Cycle 4 National Investigation of Cause Project
Final Report

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Executive Summary

The work described in this report is the first part of a project aimed at selecting the most appropriate laboratory tests for conducting Investigation of Cause (IOC) and Investigation of Solution (IOS) studies meant to eliminate effluent-related effects on fish reproduction. The full project calls for similar investigations at two other mill sites followed by the use of the selected laboratory tests with effluents from five mills before and after biotreatment.

The work described in this report represents an unprecedented volume of work done on one mill (Smurfit-Stone bleached kraft mill at La Tuque, QC) effluent in a relatively short timeframe (May 2006 to January 2007). The work included an assessment of wild fish in the actual river receiving the mill effluent as well as a series of laboratory tests with four species of fish, coupled with extensive chemical analyses of the effluents tested. The laboratory tests ranged in duration from just a few days to over six months and covered an assessment of reproductive indicators in fish ranging from the biochemical level to egg production. The results of the work with the LaTuque mill effluent allowed the following conclusions to be made:

- Field work and recently submitted Cycle 4 EEM data showed that the La Tuque mill effluent did not cause reduced gonad size in wild fish as was the case in previous EEM cycles.
- The minimal responses of fish in all the laboratory tests evaluated in this study were consistent with the observations on wild fish.
  - The lifecycle test with fathead minnow found that the overall condition of the fish exposed to the La Tuque mill effluent was similar to the condition of wild fish living downstream from the mill and the various medium- and short-term tests with three different species provided accurate assessment of the reproductive status of fish exposed to the mill effluent.
- Despite the minimal responses observed in laboratory fish, the effects on some reproductive endpoints at high concentrations of the La Tuque mill effluent illustrated the potential value of these endpoints for IOC/IOS work.
- Based on the results of this study, the egg production endpoint in laboratory tests appears to have the greatest potential for assessing an effluent's ability to affect fish reproduction as effluent-related effects were found to be similar (i.e., slight stimulation at low and intermediate effluent concentrations and reduced egg production at 65% and 100% effluent concentrations) in three species of fish.
- There were indications that the effect of a mill effluent on egg production can be reliably assessed in a shorter timeframe than previously thought and, if confirmed in future studies, this would make tests using the egg production endpoint (possibly using a variety of species) as one of the tools for IOC/IOS work.
- Chemical fingerprinting and differential responses of fathead minnow in tests done during the course of the project suggested that effluent quality from a mill may be somewhat variable indicating the necessity for continued chemical characterization of effluents and the complexity of the task to identify causative agent(s).
- While the results of the first phase of the project described in this report provided useful information, the final selection of diagnostic tools for IOC/IOS work will only be possible upon the completion of the full project.

Introduction

The national assessment of the first three cycles of the Environmental Effects Monitoring (EEM) program showed that pulp/paper mill effluents are causing general nutrient enrichment in the receiving environment as well as metabolic disruption in fish (Lowell et al., 2005). The consequence of metabolic disruption is that fish are allocating less energy towards reproduction. This is evidenced by larger condition factor and liver size as well as smaller gonads in fish inhabiting effluent-contaminated waters. When effects are identified, the EEM program calls for Investigation of Cause (IOC) and Investigation of Solution (IOS) studies so that the effects are reduced or entirely eliminated. In the case of enrichment, the causes are known to be effluent carbon, nitrogen and phosphorous, although the key element or combination of elements causing enrichment may be site and time specific. In the case of nutrient enrichment, because the causative agents are known, mills can proceed directly to IOS studies with the goal of minimizing nutrient input.

In the case of metabolic disruption in fish, the causative agent(s) are not known and more detailed and exhaustive IOC and IOS studies will be required to remedy the situation. To facilitate this process, a research consortium, consisting of scientists from academia, government and industry, was formed. This consortium developed a roadmap for dealing with this issue. This roadmap consists of five activities: i) preparation of a document reviewing the current state of knowledge and the associated knowledge gaps (completed in 2005; see Kovacs et al., 2006) ii) selection and/or development of diagnostic tools for IOC/IOS studies; iii) IOC studies; iv) IOS studies; and v) confirmation studies of implemented solutions.

The current focus of the five-activity roadmap is on selection and/or development of diagnostic tools that could be used for IOC/IOS work. The IOC/IOS work needs to be done under controlled conditions in the laboratory. Thus, the laboratory tests used for this purpose must be able to show the same response pattern as is observed in wild fish during the EEM studies. At the same time, the laboratory tests must be practical in terms of duration and volumes of effluent needed. With these requirements, a research project was developed which calls for field work on wild fish to be conducted at three mill sites (representative of the effects seen in wild fish and the major pulping process types in Canada) as well as a series of laboratory tests with effluents from each of the mills. The laboratory tests involve different species of fish and examination of the effect of effluents on several reproductive endpoints. As well, the tests have different duration time and effluent volume requirements. Based on the results of the work done at the three mill sites, laboratory tests will be selected for testing effluents from five mills before and after biotreatment. The overall aim is to identify the laboratory test or group of tests that can be used in the most cost-effective manner for IOC/IOS work directed towards eliminating the mill effluent-related metabolic disruption in wild fish. This report describes the work done using effluent from the first mill site.

Mill Selected for Study and Project Outline

The bleached (DNED, DED) kraft mill in La Tuque Quebec, producing about a 1200 t/d of bleached linerboard, food-grade and White Top boxes was selected for this phase of the project. The products are made from a furnish of 23% softwood, 60% sawdust and 17% hardwood. The

water usage at the mill is 87,000 m^3/d for process and 17,000 m^3/d for cooling (non-contaminated water). The mill effluent (process water) is treated in an oxygen-activated sludge plant, with an hydraulic retention time of about 9.8 h, prior to discharge into the St. Maurice River.

Wild fish were sampled upstream and downstream from the effluent discharge during September 2006 and laboratory tests with effluent from the mill were conducted between May 2006 and January 2007 as per the project outline described below:

Lead Investigator(s) & Organization	Wild Fish	Fathead Minnow Life-Cycle	Fathead Minnow Short- & Medium-Term	Rainbow Trout VTG	Mummichog Life-Cycle	Mummichog Short-term	Zebrafish	Stickleback	Chemistry
1. McMaster NWRI	Fall 06								
2. Parrott NWRI		Spring 06 to Jan. 07							
3. Kovacs/Martel Paprican			Spring & Fall 06	Spring 06					
4. MacLatchy UNB					Spring to fall 06	Spring 06			
5. Van Der Kraak U. of Guelph							Spring 06		
6. van den Heuvel UPEI								Winter 06/07	
7. Hewitt/Sherry & Parrott NWRI, O'Connor Paprican									Spring 06 to Jan 07

The findings by each of the lead investigators/organizations are described in the next seven sections.

Findings

1. Field Studies at La Tuque

The purpose of the wild fish collections was: (1) to conduct an EEM-like study with fish at the same site which was the source of the effluent for laboratory tests and (2) to evaluate additional reproductive endpoints in the wild fish that correspond to the endpoints being used in the laboratory studies for IOC. These included measurement of circulating reproductive steroid and vitellogenin (VTG) levels, *in vitro* steroid hormone production, secondary sex characteristics, gonadal histology and hepatic mixed function oxygenase activity.

