FINAL REPORT
2003 Kennebec River Caged Mussel Study

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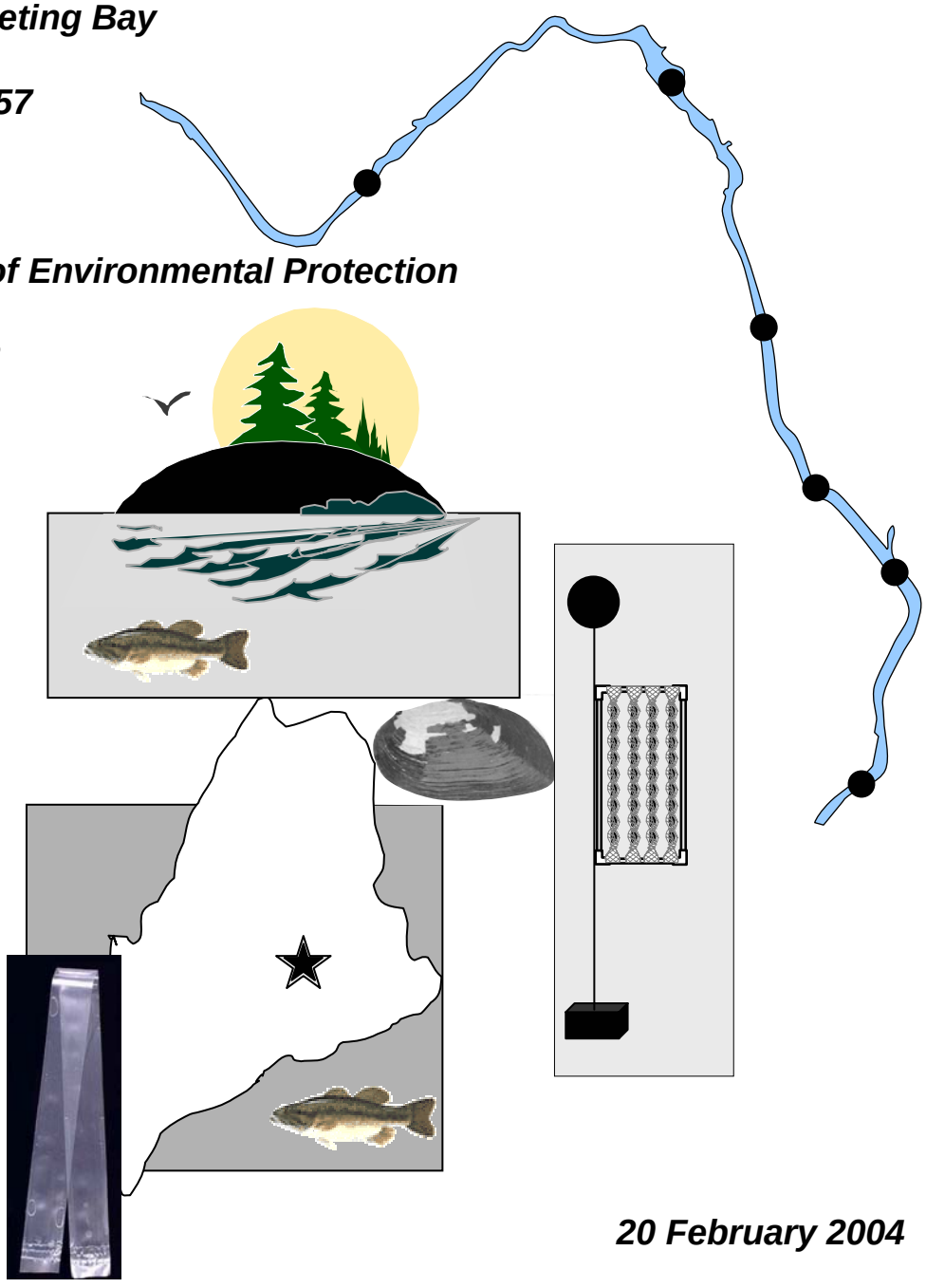

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1.0 EXECUTIVE SUMMARY

A caged mussel study was conducted in the Kennebec River, Maine during the summer of 2003 to determine the feasibility and scientific value of using transplanted mussels to monitor the effluent from the SAPPI mill at Hinckley, Maine. The study was designed to test whether caged mussels are a viable fish surrogate for monitoring the effluent discharged by kraft mills. Results suggest that caged mussels are a viable option and can provide more detailed information over fine spatial scales that cannot be provided by collecting fish in the impoundments above and below the mill. Although the tissue chemistry results suggest that neither 2,3,7,8-TCDD or 2,3,7,8-TCDF, the most toxic dioxin-furan congeners, are currently being discharged by the mill, growth rate and vitellin induction results suggest that the effluent could be causing some adverse effects on the environment. There were substantial uncertainties associated with the tissue chemistry results, which limits their use for determining whether or not the mill is in compliance. Assuming the tissue chemistry data are correct, the mill is in compliance. The caged mussels survived, grew, and demonstrated the ability to accumulate dioxins and furans in their tissues if these compounds were present in the water column.

The ***primary*** objective was to determine whether the mill is ***currently discharging*** dioxins and furans, particularly 2,3,7,8-TCDD and 2,3,7,8-TCDF, into the Kennebec River by measuring the accumulation of these compounds in mussel tissues. The ***secondary*** objective was to determine if there are any ***adverse ecological effects*** associated with the discharge of mill effluent to the Kennebec River. Potential ecological effects were assessed using a suite of mussel growth rate metrics and the vitellin assay for reproductive status and potential endocrine disruption. An ecological risk assessment approach was used to characterize potential exposure and effects of dioxin-furan congeners, particularly 2,3,7,8-TCDD and 2,3,7,8-TCDF. The main emphasis was on the use of a gradient design to identify potential sources of these chemicals on the river and a weight of evidence approach for reaching conclusions. The working hypothesis of the gradient design was that increasing and decreasing concentrations of chemicals in mussels deployed along the gradient can be used to indicate potential sources.

Caged freshwater mussels (*Elliptio complanata*) were deployed in the Kennebec River at 6 stations over a distance of approximately 24 miles. Two stations were positioned above the mill discharge, three stations within the mixing zone, and one station below the Shawmut Dam. A total of 432 freshwater mussels were used. Average mussel survival was 99%. Increases in shell lengths and whole-animal wet-weights were small, but statistically significant at all stations. Mean percent increase in shell length was about 1% while mean percent changes in whole-animal wet-weight (WAWW) were 6%. Of all growth metrics, tissue weights had the greatest increases, based on comparing the end-of-test (EOT) tissue weights with the estimated tissue weight determined from the beginning-of-test (BOT) mussels used for tissue chemistry analysis. Estimated mean tissue weight increased by 43% over the study period. Although increases in shell lengths and whole-animal wet-weights were small, they were statistically significant at all stations. Some statistically significant differences were found in mussel growth (i.e., changes in shell length and WAWW) among stations and along the suspected chemical gradients.

Mussels accumulated a limited number of congeners at all stations in the low to sub-parts-per-trillion range. A total of three congeners were detected at all six stations, two dioxins (1,2,3,4,6,7,8-HpCDD, OCDD) and one furan (2,3,7,8-TCDF). 2,3,7,8-TCDF was the most toxic congener detected. The concentrations of 2,3,7,8-TCDF were highest just above the mill discharge and 11 miles below, where the TEQs were also highest. Total PCDD-F

concentrations were driven by the presence of OCDD, and total TEQs by the presence of 2,3,7,8-TCDF. The most significant gradient detected was an increasing gradient of 2,3,7,8-TCDF with distance from the mill. The tissue chemistry data suggest that the two most toxic dioxin-furan congeners on which the regulations are based (i.e., 2,3,7,8-TCDD and 2,3,7,8-TCDF) are not being discharged by the SAPPI Mill. The only other congeners detected were octachloro dibenzo-dioxin (OCDD) and heptachloro dibenzo-dioxin (1,2,3,4,6,7,8-HpCDD), but these congeners are generally considered to originate from sources other than mill effluents. Within the impoundment, concentrations of 2,3,7,8-TCDF in mussel tissues were significantly higher above the mill diffuser than below. Concentrations of 2,3,7,8-TCDF in mussels deployed immediately below the diffuser were the lowest measured in this study. The high concentrations of 2,3,7,8-TCDF above the mill and 11 miles below the mill suggest that there may be other sources of these dioxins and furans in those areas. The distribution of lipid-normalized 2,3,7,8-TCDF was identical to the non-normalized data. There was no significant difference in 2,3,7,8-TCDF concentrations in any above-below comparisons. The increasing gradients away from the mill suggest that it is not appropriate to use stations 13 miles above and 11 miles below for the above-below comparisons to assess current mill discharges. The increasing gradients and variable concentrations of dioxins-furans at other locations within this 24 mile stretch of the river preclude an accurate assessment of current mill discharges using these stations.

The weight of evidence from the effects measurements (mussel growth rate and induction of vitellin) suggests that the mill may be discharging some chemicals with the potential for adverse effects. The caged mussel methodology provides an effective alternative for measuring effects, particularly if tissue chemistry analysis remains problematic. Because the focus of DEP's dioxin monitoring program (DMP) is on measuring chemical exposure in fish, effects have never been measured on the Kennebec River, either inside or outside the impoundment. Apparently, dioxins and furans have not been measured in fish within the impoundment either. Interestingly, the DEP has a macroinvertebrate biomonitoring program that has sampled twice within the impoundment. One of the reasons for initially proposing the caged mussel approach was that it would be consistent with DEP's current biomonitoring approach that includes rock baskets, riffle bags, and cones. These techniques are similar to the caged mussel approach in that they are experimental approaches that can be used along suspected chemical gradients but only measure effects. DEP's overall monitoring strategy would be enhanced by including caged mussels at their biomonitoring stations. This would allow for the characterization of other chemicals of concern. Mussels have been well established throughout the world as good sentinel organisms to evaluate the status and trends of chemicals in a variety of environments.

In a similar study conducted in 2000, the BOT concentrations of dioxins-furans in mussels collected from Lake Nequasset, Woolwich, Maine were below detection limits. In the 2003 study, mussels were collected from the same lake and the BOT tissue samples analyzed by two different laboratories. One laboratory reported Total PCDD-F concentrations of approximately 1 pptr while the other reported concentrations ranging from 5 to 20 pptr. This discrepancy made it difficult to clearly establish BOT concentrations of dioxins and furans in mussel tissues. In the 2000 study, approximately 15 congeners were detected in tissue samples from Stations 1 and 6, while only three congeners were detected in the 2003 study. In addition to the discrepancies between laboratories in the BOT tissue chemistry, a serious error was made for the EOT data in that 2,3,7,8-TCDD was reportedly detected at the station just above the mill. Upon request by DEP, a re-analysis of the data sheets showed that no 2,3,7,8-TCDD was accumulated. Collectively, the analytical problems suggest the data are questionable.

2.0 BACKGROUND AND INTRODUCTION

Several questions have been raised regarding the use of dioxins and furans in fish tissues to represent current discharges. This is particularly true on the Kennebec River where the fish collection sites are separated by 24 miles. A state law enacted in 1997 prohibits discharges of dioxins and furans into the rivers of Maine and required compliance by December 31, 2002. The most specific objective of that law is to determine if kraft pulp mills are ***currently*** discharging dioxins or furans, particularly 2,3,7,8-TCDD and 2,3,7,8-TCDF, two of the more toxic congeners often associated with kraft mills. The key issue is distinguishing between current and previous discharges. The existing approach used by the Maine Department of Environmental Protection (MDEP) is to collect fish from locations above and below the effluent diffuser. However, the fish tissue chemistry data do not provide direct evidence that the dioxins or furans measured in fish captured below a mill are associated with current mill discharges because of issues associated with mobility, different ages, sizes and lipid content, different species, and perhaps most importantly of all, the ability of fish to accumulate dioxins and furans from previous exposures through dietary exposure. It has been well established that the major exposure pathway for all dioxins and furans in fish is through their diet and that their food items could contain dioxins and furans acquired from sources other than current discharges. Furthermore, fish appear to preferentially accumulate 2,3,7,8-TCDD from these dietary sources which makes it even more difficult to distinguish between current and previous discharges.

In the summer of 2000, a caged mussel study was conducted to assess bioavailable dioxins and furans associated with effluent discharged frm the SD Warren/South African Paper and Pulp Industries, Ltd. (SAPPI) mill near Hinckley, Maine (Figure 1). This study used the same two fish collection sites to provide comparative tissue chemistry data for caged mussels and wild fish. The results of that caged mussel study were inconclusive because of the large distance between sampling stations and effluent diffuser (i.e., the “above” station was 13 miles upstream of the diffuser and the “below” station was 11 miles downstream), but the data successfully demonstrated that transplanted mussels are a viable option to monitor the effluent discharged by kraft mills and can provide detailed information over fine spatial scales that cannot be provided by collecting fish above and below dams creating these impoundments.

A second caged mussel study was proposed for the summer of 2003 to determine if mussels deployed along a gradient could eliminate some of the uncertainties associated with earlier efforts. The basic concept was that deploying caged mussels along a suspected chemical gradient, at locations both above and below the mill discharge, would facilitate characterizing dioxins and furans associated with the effluent, if present. An increase in dioxin-furan concentrations in mussel tissues would reflect an accumulation from a current discharge, particularly if the mussels were suspended in the water column. While the major exposure pathway for dioxins and furans in fish is through dietary exposure, mussels better represent water column exposures that include dissolved and particulate pathways associated with current mill discharges. Differences in congener distribution pattern (i.e., chemical fingerprinting) can be used to confirm potential sources.

This report summarizes the tissue chemistry, survival, and effects data collected in 2003 to assess the bioavailability of dioxins and furans, particularly 2,3,7,8-TCDD and 2,3,7,8-TCDF that may be associated with effluent discharged by the SAPPI pulp and paper mill near Hinckley.

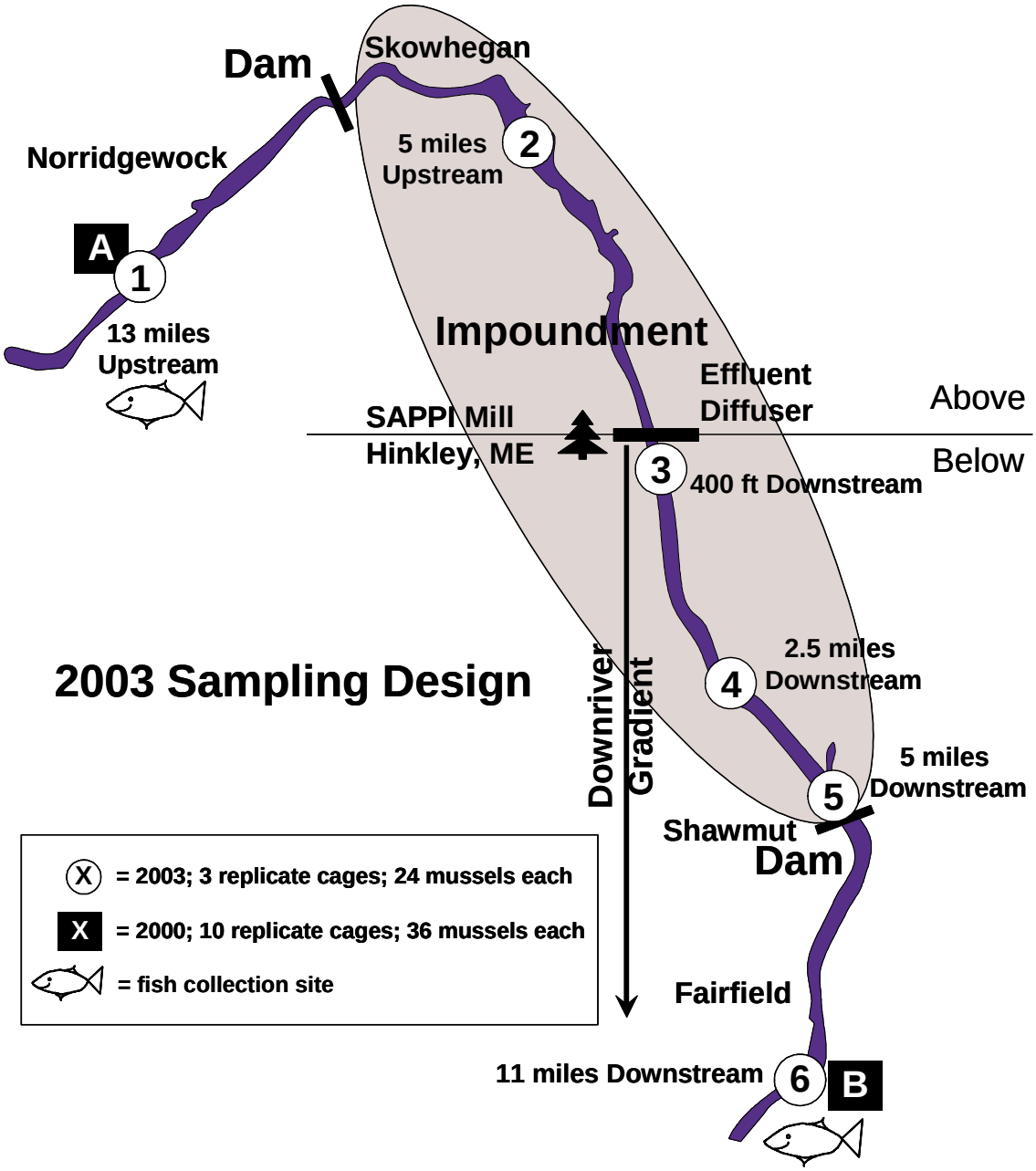


Figure 1. Kennebec River caged mussel station locations.

2.1 Purpose & Study Objectives

The purpose of this study was to determine the feasibility and scientific value of using transplanted mussels to monitor the effluent from the SAPPI kraft mill at Hinckley, Maine.

The ***primary*** objective was to determine whether the mill is ***currently discharging*** dioxins and furans, particularly 2,3,7,8-TCDD and 2,3,7,8-TCDF, into the Kennebec River by measuring the accumulation of these compounds in mussel tissues. To determine whether the mill is currently discharging these congeners, mussel tissue chemistry data were analyzed for decreasing chemical gradients of these chemicals in mussel tissues with distance. Even though it is generally assumed that the other congeners measured in this study are not generally associated with mill discharges, they were also evaluated in case the relative proportions in effluents have changed with process modifications over the years and these congeners have become more important in terms of identifying a chemical fingerprint from the mill. There are no good data available on the congener distribution of current mill discharges. Concentrations of total dioxins and furans were also examined in case the sum of the individual congeners might indicate a possible effect.

The ***secondary*** objective was to determine if there are any ***adverse ecological effects*** associated with the discharge of mill effluent to the Kennebec River. Potential ecological effects were assessed using a suite of mussel growth rate metrics and the vitellin assay for reproductive status and potential endocrine disruption. An ecological risk assessment (ERA) approach was used to characterize potential exposure and effects of dioxin-furan congeners, particularly 2,3,7,8-TCDD and 2,3,7,8-TCDF. No assumptions were made regarding the relative environmental significance of total dioxins and furans versus individual congeners or TEQs. The main emphasis was on the use of a gradient design to identify potential sources of these chemicals on the river. The working hypothesis of the gradient design was that increasing and decreasing concentrations of chemicals in mussels deployed along the gradient can be used to indicate potential sources.

3.0 METHODS

A caged mussel study was conducted in the Kennebec River, Maine during the summer of 2003 to determine the bioavailability of dioxins and furans associated with effluent discharged from the S.D. Warren/South African Paper and Pulp Industries, Ltd. (SAPPI) pulp and paper mill near Hinckley, Maine (Figure 1). American Society for Testing and Materials (ASTM) standardized protocols were followed for collection, transport, caging, and measurement of freshwater mussels. Complete details of transplant methodology used in this study are described in ASTM Standard Guide for Conducting In-situ Field Bioassays with Marine, Estuarine and Freshwater Bivalves (ASTM 2001).

Bioaccumulation in mussel tissues was used to estimate exposure to and bioavailability of dioxins and furans. This was accomplished by comparing end-of-test (EOT) concentrations in mussel tissues to concentrations in mussel tissues before deployment. Growth based on changes in whole-animal wet-weight (WAWW), shell length, tissue wet weight, and shell weight was measured to 1) to calibrate bioaccumulation (i.e., to determine if chemical dilution due to tissue increase or chemical magnification due to tissue loss has occurred), 2) to characterize the health of the mussels and determine if adverse effects are occurring as a result of exposure to site-related conditions, and 3) to evaluate whether the caged mussels meet performance criteria for a successful test. Measurements of mussel WAWW and shell length before and after deployment, and of mussel soft tissue weights at the end of the test, aid in interpreting contaminant accumulations and potential effects. The ASTM Standard Guide specifically states there will be a high degree of uncertainty in the tissue chemistry data if mussels lose more than 20% of their body weight during the study. This criterion was used to determine if the caged mussel study was successful and to aid in interpretation of the tissue chemistry data. Percent lipids was used to corroborate effects, and tissue chemistry were used to estimate exposure.

3.1 Study Design

Conceptual Approach

- Gradient design;
- Ecological risk assessment-based monitoring (exposure and effects); and
- Weight of evidence.

The gradient design is intended to identify potential sources of chemical exposure and associated biological effects by demonstrating a gradient in the exposure or effects measurements with distance from the suspected source. Ecological risk assessment-based monitoring refers to monitoring that places equal emphasis on characterizing exposure and effects. In the context of the caged mussel pilot study, weight of evidence refers to the use of multiple exposure endpoints (e.g., concentration of total dioxins and furans, congener-specific dioxins and furans, and lipid-normalized dioxins and furans), multiple effects endpoints including growth (e.g., whole animal wet weight (WAWW) and WAWW growth rate, shell length and length growth rate, shell weight, tissue weight) and vitellin (e.g., vitellin, tissue normalized, protein normalized) and the use of all of these metrics to make inferences regarding potential ecological and human health effects.

The bioavailability of dioxins/furans was quantified using the concentrations of these chemicals accumulated in mussel tissues after a 66-day exposure period. The *in situ* mussel transplant

study consisted of collecting mussels (*Elliptio complanata*) from a relatively clean source and caging individuals of a uniform size range. The caged mussels were transplanted along a suspected chemical gradient in the Kennebec River, retrieved after 66 days, and assessed for exposure and effects. Mussel tissues were removed for chemical analysis of dioxins, furans, percent lipids, and percent moisture. Table 1 summarizes the study design.

Table 1. Summary of caged mussel study experimental design

Study Summary	
Stations	6 on the Kennebec River, beginning at Norridgewock (13 miles above mill discharge) and ending downstream at Fairfield (11 miles below mill discharge)
Size range of mussels at start of test	62.4 to 66.9 mm
Number of cages per station	3
Number of mussels per cage	24 (3 bags with 8 mussels/bag)
Number of mussels deployed	432 (6 stations x 3 cages x 24 mussels/cage)
Number of mussels for T_0 tissue chemistry & biomarkers	72 (for tissue chemistry: 3 replicates x 20 mussels/replicate; for biomarkers: 3 replicates x 4 mussels/replicate)
Total number of mussels required	504
Deployment configuration	Gradient Design
Deployment period	66 days
Exposure endpoints	Dioxins and Furans
Effects endpoints	growth (Δ WAWW & length; EOT tissue & shell weight), % lipids, % water

3.2 Test Duration & Schedule

The caged mussel study was conducted from August to September 2000. A 66-day deployment period was used. The in-situ mussel study was conducted according to the following schedule:

- July 27, 2003: *Elliptio* collected from Nequasset Lake, presorted into 1-mm size groups. Sorted mussels placed in mesh bags and held overnight in Nequasset Lake.
- July 28, 2003: Distributed Kennebec River *Elliptio* to mesh bags, mesh bags attached to PVC frames, unit wrapped with predator mesh. Completed cages placed in Nequasset Lake for overnight holding. T_0 mussels shucked at Bath Water District facility.
- July 29, 2003: *Elliptio* deployed at all Kennebec River stations.
- October 3, 2003: Mussels retrieved from all Kennebec River stations. Mussels measured and shucked; tissues frozen for chemical analysis.

