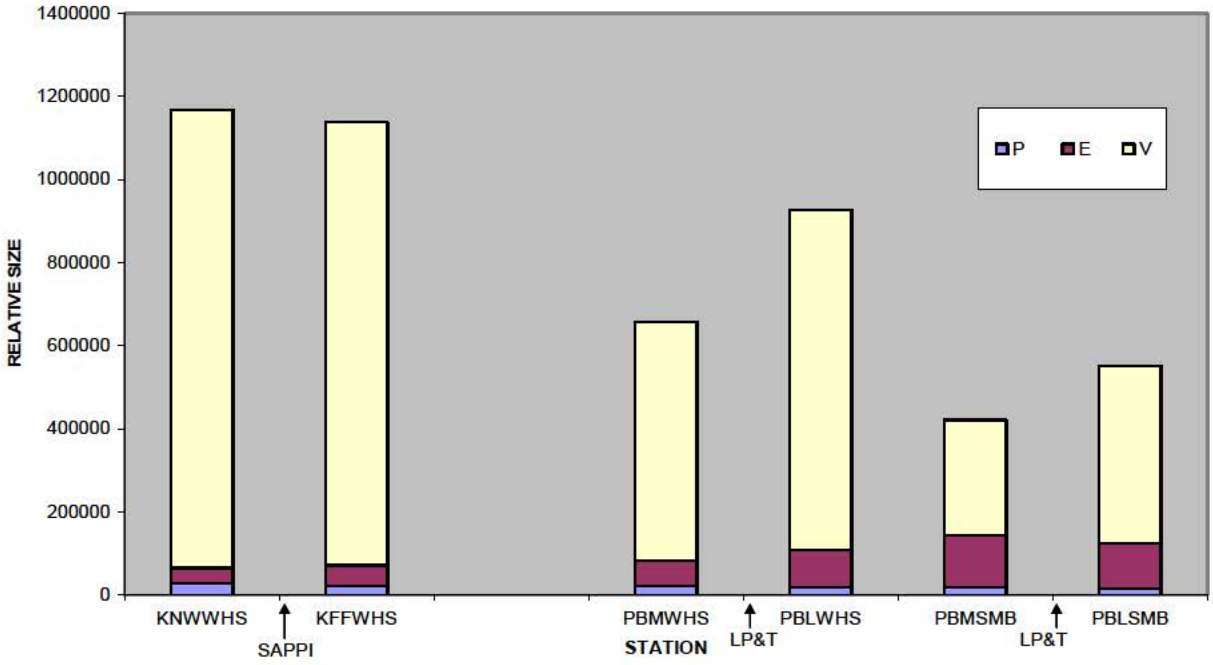
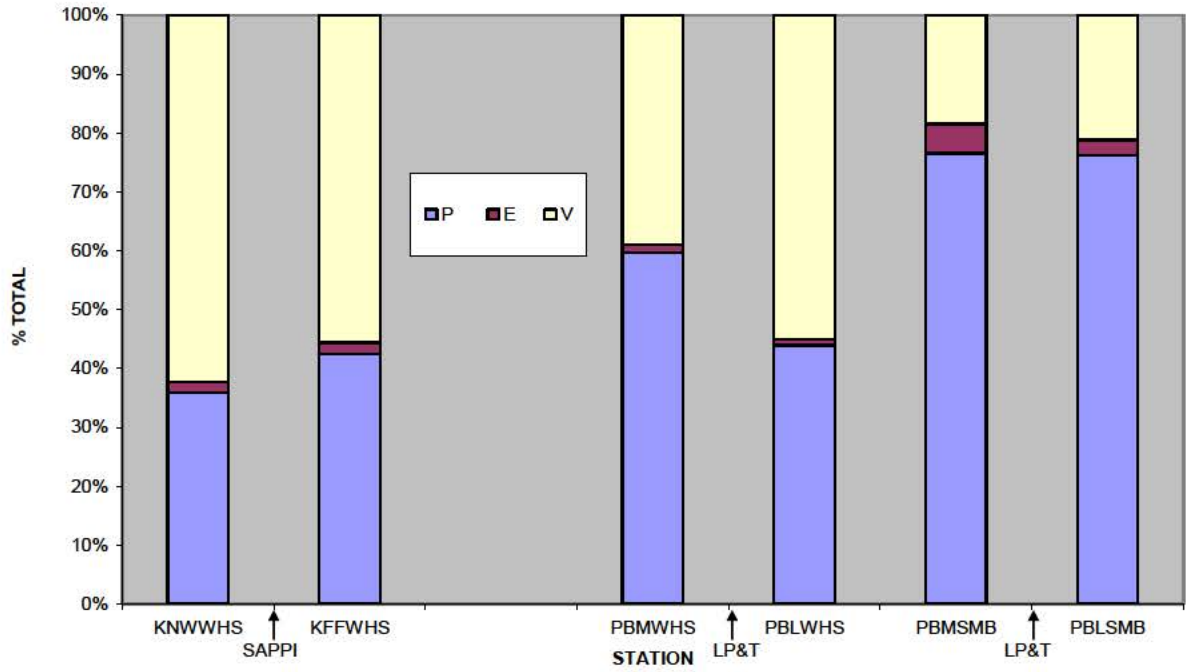


Figure 3.3.6. Mean percentage of total as primary (P), endovitellogenic (E), and vitellogenic (V) oocytes in female white suckers from the Kennebec and Penobscot rivers, 2006



Conclusions

For the Kennebec River, field data responses seen in 2004 were generally not repeated in 2006. The only response that was consistent between both years was an increase in energy storage in the liver, i.e. increased LSI, of white suckers below the SAPPI mill. The increased energy utilization, i.e. increased growth in females and increased GSI in both sexes, measured in 2004 was not observed in 2006. However, the MFO, steroid, vitellogenin, and gondad data have not been reported from the lab yet, so final conclusions cannot be made at this time.