Methods

Site selection for the wild fish studies corresponded to previous studies at this site including Hodson et al., 1992, Gagnon et al., 1994, and Alliance Environment, 2007. Three sites were sampled for fish; an upstream reference site above the Beaumont dam, a near field exposed site downstream of the effluent discharge but upstream of the dam in La Tuque and a far field site downstream of the discharge and downstream of the dam in La Tuque (Alliance Environment, 2007). White sucker (*Catostomus commersoni*) were collected from the three selected sites from September 12-22, 2006 using overnight sets of 4 inch gill net. Fishing at the immediate downstream exposed site was difficult and dangerous due to the strong currents created by the narrowing of the river immediately upstream of the dam and water release. The immediate downstream exposed site represented about 750 m of fishable area and contained habitat that differed considerably from the reference and exposed site below the dam.

Sampling Protocol

White sucker were immobilized in a foam block and blood samples were taken from the caudal vessels using a syringe and heparinized vaccutainer immediately prior to sampling. Blood was held on ice prior to separation of the plasma by centrifugation; plasma was immediately frozen in liquid nitrogen. Circulating levels of testosterone (T) (both sexes), 17β-estradiol (E2; females) and 11-ketotestosterone (11-KT; males) from the plasma samples were quantified by radioimmunoassay (RIA) procedures (McMaster et al., 1992). Each fish was rendered unconscious by concussion, and was measured for fork length (± 0.1cm), and body weight (± 0.01g). The internal organs were removed and the gonads (± 0.01g) and liver (± 0.01g) were weighed. Male fish were rated with respect to the number and distribution of nuptial tubercle expression according to a subjective scale which ranged from 0 (no tubercles) to 6 (tubercles over entire body) (McMaster et al., 1991). Both sexes of fish were also scored for visceral lipid stores using a subjective scale ranging from 1 to 5 also adapted from McMaster et al. (1991), (with 1 representing very little visceral lipid and 5 representing large amounts).

A portion of liver tissue was placed in cryovials and frozen in liquid nitrogen for transportation back to Burlington for ethoxy-resorufin-o-dethylase (EROD) analysis (Parrott et al., 1999). During the sampling, a sub-sample of ovarian tissue was taken from 12 female white sucker, and placed in separate vials with incubation media for subsequent determination of *in vitro* production of steroid hormones (McMaster et al., 1995). A sub-sample of ovarian tissue was weighed and the number of follicles were manually counted and then multiplied by the gonadal weight to estimate total fecundity (total # of eggs/fish). An additional sample of both male and

female gonadal tissue was also fixed in 10% buffered formalin for histological evaluation. Aging structures (opercula) were obtained from all fish for age analysis that was conducted at the National Water Research Institute in Burlington, ON.

Statistical Analysis

Male and female fish were analyzed separately. Examination of the potential site differences in fish length and body weight were evaluated using analysis of variance (ANOVA). Condition factor (length vs. body weight; K), gonadosomatic index (GSI) (ratio of gonad weight to body weight), liversomatic index (LSI) (ratio of liver weight to body weight) growth (age vs length and/or weight) and number of eggs (fecundity vs length, weight and gonad weight) were evaluated using analysis of covariance (ANCOVA). Data were checked for normality and evaluated for homogeneity using the Levine's test prior to analysis; and logarithmic transformations were used if data did not meet these assumptions. Nonparametric Kruskal-Wallis tests were used to compare circulating steroid, *in vitro* steroid production, fecundity and age data between sites. All data analyses were conducted using SYSTAT 9.0 statistical software (Wilkinson 1990).

Results and Discussion

Female white sucker collected downstream of the La Tuque dam were longer and heavier than the upstream reference females ($p<0.029$). The downstream females also had an increased condition relative to the upstream reference females ($p=0.041$) and were older ($p<0.001$) (Table 1.1). Examination of fish growth was evaluated relative to both fish weight and fish length. Female white sucker collected at both sites downstream of the discharge demonstrated reduced growth compared to upstream reference females in terms of both length ($p<0.004$) (Figure 1.1) and weight ($p<0.048$). Female white sucker gonadal development when expressed relative to body weight or fish length demonstrated significant site differences in the slopes of the regressions ($p=0.037$, 0.045, respectively). When the data were examined graphically, far field females appear to invest more energy into reproductive development especially in larger (heavier and longer) fish (Figure 1.2). Although absolute fecundity numbers are higher in far field females, relative to length, weight and gonad size, no site differences exist ($p>0.05$). Female white sucker collected from both exposed sites (near field and far field) had significantly larger livers relative to the upstream reference females ($p<0.001$) (Figure 1.3). Internal fat stores are significantly reduced in exposed females from both exposed sites ($p<0.027$).

Male white sucker collected from the near field exposed site were lighter ($p<0.005$) and shorter ($p<0.05$) than the reference and far field exposed males but were similar in age ($p>0.057$). There were no differences in condition of the across the sites ($p>0.107$) but near field males grew slower than reference males (age vs length $p=0.006$) (Figure 1.4). There were no differences in testicular development relative to the length of the fish across the three sites ($p=0.990$). Relative to fish weight, there were also no site differences in investment to testis size ($p=0.907$). Liver weiths in males exhibited significant site differences in the relationship between liver weight and body weight ($p<0.016$). Near field exposed fish demonstrate similar slopes but have larger livers relative to the reference males. Far field males have significantly different slopes with large fish having significantly larger livers (Figure 1.5). There were no site differences in storage

of fat for energy for males (p>0.78). Male secondary sexual characteristics also did not differ with exposure.

Table 1.1. White sucker collected from an upstream reference and two sites downstream of the pulp mill on the St. Maurice River around La Tuque, Quebec.

Sex	Parameter	Reference (Beaumont)	Downstream Mill (near field)	Downstream Dam (far field)
Female	Length (cm)	44.1 ± 0.6	42.2 ± 0.9	46.3 ± 0.6*
	Weight (g)	1206.6 ± 45.5	1138.6 ± 62.9	1462.0 ± 52.3*
	K	1.40 ± 0.02	1.51 ± 0.04	1.46 ± 0.02*
	Gonad weight (g)	45.72 ± 1.94	46.59 ± 2.86	62.16 ± 3.26
	Liver weight (g)	11.40 ± 0.54	15.18 ± 0.98*	20.05 ± 1.17*
	Fecundity	33657 ± 1444	31971 ± 2351	39267 ± 1641
	Fat index	2.6 ± 0.3	1.1 ± 0.2*	1.6 ± 0.2*
	Age (yrs)	7.3 ± 0.4	9.1 ± 1.1	11.9 ± 0.8*
	N	20	11	22
Male	Length (cm)	42.0 ± 0.6	39.4 ± 0.6*	42.4 ± 0.49
	Weight (g)	1065.9 ± 42.0	929.5 ± 38.5*	1065.9 ± 42.0
	K	1.42 ± 0.02	1.51 ± 0.03	1.48 ± 0.03
	Gonad weight (g)	65.80 ± 3.62	59.94 ± 4.02	69.33 ± 2.69^
	Liver weight (g)	7.96 ± 0.39	8.88 ± 0.62*	11.70 ± 0.85^
	Tubercle index	2.2 ± 0.2	1.9 ± 0.2	2.1 ± 0.2
	Fat index	1.5 ± 0.3	1.3 ± 0.2	1.3 ± 0.3
	Age (yrs)	8.1 ± 0.7	8.6 ± 0.7	10.9 ± 1.2
	N	20	16	15

* Significantly different than reference fish (p<0.05)
^ Significant interaction in the relationship between the ANCOVA variables between sites.