3.3 Mussel Processing Locations

The beginning-of-test (BOT) mussel sorting, measurements, and distribution took place approximately 3.5 miles East of Bath in Woolwich, at the Bath Water District treatment plant adjacent to Nequasset Lake. The lake was only about 50 meters from the treatment plant, which facilitated transportation of the mussels from the collection site to the measurement facility at the beginning of the test as well as returning all unused mussels after preparing the cages for deployment. The water quality laboratory at the Bath Water District treatment plant was used for BOT tissue removal. The end-of-test (EOT) mussel measurements, tissue

removal, and storage prior to shipment for chemical analysis occurred at a DEP laboratory in Augusta, Maine.

3.4 Mussel Collection

Mussels in the 58- to 68-mm shell length size range were collected from Nequasset Lake, an area believed to be relatively free of contamination and high in *Elliptio complanata* density. Four SCUBA divers from Friends of Merrymeeting Bay (FOMB) collected the mussels by hand. The number of mussels removed from their natural habitat was limited by keeping a running tally of the number collected.

3.5 Mussel Sorting and Distribution

Shell length (longest axis, generally from the anterior end near the beak to the leading posterior end, as determined with vernier calipers) was used to sort and select mussels to be used in the study. The final size range for *Elliptio*, 62.4 to 66.9 mm shell length, was based on obtaining the maximum number of mussels in the minimum size range.

After collection, mussels were placed in tubs without water or ice. They were then presorted into 1-mm size groups for distribution to mesh bags. They were held without water until after the presort to eliminate the potential of oxygen depletion in the holding water. Once sorted into smaller groups, the mussels were placed in mesh bags (mesh size approximately 1.25"), with one size group per bag. The mussels were then placed in Nequasset Lake until measurement and distribution the following day. All unused mussels were returned to Nequasset Lake.

Prior to distributing mussels to the mesh bags, the mussel lengths were remeasured (to nearest 0.1 mm) and weighed (to nearest 0.01 g) for the first time using ASTM (2001) procedures. The WAWWs and shell lengths were recorded by hand on data sheets and electronically by a computer connected to the electronic balance. Only live mussels that were fully closed, or those that closed immediately upon light physical stimulation were used.

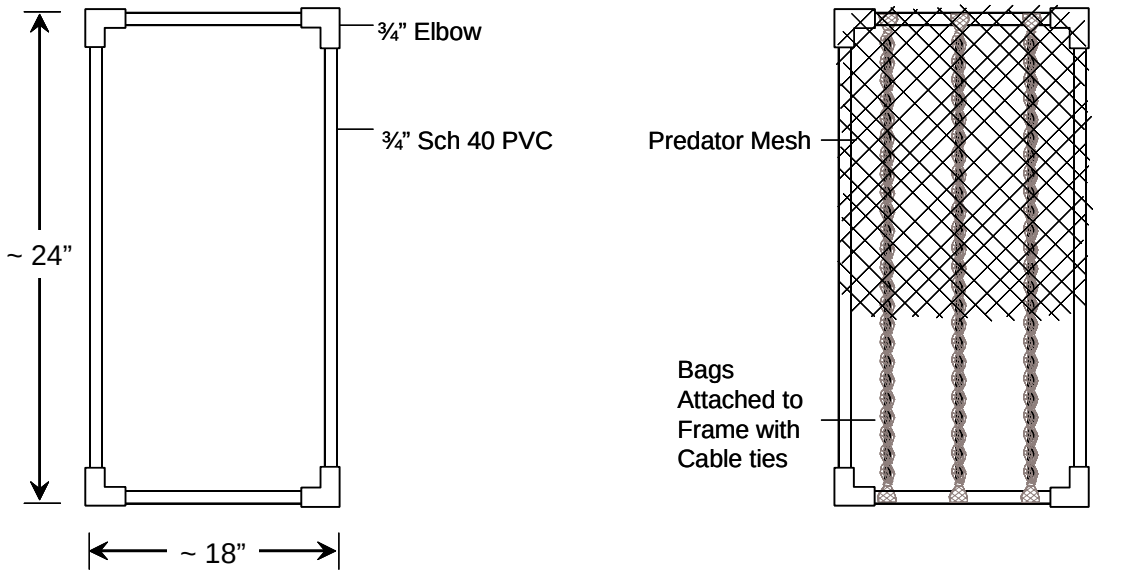
In addition to placing mussels into mesh bags for deployment, a subgroup of mussels from the same size class deployed in the field were retained in a separate compartmentalized tray. These mussels, which were used for BOT tissue weights, shell weights, tissue chemistry, and biomarker analysis, were treated in exactly the same way as those being deployed in the field, i.e., they were selected from the same size groups as the mussels deployed in the field and they were measured for length and whole-animal wet-weight at the same time and in the same order as the mussels to be deployed in the field. An Analysis of Variance (ANOVA) confirmed no statistical difference in size distribution among cages or stations (including mussels used for the BOT measurements). No significant differences were found when comparisons were made by cage or station:

	p value
WAWW by cage	0.6227
WAWW by station	0.7489
Length by cage	0.9999
Length by station	0.9796

3.6 Mesh Bags and PVC Cages

Mesh bags (approximately 4" in diameter and 5' long; 0.25" mesh size) made from plastic netting were used to hold the mussels during the deployment period. A plastic tag showing Station Number and Bag Number was attached to each bag. Mussels were placed in the mesh bags sequentially. Nylon cable ties were used to separate individuals to allow a more even exposure to environmental conditions, keep track of position, and prevent mussels from shifting position in the bag. Three bags were prepared for each cage. Each bag contained eight individuals.

Cages approximately 18" x 40" were constructed from 3/4" Schedule 40 polyvinyl chloride (PVC) pipe. The mesh bags were attached to the PVC frame with nylon cable ties approximately 6" in length. Once the mussel bags were attached to the PVC cage, the unit was wrapped with heavy duty plastic mesh (approximately 1¼" mesh size) to provide security, discourage predators, and protect the mussels during transport, deployment, and retrieval (Figure 2).



A: Schematic of PVC Frame for mussels

B: Bags attached to Frame; Predator mesh

Figure 2. Diagram of cage design.

3.7 Baseline Tissue Weight, Shell Weight and Tissue Chemistry

By random assignment, three groups of mussels, each consisting of 24 individuals, were put into separate compartmentalized trays rather than mesh bags, and used to determine baseline tissue weights and shell weights and for beginning-of-test tissue chemistry and biomarker analyses. All 72 individuals (i.e., 3 reps x 24 mussels/rep) were measured for shell length and WAWW; tissues were removed and weighed and the empty shells were weighed for 20

individuals per replicate. Because weighing tissues and shells is a destructive process and could not be made on individuals deployed in the field, the tissue and shell weight measurements made on these baseline individuals were used to estimate tissue and shell weights for mussels deployed in the field. Tissues from 20 individuals in each replicate were composited for chemical analysis. Each composite baseline tissue sample was analyzed for dioxins, furans, percent solids, and percent lipids. The remaining four individuals per replicate were wrapped in foil and frozen for biomarker analyses. Neither tissue weights nor shell weights were determined on the biomarker individuals.

3.8 Overnight Holding

Caged mussels were held in Nequasset Lake for up to 16 hours at the beginning of the test (i.e., end of the first day after collection, after filling a series of bags, and until deployed). Surface water from this lake was used during the BOT and EOT measurement activities, as required. After retrieval from deployment stations on the Kennebec River, caged mussels were returned directly to the DEP lab in Augusta for final growth measurements, removal of mussel tissues for chemical analysis, and storage of those samples until shipment for analysis. There was no overnight holding at the end of the test.

3.9 Station Locations and Deployment

Caged mussels were deployed along a gradient at 6 stations on the Kennebec River (Figure 1; Table 2). Two stations were above the effluent diffuser. The first, Station #1, was 13 miles above the diffuser at the same location used in the 2000 study. The second, Station #2, was within the impoundment, approximately 5 miles above the effluent diffuser. Three stations were situated in a gradient immediately below the effluent diffuser with the first station as close to the outfall as possible, the second approximately 2.5 miles downstream and the third approximately 5.0 miles downstream. The last station, Station #6, was located approximately 11 miles from the diffuser, the same location used in the 2000 pilot study. Surface marker buoys were used to identify the deployment locations. Three cages of approximately 24 mussels each were deployed at each of these stations so they floated approximately 1 meter below the surface. In addition to deploying caged mussels near the diffuser, two additional temperature monitors were also deployed in this area. One monitor was deployed immediately adjacent to the diffuser (see Table 2 for location) and the second was deployed approximately 100 feet below the diffuser. Station locations were identified by DEP and FOMB, with input from Applied Biomonitoring, and confirmed with GPS.

A random number table was used to assign cages to stations. Station 1 was furthest upstream (up) on the river and Station 6 furthest downstream (dn). Cages were deployed according to the distribution provided in Table 2.

The attachment of weights, lines, and buoys occurred just prior to deployment. Heavy (i.e., 25-lb) cylindrical blocks with eye-bolts were used as anchors. The caged mussels were deployed from boats provided by DEP, and SAPPI. FOMB, DEP, and Applied Biomonitoring staff deployed all caged mussels.

Table 2. Station locations for the caged mussel study.

Station	Distance from Diffuser	Cage Number	Depth (Ft)	North Latitude (deg/min)	West Longitude (deg/min)
1	13 mi -up	2	23	44° 43.836'	69° 46.391'
		10*	23	44° 43.830'	69° 46.395'
		13	23	44° 43.819'	69° 46.400'
2	5 mi - up	6	13	44° 45. 358'	69° 39. 940'
		17*	16	44° 45. 364'	69° 39. 916'
		19	16	44° 45. 369'	69° 39. 895'
3	0.08 mi - dn	7	14	44° 41. 665'	69° 37. 909'
		21*	nm	44° 41. 667'	69° 37. 908'
		5	nm	44° 41. 664'	69° 37. 940'
4	2.5 mi - dn	11	33	44° 39. 249'	69° 36. 877'
		15*	27	44° 39. 266'	69° 36. 862'
		16	20	44° 39. 281'	69° 36. 848'
5	5.0 mi - dn	1	20	44° 37. 991'	69° 35. 146'
		9*	20	44° 38. 009'	69° 35. 139'
		18	22	44° 38. 039'	69° 35. 108'
6	11 mi - dn	4	18	44° 34.870'	69° 35.830'
		8*	15	44° 34.869'	69° 35.789'
		14	13	44° 34.875'	69° 35.777'
		Diffuser Coordinates		44° 41. 756'	69° 37. 951'
	Temperature Monitor deployed at plume			44° 41. 752'	69° 37. 928'
	Temperature Monitor deployed 100' downstream of plume			44° 41. 735'	69° 37. 910'

*Cage with temperature monitor; NM = not measured

3.10 End-of-Test Retrieval and Measurements

Retrieval and end-of-test mussel measurements were made on October 3rd. Cages were retrieved by Applied Biomonitoring, FOMB, and DEP. Boats were supplied by DEP and the SAPPI mill. All cages were retrieved and delivered to the DEP laboratory by 10:30 am.

During transportation from field stations, the caged mussels were placed on tarps to avoid exposure to chemicals on the ground and covered with additional tarps to minimize exposure to sun and wind. The mesh bags were removed from the PVC cages and placed in an ice chest containing wet ice. At the DEP lab, mussels were allowed to equilibrate (i.e., replace any air between shells with water) in water for a minimum of 10 minutes before measuring WAWW.

End-of-test measurements were made using live mussels only according to procedures in ASTM (2001). The number of survivors per cage was recorded. Mussels with broken shells or those that did not close upon light physical stimulation were considered dead. Mussels were placed into compartmentalized trays to keep their order during measurements. The trays containing mussels to be measured were placed in water so that the mussels were completely submerged. Mussels were then measured for change in size: individuals were measured for

WAWW, shell length, shell weight, and soft-tissue weight. Once the WAWW and shell length measurements were made, the mussels were placed in another compartmentalized tray and set in a cooler until tissue removal. The mussels were not kept in water once the growth measurements were made. For each cage, tissues from all surviving mussels within the first 20 individuals were shucked for chemical analysis. The remaining four mussels from each cage were wrapped in foil, labeled, and frozen for biomarker analyses. The frozen mussels were in the custody of Ed Friedman, FOMB, prior to shipment to Environment Canada's St. Lawrence Center laboratory for analysis.

3.11 Collection and Preparation of Bivalve Tissues for Chemical Analysis

Tissue removal, which occurred after all WAWW and shell length measurements were made, was conducted according to ASTM (2001). All shucking knives used in tissue removal were stainless steel. Cutting boards, plastic trays, and weigh boats were covered with aluminum foil prior to cleaning. Prior to shucking mussels from a given cage, the knife and foil-covered implements were decontaminated by (1) washing with a soap-free biological cleaning solution, (2) rinsing with hot tap water, (3) rinsing with distilled water, (4) rinsing with acetone, and (5) a final rinsing with hexane. Worn or ripped foil was replaced before shucking mussels from a new cage. Decontamination was overseen by Barry Mower (DEP). Gloves were worn during the shucking process to reduce the potential for cross contamination.

Once detached, the mussel's shell was used as a "holding dish" to prevent contact with other surfaces until the tissues were weighed. Caution was used to minimize contact of tissue with surfaces other than the interior of its shell. Shucked mussels were placed in order on a foil-lined tray. All mussels from one cage were shucked before weighing the tissues and the shells. Tissues from all individuals within one cage were composited for chemical analysis. Sample jars were provided by DEP. Sample labels were affixed to the outside of the jar. These tissue composites were frozen at -20°C prior to shipment to PACE Analytical Laboratory for analysis of dioxins, furans, percent lipids, and percent solids. DEP was responsible for shipment and delivery of tissues to the analytical laboratory. Appropriate chain-of-custody forms were completed and accompanied the tissue samples.

Shells were weighed after the tissues were removed and weighed. Tissue and shell weights were recorded for each individual mussel to allow pairing with WAWW, shell length, and other growth metrics. The tissue and shell weights were recorded electronically to an Excel spreadsheet and by hand to a hard copy.

3.12 Mussel Tissue Chemistry

Tissues were analyzed for dioxins, furans, and percent lipids. All analyses were conducted by PACE Analytical Laboratory, Dioxin Laboratory, Minneapolis, MN. All dioxin-furan analyses were conducted according to USEPA Method 1613B.

3.13 Temperature Measurements

Water temperature was recorded at 15-minute intervals during the entire test with *in situ* temperature monitors (Onset® Tidbit). One temperature monitoring device was deployed at each station by attaching it directly to one of the cages deployed at the station.

3.14 Vitellin Analysis

Increased vitellin production is an indication of potential endocrine disruption and reproductive effects in bivalves and is comparable to the induction of increased vitellogenin in fish. These chemical inducers mimic or interfere with endogenous hormones in vertebrates and invertebrates and may cause adverse biological effects. It was expected that the females would have some increase in vitellin because they were preparing for the next spawning cycle. However, excessive vitellin production in the females and the males, is an indication of adverse effects. The vitellin assays were conducted as a professional courtesy by Francois Gagne, Christian Blaise, and Chantale Andre of Environment Canada's St. Lawrence Center, the developers of this biochemical assay.

Methods used in this study were similar to those developed over the past several years (Blaise et al 1999, 2003; Gagne et al 2001, 2002). Vitellogenin-like proteins were measured indirectly using the alkali-labile phosphate (ALP) assay. ALP was normalized for proteins, but these data were not as responsive. The ALP assay is an indirect method to determine the relative levels of vitellin in biological tissues. The ELISA was not performed because the available kits are for fish vitellogenin and the appropriate antibodies do not cross-react well with bivalves. The ALP assay, because it is indirect, is validated with gel electrophoresis where vitellogenin-like protein bands are quantified by densitometric analysis. Four mussels from each cage were wrapped in aluminum foil and frozen prior to shipment to the St. Lawrence Center for analysis.

3.15 Data Analysis

A practical, step-wise approach to data analysis was used for this study. First, the tissue chemistry and growth data were summarized by station, and descriptive statistics such as mean, standard deviation, and 95% confidence interval (CI) were calculated. Bar graphs, with 95% CIs, were used to identify stations with the highest and lowest means as well as possible gradients. Comparative statistics (i.e., t-test or Analysis of Variance (ANOVA), depending on the hypothesis) were used to help confirm general differences identified by examining the graphs. Several parameters were regressed against distance from the diffuser to determine whether the mill was a potential source of chemicals measured in mussel tissues or if growth affects could be associated with exposure to the effluent. These regressions, and the subsequent statistical analyses, only represent a first order approximation because mean values were used. Means were used rather than the individual data points to minimize the effects of variability in the data.

For tissue chemistry, the normal approach to data analysis begins with a comparison to beginning-of-test (T_0) conditions. However, discrepancies in the T_0 results for Lake Nequasset mussels precluded the usual practice of comparing beginning- and end-of-test concentrations to determine net accumulation during the exposure period (see Discussion Section 5.7). Because of these discrepancies and associated concerns with some of the methods used in the chemical analyses, mussels were assumed to have initial dioxin-furan tissue burdens that were similar to those measured for the 2003 study by Columbia Analytical (Applied Biomonitoring 2004).

For the growth metrics, each individual mussel was considered a replicate. If all mussels survived, the level of replication at each station was 72 (i.e., 24 mussels/cage x 3 cages) for WAWW and shell length. The level of replication at each station was 60 (i.e., 20 mussels/cage x 3 cages) for tissue weight and shell weight. The level of replication for the biomarker analyses

was 4. For the bioaccumulation portion of the study, the level of replication at each station was 3, because three composite samples were prepared for each station.

All statistical analyses were conducted using GraphPad InStat software (version 3.05, Win 95/NT; GraphPad Software, San Diego, California, www.graphpad.com). InStat automatically assesses data for normality and common variances, and recommends alternative approaches if the data failed to meet the assumptions. For the ANOVAs, data that failed to meet these assumptions were analyzed with the nonparametric Kruskal Wallis test. For the t-tests, a Welch correction was applied to data that failed to these assumptions. All tests were conducted at the 95% confidence level ($\alpha = 0.05$).

3.15.1 Bioaccumulation Data

Only wet-weight tissue concentrations are included because the tissue samples were not analyzed for percent moisture by the analytical laboratory.

The following conventions were used for all tissue chemistry data:

- A zero ("0") was used for all concentrations reported as <DL.
- All data, including zeros, were used when calculating means and 95% confidence intervals.

The following process was used in data analysis:

- For each station, calculate mean, standard deviation, and 95% confidence interval for each congener
- For each station, calculate mean, standard deviation, and 95% confidence interval for Total PCDD-F
- Lipid-normalize congener data using the percent lipid value reported for each sample by the laboratory
- Calculate mean, standard deviation, and 95% confidence interval for each congener and Total PCDD-F on a lipid-normalized basis
- Using replicate tissue chemistry results, test for differences among all six stations with ANOVA and multiple range tests
- Using bar graphs, identify potential gradients
- Using regression analysis on mean data, test for significant regressions and gradients
- Using a t-test, test for differences between above and below the diffuser, for the following comparisons:
  - Station 1 (Above) versus Station 6 (Below)
  - Station 2 (Above) versus Station 3 (Below)
  - Station 2 (Above) versus Stations 3, 4, 5 (Below)

Three different types of above-below mill comparisons were made to satisfy different requirements. Stations 1 and 6 were compared because mussels were deployed at these same locations in the 2000 study, and fish are routinely collected from these locations as part of DEP's monitoring effort. Stations 2 and 3 were compared to test for differences directly above and below the discharge. Data for Stations 3, 4, and 5 were pooled and compared to Station 2 to determine if there was any difference within the impoundment between above and below the discharge.

Toxicity equivalent concentrations (TEQs) were calculated using toxicity equivalence factors (TEFs) provided by the World Health Organization (WHO; Vanden Berg et al. 1998). For the human health component, the human-mammal TEFs were used. To assess for potential ecological impacts, the fish TEFs were used. The TEQs were calculated for each sample using the detected concentrations; as with the calculation of Total PCDD/PCDF, a “0" was used for concentrations reported as <DL.

3.15.2 Survival & Mussel Health Metrics

Percent survival was calculated as initial number deployed minus number dead divided by number deployed. Dead mussels were defined as those with empty shells. No statistical comparisons were conducted on survival by station because survival at all stations was similar and very high. Growth was measured to calibrate bioaccumulation (i.e., to determine if chemical dilution due to tissue increase or chemical magnification due to tissue loss has occurred) and to determine the health of the mussels after the exposure period. Four growth metrics were used: shell length, WAWW, wet tissue weight, and shell length. Percent lipids were also used as an indication of mussel health.

Descriptive summary statistics (i.e., mean, minimum, maximum, and percent change) were calculated for all growth metrics. Using these data, the end-of-test growth metrics were compared to beginning of test to determine if there was measurable growth during the deployment periods. Particular attention was given to changes in tissue weight, as this metric is critical for evaluating and interpreting the tissue chemistry data. A one-way ANOVA and a multiple range test were used to test for differences in growth metrics (i.e., whole-animal wet-weights, shell length, tissue weight, or shell weight) among stations.

3.15.3 Water Temperature Data

Water temperature was measured to help understand the bioaccumulation and growth results and to determine if there were any differences among stations that could be attributed to temperature. Maximum, minimum, mean, and range in water temperature were calculated for each station. Water temperature profiles based on all the data collected during the field deployment were made and used to identify trends in water temperature. To reduce variability and autocorrelation in the temperature data for statistical analyses, the temperature series for each station was reduced to average daily temperatures. In addition, the range in daily temperature was determined by subtracting the lowest measurement within a 24-hour period from the highest measurement within the same period. Differences in daily average temperatures among stations and daily temperature ranges among stations were tested with an ANOVA and a multiple range comparison test.

3.16 Data Quality Review & Acceptability

The ASTM standard guide (ASTM 2001) suggests that two criteria be used to determine bioaccumulation data acceptability: 1) There should be no significant loss in tissue weight during the exposure period; and 2) If survivors have not lost significant tissue mass, a survival criterion of >45% may be acceptable to interpret the bioaccumulation data. The lowest survival in any cage was 95%; lowest mean survival at any station was 97.5%. There were no significant losses in tissue weight, so all the *Elliptio* effects data were considered acceptable for data analysis.

4.0 RESULTS

The relative contribution of each congener by station, on a concentration basis and on a percentage basis of both total PCDD-F concentration and total TEQ, provide the best overview of the results. They show the chemical gradients, or lack thereof, potential sources, the influence of the dams, and the limited utility of the fish data because of where the fish have routinely been collected. For example, the relative contribution of 2,3,7,8-TCDF to the total TEQ shows the obvious increasing gradient with distance from the mill as well as the step increase below the Shawmut Dam. In general, statistical results for the above-below comparisons and significant gradients were virtually identical for both non-normalized and lipid-normalized data.