For the Penobscot River, responses were generally similar to those of 2005 for energy utilization as indicated by increased GSI and for energy storage as indicated by increased K for males and LSI for both sexes below the mill at PBL. Responses were also similar to those of 2005 with no change in growth and no increase in K in females. There were some differences from 2005, namely direction of energy to growth (size at age) in males. The MFO, steroid, vitellogenin and gonad data have not been reported from the lab yet, so final conclusions cannot be made at this time.

The 2005 gonad data from white suckers from the Penobscot River below Millinockett and Lincoln showed intersex in a small (10%) incidence, that is reported to be not uncommon in the literature. Gonadal staging showed some advanced development of oocytes below Lincoln as well. These data need to be compared to the 2006 data, when available, before the significance is known.

These responses for male white suckers in the Penobscot River fit a pattern of exploitation for 2006, caused by mortality or eutrophication. Since there is no directed fishery for white suckers on the Penobscot, these responses are more likely caused by eutrophication. Increased productivity of the system at PBL is likely due to nutrient discharges from the mill and treated municipal wastewater from the Town of Lincoln.

The responses also fit a pattern of metabolic disruption, but the steroid data are needed for a final determination. For suckers the pattern of responses most closely resembles one of metabolic disruption with energy directed mostly toward reproduction and little toward growth in females and both in males.

There was no difference in head kidney somatic index (KSI) or spleen somatic index (SSI) between PBM and PBL for males, but SSI increased in females below the mill at PBL in 2006 (Figure 3.3.7), unlike 2005 when there was no difference above and below the mill for either sex. This finding is unlike that from the Kennebec and Androscoggin rivers in previous years where SSI was significantly lower below the mills and host municipalities, both of which were larger than Lincoln on the Penobscot River.

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3.4

Caged Mussel Vitellin Study

CAGED MUSSEL VITELLIN STUDY -DEP

In 2003 a study with caged mussels detected a significant induction of vitellin, a vitellogenin-like reproductive protein normally found in females, in a subsample of both males and females at stations 3 and 4, ~ 0.08 miles and 2.5 miles below the SAPPI bleached kraft pulp and paper mill on the Kennebec River respectively compared to stations KR1 and KR2, ~ 13 and 5 miles above the mill respectively. Growth of whole animal length and weight, shell weight, and wet tissue weight were elevated at station KR5, ~ 5 miles below the mill. A repeat study in 2004 found no such induction at station KR6, ~ 11 miles below the mill, compared to KR2 and there was no difference in condition factor or relative gonad size (GSI) between the stations. A repeat study in 2005 found increased growth in shell length and whole animal wet weight at all stations sampled below the mill (KR3-5). ALP (vitellin) total mass was greater at KR5 than the upstream station KR2, but ALP normalized to protein was no different between the two. In 2005 there was a significant increase in lipid peroxidation at all stations below the mill. The study was repeated in 2006 including all stations 2-6. A similar study was attempted in the Penobscot River above (PBW) or below (PBL and PBC) the Lincoln Paper and Tissue bleached kraft pulp and paper mill at Lincoln in 2005, but samples were lost in a flood. The study was repeated in 2006.

Results

In the Kennebec River, unlike 2005, growth rates for length were no different among the stations (Figure 3.4.1). Growth rates for whole animal wet weight were significantly greater below the mill than above dropping off at KR6 (Figure 3.5.2) similar to that for KR5 in 2003 all stations below the mill in 2005. In the Penobscot River there was no difference in growth for either shell length or whole animal wet weight among the stations, due to high variability among the stations (Figures 3.4.3 and 3.4.4). The indication of enrichment in the Kennebec below the mill and not in the Penobscot below the mill was opposite that seen with the fish for both rivers. The vitellin and other lipid peroxidation data have not yet been received from the lab.

Figure 3.4.1. Growth rates in length of caged mussels in the Kennebec River, 2006