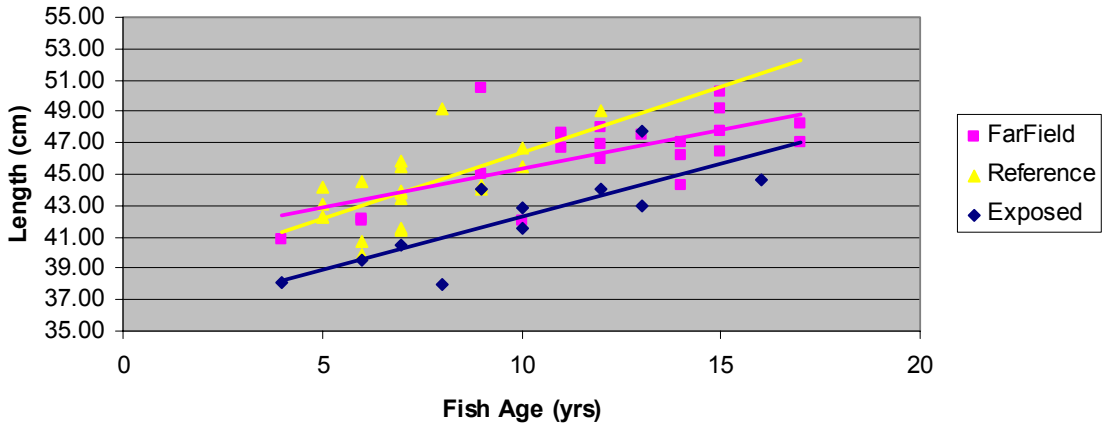

Figure 1.1. Female white sucker growth (fish length vs fish age) demonstrating reduced growth in females collected downstream of the effluent discharge relative to upstream reference females.

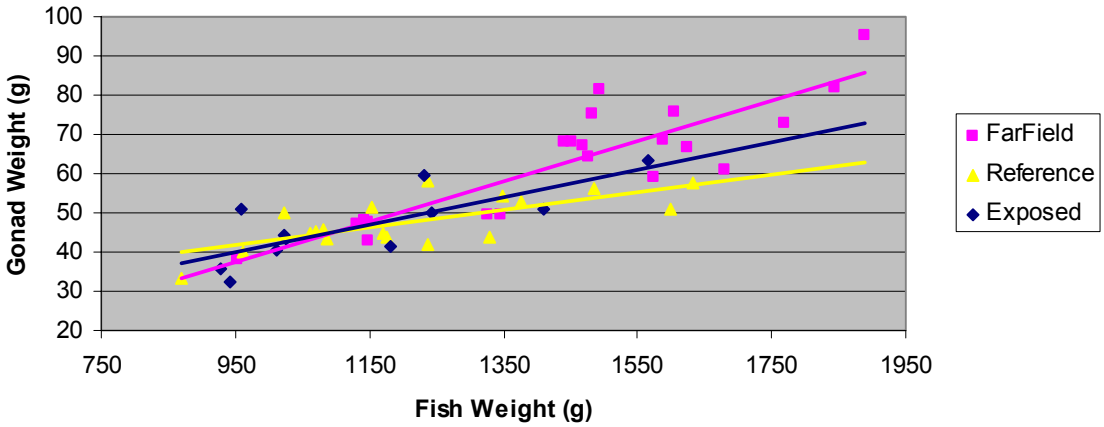

Figure 1.2. Female white sucker investment in reproductive growth (gonad weight vs body weight) indicating that far field exposed females invest more energy in reproductive tissue when they are larger.

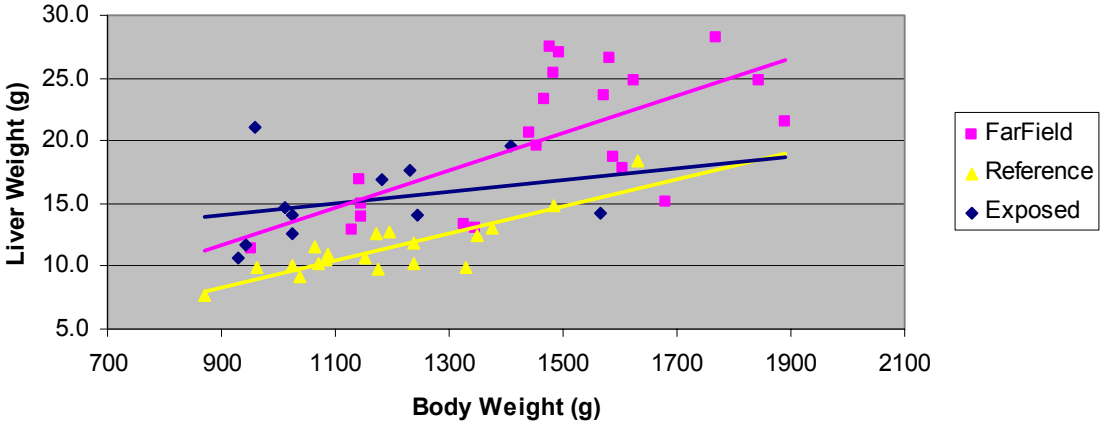

Figure 1.3. Female white sucker liver weight vs body weight indicating that exposed fish from both sites have larger livers.

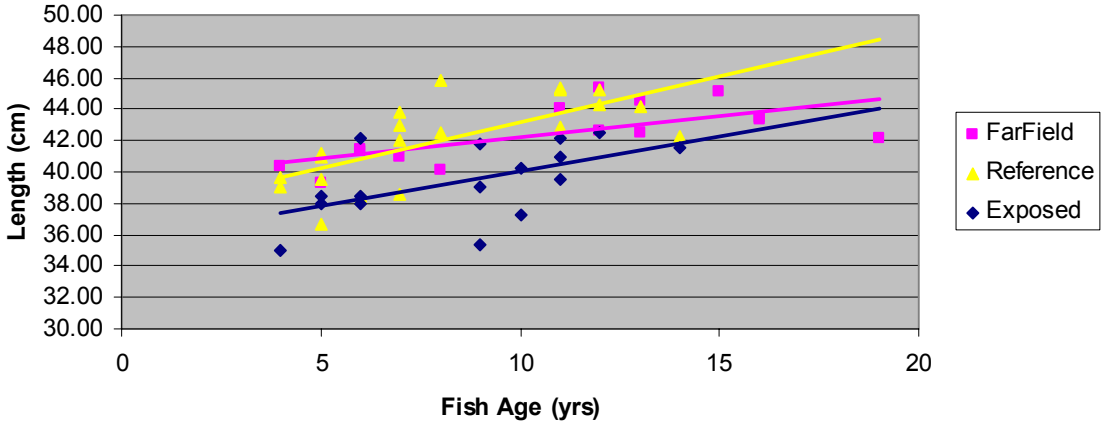

Figure 1.4. Male white sucker growth (fish length vs fish age) demonstrating reduced growth in male white sucker at the near field exposed site.