4.1 Tissue Chemistry – Dioxin/Furans in Mussel Tissues

Mussels accumulated a limited number of congeners at all stations in the low parts-per-trillion (pptr) range (Table 3; Figure 3A). 2,3,7,8-TCDD, the most toxic dioxin congener, was not detected at any station. 2,3,7,8-TCDF, the most toxic furan congener, was measured in mussels from all six stations. The only other congeners detected were octachloro dibenzo-dioxin (OCDD) and heptachloro dibenzo-dioxin (1,2,3,4,6,7,8-HpCDD), but these congeners are generally considered to originate from sources other than mill effluents. Mussels from all six stations accumulated all three congeners.

Total PCDD-F concentrations (Figures 3A, 3B) were influenced primarily by the presence of octachloro dibenzo-dioxin (OCDD), 1,2,3,4,6,7,8-HpCDD, and 2,3,7,8-TCDF (Table 3; Figures 3A, 3B). Total PCDD-F concentrations were driven by the presence of OCDD that ranged from a low of 82% at Station 1 to a high of 89% at Station 6 with a mean of 85%. Conversely, 2,3,7-TCDF ranged from a low of less than 1% at Station 3 to a high of approximately 2.5% at Station 1 and a mean of 1.8%.

4.1.1 TEQs

Using all data and both the human health and fish TEFs, TEQs for all stations were on the order of 0.005 to 0.008 (Figures 4A, B). There was no difference in the total TEQ value using TEFs for either human health or fish. Total TEQs at all stations were driven by 2,3,7,8-TCDF. In fact, the pattern of relative total TEQ by station is very similar to the pattern found for the concentration of 2,3,7,8-TCDF by station (Figure 5A).

Table 3. Dioxin-furan congeners (ng/kg-ww) in mussel tissue samples. "0" substituted for non-detects; DL = detection limit.

Compound	DL (ng/Kg)	Station 1 13 m up	Station 2 5 m up	Station 3 400 ft down	Station 4 2.5 m down	Station 5 5 m down	Station 6 11 m down
Non-normalized data							
2,3,7,8-TCDF	0.1	0.091	0.130	0.038	0.082	0.090	0.131
1,2,3,7,8-PeCDF	0.1	0.000	0.000	0.000	0.000	0.000	0.000
2,3,4,7,8-PeCDF	0.1	0.000	0.000	0.000	0.000	0.000	0.000
1,2,3,4,7,8-HxCDF	0.25	0.000	0.000	0.000	0.000	0.000	0.000
1,2,3,6,7,8-HxCDF	0.25	0.000	0.000	0.000	0.000	0.000	0.000
2,3,4,6,7,8-HxCDF	0.25	0.000	0.000	0.000	0.000	0.000	0.000
1,2,3,7,8,9-HxCDF	0.25	0.000	0.000	0.000	0.000	0.000	0.000
1,2,3,4,6,7,8-HpCDF	0.5	0.000	0.000	0.000	0.000	0.000	0.000
1,2,3,4,7,8,9-HpCDF	0.5	0.000	0.000	0.000	0.000	0.000	0.000
OCDF	1	0.000	0.000	0.000	0.000	0.000	0.000
2,3,7,8-TCDD	0.1	0.000	0.000	0.000	0.000	0.000	0.000
1,2,3,7,8-PeCDD	0.1	0.000	0.000	0.000	0.000	0.000	0.000
1,2,3,4,7,8-HxCDD	0.25	0.000	0.000	0.000	0.000	0.000	0.000
1,2,3,6,7,8-HxCDD	0.25	0.000	0.000	0.000	0.000	0.000	0.000
1,2,3,7,8,9-HxCDD	0.25	0.000	0.000	0.000	0.000	0.000	0.000
1,2,3,4,6,7,8-HpCDD	0.5	0.603	0.922	0.647	0.630	0.575	0.554
OCDD	1	3.077	5.010	4.460	4.487	3.723	5.400
Total PCDD/PCDF		3.770	6.062	5.145	5.198	4.388	6.085
Percent Lipids		0.75	0.70	0.50	0.63	0.61	0.58
Lipid-normalized data							
2,3,7,8-TCDF		12.2	20.3	8.0	12.4	14.5	22.7
1,2,3,7,8-PeCDF		0	0	0	0	0	0
2,3,4,7,8-PeCDF		0	0	0	0	0	0
1,2,3,4,7,8-HxCDF		0	0	0	0	0	0
1,2,3,6,7,8-HxCDF		0	0	0	0	0	0
2,3,4,6,7,8-HxCDF		0	0	0	0	0	0
1,2,3,7,8,9-HxCDF		0	0	0	0	0	0
1,2,3,4,6,7,8-HpCDF		0	0	0	0	0	0
1,2,3,4,7,8,9-HpCDF		0	0	0	0	0	0
OCDF		0	0	0	0	0	0
2,3,7,8-TCDD		0	0	0	0	0	0
1,2,3,7,8-PeCDD		0	0	0	0	0	0
1,2,3,4,7,8-HxCDD		0	0	0	0	0	0
1,2,3,6,7,8-HxCDD		0	0	0	0	0	0
1,2,3,7,8,9-HxCDD		0	0	0	0	0	0
1,2,3,4,6,7,8-HpCDD		80.4	132	133	99.3	94.9	96.9
OCDD		410	709	908	707	613	922
Total PCDD/PCDF		503	861	1049	819	722	1041

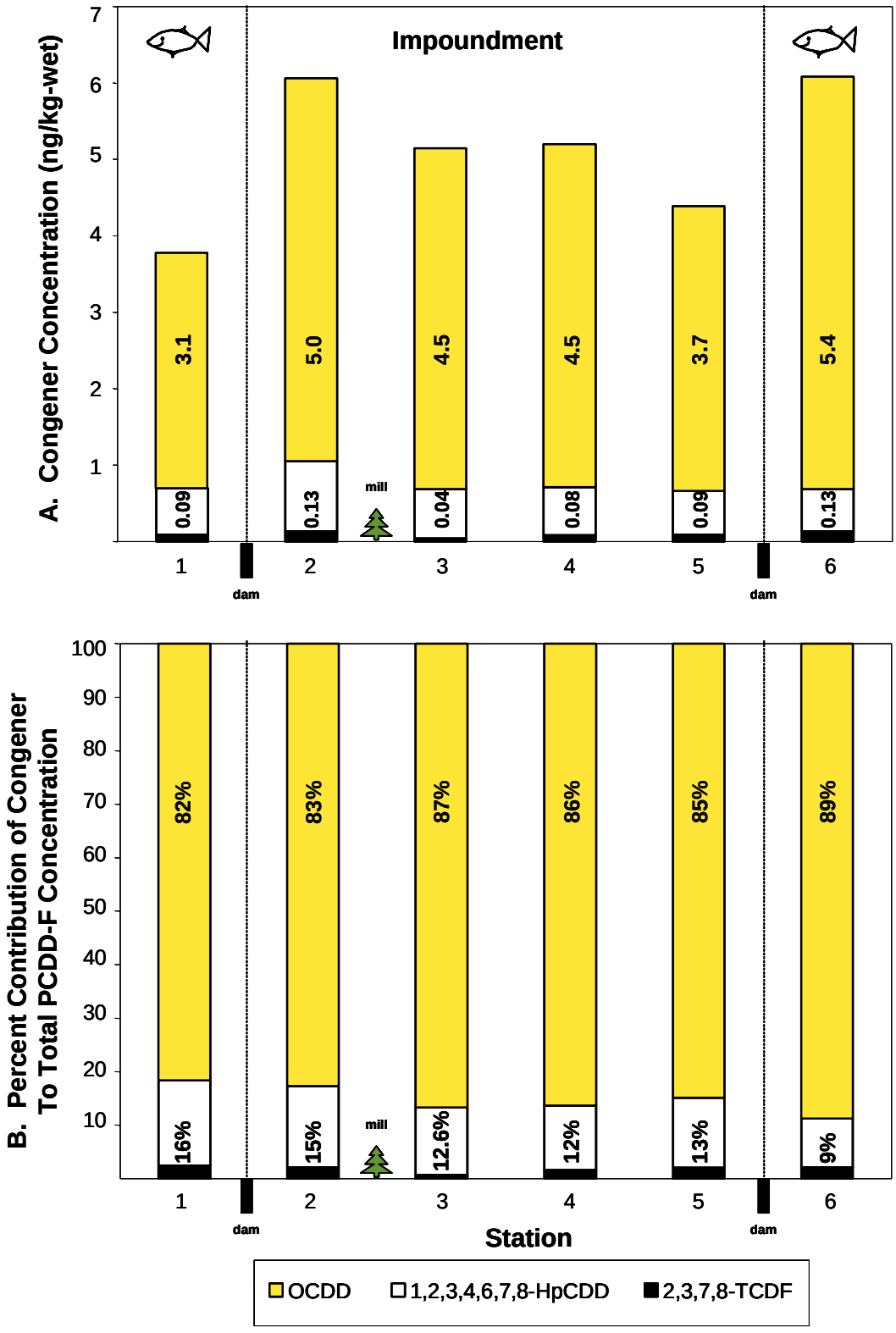


Figure 3. (A) Concentration of three congeners detected in mussel tissues. (B) Percent contribution of each congener to Total PCDD-F concentration.

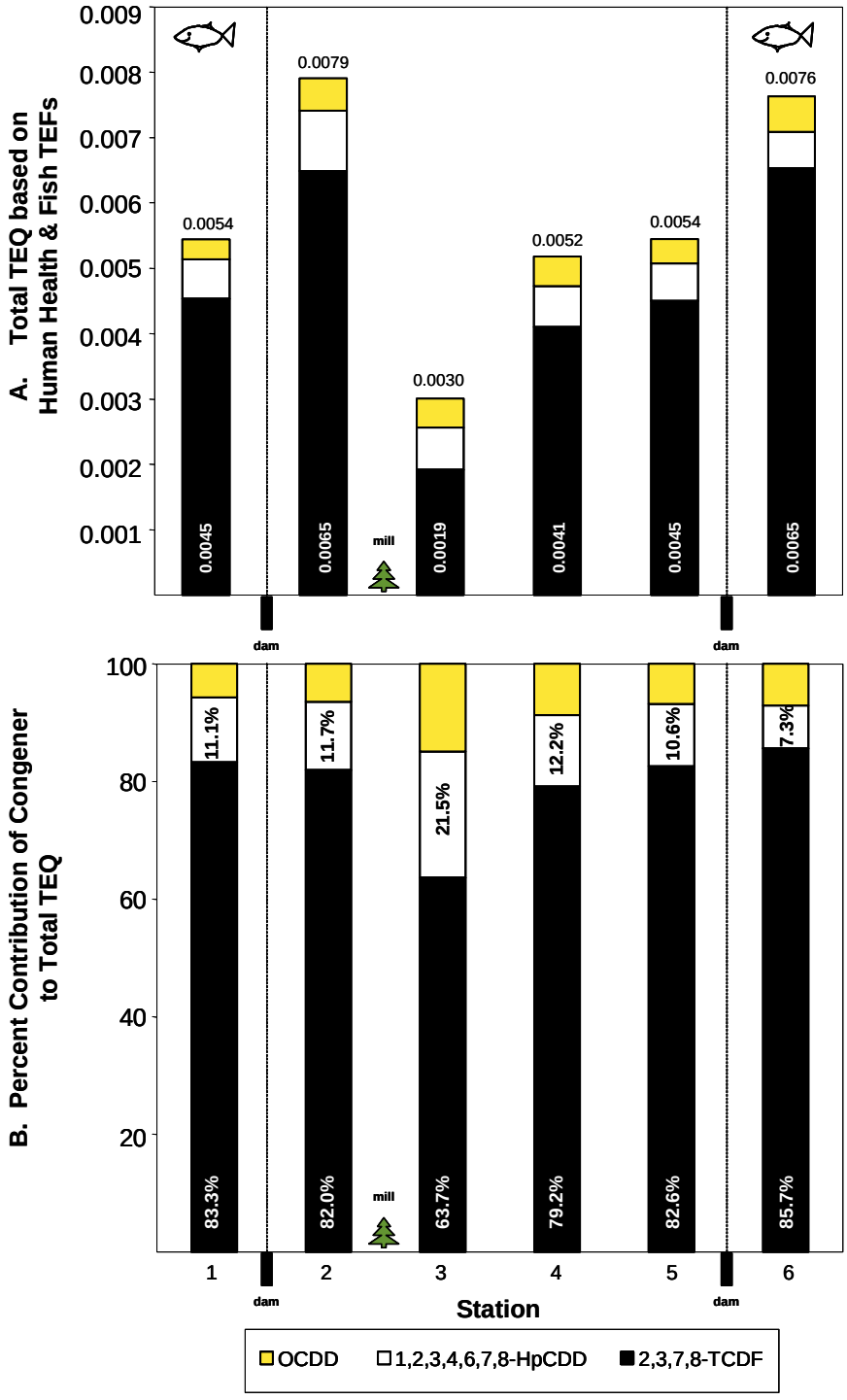


Figure 4. (A) Total TEQs by station. (B) Percent TEQ contribution by station.

4.1.2 2,3,7,8-TCDF

2,3,7,8-TCDF was accumulated by mussels at all stations (Table 3, Figure 5A) with the highest concentrations measured for Stations 2 and 6. The distribution of lipid-normalized 2,3,7,8-TCDF was identical to the non-normalized data (Table 3, Figure 5B). The above-below comparisons showed the following:

	Non-Normalized	Lipid-normalized
Station 1 vs Station 6	NSD (p = 0.49)	NSD (p = 0.19)
Station 2 vs Station 3	NSD (p = 0.14)	NSD (p = 0.26)
Station 2 vs Pooled Stations 3, 4, 5	Significantly higher above (p = 0.03)	NSD (p = 0.25)

The distribution of mean values shown in Figures 5A, B suggest an increasing gradient with distance away from the mill for 2,3,7,8-TCDF. A regression analysis conducted on these mean values resulted in a significant gradient for both non-normalized and lipid-normalized data (Figures 6A, B). The high coefficient of variation (R^2=0.92) shows that approximately 92% of the total variation in 2,3,7,8-TCDF can be explained by distance. Approximately 99% of the total variation in lipid-normalized 2,3,7,8-TCDF can be explained by distance. The lipid-normalized data may be more descriptive of the congener distribution than the non lipid-normalized data because the relationship improved with lipid normalization.

The increasing gradient with distance from the mill strongly suggests that the mill is not a source of 2,3,7,8-TCDF. The high concentrations of this congener at Stations 2 and 6 suggest that there are other potential sources of 2,3,7,8-TCDF in these areas.

4.1.3 2,3,7,8-TCDD

As mentioned previously, 2,3,7,8-TCDD was not detected in mussel tissues from any station. These data show that 2,3,7,8-TCDD is not being discharged by the mill.

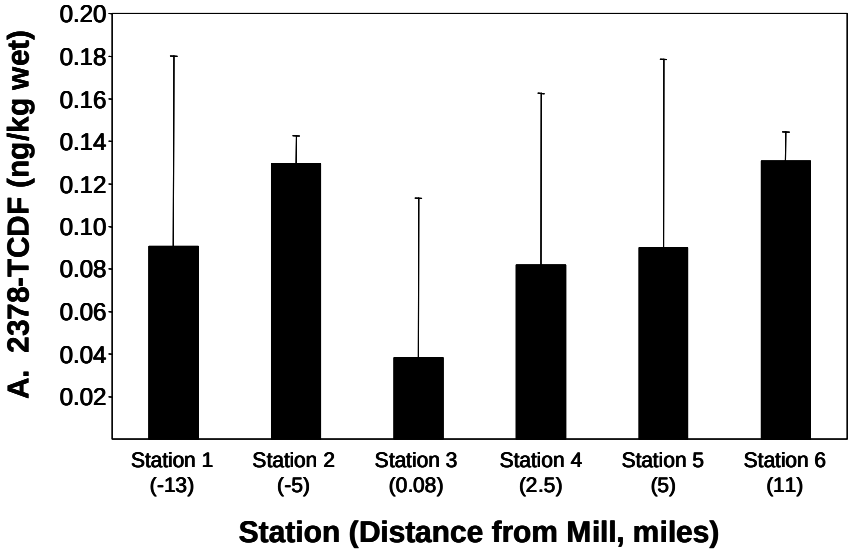
4.1.4 HpCDD

The distribution of HpCDD is very different than that for 2,3,7,8-TCDD (Table 3, Figures 7A,B). For both the non-normalized and lipid-normalized data, there was a decreasing gradient with distance from the mill with the highest concentration measured in mussels from Station 2.

The above-below comparisons showed the following:

	Non-Normalized	Lipid-normalized
Station 1 vs Station 6	NSD (p = 0.88)	NSD (p = 0.77)
Station 2 vs Station 3	NSD (p = 0.26)	NSD (p = 0.98)
Station 2 vs Pooled Stations 3, 4, 5	NSD (p = 0.26)	NSD (p = 0.23)

The mean values across stations shown in Figures 7A, B show a decreasing gradient with distance away from the mill for HpCDD. Based on a regression analysis conducted on these mean values, the gradient was not significant for either the non-normalized or the lipid-normalized data (Figures 8A, B). The regression line for HpCDD with distance from the mill



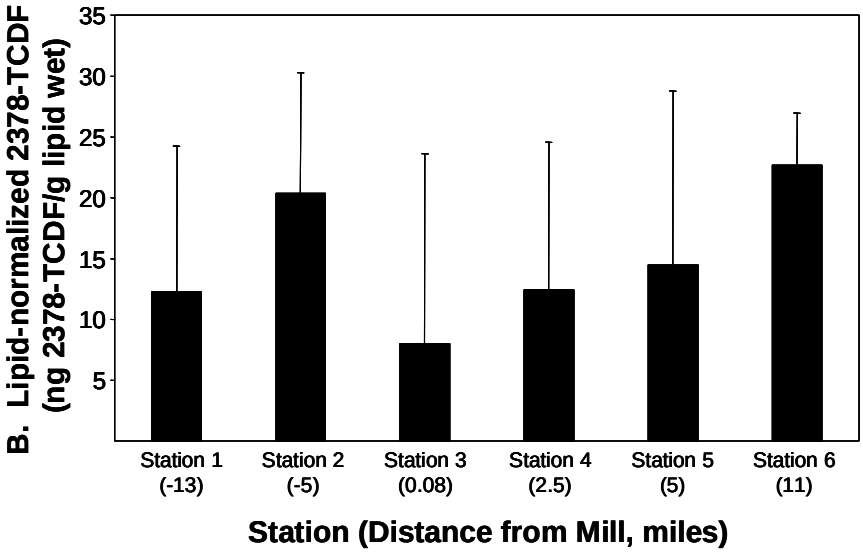
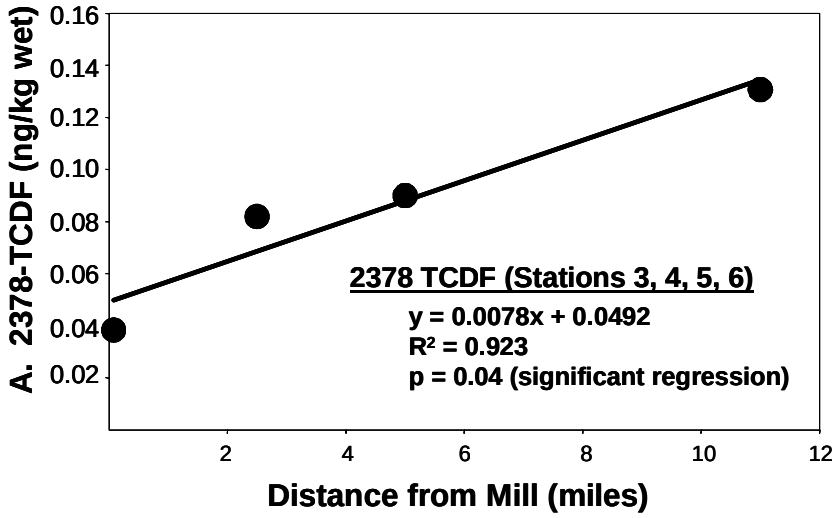


Figure 5. (A) 2,3,7,8-TCDF concentration by station. (B) Lipid-normalized 2,3,7,8-TCDF concentration by station.
Error bars represent 95% confidence interval.



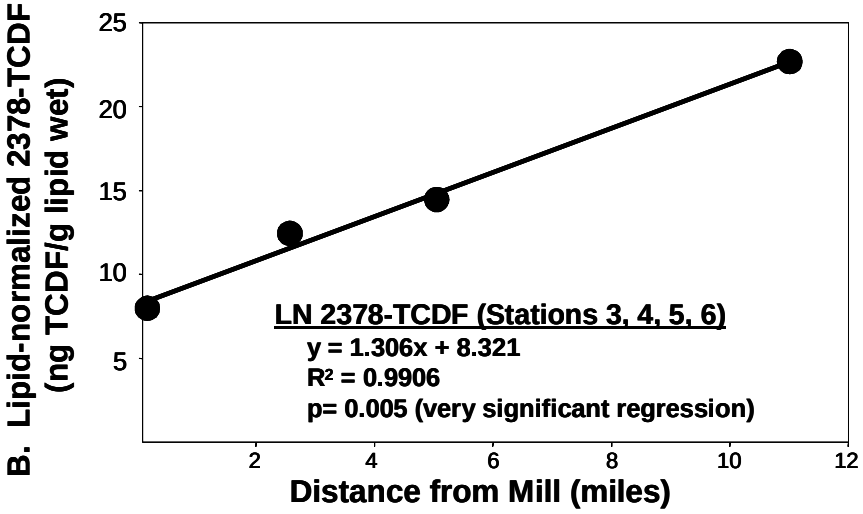
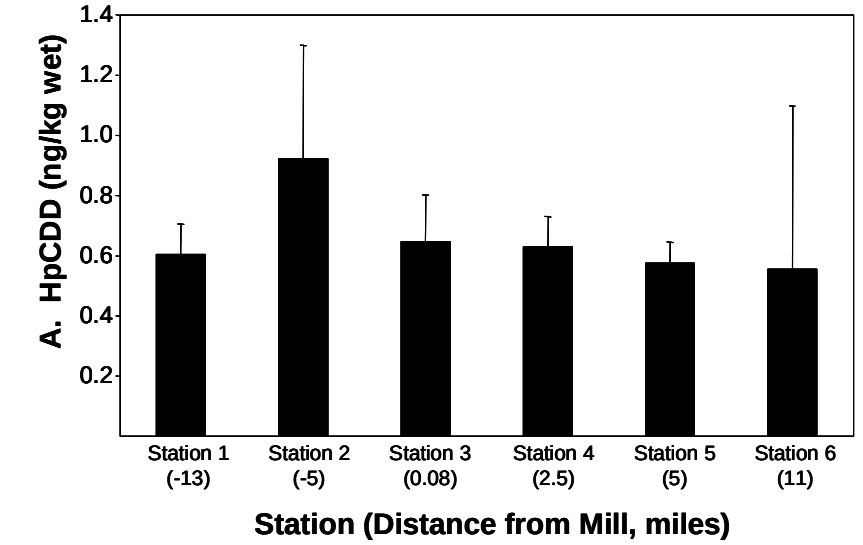


Figure 6. (A) Relationship between 2,3,7,8-TCDF concentration and distance from mill. (B) Relationship between lipid-normalized 2,3,7,8-TCDF concentration and distance from the mill.