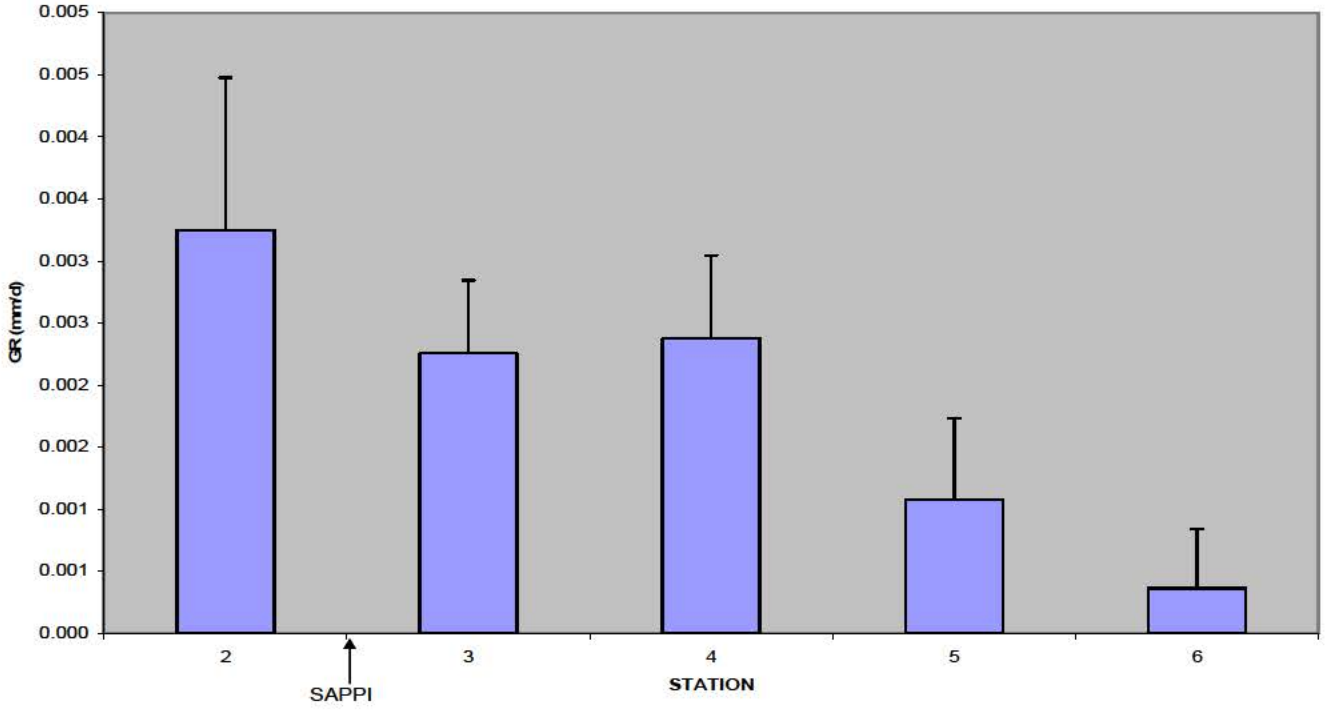


Figure 3.4.2. Growth rate in weight of caged mussels in the Kennebec River, 2006

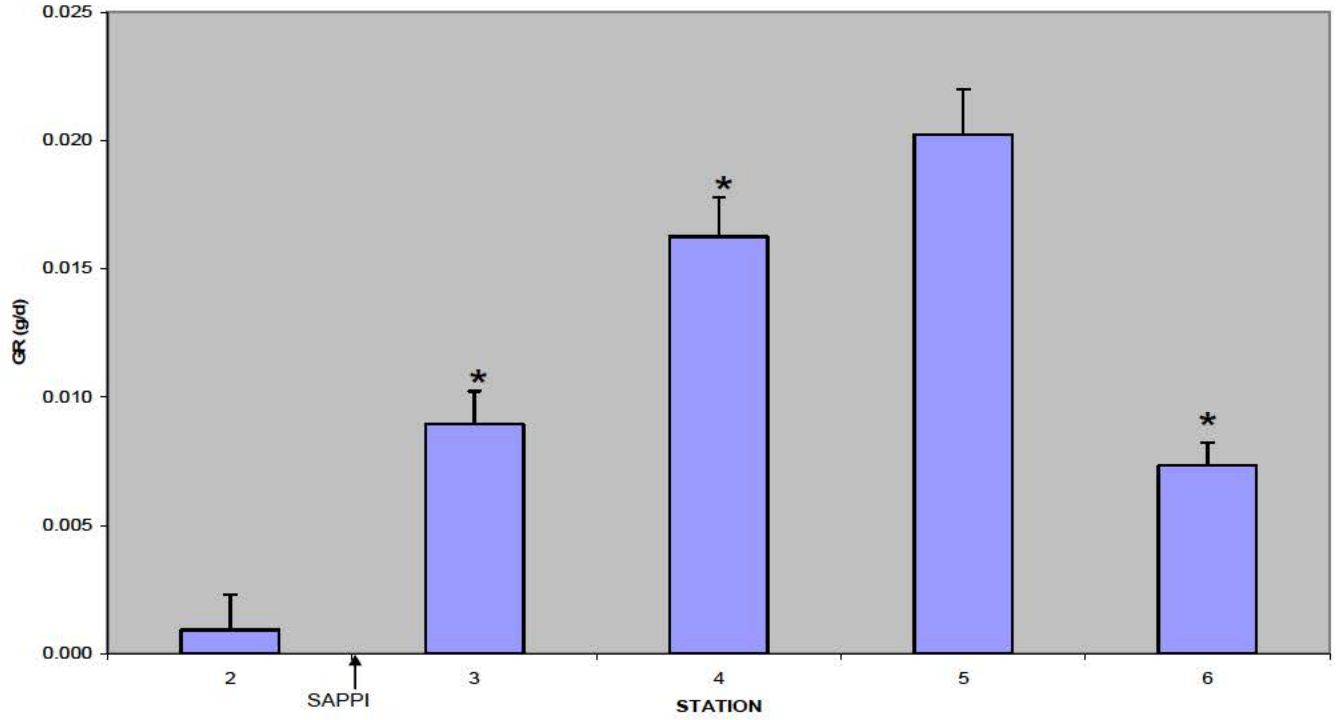


Figure 3.4.3. Growth rate in length of caged mussels in the Penobscot River, 2006 (mean+se)