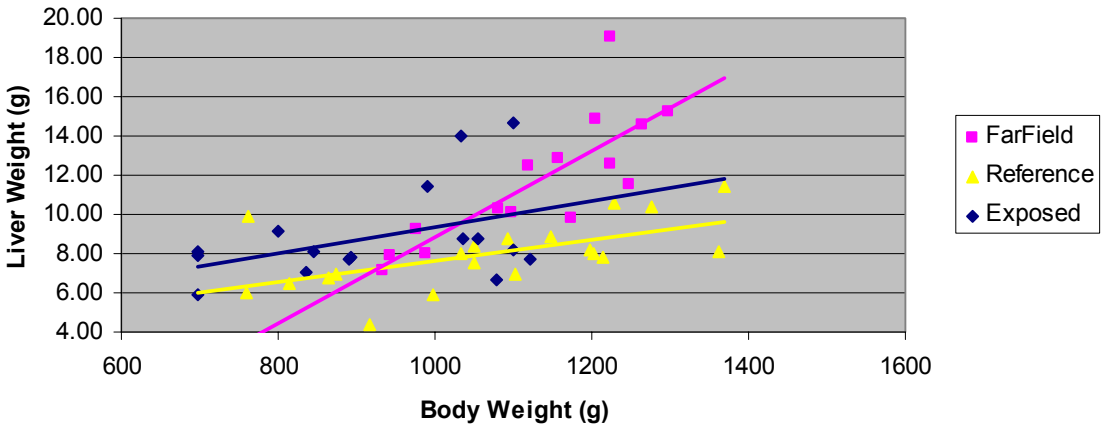

Figure 1.5. Male white sucker liver weight vs body weight demonstrating increased liver weight in near field exposed fish and a different relationship between liver weight gain and body weight gain in far field exposed males.

Circulating and *In Vitro* Production of Steroid Hormones

Circulating levels of the two major biologically active reproductive steroids were measured in both male and female white sucker. Circulating levels of both E2 and T were similar among females collected from the three sites ($p>0.05$) (Table 1.2). Although circulating levels of 11-KT were also similar between the three sites, male white sucker collected downstream of the effluent discharge demonstrated reduced circulating levels of T ($p<0.05$).

Follicles from female white sucker were incubated *in vitro* under either basal (nutrient media alone) or stimulated conditions (human chorionic gonadotropin (hCG)). Follicles from all sites responded in a positive fashion to hCG stimulation in terms of both E2 and T production ($p>0.05$) (Table 1.3). Basal production of E2 was reduced in follicles collected from females downstream of the dam at the far field site and stimulated production of E2 was increased in near field exposed females ($p<0.05$). No other site differences in steroid production were found.

Table 1.2. Circulating levels of testosterone (both sexes), 17β-estradiol (females) and 11-ketotestosterone (males) in plasma collected from white sucker from three sites on the St. Maurice River around La Tuque, Quebec.

Sex	Steroid (pg/ml)	Reference (Beaumont)	Downstream Mill (near-field)	Downstream Dam (far-field)
Female	17β-estradiol	558.4 ± 96.1	589.2 ± 121.4	621.1 ± 149.9
	Testosterone	273.0 ± 44.9	267.4 ± 61.9	225.8 ± 54.7
Male	11-ketotestosterone	1756.9 ± 382.8	2204.4 ± 531.9	1381.9 ± 222.2
	Testosterone	482.0 ± 78.0	289.1 ± 68.0*	303.7 ± 88.7*

Table 1.3. *In vitro* steroid production by ovarian follicles collected from white sucker from three sites on the St. Maurice River around La Tuque, Quebec. Production was measured under basal incubation conditions or following stimulation with human chorionic gonadotropin (hCG).

Steroid (pg/10 follicles)	Treatment	Reference (Beaumont)	Downstream Mill (near-field)	Downstream Dam (far-field)
17β-estradiol	Basal	55.88 ± 7.76	83.11 ± 13.46	28.4 ± 4.09*
	hCG	90.97 ± 11.86	169.27 ± 21.56*	80.23 ± 12.62
Testosterone	Basal	21.27 ± 3.1	26.48 ± 7.4	14.83 ± 1.59
	hCG	47.89 ± 12.34	79.46 ± 15.86	36.36 ± 5.08

The analysis of circulating VTG levels as well as hepatic mixed function oxygenase activity has not yet been completed.

Conclusions

Although previous research studies (Hodson et al., 1992; Gagnon et al., 1994) and EEM cycles (Cycles 2 and 3) demonstrated reproductive effects of the effluent from the La Tuque mill, no negative effects on gonad size were found in this study. This corresponds well with the recently submitted Cycle 4 studies conducted in the fall of 2005 where no reductions in gonadal development were found (Alliance Environment, 2007). Of the EEM endpoints that were measured, white sucker downstream of the effluent discharge grow slower and have increased liver sizes relative to the upstream reference fish. There were some indications of reproductive alterations as male fish downstream of the discharge had reduced circulating levels of T, but this did not translate to alterations in the expression of male secondary sexual characteristics. *In vitro* production of steroids by female ovarian tissue failed to demonstrate any consistent trends which was similar to what was found for circulating steroid levels.

2. Life-cycle Test with Fathead Minnow

The life cycle test is the second anchor of the study. In this test, fish are exposed from the egg stage, through hatching, and juvenile stages until sexual maturity and the reproductive capacity of the fish is monitored for approximately three months. Because of this, it is considered to have the best potential in replicating the condition of wild fish. Endpoints include growth, egg production, the development of secondary sexual characteristics, time to maturity, spawning events as well as biochemical indicators. The duration of the test is approximately six months.

The test was started during July 20, 2006 and day 1 post hatch was July 25 2006. The test ended January 29-Feb 1, 2007.

Methods

Effluent exposures of fish

Effluent (2,000 L) was shipped weekly from La Tuque to Burlington, ON. Shipping took 1 to 3 days. Effluent was stored at 4°C until ready to use. Effluent flowed through a diluter which mixed it with Burlington laboratory water. Exposure concentrations were 0, 1, 3, 10, 30 and 100% effluent. There were 4 replicates of each effluent concentration and 8 replicates of control (lab water) tanks. Flows were 30 mL/min to each aquarium, which provided about 4 solution turnovers per 24 h (Table 2.1).