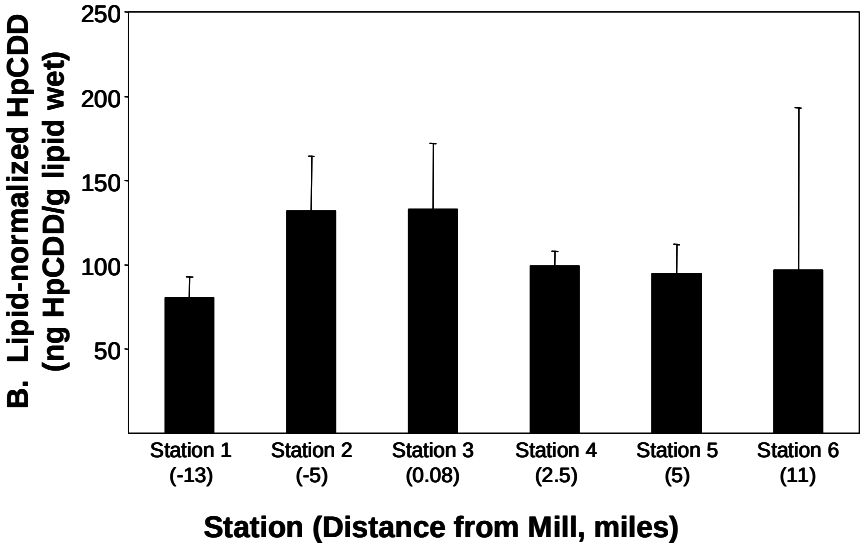
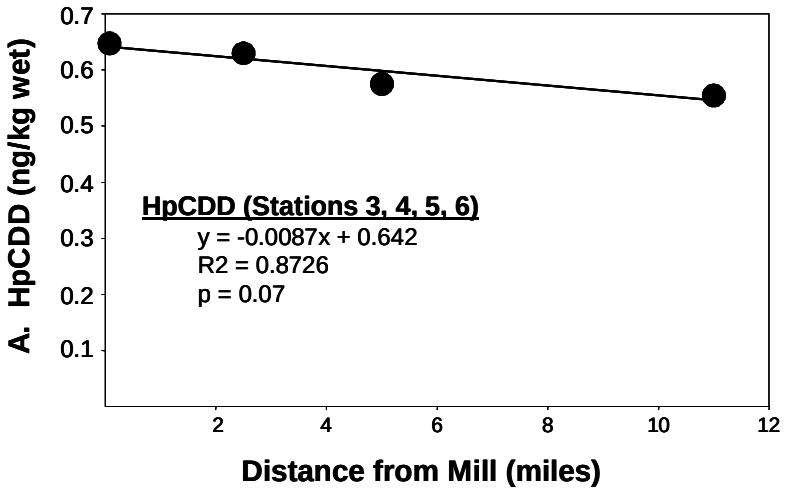


Figure 7. (A) HpCDD concentration by station.
(B) Lipid-normalized HpCDD concentration by station.
Error bars represent 95% confidence interval.



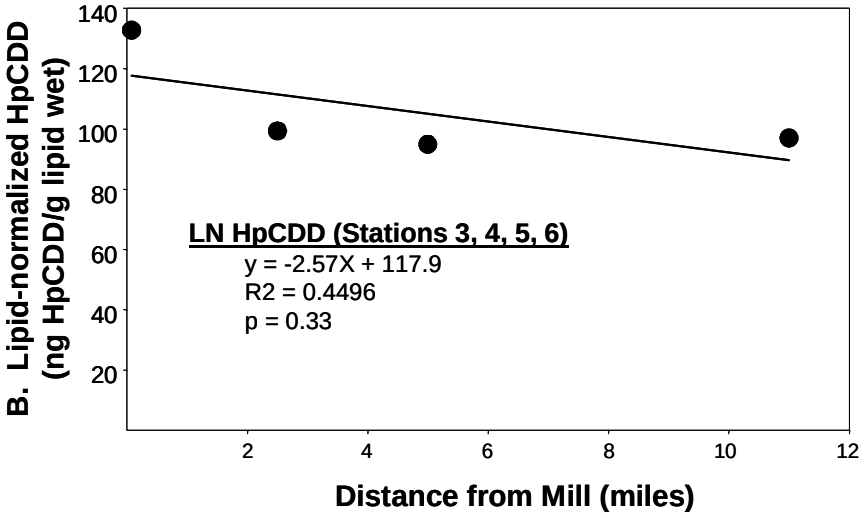


Figure 8. (A) Relationship between HpCDD concentration and distance from mill. (B) Relationship between lipid-normalized HpCDD concentration and distance from the mill.

(i.e., for Stations 3, 4, 5, and 6) is not statistically significant (p=0.07) at the 95% confidence level but, it would be at the 90% confidence level. The good coefficient of variation (R^2=0.87) shows that approximately 87% of the total variation in HpCDD can be explained by distance. Although the mill could be a source of HpCDD, the high concentration measured in mussels deployed at Station 2 suggest that there may be a source of HpCDD in that area.

4.1.5 OCDD

OCDD was also detected in mussels from all stations (Table 3, Figures 9A,B). The distributions for the non-normalized and lipid-normalized data are different from each other. The non-normalized data show that the highest concentrations were measured in mussels from Stations 2 and 6, and that there was a small decreasing gradient with distance from Stations 2 to 5, with the concentration at Stations 3 and 4 very similar. The lipid-normalized data show a much stronger gradient over Stations 3, 4 and 5, with the highest concentrations measured in mussels from Stations 3 and 6. Based on a regression analysis conducted on the mean values, the gradient was not significant for either the non-normalized or the lipid-normalized data (Figures 10A, B). However, the concentration at Station 2 is also elevated. Both the non-normalized and lipid-normalized data suggest that there could sources of OCDD near Stations 2, 3, and 6. The regression line for OCDD with distance within the impoundment (i.e., Stations 2, 3, 4 and 5) is not statistically significant (p=0.07) at the 95% confidence level but that it would be at the 90% confidence level. The good coefficient of variation (R^2=0.86) shows that approximately 86% of the total variation in OCDD can be explained by distance.

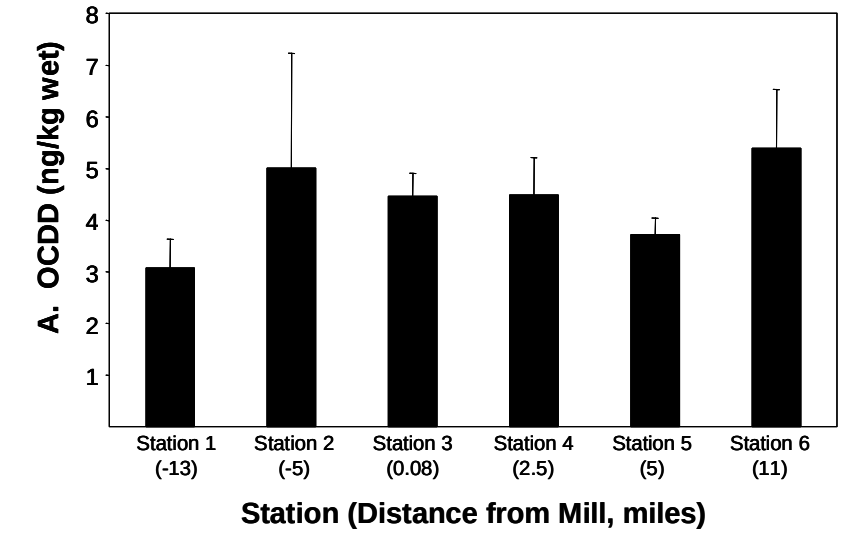
It was more difficult to fit a regression line to the lipid-normalized data because of the high concentrations at Stations 3 and 6. The best-fit regression line for lipid-normalized OCDD with distance from the mill is not statistically significant (p=0.14), but the high coefficient of variation (R^2=0.96) shows that approximately 96% of the total variation in OCDD can be explained by distance. The increasing gradient with distance approaching the mill and the decreasing gradient with distance away from the mill suggest that there may be sources of OCDD near both Stations 2 and 3.

The above-below comparisons showed the following:

	Non-Normalized	Lipid-normalized
Station 1 vs Station 6	Below significantly higher (p = 0.02)	Below significantly higher (p = 0.0007)
Station 2 vs Station 3	NSD (p = 0.68)	NSD (p = 0.14)
Station 2 vs Pooled Stations 3, 4, 5	NSD (p = 0.56)	NSD (p = 0.74)

4.1.6 Total PCDD-Fs

The concentration of Total PCDD-Fs were highest in mussels deployed at Stations 2 and 6 (6.1 ng/kg-ww; Table 3, Figure 11A). The lowest concentration was measured in mussels deployed at Station 1 (3.8 ng/kg-ww; Table 3, Figure 11A). There was no significant difference in Total PCDD-F concentration among the six stations (p = 0.15). For lipid-normalized Total PCDD-F, the highest concentration found at Station 3 (1049 ng Total PCDD-F/g lipid - ww; Table 3, Figure 11B) and Station 6 (1042ng Total PCDD-F/g lipid - ww; Table 3, Figure 11B). There was a statistically significant difference in lipid-normalized Total PCDD-F concentration across stations, with the concentration in mussels from Station 1 (upstream, above the dam, and



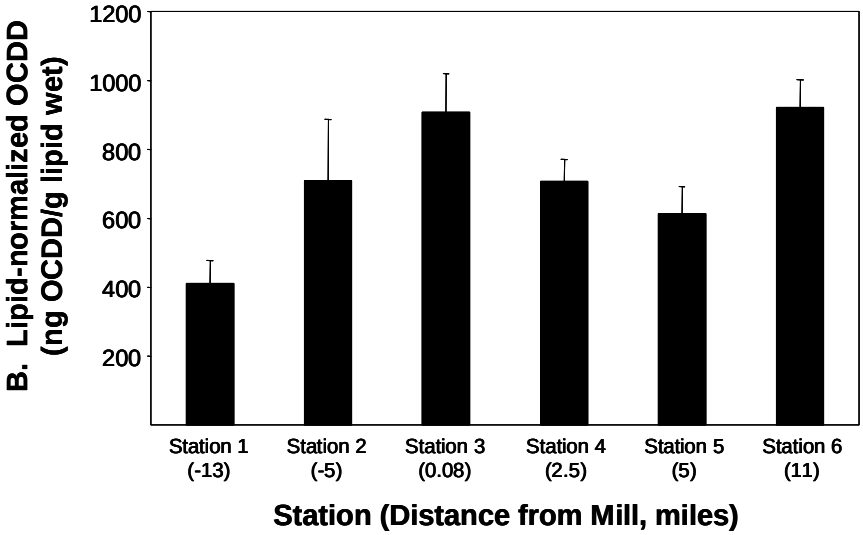


Figure 9. (A) OCDD concentration by station. (B) Lipid-normalized OCDD concentration by station.
Error bars represent 95% confidence interval.

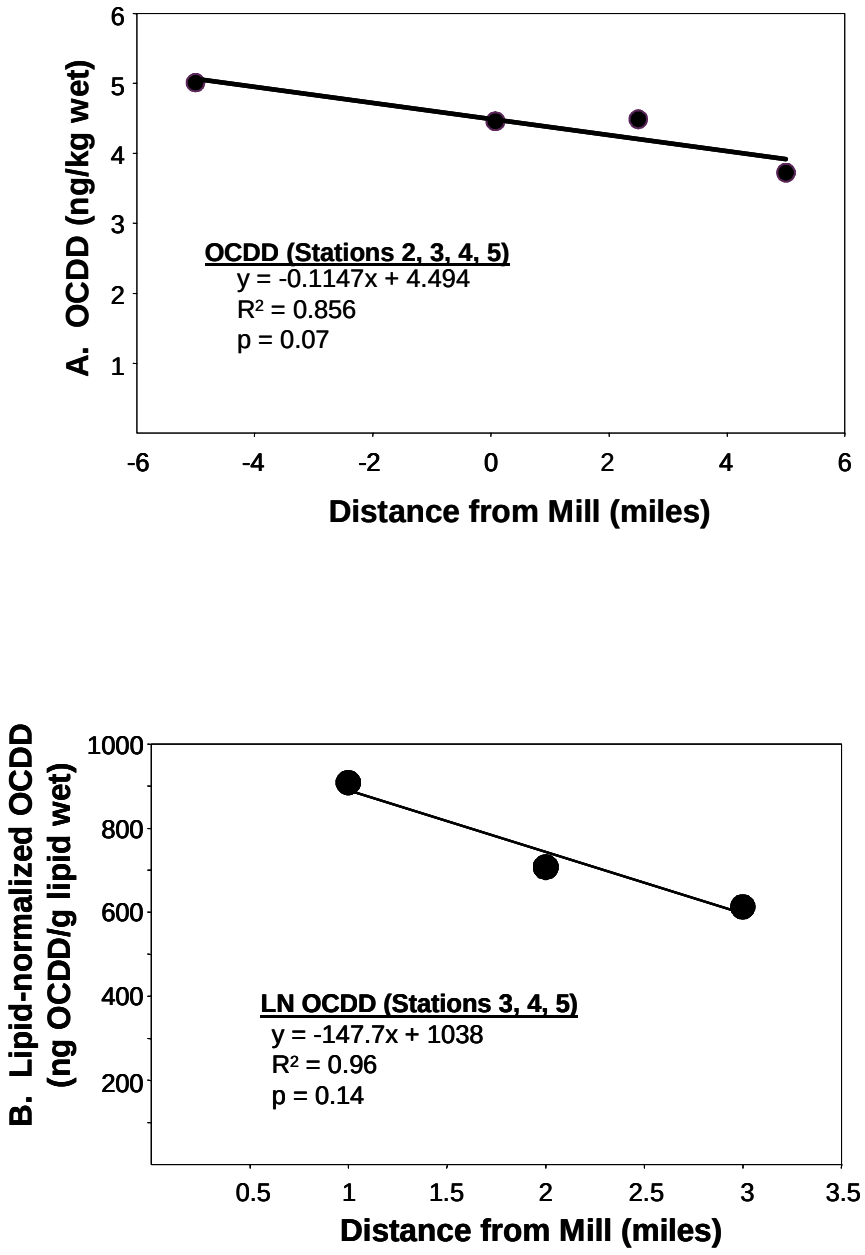
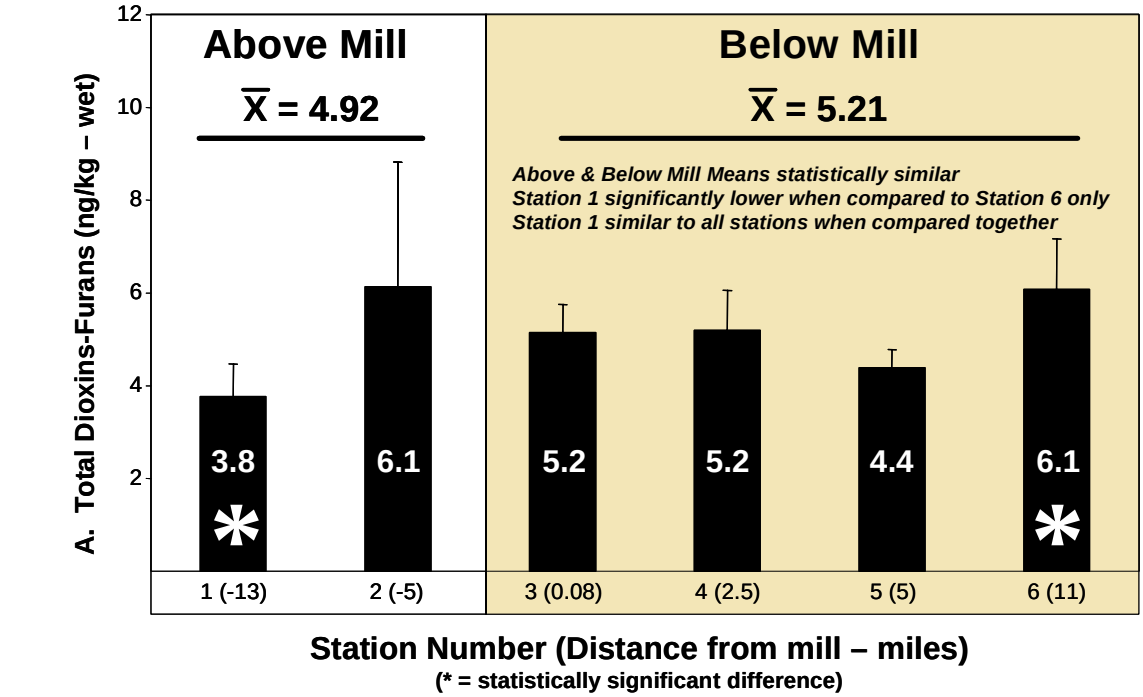


Figure 10. (A) Relationship between OCDD concentration and distance from mill. (B) Relationship between lipid-normalized OCDD concentration and distance from the mill.



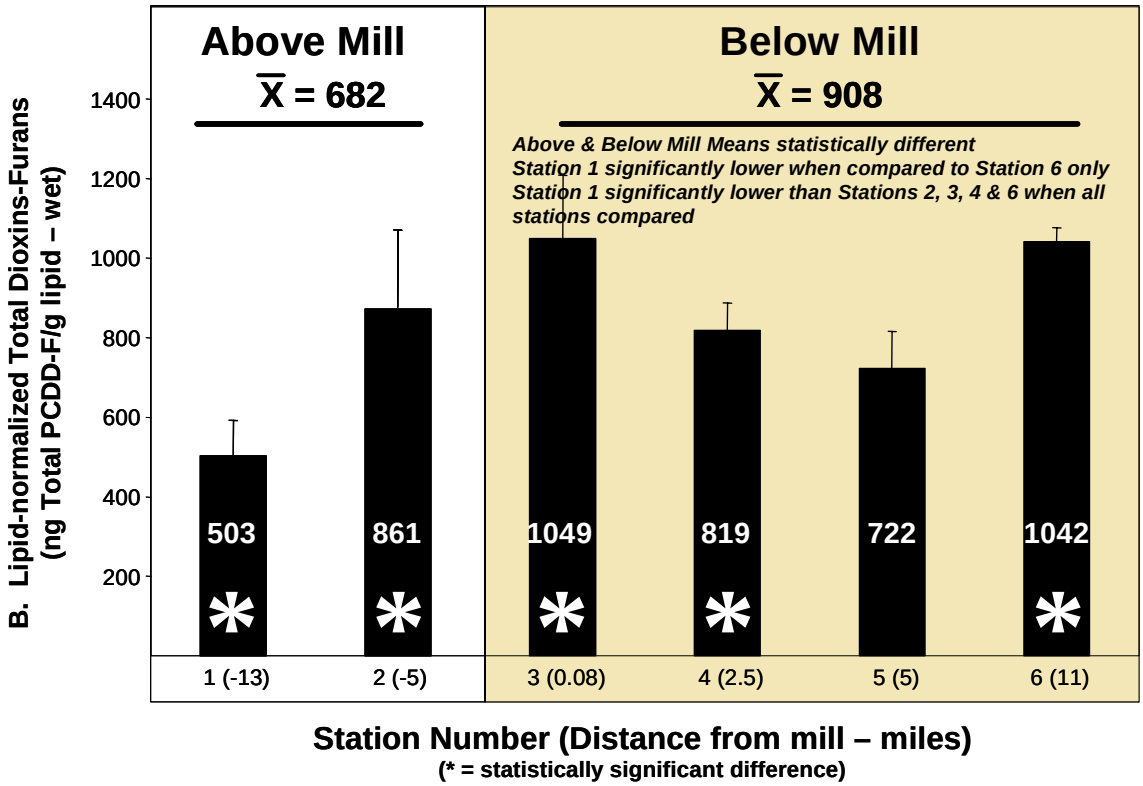


Figure 11. (A) Total dioxin-furan concentration by station.
(B) Lipid-normalized Total dioxin-furan concentration by station.
Error bars represent 95% confidence interval.

outside the impoundment) significantly lower than those measured in mussels from Stations 2, 3, 4, and 6 ($p = 0.0004$).

There was a decreasing gradient within the impoundment (i.e., across Stations 2, 3, 4, and 5) in Total PCDD-F concentration (Figures 11A, 12A). The gradient is significant ($p = 0.04$, $R^2 = 0.91$) and 91% of the total variation is explained by the regression. These results suggest that something in the vicinity of Skowhegan may be a source of dioxins and furans on the Kennebec River.

There was also a decreasing gradient in lipid-normalized Total PCDD-F across Stations 2, 3, 4 and 5 (Figure 12B). The regression analysis conducted on all stations within the impoundment resulted in a non-significant line ($p = 0.55$, $R^2=0.20$) with only 20% of total variation in Y explained by the regression. If Station 2 is excluded from the regression analysis, the line remains non-significant ($p = 0.15$), but the R^2 value increases to 0.94.

The lipid-normalized data suggest that the mill and perhaps Skowhegan are both sources of PCDD-F. ANOVA results suggest higher lipid-normalized Total PCDD-F concentrations at Stations 2, 3, and 6 when compared to Station 1 upstream, above the dam, and outside the impoundment.

4.2 Survival

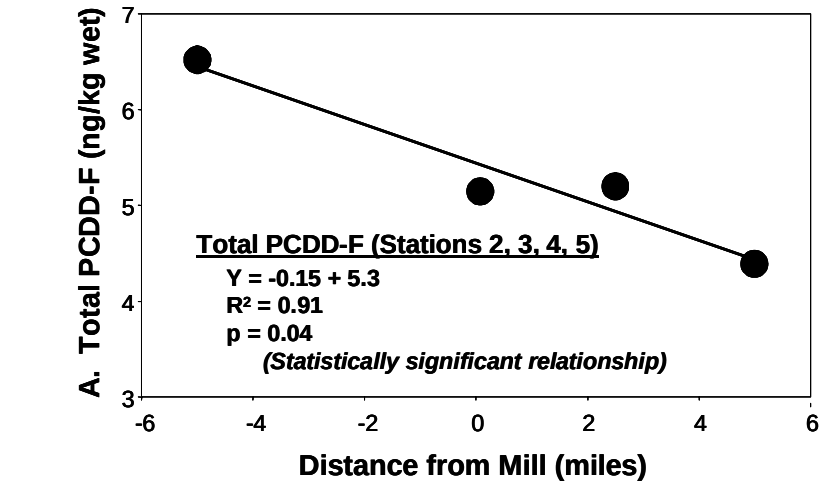
Mean *Elliptio* survival for all stations was 98.8% (Table 4). Of the 432 mussels deployed (24 mussels/cage x 18 cages), 427 mussels survived and only 5 mussels died. Survival by cage was 100% for 15 of the 18 cages; one individual died in Cages 6, 15, and 21 and two individuals died in Cage 18.

The very high survival measured for each of the cages indicates that the caging process, suspension in the river, and exposure to high currents, changing water levels, high summer temperatures, and potentially high temperatures associated with the mill effluent did not have an adverse effect on the mussels.

Table 4. Percent survival by cage and station.