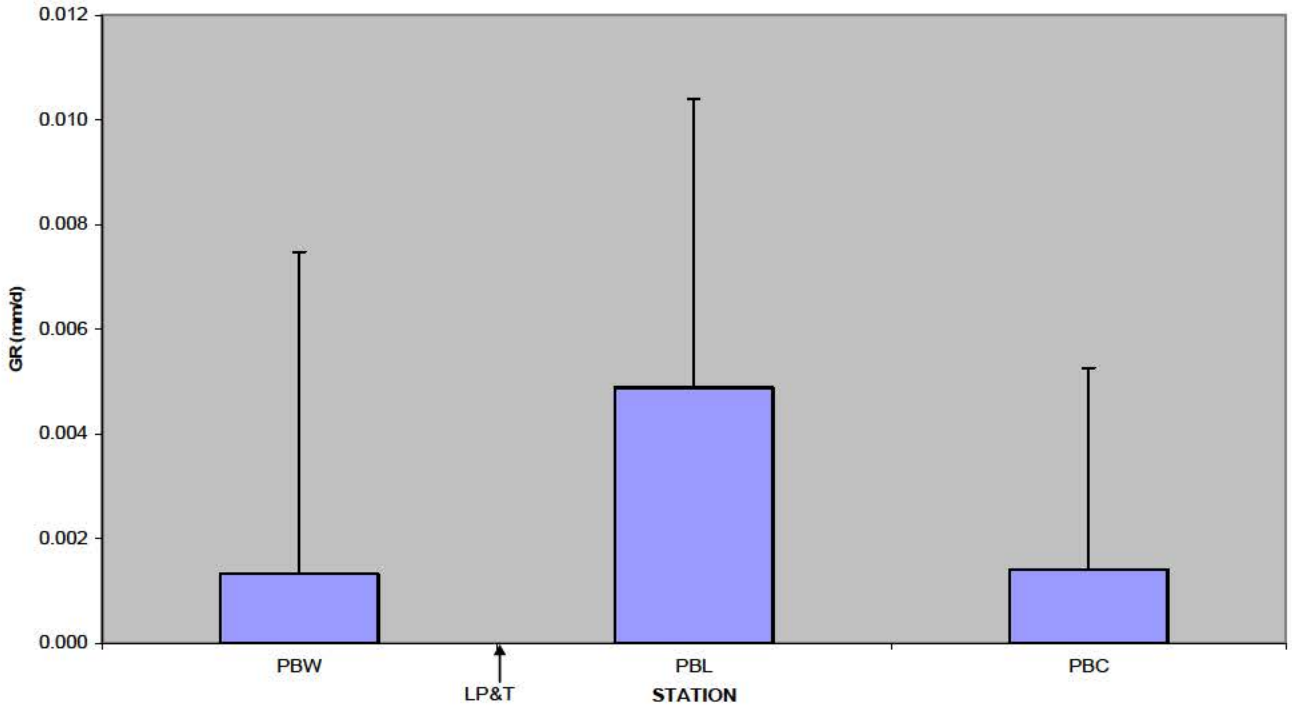
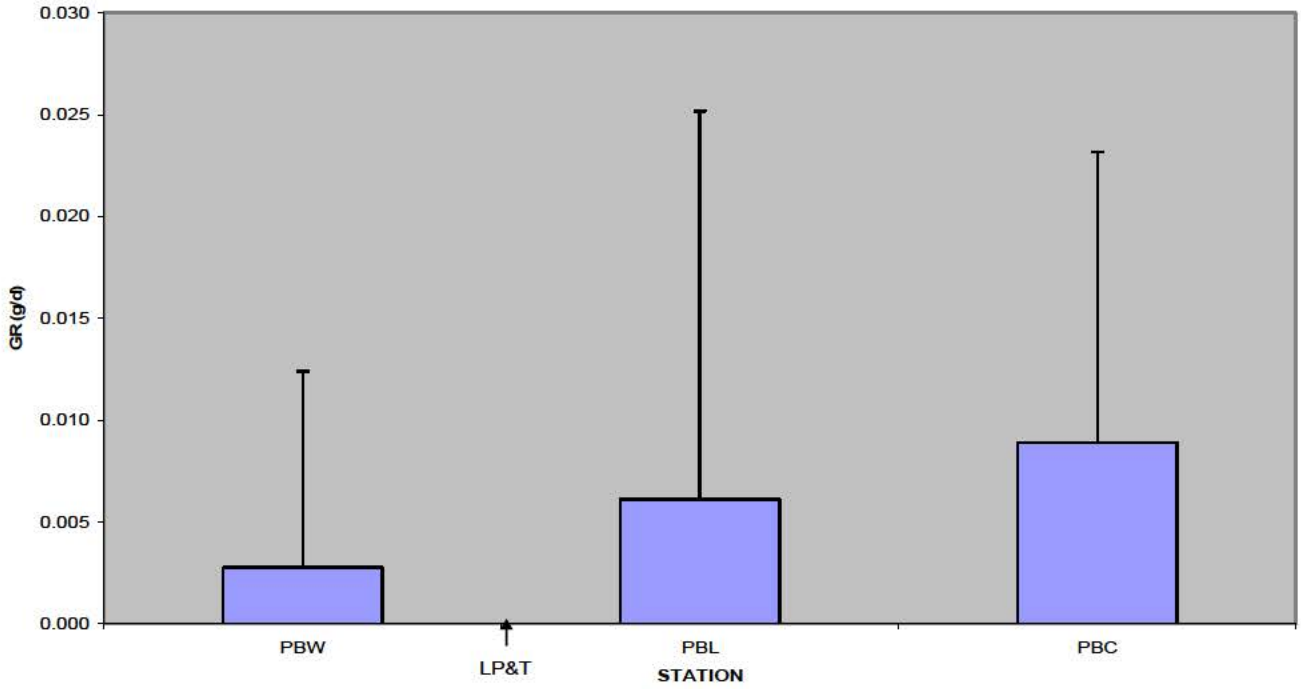


Figure 3.4.4. Growth rate in weight of caged mussels in the Penobscot River, 2006 (mean+se)



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

A portion of this work was funded by:

SURFACE WATER AMBIENT TOXIC MONITORING PROGRAM
DIVISION OF ENVIRONMENTAL ASSESSMENT
MAINE DEPARTMENT OF ENVIRONMENTAL PROTECTION
AUGUSTA, MAINE 04333
June 2007

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.