Egg hatching and fish growth

Methods for egg hatching and care of growing fish are detailed in Parrott and Blunt (2005). Fertilized fathead minnow eggs were placed in 200 ml mesh-bottomed hatching cups within 12 L aquaria. Hatching was monitored and fish were fed 2 times per day newly-hatched brine shrimp (HBS) and more as they grew (Table 2.1). On day 7, post-hatch larvae were transferred to aquaria. Measures of fish weight were taken on 32, 46, 54, 77, 90, 102, 133 days post hatch (dph). Fish were culled to 20 on 32 dph, then to 15 on 46 dph, then to 12 on 54 dph.

Fish maturation and breeding

Secondary sex characteristics started to develop and about 30% of fish could be distinguished externally as male or female at 77 dph. Three breeding tiles were added to each aquarium to promote maturation and reproductive behaviours. Secondary sex characteristics of 55% of the fish were evident at 90 dph. On day 102 fish were culled to 8, selecting 5 females and 3 males per aquaria for the breeding phase of the experiment. Eggs were counted, assessed for fertilization, rolled off tiles and removed to hatching cups in aerated clean water. Hatching success was monitored as well as deformities and abnormalities in larvae. Egg diameter was measured in several batches of eggs from each treatment.

Table 2.1. Test conditions for lifecycle test with fathead minnow.

Date(s) effluent sampled	Weekly, July 12, 2006 to January 29, 2007
Pre-exposure phase	None
Effluent exposure, d	Lifecycle 189 to 192 days post hatch, 194-197 d total
Tank Turnovers (#/d)	4 volumes / day
Quantity of effluent used	28,000 L (29 weeks)
Replicates	8 for control, 4 for each effluent concentration
Loading density, g/L/day	0.3 g/L/day
Feeding	fed twice daily newly-hatched brine shrimp, with added frozen (and thawed) brine shrimp slurry as they grew older
Endpoints measured	• Hatching success, larval survival, growth over time • length, weight, condition factor of adults • liver-somatic index, gonadosomatic index of adults • secondary sexual characteristics • egg production, number of spawns, • egg fertilization, egg hatching, • in males: testosterone production by testes • in females: testosterone and estradiol production by ovaries
pH	7.82 ± 0.012 (n = 420)
Dissolved oxygen, mg/L	7.58 ± 0.024 (n = 420)
Temperature, °C	24.2 ± 0.023 (n = 420)

Fish sampling and grading of sex characteristics

At 189-192 dph fish were sampled as described in Parrott and Blunt (2005) with the following exceptions. Blood samples were taken by caudal peduncle severance after anesthesia in clove oil.

Male fish secondary sex characteristics were assessed as follows: Dorsal fin dot, was graded as absent (0 points) or present (1 point). Dorsal fatpad, graded on scale of 0 (no pad) to 5 (very well-developed pad). Nuptial tubercles were counted under a dissecting microscope, and the number of large tubercles was noted. Banding was assessed on a scale of 0 (no banding) to 5 (very dark pronounced banding). Fish with a black head and no bands received a score of 2. Male Index was calculated as sum of fin dot score + dorsal fatpad score + (tubercles and large tubercles/5). Male Total Index was the Male Index plus the score for banding. This separation of banding was done as this characteristic may change (banding may fade) when fish were sampled.

For female fish, ovipositor length and width were measured under a dissecting microscope, and triangular ovipositor area was calculated as length x width / 2. All fish were sexed externally except one immature fish.

Statistical analyses

Data were analyzed using Systat 10.2. Growth parameters of length (mm), weight (g), condition factor (CF), LSI, GSI, ovipositor length (mm), ovipositor width (mm), ovipositor area (mm^2), band index, male index and male total index, were assessed for differences among treatments using ANOVA. Significant differences from controls were assessed using paired t tests (separate variances) to determine levels of significance. P values were depicted in figures and tables as asterisks: * $p < 0.05$, ** $p < 0.009$, ***$p < 0.001$.

Results and Discussion

Egg hatching and fry survival

There were no differences in hatching of eggs with exposure to pulp mill effluent. Hatching success was over 90% and survival of fish up to 32 dph ranged from 68% in 30% effluent, to 85% in 100% effluent (data not shown).

Fish growth

During the experiment fish were occasionally weighed as groups to assess progression of growth over time (Figure 2.1). Juvenile fish growth was similar in all effluent treatments up to 77 dph. Fish growth at 90 dph showed slight decreased weights of fish from 30% and 100% effluent. This trend continued and was statistically significant at 102 dph. At 133 dph fish could be sexed and males and females could be weighed separately. Weight of males was significantly reduced with exposure to 30% and 100% effluent at 133 dph.

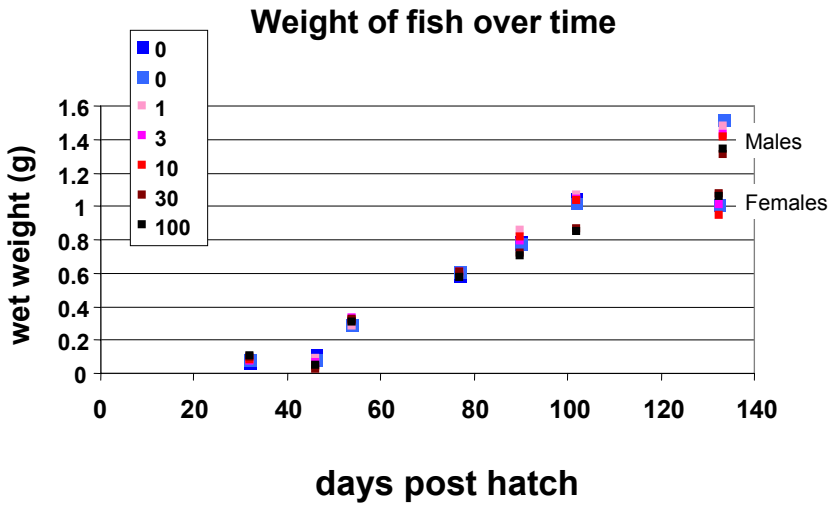

Figure 2.1. Weight of fathead minnows exposed to various concentrations of pulp mill effluent.

At the end of the experiment when individual fish weights and lengths were determined, fish growth was reduced by exposure to effluent. Males exposed to as little as 1% effluent were significantly shorter in length than control males (Figure 2.2). Male weight was decreased at effluent concentrations of 30 and 100% (Table 2.2). Females also showed this trend of decreased length, with statistically significant decreases seen at effluent concentrations of 10% and above (Figure 2.2). Condition factor (CF) was elevated in male fish exposed to effluent concentrations of 3 to 100% and in female fish exposed to 30 and 100% effluent (Table 2.2).