Station	% Survival	Station	% Survival	Station	% Survival
Station 1 (13 mi above)		***Station 2 (5 mi above)***		***Station 3 (0.08 mi below)***	
Cage 2	100	Cage 6	95.8	Cage 5	100
Cage 10	100	Cage 17	100	Cage 7	10
Cage 13	100	Cage 19	100	Cage 21	95.8
Station Mean:	***100***		***98.6***		***98.6***
Station 4 (2.5 mi below)		***Station 5 (5 mi below)***		***Station 6 (11 mi below)***	
Cage 11	100	Cage 1	100	Cage 4	100
Cage 15	95.8	Cage 9	100	Cage 8	100
Cage 16	100	Cage 18	91.7	Cage 14	100
Station Mean:	***98.6***		***97.2***		***100***

Overall Mean Survival for all Stations = 98.8%



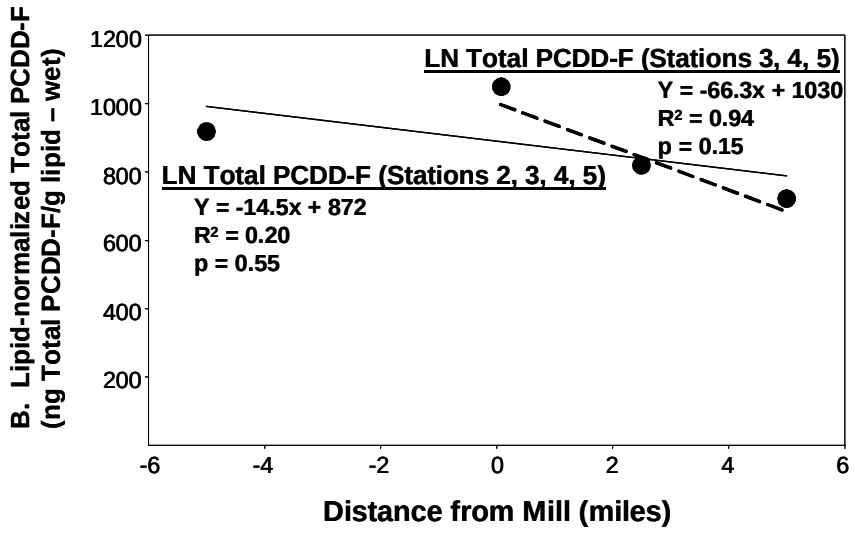


Figure 12. (A) Relationship between Total dioxin-furan concentration and distance from mill. (B) Relationship between lipid-normalized Total dioxin-furan concentration and distance from the mill.

4.3 Mussel Growth Metrics

Elliptio deployed on the Kennebec River had very small increases in shell length and modest increases in WAWW during the 66-day exposure period. Percent increase in shell length was generally less than 2.2% while percent changes in WAWW were less than 10.2% (Table 5). Of all growth metrics, tissue weights had the greatest increases, based on comparing the end-of-test tissue weights with the estimated tissue weight determined from the T_0 tissue chemistry individuals. Tissue weights increased by up to 43.6%. The data suggest that none of the mussels lost a significant amount of tissue weight, and therefore, were in good health so that the tissue chemistry data can be used with confidence.

4.3.1 Shell Length

At the start of the test, shell lengths for individual mussels ranged from 62.4 to 66.9 mm, a range of 4.5 mm (Table 5). Mean initial shell length for all stations was 64.8 mm. There was no statistically significant difference in mean shell lengths among individual cages or among stations at the beginning of the test (see Section 3.5).

Mean shell length increased at all stations during the 66-day exposure period. When compared to the beginning-of-test measurements, there was a significant increase in shell length at all stations ($p < 0.0001$), with an average increase in shell length across all stations of approximately 0.74 mm. EOT shell lengths for individual mussels ranged from 62.9 to 68.7 mm (Table 5). Mean EOT shell length by station ranged from 65.1 mm at Station 1 to 66.30 mm at Station 5 (Table 5, Figure 13A). The mean percentage increase in shell length across stations ranged from 0.6% at Station 1 to 2.2% at Station 5 (Table 5). Statistically significant differences in shell length were found among stations at the end of the test (Table 6). Shell length at Station 5 was significantly higher than at any other station. Shell length was similar among Stations 1, 2, 3 and 6. Shell lengths at Station 4 were also similar to those at Stations 2, 3, and 6, but not Station 1.

EOT length growth rates by station ranged from 0.04 mm/wk at Station 1 to 0.15 mm/wk at Station 5 (Table 5; Figure 13B). Several statistically significant differences in length growth rates were found ($p < 0.0001$; Table 6). As with shell length, length growth rates were significantly higher at Station 5 when compared to all other stations. Several other differences were found, with Station 1 similar to Stations 2 and 6; Station 2 similar to Stations 3 and 6, and Station 3 similar to Station 4.

4.3.2 Whole-Animal Wet-Weight (WAWW)

At the start of the test, WAWWs for individual mussels ranged from 16.56 to 33.48 g-wet, a range of 16.92 g (Table 5). Mean initial WAWW for all stations was 23.12 g-wet. There was no statistically significant difference in mean WAWWs among individual cages or among stations at the beginning of the test (see Section 3.5).

Mean WAWW increased at all stations during the 66-day exposure period. When compared to the beginning-of-test measurements, there was a significant increase in WAWW at all stations ($p < 0.0001$), with an average increase in WAWW across all stations of approximately 0.74 mm. EOT WAWWs for individual mussels ranged from 17.85 to 34.26 g-wet (Table 5). Mean EOT

Table 5. Summary of mussel growth metrics.

Station (Distance Above/Below Mill)	Station 1 (13 m above)	Station 2 (5 mi above)	Station 3 (0.08 m below)	Station 4 (2.5 mi below)	Station 5 (5 mi below)	Station 6 (11 mi below)	T_0 Chems	All Data
Percent Survival	100%	99%	99%	99%	97%	100%	na	98.8%
% Change Shell Length	0.6%	0.8%	1.1%	1.4%	2.2%	0.8%	na	1.1%
% Change Weight	3.9%	4.3%	6.1%	7.9%	10.2%	3.7%	na	6.0%
Est % Change in Tissue Weight	12.5%	15.7%	25.9%	40.8%	43.6%	14.0%	na	25.3%
Est % Change in Shell Weight	0.8%	1.9%	7.3%	7.0%	9.2%	3.2%	na	4.9%
Est % Change in Lipids	33.1%	23.6%	-11.9%	12.4%	8.2%	3.5%	na	11.5%
Initial Length (mm)								
mean	64.75	64.74	64.76	64.79	64.88	64.79	64.69	64.77
min	63.02	62.47	63.02	62.61	63.08	62.62	62.37	62.37
max	66.96	66.99	66.77	66.82	66.92	66.82	66.70	66.99
count	72	72	72	72	72	72	72	504
95% CI	0.265	0.275	0.256	0.267	0.247	0.243	0.244	0.097
EOT Length (mm)								
mean	65.12	65.24	65.45	65.69	66.30	65.28	na	65.51
min	63.10	62.92	63.70	63.27	64.20	63.17	na	62.92
max	67.46	67.56	67.99	68.74	68.71	67.38	na	68.74
count	72	71	71	71	70	72	na	427
95% CI	0.28	0.27	0.27	0.30	0.25	0.24	na	0.12
Length Growth Rate (mm/wk)								
mean	0.04	0.06	0.08	0.10	0.15	0.05	na	0.08
min	-0.02	-0.04	-0.01	-0.01	0.02	-0.03	na	-0.04
max	0.19	0.19	0.24	0.24	0.33	0.16	na	0.33
count	72	71	71	71	70	72	na	427
95% CI	0.008	0.010	0.011	0.013	0.017	0.009	na	0.006
Initial WAWW (g-wet)								
mean	22.85	23.10	23.64	23.25	23.12	22.96	22.90	23.12
min	17.77	16.56	16.57	17.47	17.84	17.03	16.96	16.56
max	28.41	31.24	30.89	30.28	33.48	30.70	31.60	33.48
count	72	72	72	72	72	72	72	504
95% CI	0.56	0.73	0.77	0.67	0.75	0.56	0.64	0.25
EOT WAWW (g-wet)								
mean	23.72	24.02	24.95	24.96	25.44	23.80	na	24.48
min	18.71	17.85	19.31	19.07	20.31	18.15	na	17.85
max	30.90	31.98	32.29	31.47	34.26	31.30	na	34.26
count	72	71	71	71	70	72	na	427
95% CI	0.54	0.69	0.74	0.66	0.71	0.56	na	0.27
WAWW Growth Rate (mg/wk)								
mean	93	101	147	189	241	89	na	143
min	-11	-34	-75	33	-69	-63	na	-75
max	272	252	340	362	367	351	na	367
count	72	71	71	71	70	72	na	427
95% CI	11.6	13.1	17.5	16.3	20.4	15.2	na	8.4
Tissue Weight (g-wet)								
mean	6.17	6.35	6.91	7.72	7.88	6.25	5.49	6.87
min	4.76	5.21	5.00	6.19	6.50	4.53	4.11	4.53
max	7.38	8.27	10.51	9.33	10.40	10.27	7.29	10.51
count	60	60	60	59	58	60	60	357
95% CI	0.16	0.14	0.27	0.19	0.26	0.21	0.16	0.11
Shell Weight (g-wet)								
mean	10.64	10.75	11.33	11.28	11.52	10.88	10.56	11.07
min	6.90	7.52	7.96	5.22	8.50	7.40	7.21	5.22
max	13.14	15.44	16.48	17.06	17.13	16.00	14.83	17.13
count	61	61	61	60	59	61	60	363
95% CI	0.35	0.44	0.45	0.51	0.46	0.41	0.43	0.18
Percent Lipids								
mean	0.75	0.70	0.50	0.63	0.61	0.58	0.56	0.63
min	0.72	0.47	0.42	0.58	0.58	0.50	0.39	0.42
max	0.77	0.88	0.59	0.70	0.65	0.66	0.78	0.88
count	3	3	3	3	3	3	3	18
95% CI	0.03	0.24	0.10	0.07	0.04	0.09	0.22	0.06

Table 6. Summary of statistical results (p values) for mussel growth metrics. (* = statistically significant)

Station No. & Distance to Outfall	Station 1 (13 mi above)	Station 2 (5 mi above)	Station 3 (0.08 mi below)	Station 4 (2.5 mi below)	Station 5 (5 mi below)	Station 6 (11 mi below)
Length: EOT vs BOT	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Length: EOT comparisons	p < 0.0001*; Station 1 = Stations 2, 3, 6; Station 4 = Stations 2, 3, 6; Station 5 different than all other stations					
Length GR: EOT comparisons	p < 0.0001*; Station 1 = Stations 2, 6; Station 2 = Stations 3, 6; Station 3 = Station 4; Station 5 different than all other stations					
WAWW: EOT vs BOT	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
WAWW: EOT comparisons	p = 0.0006*; All stations similar except: Station 5 different than Stations 1, 2 and 6					
WAWW GR: EOT comparisons	p < 0.0001*; Station 1 = Stations 2, 6; Station 2 = Station 6; Station 3 = Station 4; Station 4 = Station 5					
Tissue: EOT vs BOT	< 0.01*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Tissue: EOT comparisons	p < 0.0001*; Station 1 = Stations 2, 6; Station 4 = Station 5					
Shell: EOT vs BOT	>0.05	>0.05	>0.05	>0.05	<0.05*	>0.05
Shell: EOT comparisons	p = 0.0273*; no significant differences were identified with the Tukey-Kramer Multiple Comparison test.					
Percent Lipids: EOT vs BOT	p = 0.2577; no significant difference between BOT and EOT					
Percent Lipids: EOT comparisons	p = 0.1174; no significant difference among stations					
Vitellin in Females: gradient (Stations 1, 2, 3, 4)	p = 0.03 (significant regression), R^2=0.94 (94% of total variation in Y explained by the regression) straight line relationship that is significant					

WAWW by station ranged from 23.72 g-wet at Station 1 to 25.44 g-wet at Station 5 (Table 5, Figure 14A). The mean percentage increase in WAWW across stations ranged 3.7% at Station 6 to 10.2% at Station 5 (Table 5). Statistically significant differences in WAWW were found among stations at the end of the test (Table 6). WAWW was similar at all stations except Station 5, which had WAWWs that were significantly higher than at Stations 1, 2, and 6.

EOT WAWW growth rates by station ranged from 89 mg/wk at Station 6 to 241 mg/wk at Station 5 (Table 5; Figure 14B). Several statistically significant differences in WAWW rates were found among stations ($p < 0.0001$; Table 6), with WAWW growth rates significantly higher at Stations 4 and 5 when compared to Stations 1, 2, and 6.

Figure 15 shows the negative relationship between Total PCDD-F and WAWW growth rate between Stations 2 and 5 within the impoundment. This is the first piece of evidence to suggest that dioxins and furans are affecting mussel growth rates. Although there are only four points and this relationship would also need to be verified with additional studies, the coefficient of variation is good (R^2=0.86), but the regression line is not statistically significant (p= 0.07; Table 6) at the 95% confidence level. It is significant at the 90% confidence level. This is probably a pseudo-correlation in that it is very unlikely that these low concentrations are directly affecting mussel growth rates. Total dioxins and furans are probably only acting as a surrogate for some other unmeasured factor in the river or associated with the effluent that was not measured.

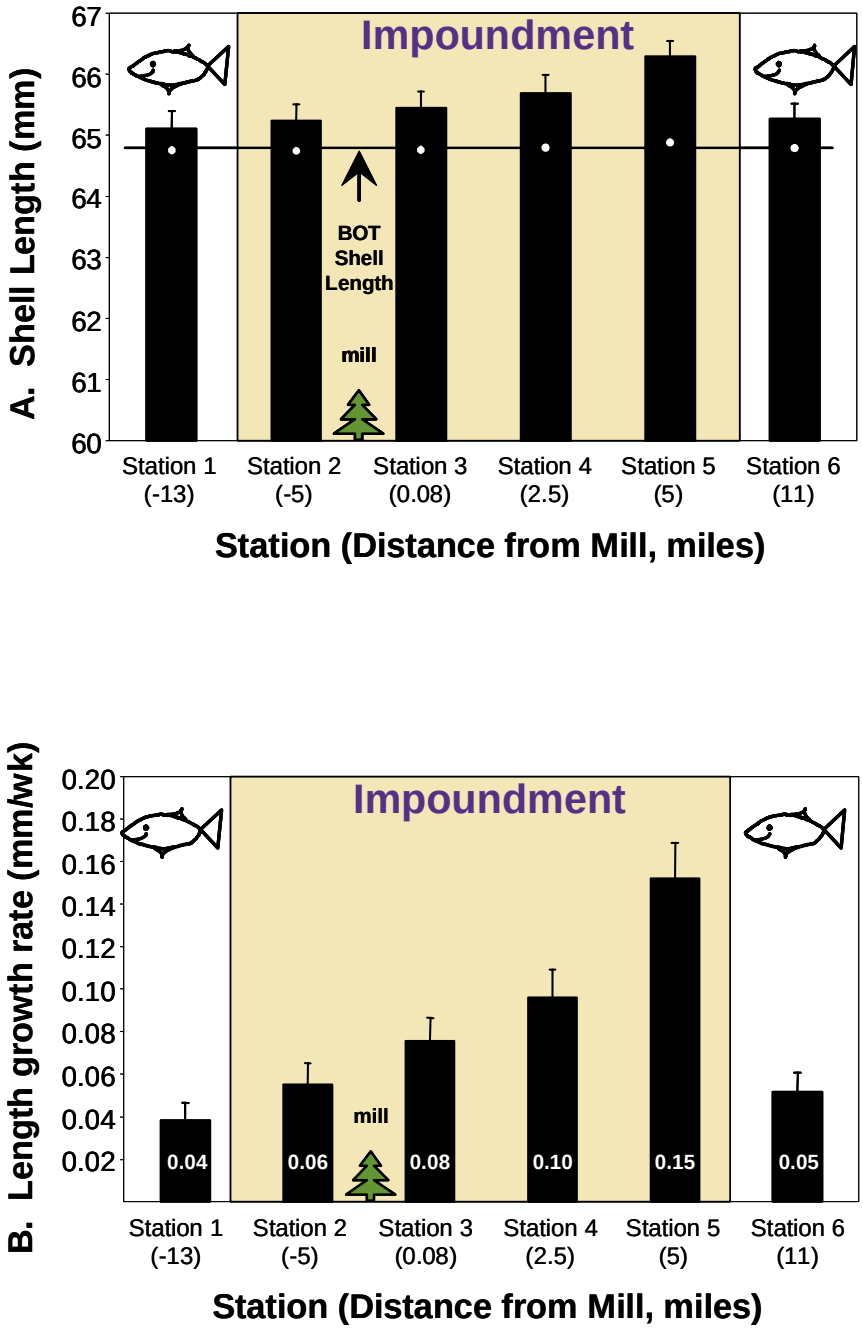
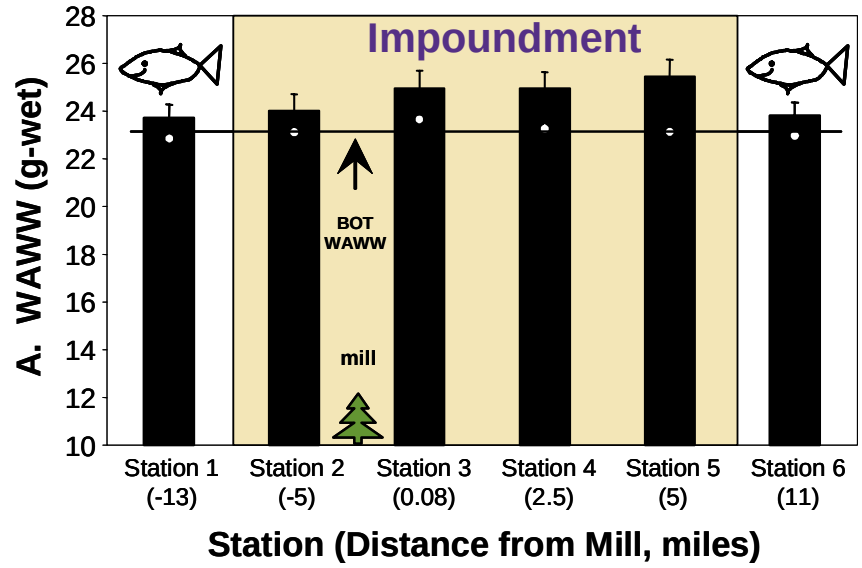


Figure 13. (A) EOT mussel shell length by station.
(B) Length growth rate by station.
Error bars represent 95% confidence interval.



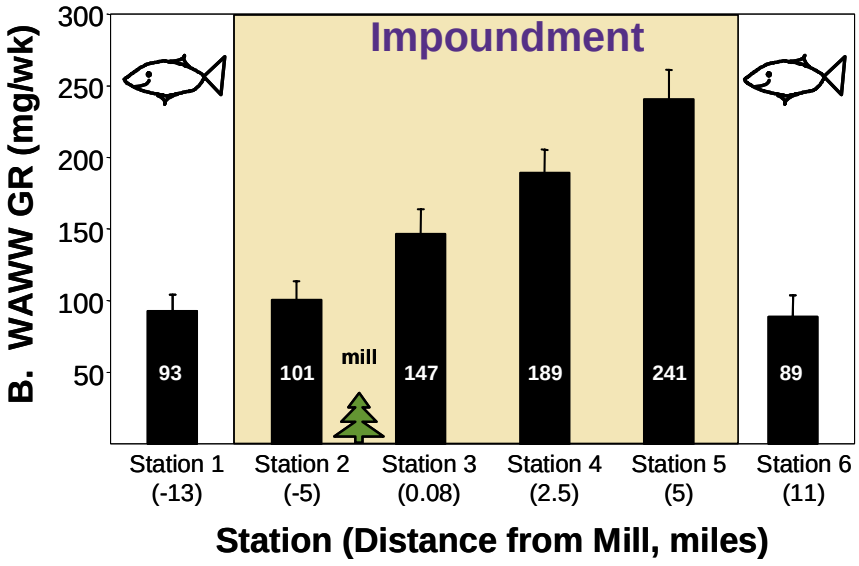


Figure 14. (A) EOT mussel WAWW by station.
(B) WAWW growth rate by station.
Error bars represent 95% confidence interval.

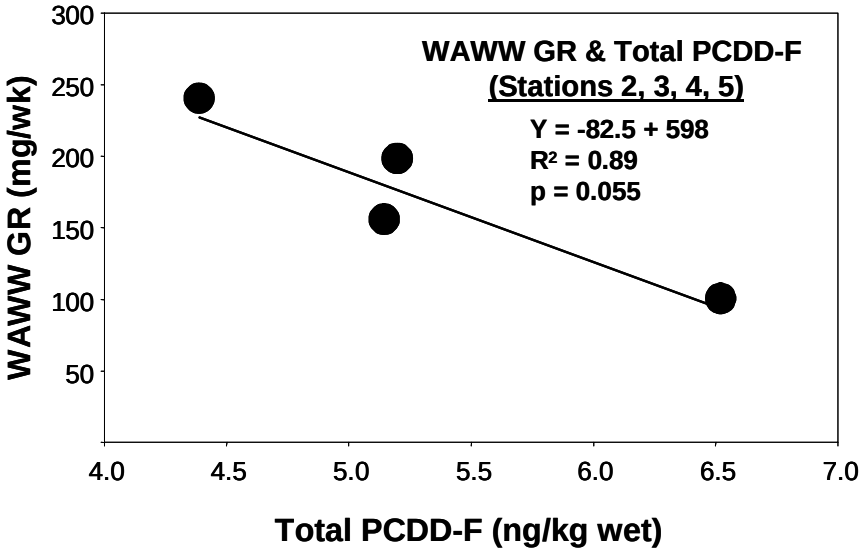


Figure 15. WAWW growth rate versus Total PCDD-F concentration.

4.3.3 Wet Tissue Weights

Mean whole soft tissue weight at the start of the test was estimated at 5.49 g-wet (Table 5) based on the tissue weights from the 60 baseline BOT measurements. Based on this estimated BOT value, mean whole soft tissue weights increased at all stations during the 66-day exposure period. Mean EOT wet tissue weights by station ranged from 6.17 g-wet at Station 1 to 7.88 g-wet at Station 5 (Table 5, Figure 16). The percentage change in wet tissue weight across stations ranged from 12.5% at Station 1 to 43.6% at Station 5 (Table 5).

There was a significant increase in tissue weight at all stations when compared to the BOT tissue weights (Table 6). EOT tissue weights were significantly higher at Stations 4 and 5 when compared to all other stations. EOT tissue weights were similar at Stations 1, 2 and 6.

4.3.4 Shell Weight

Mean shell weight at the start of the test was estimated at 10.56 g-wet (Table 5) based on the shell weights from the 60 baseline BOT measurements. Based on this estimated BOT value, mean shell weights increased at all stations during the 66-day exposure period. Mean EOT shell weights by station ranged from 10.64 g-wet at Station 1 to 11.53 g-wet at Station 5 (Table 5, Figure 17). The percentage change in shell weight across stations ranged from 0.8% at Station 1 to 9.2% at Station 5 (Table 5).

There was not a significant increase in shell weight at all stations when compared to the BOT shell weights. Only mussels at Station 5 had a significant increase in shell weight when compared to the BOT estimate (Table 6). Although results of the ANOVA on EOT shell weights indicated a statistically significant difference among stations, no significant differences were found with the Tukey-Kramer multiple comparison test (Table 6).

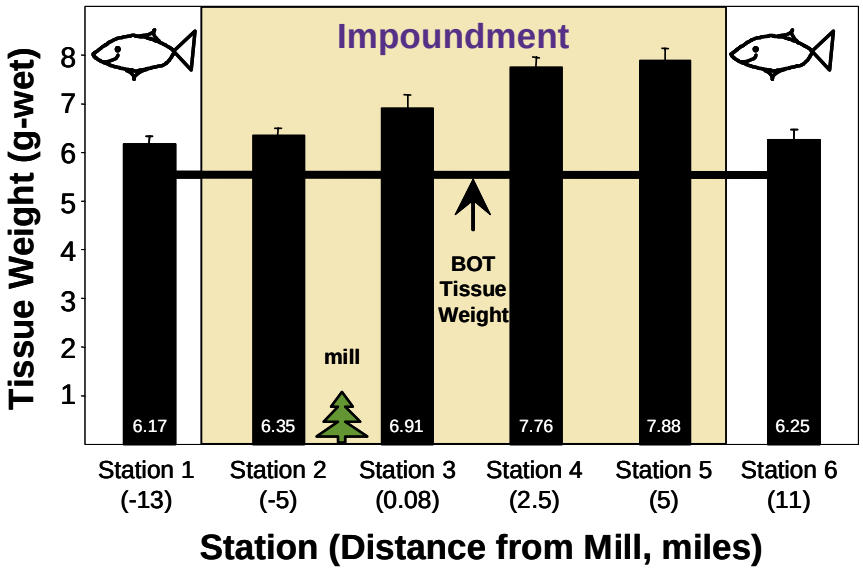


Figure 16. EOT mussel tissue weight by station.
Error bars represent 95% confidence interval.