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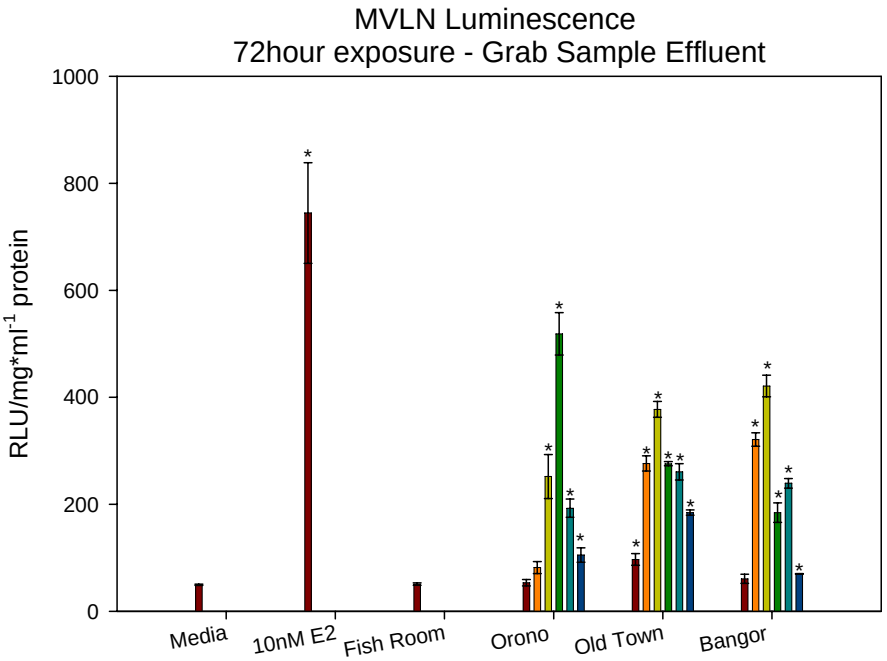

Figure 1. Reporter gene analysis showed that effluent from Orono, Old Town, and Bangor wastewater treatment facilities elicited an estrogen receptor mediated response *in vitro*. This response varied between sampling periods, with a trend in higher responses over the winter months. At no sampling period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

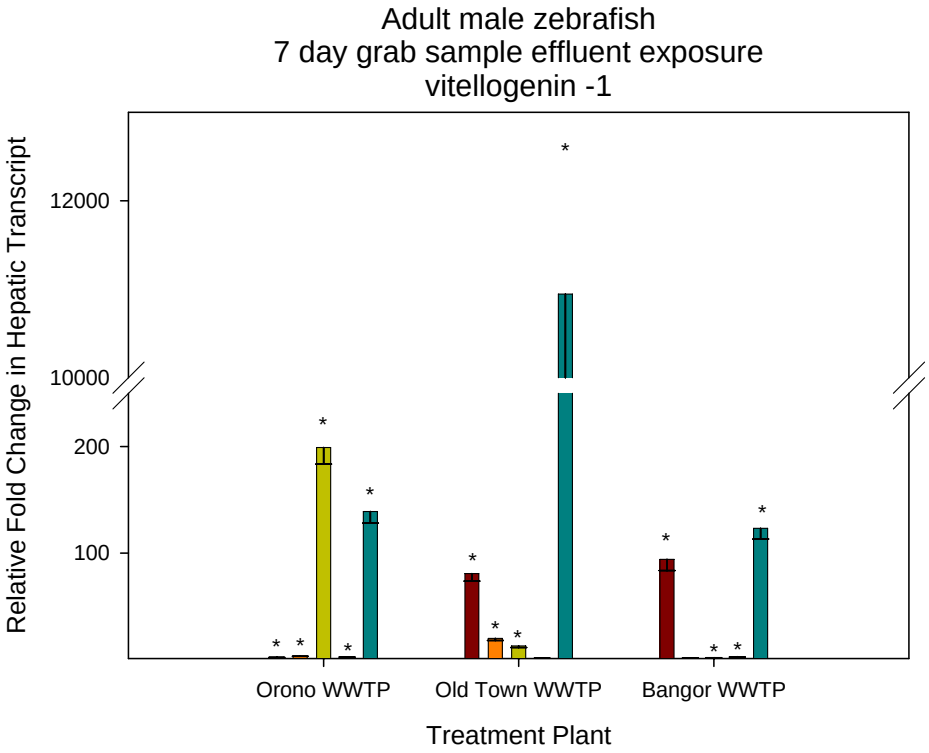

Figure 2. Adult male zebrafish exposed to wastewater effluent exhibited a mixed vitellogenic response. Only in February at the Old Town treatment facility did hepatic vitellogenin mRNA concentration rise in great enough magnitude to warrant concern. For comparison, increases in hepatic vitellogenin