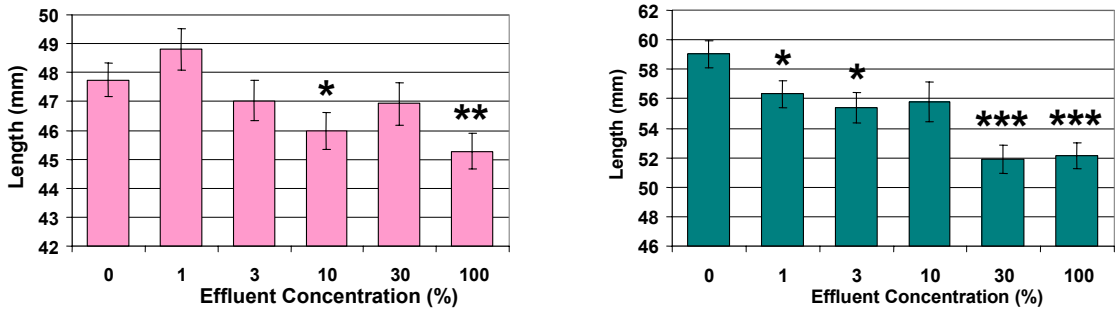

Figure 2.2 Mean length (± standard error) of female (left panel) and male (right panel) fathead minnows exposed to pulp mill effluent (asterisks defined below Table 2.2)

Organ weights

Livers were enlarged in fish exposed to high concentrations of pulp mill effluent. LSI in male and female fish was elevated by exposure to La Tuque effluent at concentrations of 30% and above (Table 2.2). Gonad size was unaffected by exposure to pulp mill effluent, except for females exposed to 100% effluent that had significantly increased GSI.

Secondary sex characteristics

There were no occurrences of male sex characteristics in female fish, or female sex characteristics in male fish (as was seen previously in pulp mill effluent exposures, Parrott et al., 2004).

High effluent concentrations reduced male secondary sex characteristics, while exposure to low effluent concentrations increased male sex characteristics (Table 2.2). Male total index was significantly reduced in male fish exposed to 100% effluent. This was due to a significant decrease in the band index of males exposed to 100% effluent. Male Index (a sum of fin dot, dorsal pad and tubercle score) was increased in males exposed to 1% to 10% effluent, and male total index was increased in males exposed to 1 and 10% effluent.

Egg production

There were no effluent-related differences in time to first spawning. Breeding began at 89 dph in one aquarium from each of 0, 3% and 30% effluent exposure concentrations. Other exposure concentrations started breeding at 90 dph (1%) or 93 dph (10% and 100%).

Total egg production was significantly increased in fish exposed to 1% to 30% La Tuque effluent (Figure 2.3). There was an indication of decreased egg production in two of four replicates of fish exposed to 100% effluent, but when all replicates were pooled, variability in egg production among replicates in the 100% effluent exposure was high, and so significant differences from control egg production were not detected.

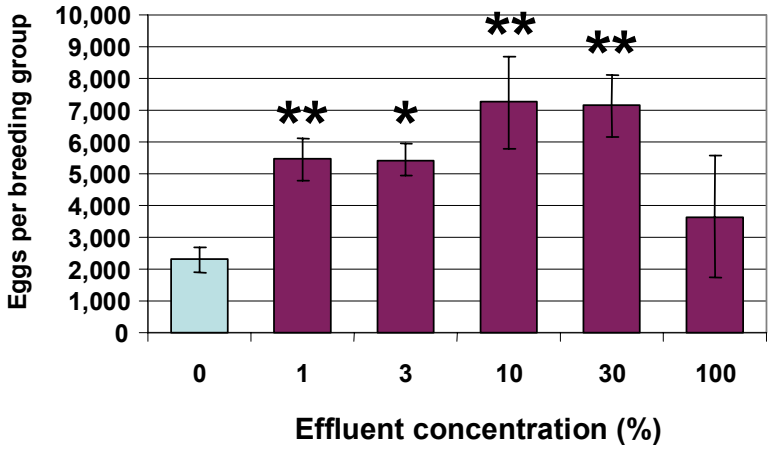

Figure 2.3 Mean egg production (± standard error) per breeding group (3 males and 5 females per aquarium) of fathead minnows exposed to pulp mill effluent (asterisks defined below table 2.2).

Conclusions

- Low concentrations (1 and 3%) of La Tuque effluent decreased length of male fish, but increased condition factor and male secondary sex characteristics. No changes were seen in female fathead minnows exposed to low effluent concentrations.
- Most effluent concentrations (1% to 30%) significantly increased egg production of fathead minnows.
- Exposure to high effluent concentrations (30 to 100%) caused decreased growth and increased condition, and increased liver-somatic indices in both males and females.
- The fathead minnow lifecycle assay was able to mirror the effects seen in wild fish captured downstream of La Tuque effluent outfall: smaller fish with enlarged livers

Table 2.2. Mean values and standard error for growth parameters and secondary sex characteristics of mature fathead minnow females and males. Growth parameters were length (mm), weight (g), condition factor (CF), liver-somatic index (LSI), gonadosomatic index (GSI), ovipositor length (mm), ovipositor width (mm) and ovipositor area (mm^2), band index, male index and male total index.

FEMALES

Concn (%)	n	Length (mm)	se	Weight (g)	se	CF	se	LSI	se	GSI	se
0	34	47.8	0.58	1.25	0.04	1.14	0.022	2.90	0.13	15.5	0.732
1	18	48.8	0.71	1.34	0.06	1.14	0.022	3.21	0.333	15.7	0.987
3	14-15	47.0	0.71	1.17	0.05	1.12	0.033	3.47	0.322	15.4	0.960
10	17	**46.0***	0.63	1.15	0.06	1.17	0.037	3.37	0.249	14.5	1.09
30	19	46.9	0.74	1.31	0.07	**1.26***	0.038	**4.29*****	0.194	17.3	0.907
100	18	**45.3****	0.62	1.30	0.06	**1.38*****	0.029	**3.69***	0.288	**19.9****	1.25

	n	Ovi Length (mm)	se	Ovi Width (mm)	se	Ovi Area (mm2)	se
0	30-35	2.38	0.092	1.16	0.06	1.42	0.10
1	18-19	2.26	0.076	1.19	0.08	1.37	0.12
3	15	2.25	0.091	1.16	0.07	1.30	0.078
10	16-17	2.18	0.10	1.16	0.06	1.28	0.10
30	14-19	**1.97***	0.12	1.04	0.08	1.10	0.15
100	17-18	**2.13***	0.082	1.19	0.05	1.29	0.085

MALES

Concn (%)	n	Length (mm)	se	Weight (g)	se	CF	se	LSI	se	GSI	se
0	23-25	59.0	0.91	2.72	0.15	1.30	0.029	2.59	0.103	1.30	0.086
1	11-12	**56.3***	0.90	2.61	0.12	1.43	0.057	3.07	0.187	1.21	0.112
3	11	**55.4***	1.0	2.59	0.14	**1.52****	0.045	2.88	0.239	1.41	0.124
10	12	55.8	1.3	2.60	0.21	**1.47****	0.042	2.94	0.206	1.07	0.099
30	11-12	**51.9*****	0.96	**2.05*****	0.14	**1.46***	0.054	**3.98*****	0.204	1.56	0.147
100	11	**52.1*****	0.88	**2.01*****	0.11	**1.41***	0.031	**4.06****	0.44	1.66	0.166

	n	Band Index	se	Male Index	se	Male Total Index	se	Ovi Area (mm2)	se
0	21-25	4.29	0.23	4.15	0.33	8.51	0.44	0.153	0.021
1	10-12	4.20	0.29	**6.20****	0.52	**10.5***	0.77	0.197	0.034
3	12	4.08	0.31	**5.74***	0.50	9.83	0.66	0.153	0.027
10	11-12	4.18	0.33	**5.78***	0.65	**10.2***	0.66	0.179	0.023
30	11-12	3.46	0.37	4.32	0.42	7.92	0.50	0.204	0.028
100	10-11	**1.90*****	0.46	3.34	0.34	**5.18*****	0.63	0.222	0.042