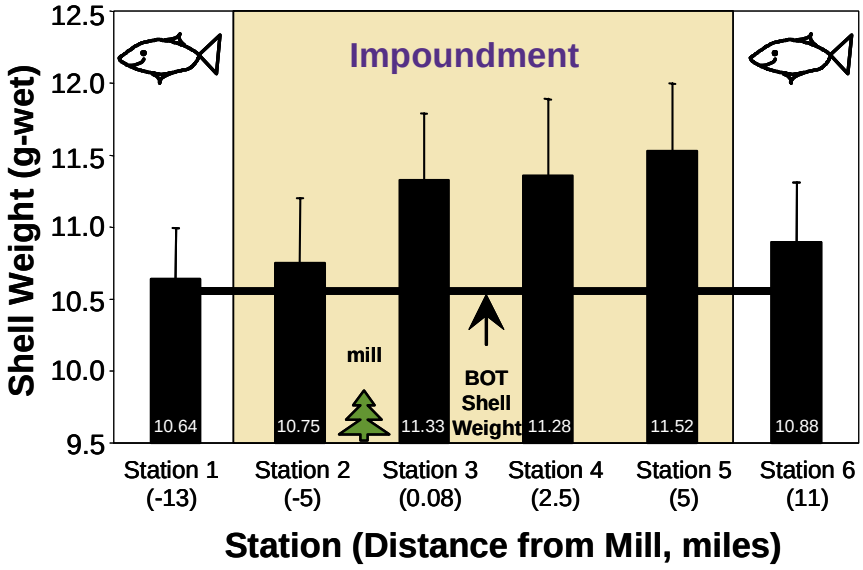


Figure 17. EOT mussel shell tissue weight by station.
Error bars represent 95% confidence interval.

4.3.5 Percent Lipids

Mean percent lipids at the start of the test was estimated at 0.56% (Table 5) based on the analysis of the three composite BOT tissue samples. Based on this estimated BOT value, mean percent lipids increased at all stations except Station 3 during the 66-day exposure period. Mean EOT percent lipids by station ranged from 0.50% at Station 3 to 0.75% at Station 1 (Table 5). These increases were not statistically significant at ($p = 0.2577$; Table 6) when compared to the BOT lipid content. Similarly, there was no significant difference in percent lipids among stations at the end of the test ($p = 0.1174$; Table 6)

4.3.6 Mussel Growth and Distance from the Mill

Mussel shell length, WAWW, and tissue weight were the most informative mussel metrics measured in this study. As shown in Figures 13, 14, and 16, there appears to be a relationship between these growth metrics and distance from the mill. A regression analysis showed that each metric was well correlated with distance (Figure 18), with WAWW having the largest R^2 value of 0.99.

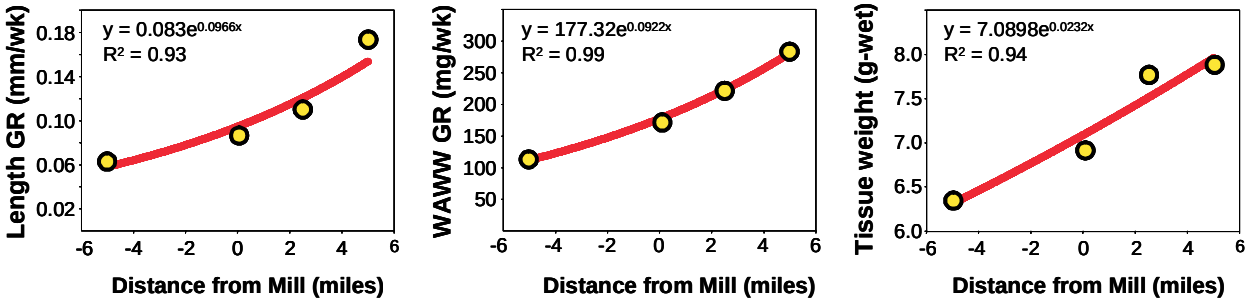
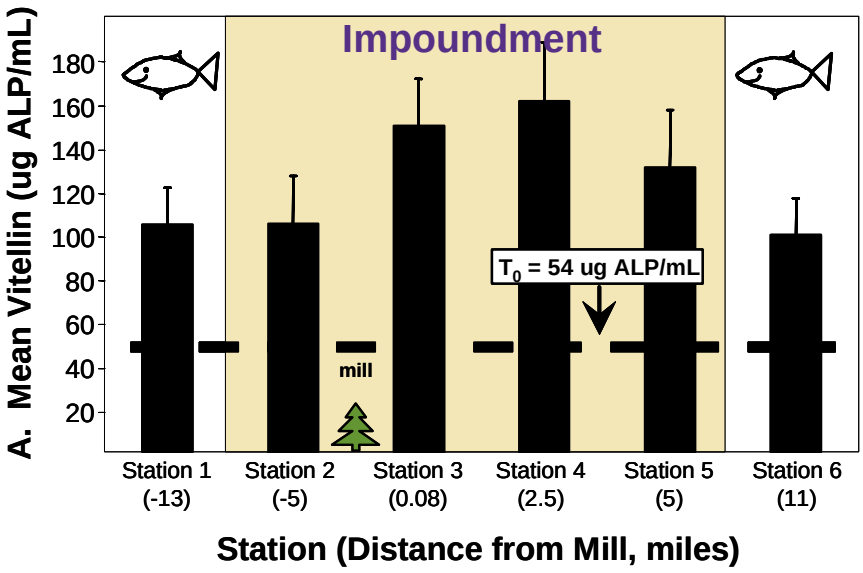


Figure 18. Relationship between length growth rate, WAWW growth rate, and EOT tissue weight and distance from the mill.

4.4 Vitellin Analysis

Figures 19A,B show mean vitellin (average of all females and males at a given station) concentration by station and vitellin normalized to tissue mass. Figures 20A,B show vitellin in females and males by station, respectively. EOT vitellin concentration was significantly higher than at the beginning of the test at all stations ($p < 0.0001$). Not only was vitellin significantly higher at Stations 3 and 4, immediately below the mill, but both female and male vitellin were higher there as well. Vitellin concentrations at pooled Stations 3, 4, and 5 were significantly higher when compared to pooled Stations 1 and 2 ($p = 0.0001$). Significantly higher concentrations were also found for pooled Stations 3 and 4 when compared to pooled Stations 1, 2, 5, and 6 ($p = 0.0001$). The figures also show the increase in vitellin compared to beginning-of-test vitellin for ug ALP/mL and ug ALP/mL normalized for tissue mass. ALP was also normalized for proteins, but these data were not as responsive and are not shown here.

Figure 21 shows the regression relationship between female vitellin and distance from the mill for Stations 1, 2, 3, and 4. The regression line is statistically significant ($p=0.03$) and the high coefficient of determination ($R^2=0.94$) suggests that the equation is reasonably predictive. The



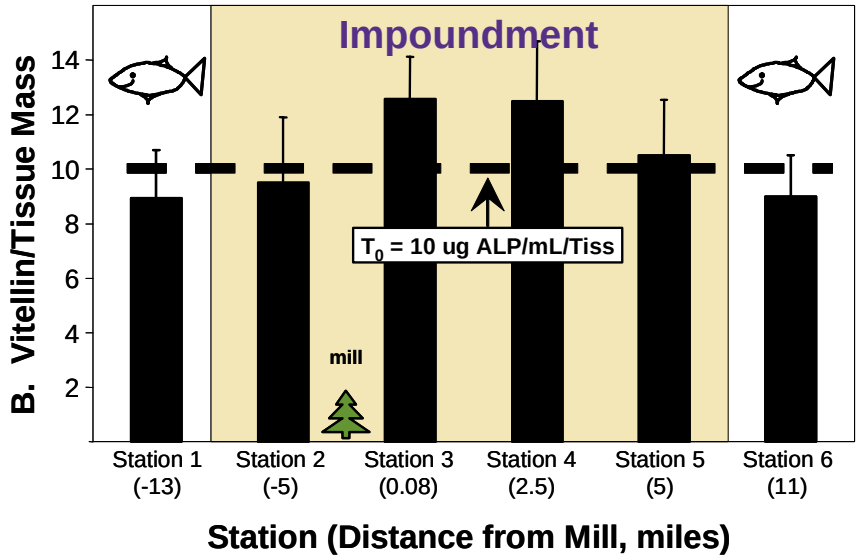


Figure 19. (A) Mean vitellin concentration in mussel tissues by station.
(B) Mean normalized vitellin concentration by station.
Error bars represent 95% confidence interval.

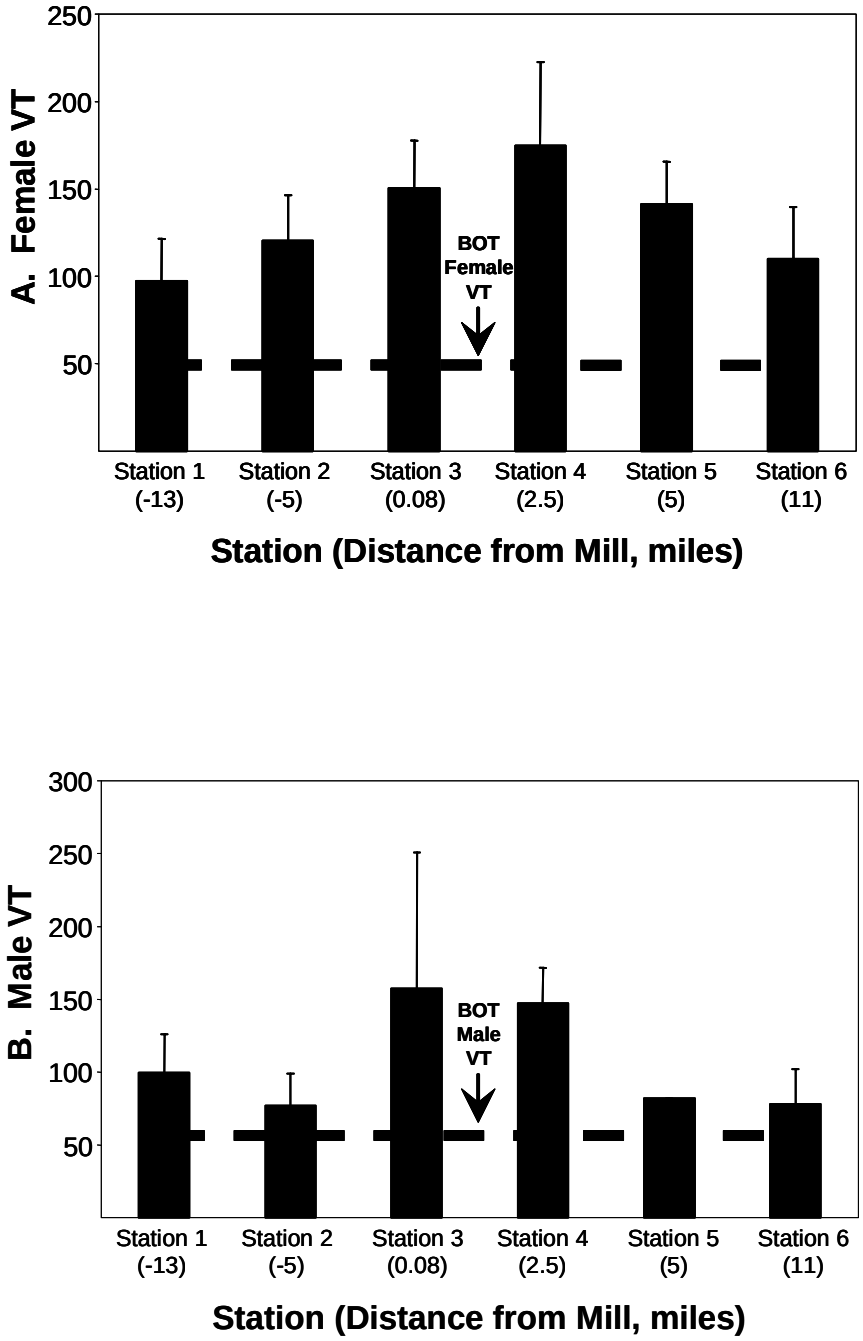


Figure 20. (A) Mean vitellin concentration in female mussels by station.
(B) Mean vitellin concentration in male mussels by station.
Error bars represent 95% confidence interval.

increasing gradient with distance approaching the mill further suggest the mill as a source of endocrine disrupting compounds and potential reproductive effects in mussels based on the induction of vitellin in females. The regression line for males is not shown because it is insignificant and there is no gradient, only a large spike of vitellin for male mussels at Stations 3 and 4. Figure 22 shows the similarity in the relationship between vitellin induction in males and females and why it was appropriate to pool those samples for an overall trend analysis. The error bars are relatively large because of the relatively small number of animals used for each analysis. It should be remembered that we only used 4 mussels from each cage for the vitellin analysis since it was not originally considered as a major part of this study. Interestingly, we now believe that the vitellin assay provided information that was just as useful, if not more useful than the growth rate data.

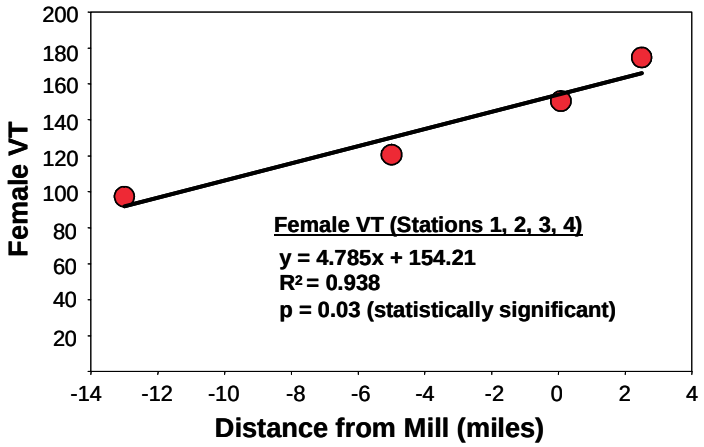


Figure 21. Relationship between vitellin in females and distance from the mill.

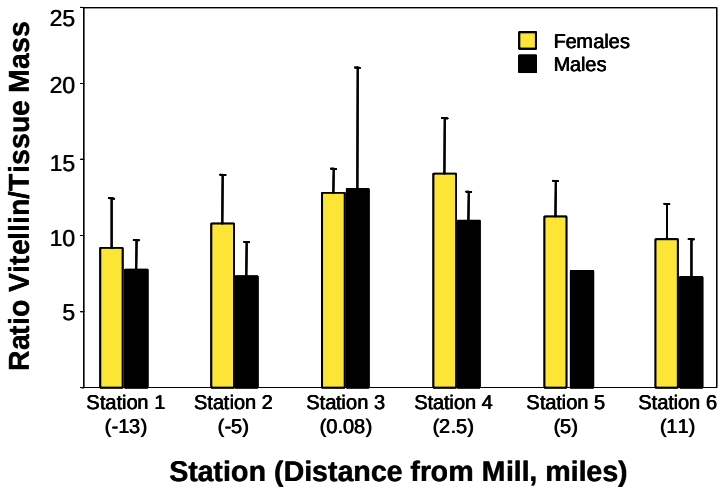


Figure 22. Comparison of normalized vitellin between females and males by station.
Error bars represent 95% confidence interval.

4.5 Water Temperature

All water temperature monitors attached to the mussel cages were retrieved, as well as those deployed at the effluent diffuser and 100 feet downstream of the diffuser. Water temperature profiles by station are shown in Figure 23. The mean, minimum, maximum, and range in water temperature at each station over the course of the study are provided in Table 7. Daily average water temperatures were determined to lessen the variability present on a daily basis (Figure 24). Water temperatures ranged between 14.3 and 25.8°C over the course of the entire study at the six deployment stations. The highest temperatures were measured in August, approximately 20 days after deployment. Water temperatures were lower at Station 1 when compared to the other stations where mussels were deployed, with the difference being between 0.5 and 2.7°C. Water temperature was highest at the diffuser, approximately 1 to 3°C higher than at Station 1, and approximately 0.2 to 0.7°C higher than other stations in the immediate vicinity (i.e., Station 3). Water temperature increased slightly over the first 25 days, and then decreased fairly consistently over the remaining test, with the exception of a slight elevation occurring in mid-September (Figures 23, 24).

Table 7. Mean, minimum, maximum, and range in water temperature (°C).

	Station 1	Station 2	Station 3	Station 4	Station 5	Station 6	At Outfall	Outfall +100'
Mean	20.51	21.46	21.72	21.94	21.86	21.86	22.30	21.84
Min	14.28	15.47	16.44	16.54	16.56	16.02	16.87	16.51
Max	24.21	25.52	25.73	25.83	25.67	25.77	26.24	25.81
Range	9.93	10.05	9.29	9.29	9.11	9.75	9.37	9.30

Results of the ANOVA on daily average water temperatures showed that water temperatures at Station 1 were significantly lower than those at Stations 3 through 6; there was no statistically significant difference between daily average water temperature at Stations 1 and 2. Similarly, there was no statistically significant difference in daily average water temperature among Stations 3 through 6.

Several statistically significant differences were found in the range of daily water temperature among stations. Daily ranges were different between the following stations:

Station 3 ≠ Stations 1, 2, and 6
Station 6 ≠ Stations 4 and 5

The range in daily water temperature at Station 6 was also significantly different than that measured at the plume and 100' downstream from the diffuser.

In addition to the statistical analyses, Figures 25A, B graphically demonstrate that there is virtually no difference in temperature within the impoundment, particularly below the mill.

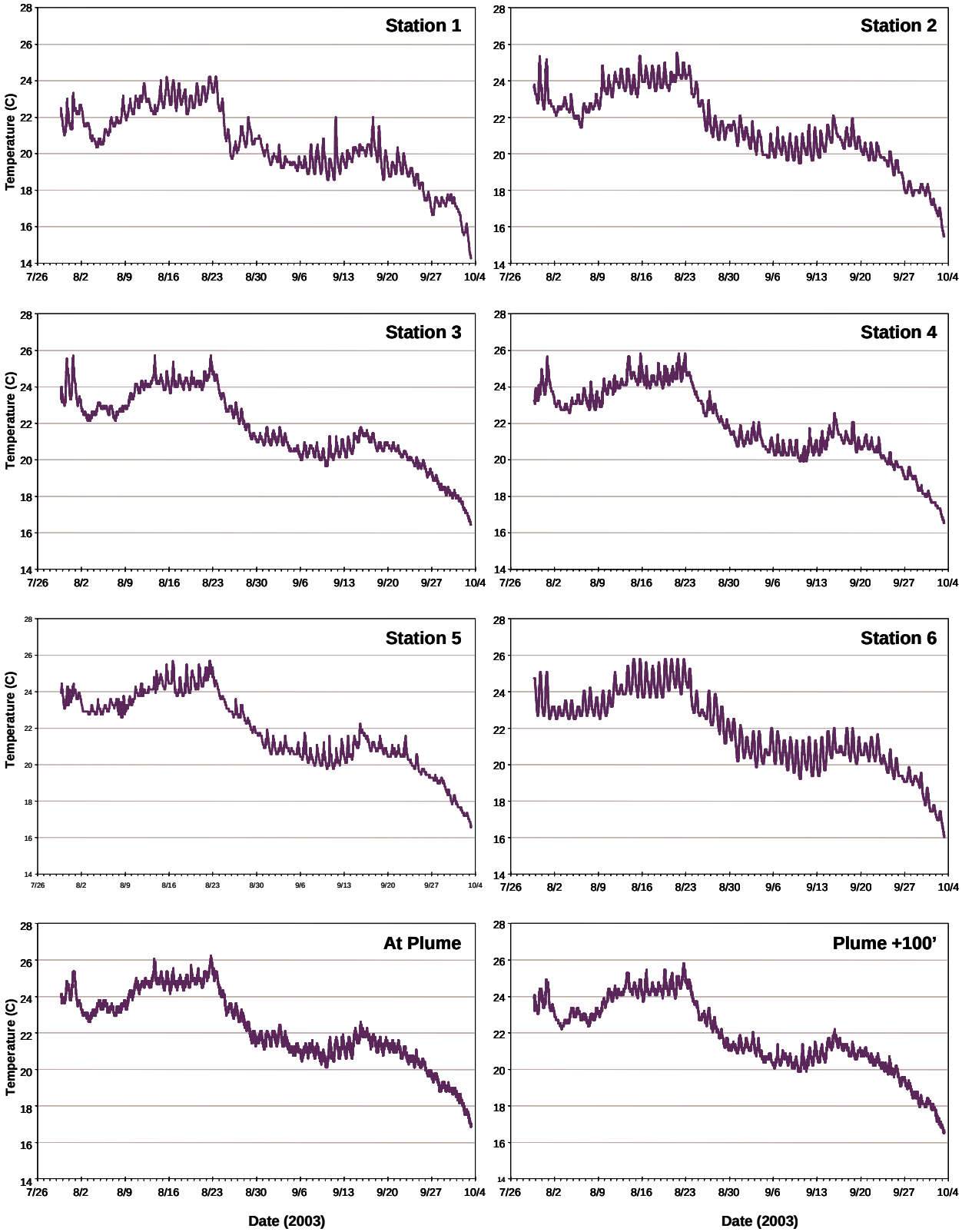


Figure 23. Water temperature profiles by station and time.

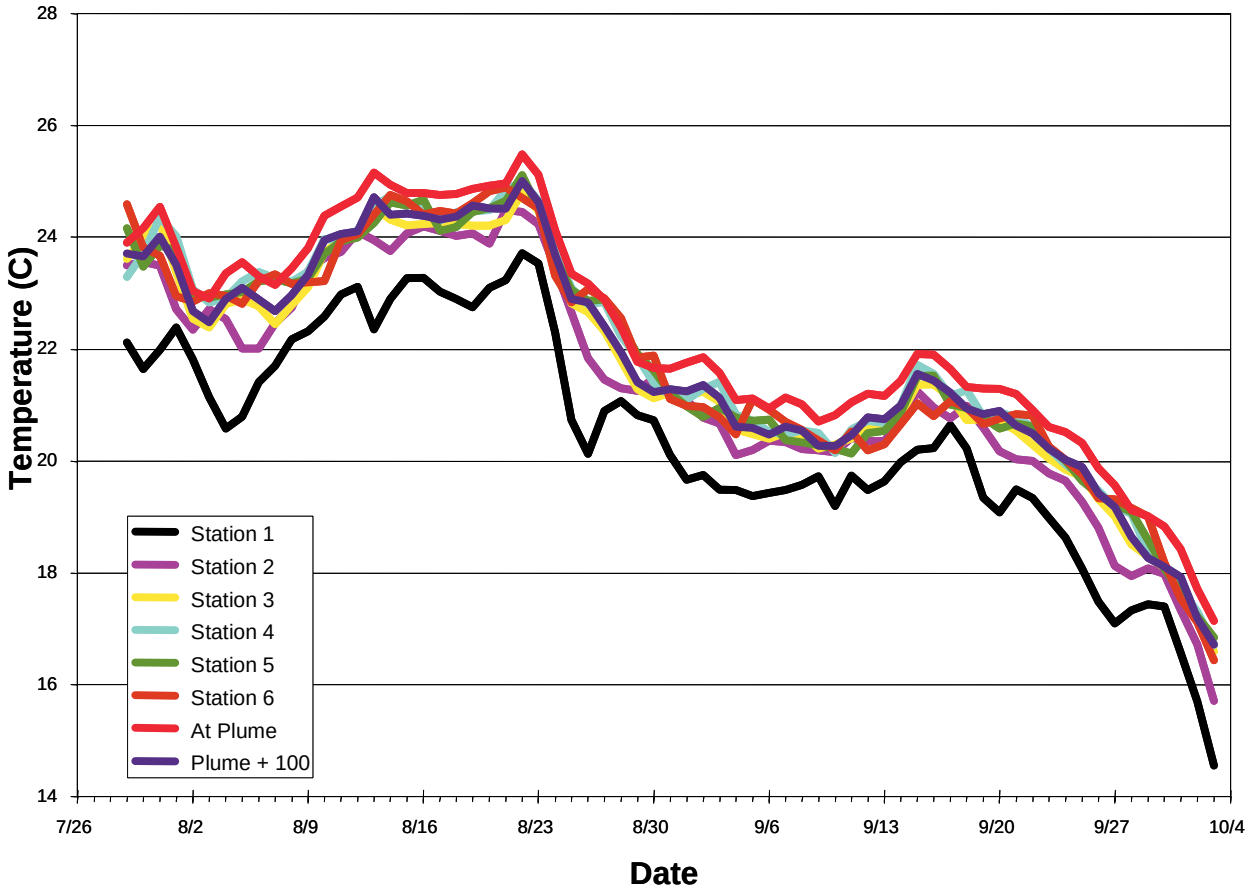


Figure 24. Daily average water temperature by station.