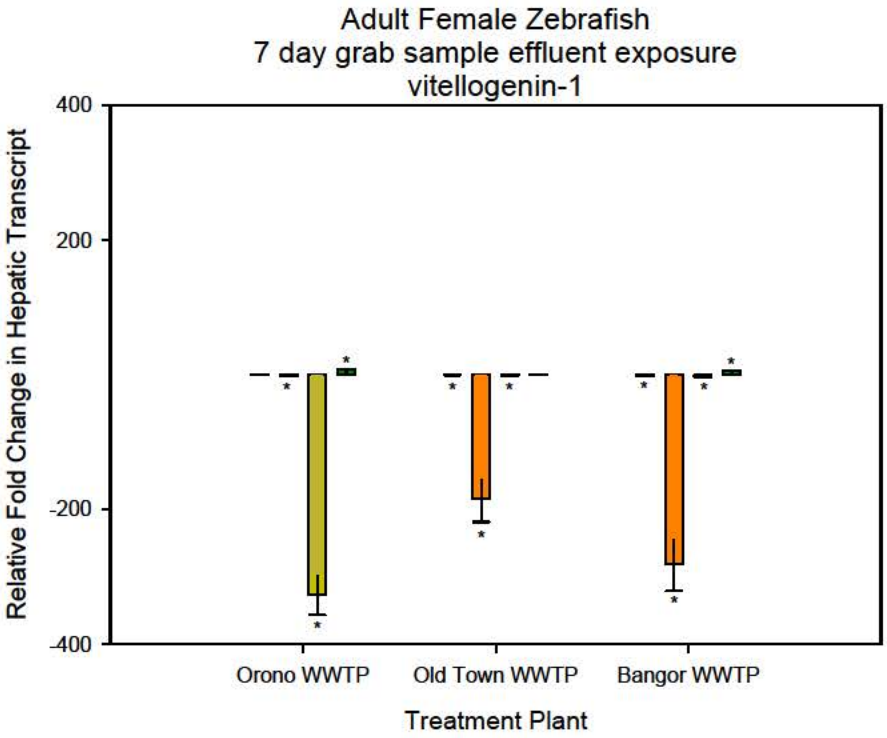

Figure 3.: Adult female zebrafish vitellogenin mRNA levels were drastically decreased after exposure to wastewater effluent at Dec. and Jan. samplings. This is a unknown phenomenon that the authors cannot explain except to attribute this effect to some other compound in the effluent. Little to no change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

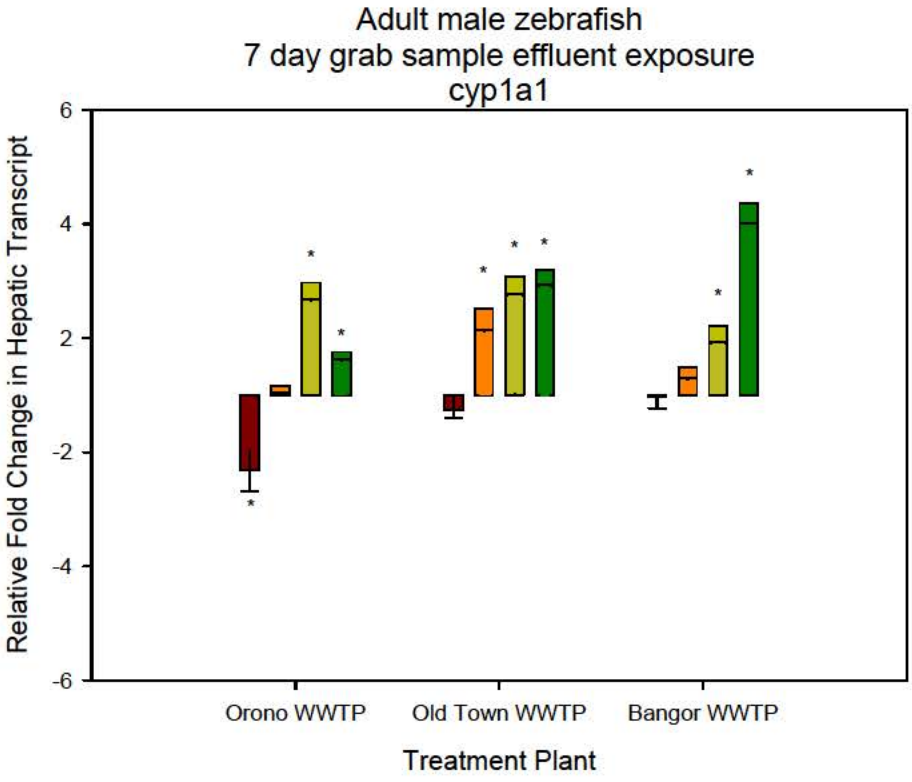

Figure 4. Adult male zebrafish are known to have reduced mRNA abundance of cyp1A1 after estrogen exposure. However, estrogen does not exhert this effect when a P450 inducer is also present in the exposure. We suggest that the increase in cyp1A1 expression is due to P450 inducers in the wastewater. This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