* $p < 0.05$, ** $p < 0.009$, *** $p < 0.001$

3. Short- and Medium-Term Tests with Fathead Minnow and Rainbow Trout

The objective of this work was to assess the applicability of short- (<12 d) and medium-term (<30 d) tests for IOC and IOS work aimed at eliminating/reducing mill effluent-related effects on fish reproduction. Two freshwater species, the fathead minnow (*Pimephalas promelas*) and the rainbow trout (*Oncorhynchus mykiss*), were used for this portion of the project.

Methods

Adult fathead minnow reproduction tests

The adult (12 month old) fathead minnow was used in both short- (September 2006) and medium-term (May-June 2006) tests. The fish, separated by sex, were raised in laboratory well water (also used for making effluent dilutions) as described in Martel et al. (2004).

Both the short- (six days pre-exposure and five days effluent exposure) and medium-term (seven days pre-exposure and 21 days effluent exposure) tests are adaptations of a test developed by Ankley et al. (2001) and have been used at Paprican for previous work with mill effluents (Martel et al., 2004; Kovacs et al., 2005). For the pre-exposure phase, the fish were distributed in groups of two males and four females in each aquarium that contained two spawning substrates. The effluent-exposure phase (0%, 1%, 10%, 30% and 100% effluent) of the tests was initiated by selecting groups of fish that demonstrated good reproductive performance (≥18 eggs/female/day, ≥3 spawning events over 7 days) during the pre-exposure phase and these groups were randomly assigned to one of the four replicates for each of the treatments. The detailed test conditions are given in Table 3.1.

The fish were monitored for the number of spawns, egg production and egg fertilization. Hatching success of the eggs was only monitored in the medium-term test. At the end of the tests, each fish was weighed and measured for length (condition factors or weight/length^3 were calculated) and examined for secondary sexual characteristics using criteria described earlier (Martel et al., 2004). At the completion of the medium-term test, the fish were sacrificed and the gonads were excised and weighed to calculate gonad somatic indices (gonad weight/body weight x 100). The rest of the fish (including head, tail and internal viscera except for gonads) were homogenized and assayed for T (males and females), E2 (females) and vitellogenin activity (males) as described previously (Martel et al., 2004).

Test with immature rainbow trout

Immature rainbow trout were used in a 7 d test to measure levels of whole body vitellogenin (VTG), a protein normally produced only by mature females during oogenesis. The fish were exposed in 15 L containers for seven days with daily effluent solution renewals at concentrations of 0% (control), 10%, 30% and 100% v/v . The conditions for the test are given in Table 3.2.

At the end of the exposure period, the trout were weighed, homogenized in phosgel buffer at 4ºC and centrifuged at 3100 g for 10 minutes. The resulting supernatants were stored at – 85°C until

analysis with the rainbow trout vitellogenin enzyme immunoassay (EIA) kit from Biosense Laboratories (Bergen, Norway). All samples were assayed in duplicate.

Table 3.1. Test conditions for short- and medium-term tests with fathead minnow.

Date(s) effluent sampled	Medium-term test: May 30, June 6 and June 13, 2006 Short-term test: September 5, 2006
Pre-exposure phase	7 d for medium-term test and 6 d for short-term test
Effluent exposure, d	21 d for medium-term test and 5 d for short-term test
Tank Turnovers (#/d)	4
Quantity of effluent used	Medium-term test: 5922 L Short-term test: 1340 L
Replicates	4 (2 males and 4 females in 12.5 L)
Loading density, g/L	0.33 to 0.40
Feeding	*Ad libitum*; Freshly hatched *Artemia* (brine shrimp) three-times a day
Endpoints measured	Medium-term test • egg production, number of spawns, • egg fertilization, egg hatching, • length, weight, • secondary sexual characteristics • in males: testosterone and vitellogenin • in females: testosterone and estradiol Short-term test: • egg production, number of spawns, egg fertilization • length and weight
pH	7.4 to 8.5
Dissolved oxygen, % saturation	55 to 92
Temperature, °C	24 to 26

Table 3.2. Test conditions for short-term test with immature rainbow trout.

Date effluent sampled	June 13, 2006
Effluent exposure, d	7
Tank turnovers (#/d)	1
Quantity of effluent used	148 L
Replicates	1 (8 fish in 15 L)
Loading density, g/L	0.33 to 0.49
Feeding	None
Endpoint measured	Vitellogenin
pH	7.1 to 8.6
Dissolved oxygen, % saturation	75 to 96
Temperature, °C	12 to 13

Statistical analyses

Statistical analyses were carried out with STATGRAPHICS Centurion XV Professional (StatPoint Inc., Herndon, VA) and TOXSTAT version 3.5 (1996, Lincoln Research Associates, Bisbee AZ), following the Environment Canada (2005) guidance document on statistical methods for toxicity tests. All statistical comparisons were made at the 5% significance level ($p<0.05$). When necessary, the data were log transformed to meet assumptions of normality and homogeneity. When the data met assumptions of normality and homogeneity, the mean eggs produced per female per day, number of spawns, % fertilization and % hatching from fertilized eggs were compared for significant differences by analysis of variance (ANOVA) with the aquarium being the experimental unit of replication. Total body weight and length, gonad weight, and whole-body E2, T, and vitellogenin were compared for significant differences using an ANOVA model with aquariums as a nested factor. For gonad weight a covariate of body weight and for fish weight a covariate of length were added. When the data did not meet assumptions of normality and homogeneity, the non-parametric Kruskal-Wallis test, using the means of the particular endpoints from each replicate, was used to determine if the effluent exposure had a significant effect. In cases when the ANOVA indicated a significant effluent-related effect, the Dunnett's test or the Least Significant Difference (LSD) test was used to identify the specific effluent concentrations that were statistically significantly different from the control. When the Kruskall-Wallis test indicated significant effluent-related effects, the Steel's Many-One Rank test was used to identify the specific effluent concentrations that caused a significant difference from the control.