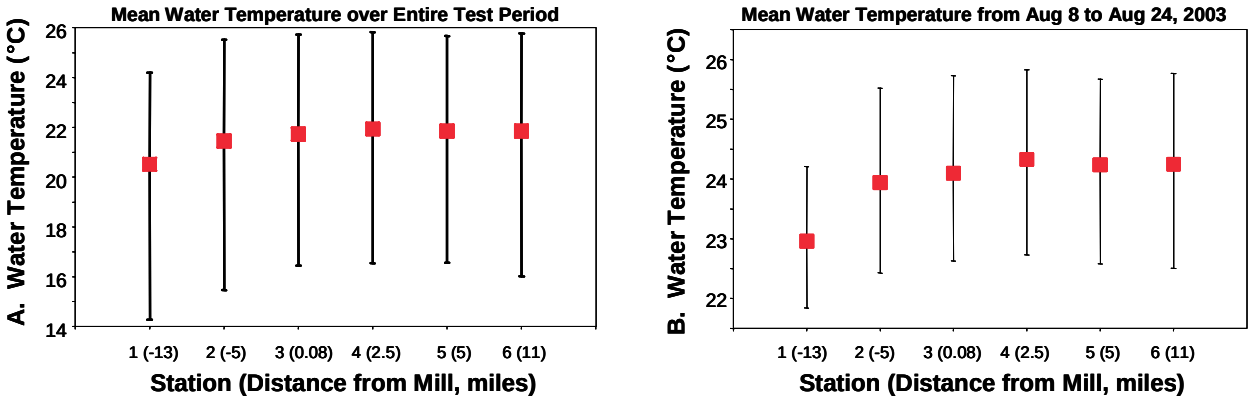


Figure 25. (A) Mean water temperature and range for entire test period, by station. (B) Mean water temperature and range for the warmest exposure period, August 8 to August 24, 2003, by station.

4.6 Correlation Analysis

Correlation analyses were run on all the major parameters measured in this study including percent survival, length, length growth rate, whole animal wet weight (WAWW), WAWW growth rate, whole soft tissue weight, shell weight, temperature, vitellin, tissue-normalized vitellin, female vitellin, male vitellin, total PCDD-F, lipid normalized PCDD-F, 2,3,7,8-TCDF, HpCDD, OCDD, and percent lipids (Table 8). In addition to using all these data, the correlation analyses were also run using only data from within the impoundment (Table 9). Using only the data from Stations 2, 3, 4, and 5 improved many of the relationships dramatically. An arbitrary r value of 0.69 was used to identify potentially significant correlations (as shown in bold in the shaded boxes). Some values exceeding 0.69 are not bolded/shaded because the parameter is an integral part of the comparative parameter (i.e., WAWW is used to calculate WAWW growth rate, and shell weight is an integral part of WAWW). Using all data, 17 potentially significant correlations were identified. Limiting the analysis to data for only Stations 2, 3, 4, and 5 (i.e., the impoundment stations), resulted in 40 potentially significant correlations. This provides additional evidence that the impoundment should be considered as a separate unit when analyzing the exposure and effects data. Again, the highlighted potentially significant correlations do not include the more obvious correlations between metrics that are directly related such as different growth metrics, different normalization metrics for tissue chemistry, and related vitellin metrics.

Table 8. Results of correlation analysis performed on all data, all stations.

	Percent Survival	Length	Length Growth Rate	WAWW	WAWW Growth Rate	Whole Soft Tissue Wt.	Shell Weight	Water Temp	Vitellin	Normed Vitellin	Female Vitellin	Male Vitellin	Total PCDD/PCDF	LN Total PCDD/PCDF	2378 TCDF	HpCDD	OCDD	Percent lipids
Percent Survival	1.00																	
Length	-0.87	1.00																
Length Growth Rate	-0.90	1.00	1.00															
WAWW	-0.88	0.89	0.91	1.00														
WAWW Growth Rate	-0.87	0.97	0.98	0.95	1.00													
Whole Soft Tissue Wt.	-0.82	0.92	0.93	0.94	0.98	1.00												
Shell Weight	-0.79	0.88	0.89	0.97	0.91	0.91	1.00											
Temperature	-0.52	0.57	0.58	0.61	0.53	0.60	**0.71**	1.00										
Vitellin	-0.53	0.51	0.54	**0.81**	**0.69**	**0.78**	**0.79**	0.50	1.00									
Normd Vitellin	-0.49	0.41	0.45	**0.76**	0.59	0.68	0.75	0.53	0.98	1.00								
Female Vitellin	-0.62	0.58	0.61	**0.82**	**0.72**	**0.83**	**0.80**	**0.69**	0.95	0.93	1.00							
Male Vitellin	-0.08	0.03	0.07	0.44	0.25	0.36	0.44	0.19	0.85	0.89	0.71	1.00						
Total PCDD/PCDF	0.10	-0.26	-0.24	-0.22	-0.33	-0.23	-0.13	0.57	-0.14	-0.01	0.09	-0.14	1.00					
LN Total PCDD/PCDF	-0.01	-0.07	-0.05	0.11	-0.10	-0.04	0.26	**0.72**	0.19	0.34	0.30	0.23	0.81	1.00				
2378 TCDF	0.29	-0.25	-0.29	-0.60	-0.42	-0.42	-0.58	-0.05	**-0.76**	**-0.78**	-0.55	-0.85	0.47	-0.05	1.00			
HpCDD	-0.14	-0.33	-0.28	-0.22	-0.30	-0.28	-0.36	-0.11	-0.19	-0.09	-0.05	-0.17	0.45	0.08	0.31	1.00		
OCDD	0.12	-0.22	-0.21	-0.18	-0.29	-0.19	-0.06	0.64	-0.09	0.04	0.13	-0.09	0.99	**0.86**	0.42	0.32	1.00	
% lipids	0.29	-0.32	-0.33	-0.53	-0.32	-0.33	**-0.68**	**-0.72**	-0.51	-0.61	-0.51	-0.47	-0.33	**-0.81**	0.51	0.32	-0.43	1.00

Table 9. Results of correlation analysis performed on data for Stations 2, 3, 4, and 5 only.

	Percent Survival	Length	Length Growth Rate	WAWW	WAWW Growth Rate	Whole Soft Tissue Wt.	Shell Weight	Water Temp	Vitellin	Normd Vitellin	Female Vitellin	Male Vitellin	Total PCDD/PCDF	LN Total PCDD/PCDF	2378 TCDF	HpCDD	OCDD	Percent lipids
Percent Survival	1.00																	
Length	-0.91	1.00																
Length Growth Rate	-0.92	1.00	1.00															
WAWW	-0.67	0.87	0.87	1.00														
WAWW Growth Rate	-0.80	0.97	0.97	0.93	1.00													
Whole Soft Tissue Wt.	-0.62	0.88	0.88	0.89	0.96	1.00												
Shell Weight	-0.61	0.81	0.82	0.99	0.89	0.85	1.00											
Temperature	-0.36	**0.71**	**0.70**	**0.86**	**0.85**	**0.95**	**0.86**	1.00										
Vitellin	0.16	0.22	0.23	0.61	0.44	0.60	0.66	**0.83**	1.00									
Normd Vitellin	0.34	0.01	0.01	0.47	0.22	0.38	0.54	0.66	0.96	1.00								
Female Vitellin	0.16	0.25	0.24	0.55	0.47	0.66	0.57	**0.86**	0.96	0.88	1.00							
Male Vitellin	0.54	-0.23	-0.22	0.26	-0.01	0.16	0.35	0.47	0.88	0.97	0.78	1.00						
Total PCDD/PCDF	**0.79**	**-0.92**	**-0.93**	**-0.98**	**-0.94**	**-0.86**	**-0.97**	**-0.78**	-0.46	-0.31	-0.40	-0.09	1.00					
LN Total PCDD/PCDF	**0.69**	**-0.69**	**-0.68**	-0.28	**-0.61**	**-0.58**	-0.18	-0.32	0.23	0.48	0.03	0.64	0.37	1.00				
2378 TCDF	-0.09	-0.11	-0.13	-0.59	-0.25	-0.28	-0.67	-0.50	**-0.77**	**-0.86**	-0.58	-0.83	0.48	-0.61	1.00			
HpCDD	0.51	**-0.77**	**-0.77**	**-0.98**	**-0.87**	**-0.88**	**-0.99**	**-0.92**	**-0.76**	-0.64	**-0.70**	-0.45	0.93	0.16	0.69	1.00		
OCDD	**0.88**	**-0.96**	**-0.97**	**-0.94**	**-0.95**	**-0.84**	**-0.91**	**-0.71**	-0.32	-0.15	-0.27	0.07	0.99	0.47	0.35	0.86	1.00	
% lipids	-0.01	-0.10	-0.12	-0.54	-0.19	-0.16	-0.62	-0.34	-0.60	**-0.71**	-0.36	**-0.69**	0.47	-0.65	0.97	0.61	0.36	1.00

One of the more interesting relationships was observed for temperature. Using all data, only three relationships $\geq$ 0.69 were found, while using the data for Stations 2, 3, 4 and 5 only, temperature was positively related (i.e., r values $\geq$ 0.69) to six parameters, including almost every growth metric, female vitellin, and several tissue chemistry metrics. While the correlations suggest several relationships that might be environmentally significant, there are probably no causal relationships. This interpretation is supported by the temperature comparisons which show no statistically significant difference among mean temperatures at any of the stations within the impoundment, even when the highest summer temperatures are analyzed separately. We believe that many of the correlations suggested through the correlation analysis are pseudo-correlations that should be tested with more rigorous regression analyses to demonstrate a high coefficient of determination and a statistically significant regression line. There are far fewer relationships that meet these two criteria. This is also the approach that was used to analyze the relationships between many of these metrics and distance from the effluent diffuser.

5.0 DISCUSSION

The caged mussel study should be considered successful because the test animals survived, grew, and accumulated dioxin-furan congeners in their tissues, and the study objectives were accomplished. With respect to the purpose and objectives of this study, the results demonstrate that transplanted mussels are a viable option to monitor the effluent discharged by kraft mills and that they provide detailed information over fine spatial scales that cannot be provided by collecting fish above and below the dams creating these impoundments. This confirms the feasibility and scientific value of the caged bivalve approach for effluent monitoring. Based on the survival and growth data, the mussels were in sufficiently good health to accumulate dioxin-furan congeners, if present. The mussels were successful at characterizing the mill effluent with respect to the presence or absence of dioxins and furans. The mussel data show differences in bioaccumulation and effects among stations separated by 2 to 5 miles within the impoundment and within 400 feet of the mill discharge. There were also differences in tissue chemistry above and below the mill, but these results were dependent on which stations were used for the comparisons.

Probably the most constructive information came from the gradient analysis. Several statistically significant relationships were found along the suspected chemical gradient, but many of these were different than expected. The gradient design facilitated establishing and refuting some expected chemical and biological relationships. New information was provided regarding chemical exposure and possible effects that would not have been available using current approaches. However, it should be made clear that significant questions remain regarding the precision and accuracy of the mussel tissue chemistry results. For these reasons neither the 2000 nor the 2003 caged mussel tissue chemistry results should be used to determine compliance with the regulations.

Future studies should emphasize ERA-based approaches, weight of evidence interpretation and the gradient design and should focus more on the impoundment where the mill is located. The limitations of using a reference site and the advantages of a gradient design for ERA-based approaches have been identified (Landis 2000, Preston 2002). Unfortunately, the biggest problem in the dioxin monitoring program (DMP) studies conducted in 2000 and 2003 is the lack of confidence in the tissue chemistry data. By inference it seems likely that the same problems are inherent in all the fish tissue chemistry data since the inception of the DMP and the above-below test. It appears that the results of the caged mussel studies have helped identify the need for better tissue chemistry data. Conversely, there should be no question regarding the ability of mussels to accumulate all of the 2,3,7,8-substituted congeners, including 2,3,7,8-TCDD, the most toxic dioxin, and 2,3,7,8-TCDF, the most toxic furan (Abad et al. 2003, Loganathan et al. 2001). Similarly, some of the effects measured in mussels in this study have been reported in other studies for mussels deployed below mill discharges (Martel et al. 2003).

5.1 Characterizing Exposure to & Identifying Sources of Dioxins and Furans

The discussion will focus on the dioxin-furan congeners generally believed to be the most representative of kraft mill processes: 2,3,7,8-TCDD and 2,3,7,8-TCDF, and possibly 1,2,3,7,8-PeCDD and 1,2,3,7,8-PeCDF (Adams et al. 2004). The mill is probably not discharging the most toxic dioxins or furans because no decreasing gradients away from the mill were found for 2,3,7,8-TCDF, and 2,3,7,8-TCDD was not accumulated by mussels at any station. The weight of evidence suggests that those dioxin and furan compounds accumulated by mussels within

the impoundment (i.e, the area associated with Stations 2, 3, 4, and 5) most likely originated above of the mill. If 2,3,7,8-TCDF, and 2,3,7,8-TCDD were being discharged by the mill, mussels deployed at Station 3, nearest the mill discharge, would have accumulated the highest concentrations. Although OCDD was the predominant congener in all tissue samples, it is primarily associated with combustion processes and not necessarily an indication of dioxins-furans present in mill effluent (Adams et al. 2004). However, it should be noted that some studies have suggested that marine bivalves such as mussels, oysters, and clams show a tendency to preferentially accumulate 2,3,7,8-TCDF and OCDD, and that there is a difference in the accumulated congener pattern in different bivalve species from the same site (Abad et al. 2003). That pattern is somewhat consistent with Kennebec results.

It has been demonstrated that the same fish species having different diets or feeding areas, and even different fish species, have variable accumulation patterns. For example 2,3,7,8-TCDD was elevated in mountain whitefish below a pulp mill discharge because the fish were feeding on filter-feeding caddisfly larvae. In contrast, longnose suckers feeding on benthic organisms with relatively uncontaminated sediments did not accumulate significant amounts of 2,3,7,8-TCDD (Carey et al. 1996, Hodson 1996). Again, this represents a major disadvantage of attempting to use fish to determine exposure from current versus historical discharges. Similarly, for caged fish deployed below a mill discharge, only those located closest to the diffuser showed induced biochemical alterations whereas there were no observed effects in those deployed further downstream (Munkittrick et al. 1994). These results suggest there were either insufficient concentrations of these biochemical inducers in stream sediments or that benthic invertebrate food sources in those areas were not being contaminated by those sediments (Hodson 1996).

The above-below comparisons show how little information is provided by comparing only two data points relative to the additional information provided by the gradient design. For 2,3,7,8-TCDF, no statistically significant differences were found between above and below when the comparisons consisted of Stations 1 and 6 or Stations 2 and 3. The comparison using Station 2 (above) versus pooled Stations 3, 4, and 5 (below) shows that concentrations above the discharge are significantly higher than below the diffuser and within the impoundment. These results suggest that the mill is not discharging 2,3,7,8-TCDF. The mean concentrations of 2,3,7,8-TCDF and other congeners in mussel tissues, which represent integrated dioxin-furan exposures in mussel tissues at specific locations, also support the conclusion that the mill is not the source of the accumulated compounds. The inclusion of Station 2 in the 2003 study was a significant advancement, as data for this station suggest a dioxin-furan source above the SAPPI mill. For most congeners, concentrations below the mill and within the impoundment, the area expected to be most affected by the mill effluent, were lower than above the mill and below the impoundment at Shawmut. The gradient analysis shows several significant relationships with distance from the mill, some of which include Station 2. The weakest relationships are for 2,3,7,8-TCDF. The increasing gradient for 2,3,7,8-TCDF between Stations 3 and 5 is difficult to explain without speculation. The consistently high concentrations of nearly all congeners below the Shawmut dam suggest that there is an additional dioxin-furan source in that region of the Kennebec River.

5.2 TEQ's

The highest Total TEQs on a wet weight basis were one to two orders of magnitude lower than those reported in the literature for other bivalves in other studies (Abad et al. 2003, Loganathan

et al. 2001). This suggests that there should be some threshold for environmental significance, or the above below comparisons could continue forever, regardless of whether the results are meaningful (Woodard and Curran and ENTRIX 2002). The calculated TEQs are virtually identical when using either the fish TEFs or the human health TEFs. They demonstrate that in this case, the interpretation remains the same and that the fish and human health values are equally protective. The human health TEFs for 2,3,7,8-TCDF, HpCDD, and OCDD are 0.1, 0.01, and 0.001, respectively. The concentrations of HpCDD and OCDD would have to be approximately 100 and 1000 times higher than 2,3,7,8-TCDF to make the same relative contribution to Total TEQ. In other words, total toxicity across stations could have been predicted by just looking at the 2,3,7,8-TCDF concentration gradients, in the same way they were used in the gradient analysis to identify the potential sources of these congeners.

The State of Maine DMP seems to be focused on human health and the use of TEQs as part of that assessment. While this is entirely appropriate for human health assessments, it is not necessarily the best approach for ecological evaluations or source identification (Abad et al. 2003, Adams et al. 2004). Important source information is lost when using a toxicity normalization that obscures the concentration of the individual congeners that is necessary to establish the chemical fingerprint associated with a particular source. This chemical fingerprinting approach is particularly important in the use of a gradient design for purposes of source identification. While it might also be informative to evaluate sources on a TEQ basis, that is not the most efficient method for source identification and chemical fingerprinting (Abad et al. 2003, Adams et al. 2004).

This focus on TEQs has also influenced the perception regarding the percent composition of pulp mill effluents. There are several confounding factors in these analyses. Among them is the fact that each mill probably has a unique signature, and it may be inappropriate to assume that some national average based on an EPA survey conducted 8 to 10 years ago accurately represents a particular mill. Furthermore, with the recent advances in mill process technology it is likely that the composition has changed. Studies at several mills in the Pacific Northwest have shown a change in the congener distribution in sediment and crab hepatopancreas samples over the past 20 years (Yunker et al. 2002). Furthermore, the general trend over the past 20 years has been a specific reduction in the most toxic dioxin-furan congeners 2,3,7,8-TCDD and 2,3,7,8-TCDF (Abott and Hinton 1996).

5.3 Distinguishing between Current and Previous Discharges

A key issue of the current dioxin-furan monitoring program is the ability to distinguish between current and historic discharges. Several factors, including life history attributes, feeding strategy, and habitat preference, will affect the accumulation of dioxin-furan congeners by aquatic biota (Adams et al. 2004). These factors must be considered during data analysis and interpretation to ensure the appropriate conclusions are being reached. Caged mussels are a good surrogate for fish, the biological indicator of dioxin-furan discharges currently used by MDEP. Caged mussels are potentially better indicators than fish for assessing current discharges because they can be placed closer to the discharge, along suspected chemical gradients, and they better represent water column exposure (Adams et al. 2004). It is important to note another difference between caged mussels and fish is that caged mussel studies are experiments that can be manipulated to test various hypotheses while the collection of fish is an observation with no ability to vary the exposure regime. Mussels could be deployed in the water column and in bottom sediment to help differentiate the difference between water column and

sediment or benthic detrital exposures. On-going studies at several DEP stations on the Kennebec River in the vicinity of the caged mussel stations are currently being used to determine attainment of aquatic life criteria (Davies et al. 1999). The conceptual approach of using transplanted rock-filled wire baskets, mesh bags, or cones is virtually identical to the caged mussel approach. Caged mussels were proposed in 2000 because it was generally believed that their ability to combine exposure and effects measurements would be a valuable supplement to the DEP Biological Monitoring Program. The potential use of caged mussels goes beyond the Dioxin Monitoring Program in that caged mussels could be used to characterize exposure from other chemicals as well. Caged mussels were successfully used to identify potential sources of PCB contamination in the 2000 caged mussel pilot study (Applied Biomonitoring 2002).

An increase in dioxin-furan concentrations in mussel tissues reflects an accumulation from a current discharge, particularly if the mussels are suspended in the water column. It is more difficult to associate dioxin-furan tissue burdens in fish with current discharges, particularly if the fish are collected several miles away from the diffuser. The lack of 2,3,7,8-TCDD in mussels deployed below the diffuser strongly suggests that 2,3,7,8-TCDD measured in fish collected 11 miles below the diffuser probably came from another source or a previous mill discharge. In other words, both theoretical and empirical evidence is available to support the conclusion that the dioxin-furan congeners measured in fish collected 11 miles below the diffuser do not represent current mill discharges. It is more probable that these congeners are related to previous or other discharges, and were accumulated by fish directly from the sediment or from prey organisms that live in the sediments (Adams et al. 2004). In the early development of using white suckers as an integral part of Environment Canada's Environmental Effects Monitoring (EEM) program for pulp and paper mill effluents, white suckers were characterized as benthic foragers. White suckers are directly exposure to contaminated sediments as they feed and are dependent on invertebrate species that live in that sediment or organic detritus for food (Munkittrick and Dixon 1987). These characteristics may preclude the ability of white suckers to ever differentiate between current and previous discharges of kraft mill effluents. Although smallmouth bass do not have the same intimate contact with sediment and detritus, they also feed on bottom-feeding benthic organisms.

5.4 Distinguishing between Ecological and Human Health

While the distinction between current and previous discharges is critical to interpreting compliance under the current regulations with respect to ecological effects, it may be a moot point with respect to human health effects since the human health effects related to the toxic effects of dioxins and furans are the same regardless of whether the discharges are current or historical. This dichotomy between ecological and human health effects may represent the biggest dilemma in the DMP. The regulations and every dioxin monitoring report since the inception of the program have clearly stated that the primary objective of the DMP is to monitor dioxin in fish for the assessment of ecological health and human health. When coupled with the regulation specifying that mills may not discharge dioxin into receiving waters, these constraints may help explain why DEP has yet to develop a successful test. Not only is DEP faced with the difficulty of ever decreasing concentrations of dioxins in kraft mill effluents, they are attempting to distinguish between current and previous discharges by using fish tissue chemistry data that has a high degree of uncertainty for both ecological and human health applications. In practice, DEP has apparently focused on human health, but this is not clearly stated in any regulations or DMP reports.