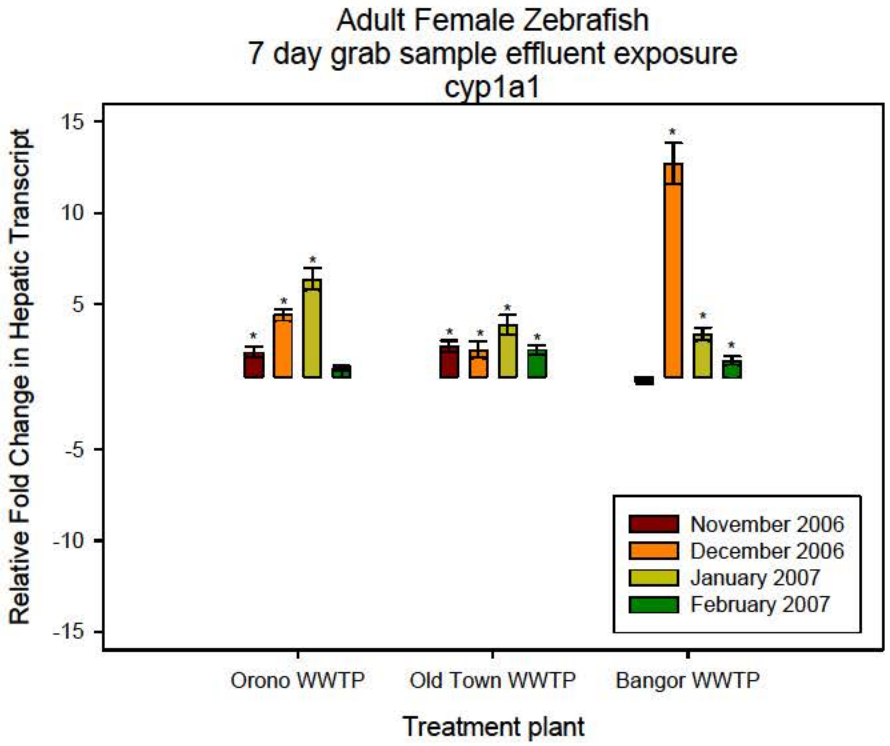

Figure 5. As in figure 4, females exhibited an increase in CYP1A1 levels after effluent exposure. Female response was generally greater than male response to these effluents.

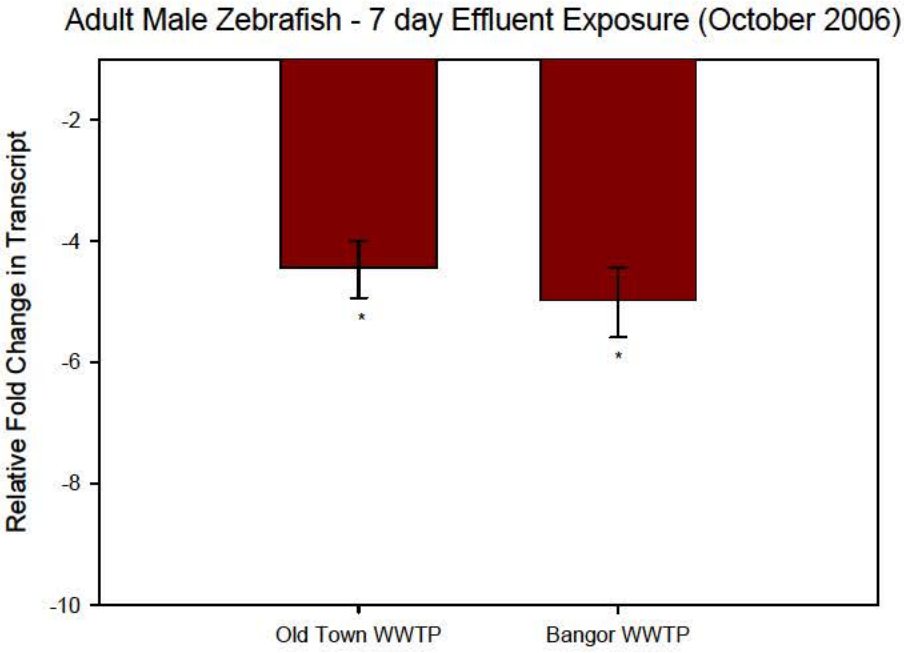

Figure 6. Male zebrafish exposed to Old Town or Bangor wastewater effluent had reduced expression of the nucleotide excision repair gene, XPC. This data corroborates our laboratories findings that 17a-ethinylestradiol dampens nucleotide excision repair. Although not a part of this project, this

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

U.S. Department of the Interior
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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.

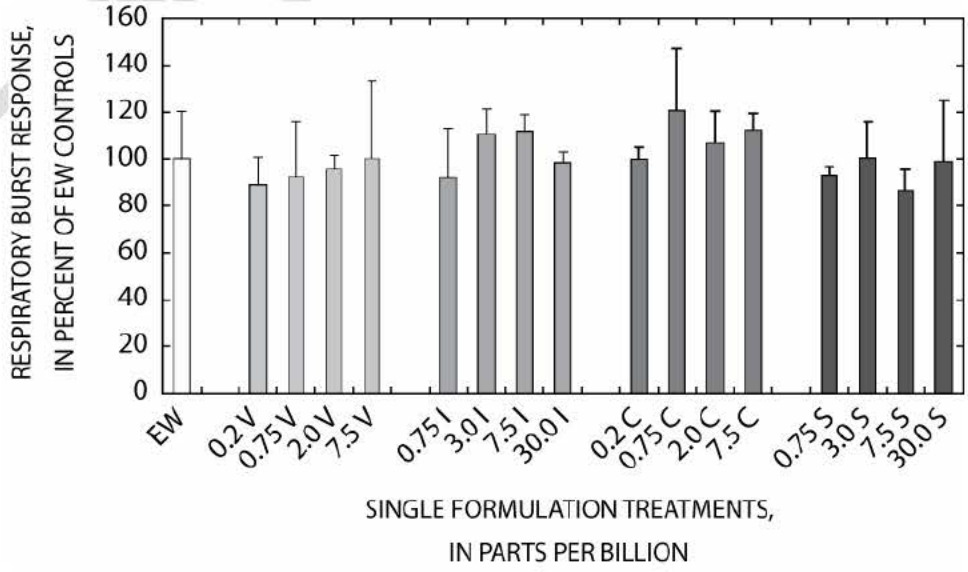

Figure 1. Preliminary data on the effects of exposure to single pesticide formulations on the innate immune system of zebrafish embryo-larvae exposed from fertilization through swim-up larvae. Bars represent means ± SD for n=2-4 replicates of 6 larvae per replicate. EW=Egg Water, V=Velpar™, I = Imidan™,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