More importantly perhaps is that the concentrations of dioxins and furans are also important in data interpretation. While there may be theoretical and empirical evidence for establishing regulations that do not allow the discharge of dioxins and furans for human health purposes, there is less justification to establish these regulations for ecological reasons alone. One of the reasons for the shift toward effects-based monitoring is the difficulty associated with measuring concentrations of dioxins and furans in the parts-per trillion range. The other is that most pulp and paper mill researchers now believe that dioxins and furans are not causing the effects that have been measured in fish (Servos et al. 2003, Stuthridge et al. 2003). Chemical inducers of biochemical changes induced in fish that have been isolated from effluents exhibit properties of polycyclic aromatic hydrocarbons (PAHs) rather than dioxins but the specific compounds causing these changes have not been identified (Hodson 1996). Biochemical changes below mill discharges have been identified at distances of up to 230 km. This suggests environmental persistence, migrating fish, or exposure via contaminated food or sediments. Other biochemical inducers appear to be metabolized and excreted by fish (Hodson 1996). This is another problem with fish monitoring. Nevertheless, if the effects measured in fish below kraft mill effluents throughout the world are not associated with dioxins and furans, there is little justification for continuing to use this exposure based monitoring to determine compliance. Currently, Canada, Sweden, and New Zealand have pulp and paper mill regulations based on measuring effects in fish (Stuthridge et al. 2003). The weight of evidence suggests that ERA-based monitoring be used with equal emphasis on measuring both exposure and effects.

There is a need to precisely state the objective of the DMP with respect to current and previous discharges and ecological versus human health before collecting additional data. This shortcoming is typical of many monitoring programs throughout the US (Carpenter and Huggett 1984, Martin and Richardson 1991, 1995; Pearce and Despres-Patanjo 19889, Perry et al. 1987, Richardson and Martin 1994). Clarification is essential because the objectives, data collection, and data interpretation are different. For example, it appears intuitively obvious that fish are better for assessing human health objectives because they are the species being consumed. Caged mussels may be better for ecological assessments because of their ability to distinguish between current and previous discharges and the ability to conduct experiments in the field. Interestingly, DEP has only applied the TEFs for humans and mammals in their calculations (i.e., to determine possible effects on human health), but they have not applied the TEFs for fish, which would identify possible ecological effects. Fish are routinely collected from areas above and below mills that are most amenable to human health assessments, but they are not collected from areas closest to the mills where the ecological effects might be most important. The emphasis on human health is obvious when considering that DEP has never collected fish from the impoundment that receives effluent from the SAPPI mill. Similarly, DEP has never measured effects in fish from the above or below locations. This is in sharp contrast to Environment Canada's environmental effects monitoring (EEM) for pulp and paper mill effluents that only measures effects. It would benefit DEP to address the dichotomy between characterizing exposure and characterizing effects, and consider using caged mussels for these assessments because they facilitate measuring both exposure and effects in a more integrated ERA-based approach.

There are other significant ramifications in focusing on human health versus ecological measurement related to the precision and accuracy of the tissue chemistry data. The human health TEQ data are based on TEFs on a wet-weight basis which introduces a potentially substantial error with respect to percent moisture in the tissue (Krahn et al. 2003). This convention is necessary because in practice, the TEFs have used the amount of fish or other

food consumed by humans that is traditionally reported on a wet-weight basis. From an ecological perspective, however, most monitoring programs report the results on a dry-weight or lipid-normalized basis to reduce these errors. This dichotomy has no doubt added to the confusion over which convention to use in the DMP. The precision and accuracy of the tissue chemistry data would be improved by using the appropriate normalization methods for ecological and human health assessments without choosing a default position for both (Adams et al. 2004, Krahn et al. 2003). Furthermore others have suggested that the data should not be lipid normalized unless it improves the observed relationships (Hebert and Keenleyside 1995).

5.5 Integrating Appropriate Monitoring Tools

There have been a number of suggestions for integrated monitoring of pulp and paper mill effluents, but integration in and of itself does not necessarily guarantee that the most appropriate monitoring elements are being integrated (Adams et al. 1992, Chapman 1996, Hall 1996, Salazar and Salazar 1997). The importance of integrating toxicology and ecology into ecological risk assessments has also been emphasized (Chapman 2002), as well as the need for multiple lines of evidence in predicting site-specific ecological effects (Hall and Giddings 2000). Appropriate integration of specific monitoring elements should be based on utilizing those approaches best suited to answer the questions being asked. The ERA framework provides a very focused approach to environmental monitoring and assessment and serves as a reminder that it is important to characterize both exposure and effects (Salazar and Salazar 1997). Characterizing exposure has been routine in Mussel Watch monitoring programs throughout the world for decades Goldberg 1975, 1976, Goldberg et al. 1978). Today, more monitoring programs are routinely measuring effects in bivalves, fish, and other organisms (Garrigues et al. 2001, Lagadic et al. 2000, Markert et al. 2003).

Others have suggested the integration of multiple chemical and biological endpoints and an integrated approach for establishing cause-and-effect relationships (Adams 2003, Adams et al. 2002). The use of bivalves for characterizing chemical exposure and associated biological effects has also been addressed (Green et al 1985, Salazar and Salazar 1998). The numerous advantages of bivalves have been discussed in detail (Phillips 1980, Phillips and Rainbow 1993, Phillips and Segar 1986). Furthermore, several authors have discussed the need for an early warning system of potential adverse effects as a key component of environmental monitoring (Parr et al. 2003, Cancio 2003). This effects element represents a key improvement to the traditional Mussel Watch monitoring programs that have only measured exposure (Goldberg and Bertine 2000). The next level of refinement is linking tissue residues to these measured effects (Hornberger et al. 2000) which has been referred to as the exposure-dose-response triad (Salazar and Salazar 1998). This will also be necessary to confirm that the effects measured in this study are being caused by the mill discharge.

With respect to the DMP, integration could take many forms. In its most simplistic form, DEP could follow the recommendation of the Review Panel and integrate two species of fish and caged mussels in an above-below test scenario (Adams et al. 2004). The integration of fish and mussel monitoring has been utilized in a number of studies (Richman 1992, 2001). However, as shown through results for the caged mussel study and inferences from the Review Panel, it is unclear whether fish can be used to distinguish current from previous discharges. A more appropriate application of integrated monitoring would be to distinguish between current and previous discharges, an outstanding and critical question of the DMP. As suggested previously, caged bivalves, deployed both in the water column and on the bottom, and the use of a gradient

design can assist in making that distinction. Appropriate integration also depends on characterizing and understanding processes before determining the most appropriate elements to include (Cowell and Monk 1981, Carpenter and Huggett 1984, Coswell 1981, White 1984). It is generally acknowledged that it is virtually impossible to identify a true reference site in the field and the advantages of a gradient design have been identified (Landis 2000).

5.6 Establishing links between effects in bivalves and fish

A concern with many monitoring programs is that bivalves are not good surrogates for fish in ecological or human health assessments because of differences in metabolism and physiological responses. Recent work by Environment Canada scientists at the St. Lawrence Center in Montreal demonstrate that most effects endpoints commonly measured in fish can also be measured in caged bivalves. They have developed a suite of biomarkers for marine and freshwater bivalves that has been tested in several locations, including upstream and downstream of a municipal effluent. These biomarkers include an assay for immunocompetence (Blaise et al 1999, Blaise et al 2002), cytochrome P450, DNA damage (Gagne et al 2002), and vitellin. The vitellin assay was used to link possible endocrine disruption and concentrations of coprostanol in caged mussel tissues (Gagne et al 2001a,b,c) and experimentally-induced sex reversal in caged mussels deployed downstream of a municipal effluent for a period of 1 year (Blaise et al 2003). Perhaps more importantly, similar effects on hepatic vitellin and reproductive function were demonstrated in spottail shiners at sites downstream of the same municipal effluent (Aravindakshan et al. In Press). These biomarkers can also be applied to pulp and paper mill effluents. The similarity between bivalves and fish was also demonstrated at a kraft mill in Florida. In that study (Kernaghan et al. In Press), caged freshwater mussels showed significant endocrine and reproductive effects that were similar to those reported for largemouth bass.

In a Canadian EEM study of a kraft mill effluent using transplanted freshwater mussels, *Elliptio complanata*, wild fish collections, and benthic community structure as effects endpoints, reduced growth in mussels and altered benthic community structure were found, but no effects were found in fish (Martel et al. 2003). This lead the authors to conclude that caged mussels were not a good surrogate for fish, but weight of evidence is not discussed. Most importantly, it is not clear if the fish were exposed to chemicals in the effluent. While the intent of “surrogate” in the State of Maine regulations is unclear, the spirit of the regulation seems similar to the Review Panel interpretation, who suggested a weight-of-evidence approach using bivalves and fish (Adams et al. 2004). Therefore, “surrogate” could mean using fish and a surrogate.

5.7 Need for Better Tissue Chemistry Measurements

The dioxin monitoring program will only be successful if the analytical data are precise and accurate (Applied Biomonitoring 2002, Adams et al. 2004, Woodard and Curran and Entrix 2002). Both the 2000 and 2003 caged mussel studies demonstrate the need for good tissue chemistry results because the results from both studies are questionable and should not be used to determine compliance. It will become increasingly more difficult to accurately and precisely quantify dioxins-furans in biological tissues as the concentrations of these chemicals decrease in effluents. In 2003, PACE Analytical detected only three congeners in these samples, while the University of Maine, Orono, detected 15 congeners in the 2000 samples. Surprisingly, PACE misread one of the chromatograms and initially reported the presence of 2,3,7,8-TCDD in two samples. Since the instrument quantifies these peaks it is unclear how

such a mistake could have been made, particularly with the most toxic dioxin congener. It was only after DEP insisted that the data were re-evaluated. Furthermore, PACE did not provide the individual data sheets to show the variability in instrument sensitivity on each sample. Their report only included the nominal detection limits specified in the contract and did not provide the details necessary to further evaluate the reasons for the large number of non-detects and the fact that their procedures only identified three congeners and at much higher concentrations than expected.

Although a number of questions have been raised about the QA/QC used by the university laboratory, it now seems possible, perhaps even likely, that the university treated the samples with greater care than the contract laboratory. The University of Maine used approximately 5 times as much tissue (150 g) as PACE Analytical in 2003, which may have increased their ability to detect more congeners. A more recent study used tissue masses that were almost 10 times higher (1500 g wet wt samples and the use of 50 to 100g of freeze-dried tissue) (Abad et al. 2003). While we have not been able to confirm the University's cleanup procedures, it is well known that just using more tissue is not enough to ensure greater detection. If the samples are not cleaned appropriately, the extra lipids and other extraneous factors in the larger tissue samples can obscure the peaks as well. It is possible that the University staff took greater care in the cleanup steps as well. Finally, it now appears possible, even likely that the standard EPA dioxin method, which does not specify either cleanup procedures or methods for determination of lipids, may not be adequate for detecting dioxins and furans with sufficient accuracy at the sub part-per-trillion level due to problems associated with cleanup, determining percent lipids, or percent moisture.

The same beginning-of-test tissue samples for the 2003 Kennebec mussel study were analyzed by two different laboratories. The samples were originally homogenized and analyzed by Columbia Analytical, and then an aliquot of each was sent to PACE Analytical for analysis. Table 10 shows that the beginning-of-test tissue chemistry data were extremely variable and had a very high degree of uncertainty which precluded using these data to determine net accumulation. Beginning-of-test Total PCDD-F concentrations reported by PACE Analytical ranged from 4.30 to 20.2 ng/kg-ww, while those reported by Columbia Analytical ranged from 0.69 to 1.23 ng/kg-ww. Sample contamination could have occurred in the transfer of BOT tissue samples from Columbia Analytical to PACE Analytical, or during PACE's handling of the samples.

Table 10. Total PCDD-F concentrations (ng/kg-ww) reported by different laboratories for the same beginning-of-test tissue samples.

	PACE Analytical	Columbia Analytical	2000 BOT
Replicate 1	20.2	0.95	0
Replicate 2	4.30	0.69	0
Replicate 3	5.64	1.23	0
Mean	***10.45 (4.97*)***	***0.96***	***0***

*Mean calculated without the 20.2 value which may be an outlier.

The dioxin-furan tissue chemistry results provided by Columbia Analytical for the caged mussels were the most believable, the most consistent and the most useful because these data provided the most reasonable explanation for accumulation of dioxins and furans after the mussels were transplanted to the Kennebec River. Even by excluding the possible outlier value of 20.2 ng/kg-

ww from the PACE data set, the calculated mean of 4.97 ng/kg-ww would indicate that mussels accumulated very little, if any dioxins or furans during the exposure period. These data appear unreasonable, particularly because dioxins-furans concentrations in all beginning-of-test mussel tissues for the 2000 Kennebec study were below the limit of detection (Applied Biomonitoring 2002). There is nothing to indicate that the tissue concentrations of dioxins and furans measured in 2000 would have, or should have changed. DEP questioned the ability of mussels to accumulate dioxins and furans in the 2000 pilot study, in part because they did not detect dioxins and furans that were expected based on previous fish sampling at other pristine locations. In summary, given the discrepancies in the PACE analytical results for mussel tissue chemistry, these data should not be used for compliance. Although there were no split samples analyzed for the fish, it seems likely that the same analytical problems are associated with the fish data and they should not be used either. Given the questions already raised about the 2000 data, any comparisons among years should be made with caution.

5.8 Need for Better Tissue Chemistry: Chemical Fingerprinting

Each mill has a unique chemical signature or "fingerprint" related to the raw materials and mill processes. The need for better analytical chemistry measurements becomes even more important as changes in mill processes result in different chemicals in the effluent. The dioxin reassessment suggests that the typical mill signature, based on TEQs, is approximately 70% 2,3,7,8-TCDD, 20% 2,3,78,-TCDF, and 1% OCDD. The dioxin reassessment also shows that the typical mill signature, based on concentration, is approximately 40% OCDD, 15% 2,3,7,8-TCDF, and only 5% 2,3,7,8-TCDD. It is critical to recognize the differences in mill signature between concentration and TEQ bases, and even more critical to have accurate, precise analytical measurements when applying TEFs because these factors have a range of four orders of magnitude (i.e., 1.0 to 0.0001).

It is possible that the congener distribution has changed since the draft dioxin reassessment report was written, and the concentrations of the most toxic congeners, 2,3,7,8-TCDD and 2,3,7,8-TCDF, have likely diminished even further with the change from elemental chlorine to chlorine dioxide. This is one of the reasons why no specific congener contribution was assumed during data analysis. In the 2003 caged mussel study, concentrations were emphasized rather than TEQs, and total concentrations were used for some of the comparisons. Total PCDD-F concentrations are not being advocated for regulatory purposes. Their use in this study was just a means to an end in order to better characterize and understand processes. Furthermore, the study was successful in increasing the understanding and providing important information that would never have been obtained through fish monitoring.

5.9 Need for Effects Measurements: Biological Fingerprinting

There are several reasons for including effects measurements in the assessment of pulp and paper mill effluents: 1) It is difficult to measure concentrations of dioxins and furans in the parts-per-trillion range; 2) Other studies have shown that dioxins and furans from pulp and paper mill effluents are probably not causing the effects in fish that have been measured throughout the world; and 3) It would be useful as part of a more integrated assessment of the potential ecological effects of pulp and paper mill effluents.

As the limitations of regulations for pulp and paper mill effluents based on water quality criteria and analytical measurements of chemicals in tissues have been reached, there has been a definite shift toward effects-based monitoring (Servos et al. 2003, Stuthridge et al. 2003). This has been the theme of the four international conferences on the effects of pulp and paper mill effluents held between 1992 and 2003. Interestingly, Mussel Watch monitoring has followed the same trend. The overall conclusion reached from international Mussel Watch workshops has been that effects measurements were essential to establish a link between chemical exposure and associated biological effects (Widdows and Donkin 1992). This further emphasizes the need to ask the "so what?" question with respect to chemicals accumulated in mussel or fish tissues (Rainbow 2002). In general however, the ERA framework is robust enough to monitor and assess chemical exposure and effects associated with these effluents (Kendall et al. 1998) and it has been suggested that neither exposure-based or effects-based monitoring is entirely appropriate for monitoring pulp and paper mill effluents or consistent with ERA-based approaches (Salazar and Salazar 2003a,b). Moreover, even ERA is being viewed in a wider context and new concepts being added to the approach (Solomon and Sibley 2002). As one example, in terms of complex mixtures, the combined concentrations of several weakly estrogenic chemicals have been shown to cause effects when their individual concentrations were below those predicted to cause effects (Silva et al. 2002). Just like the analogy to the "canary in a coal mine" sentinel often used for marine Mussel Watch programs, the same has been said for freshwater bivalves (Stolzenburg 1992). Strictly speaking, however, measuring mussel burdens is really using these sentinels, or early warning systems, as indicators of exposure and more analogous to a dosimeter used in radiation technology. It is really the measurements of effects that are analogous to the canary in the coal mine where the measurement endpoint is survival. There is still a need, however, to define the biomarkers being used for ERA and distinguish between indicators of exposure and indicators of effects (Schlenk 1999). Finally, ERA is about characterizing exposure and effects in order to understand ecological processes and define the measurement endpoints in any monitoring program (Underwood et al. 2000). Field studies with caged bivalves facilitate all of these clinical measurements of stress (ASTM 2001, Versteeg et al 1988). There is an ASTM Standard Guide for conducting in-situ field bioassays using caged bivalves (ASTM 2001), the approach has been adopted by Environment Canada for EEM at pulp and paper mills in Canada (Walker et al. 2002) and the American Public Health Association in their Standard Methods for the Examination of Water and Wastewater (APHA in press).

Nevertheless, it may be easier to establish a biological fingerprint for each mill than the chemical fingerprint. As tissue burdens decline in fish consumed by humans and that aspect of the monitoring program becomes less of an issue, the effects of mill discharges on the aquatic biota may gain importance. Current methodology can be used to characterize effects. Survival and growth are general effects endpoints, whereas biomarkers like vitellin production, are more specific. Although survival is generally not a very sensitive effects endpoint, it has significance in the context of this study with respect to the feasibility and scientific value, and repeatability. In the 2003 study, survival was very high (98.9%) and virtually identical to the pilot study conducted in 2000 (99.7%), which provides evidence that the test results are valid, comparable, and repeatable between years. A major concern with the 2003 study was that temperatures nearest the diffuser would be too high for mussels to survive. The results from this study suggest that water temperatures near the diffuser did not adversely effect survival, growth, vitellin induction, or accumulation of dioxins and furans. Similarly, there was no gradient in survival suggesting links with the mill or any other sources.

Mussel growth has more significance in the context of this study with respect to feasibility and scientific value. The similarity between the 2000 and 2003 studies provides additional evidence that the test results are valid, comparable, and repeatable among years (Applied Biomonitoring 2002). Growth provides even better evidence than survival that water temperature did not adversely affect the mussels, and that they could be placed at least within 400 feet of the mill discharge without adverse effects. The growth results from the 2003 study are important in that every growth metric showed the same increasing gradient within the impoundment. This is unusual because tissue and shell growth are often decoupled, and they do not always respond to the same environmental stressors. Even though a number of correlations and statistically significant relationships were identified, no causal relationships were established between either mussel growth and distance from the mill or mussel growth and dioxin-furan concentrations in mussel tissues. The inverse relationship between mussel growth and dioxin-furan concentrations in mussel tissues is probably a pseudo-correlation in that it is very unlikely that the low concentrations of dioxin-furans accumulated directly affected mussel growth rates. It is possible that the dioxin-furan compounds co-vary with some unmeasured parameter in the effluent, or a condition in the river that is actually causing the measured effects.

As discussed previously, the specific chemical or chemicals associated with effects measured in fish below mill effluents has not been identified, but there is consensus among pulp and paper mill researchers that it is not dioxins and furans (Servos 2003, Struthridge et al. 2003). Results from this study appear to provide additional evidence that dioxins and furans are probably not causing effects in either mussels or fish since vitellin was induced rather than suppressed. It is generally believed that dioxins and furans are anti-estrogens and would suppress vitellin production (Kendall et al. 1998). In the Kennebec study, elevated vitellin in males was not associated with elevated dioxins and furans.

In the 2003 study, vitellin was most pronounced at the two stations below the mill. This was the most important effect with respect to potential endocrine disruption and effects on mussel reproduction. In general, mussel vitellin was significantly higher below than above the mill, and there were vitellin gradients on either side of the mill. The dramatic increase in male vitellin at Stations 3 and 4 could be attributed to some other unmeasured factor in the river, but the relative specificity of vitellin induction with endocrine-disrupting compounds and the lack of a gradient suggest that this response is associated with some chemical in the mill effluent. Interestingly, vitellin was induced at all stations, which suggests that there could be some potential adverse effects. However, vitellin levels were highest and most significant at Stations 3 and 4, directly below the mill discharge. The vitellin data also show that TEQs based on the relative potency of 2,3,7,8-TCDD are not a good indicator of potential effects in mussels, because the vitellin induction at Stations 3 and 4 are most likely associated with some chemical other than dioxins and furans.

The vitellin assay is specific for endocrine disrupting chemicals, and it may be much more sensitive and easier to measure than the chemicals causing those effects in either fish or bivalves. By comparison, much less is known about the endocrine system in bivalves than in fish. Nevertheless, vitellin is a major protein found in bivalves that is synthesized from vitellogenin. Vitellin has been shown to be regulated by estrogens in both freshwater and marine bivalves (Li et al. 1998, Blaise et al. 1999). This process of endocrine disruption however appears to be similar in fish and bivalves. Vitellin induction has been demonstrated in Pacific oysters (*Crassostrea gigas*) (Li et al. 1998), marine clams (*Mya arenaria*) (Blaise et al. 1999), freshwater unionid mussels (*Elliptio complanata*) (Gagne et al. 2001a,b,c), and

freshwater zebra mussels (*Dreissena polymorpha*) (Quinn et al. 2004). Although elevated vitellin in *Elliptio complanata* was induced by exposure to a municipal effluent with secondary treatment, elevated vitellin in *Dreissena polymorpha* was induced by a municipal effluent with tertiary treatment. This demonstrates the extreme sensitivity of this assay. It also shows that even though mills have virtually eliminated dioxins and furans from their effluents they could still be causing effects that are more easily measured than measuring the chemicals that are causing the effects.

6.0 SUMMARY & CONCLUSIONS

The similarities between the 2003 and 2000 studies demonstrate the utility of caged bivalves to monitor the potential effects of effluent discharged by kraft mills. This approach provides detailed information over fine spatial scales that cannot be provided by collecting fish above and below dams creating these impoundments. Mussel survival and growth were nearly identical between years, showing the strength of the methodology and the ability to obtain consistent results across monitoring events. It should be made clear however that growth is a general indicator of stress and not specific to particular chemicals. Alternatively, the vitellin assay is directly related to endocrine disrupting chemicals in effluents and suggests that the mill discharge could be having an effect on the environment.

Collectively, the effects indicators have established a possible biological fingerprint of the discharge that will have to be confirmed with additional tests. The chemical fingerprint was less certain however due to inconsistencies in the analytical results and will require much more scrutiny to identify the problems associated with measuring parts-per-trillion levels of dioxins and furans in the tissues of biota. However, these and other data strongly suggest that the effects that have been measured in fish in the vicinity of pulp and paper mill effluents are not associated with dioxins and furans. Although effects-based monitoring has been recommended and there has been a shift in this direction for monitoring pulp and paper mill effluents, the ERA paradigm is sufficiently robust to monitor and assess these effluents. Equal emphasis should be placed on characterizing exposure and effects, using a weight-of-evidence approach, and using caged bivalves in a gradient design to help with these assessments.

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