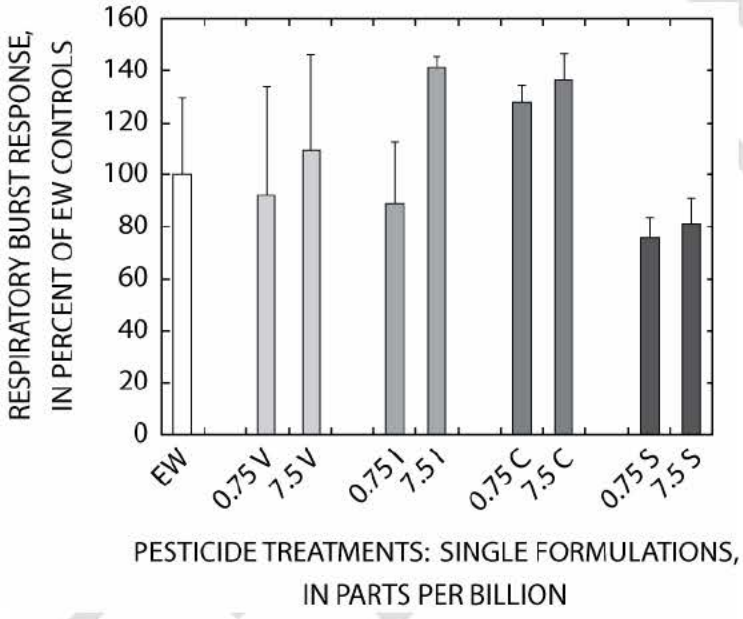

Figure 2. Preliminary data on the effects of exposure to single pesticide formulations on the innate immune system of zebrafish embryo-larvae exposed from fertilization through the swim-up larval stage. Bars represent means ± SD for n=2-4 replicates of 6 larvae per replicate. EW=Egg Water, V=Velpar™, 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.

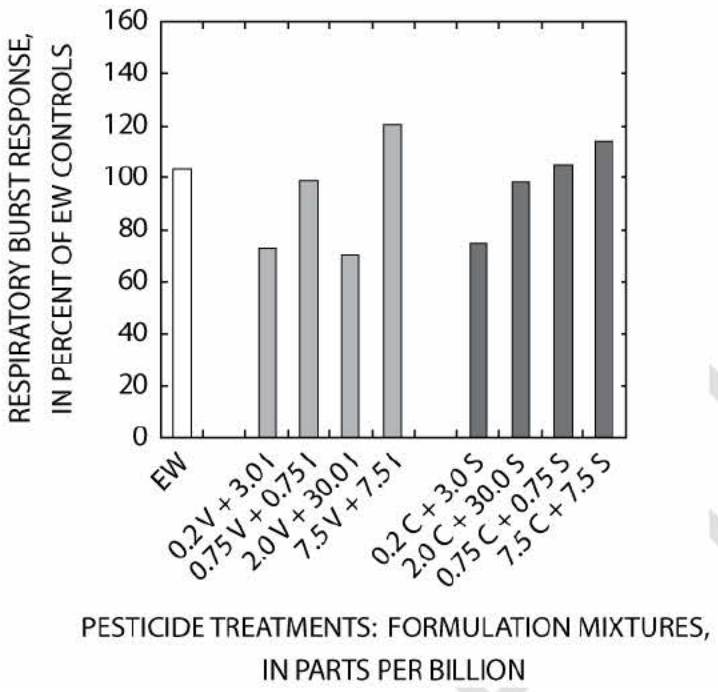

Figure 3. Preliminary data on the effects of exposure to mixtures of pesticide formulations on the innate immune system of zebrafish embryo-larvae exposed from fertilization through the swim-up larval stage. Bars represent means for one replicate, 6 larvae per replicate. EW=Egg Water, V=Velpar™, I = 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.

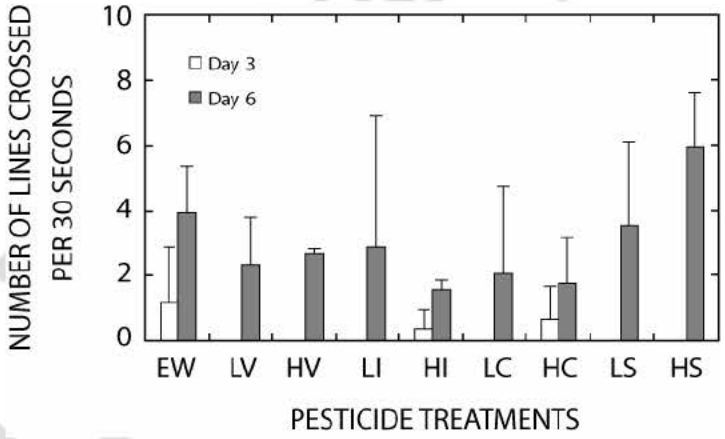

Figure 4. Preliminary data on single pesticide exposure effects on spontaneous swimming in zebrafish exposed from fertilization through swim-up. Bars represent means ± SD for n=2 replicates of 5 larvae per replicate. Swimming measured on Days 3 and 6 post-fertilization. EW=Egg Water, V=Velpar™, I = Imidan™,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

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