Results and Discussion

Medium-term adult fathead minnow reproduction test

Egg production and spawning: The egg production in fish exposed to 100% effluent was found to be significantly decreased compared to controls (Table 3.3). Lower effluent concentrations appeared to cause slight, but statistically insignificant, increases in egg production. Visual inspection of the data (Figure 3.1) shows that cumulative egg production during the pre-exposure phase and effluent exposure phase was similar in all treatments except 100% effluent where egg production was much reduced. The effluent did not significantly affect the number of spawns or the percentage of egg fertilization and hatching. This differs from previous tests at Paprican where lower egg production was always associated with reduced spawning (Martel et al., 2004, Kovacs et al., 2005 and Kovacs et al., 2007), suggesting that the La Tuque mill effluent may have affected egg production in a different manner.

Table 3.3. Egg production, number of spawning events, percent fertilized eggs and hatched larvae by fathead minnow in a medium-term test with the effluent from the La Tuque mill. Results are expressed as means (±SE, that is standard error). The numbers in brackets represent the range.

Treatment	Eggs/Female/Day	Number of Spawning Events	Fertilized Eggs, %	Hatched Larvae, %
Control	36 (4) [27-46]	14 (0.5) [13-16]	80 (2.5) [76-87]	73 (6) [65-89]
1% effluent	41 (5.5) [24-49]	16 (1.5) [12-18]	63 (8) [41-76]	57 (5.5) [48-72]
10% effluent	43 (7) [33-62]	15 (0.5) [14-16]	68 (5) [55-78]	54 (11.5) [29-74]
30% effluent	38 (7.5) [19-51]	13 (1) [10-14]	68 (6.5) [51-79]	74 (6.5) [63-92]
100% effluent	17* (4.5) [7-25]	10 (2) [5-14]	64 (18.5) [11-19]	56 (13) [29-91]

* Statistically significant difference by ANOVA and least significant differences (LSD) test ($p < 0.05$).

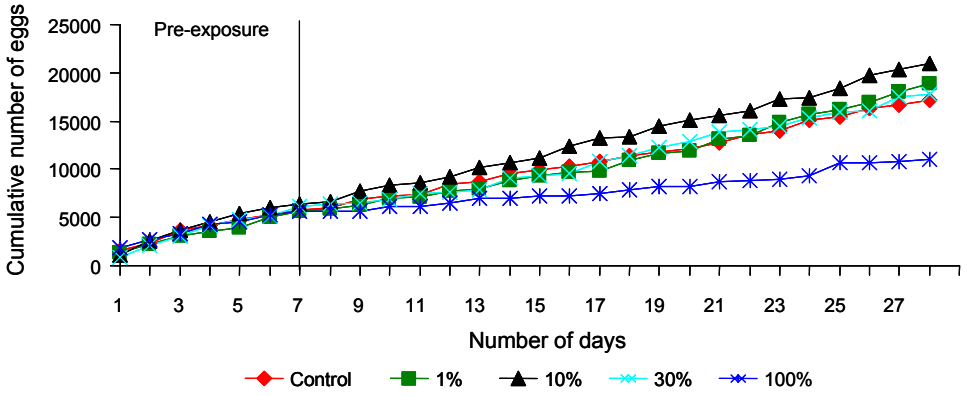

Figure 3.1. Cumulative egg production during the medium-term test with effluent from the La Tuque mill during May and June 2006.

Somatic Indices: The mean GSIs of males and females are shown in Figure 3.2. There was no significant difference between control and effluent-exposed fish. Similarly, none of the other morphometric parameters of males (detailed data not shown) were affected by the effluent. In females, body weight was significantly lower in the 10% treatment only (data not shown) and this was most likely an aberration rather than the consequence of effluent exposure.

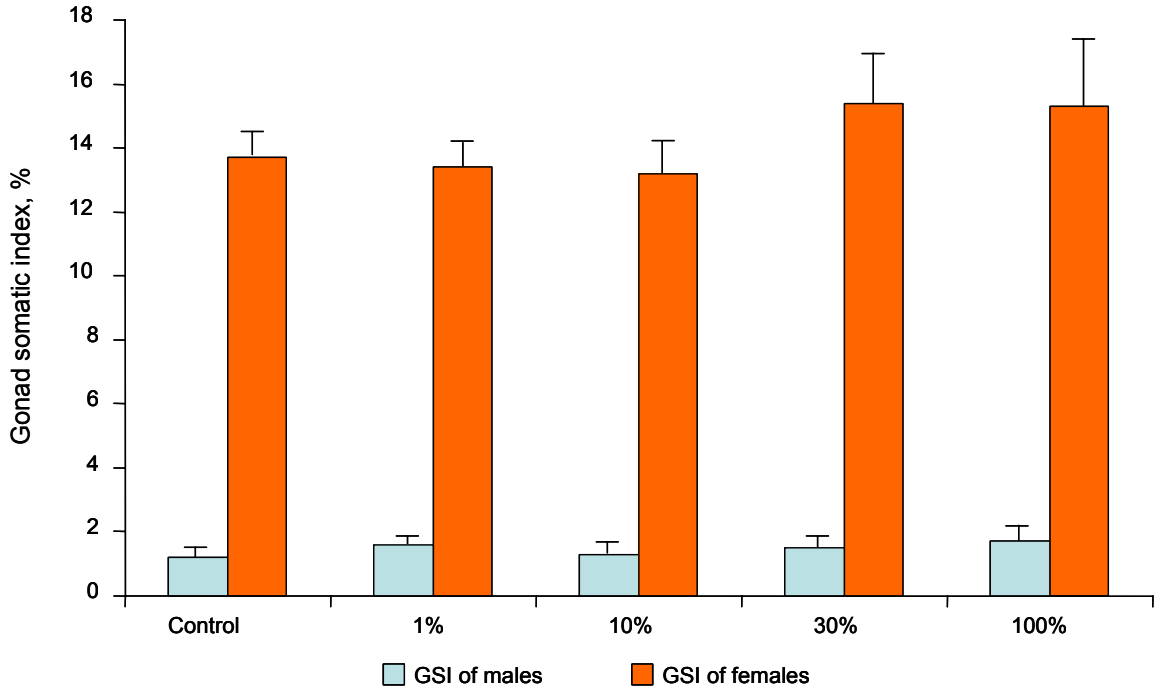

Figure 3.2. Gonad somatic indices (mean and standard error (SE)) of male and female fathead minnow in the medium-term test with effluent from the La Tuque mill.

Secondary sexual characteristics: In males, the number of tubercles, dorsal fin spot and dorsal pad size were unaffected by effluent exposure (data not shown). Ratings for overall colouration of males were found to be significantly reduced by exposure to 100% effluent. On a scale of zero to five, control fish were rated as 2.8 ± 0.16 (mean ± SE) and fish at 100% effluent were rated as 2.3 ± 0.18. The difference was attributed to lighter banding. In females, the ovipositor length was significantly shorter in the 100% treatment group (Control fish length 4.0 ± 0.13 mm; 100% effluent fish 3.3 ± 0.16 mm). The lighter banding in males and the shorter ovipositor in females at the 100% concentration corresponded to the lower egg production observed at the same concentration. There was no evidence of masculinization of females or feminization of males.

Steroid hormones and vitellogenin: In males, T and VTG were not significantly affected by effluent exposure (Figure 3.3). While the VTG in the 100% effluent fish was about 6 times greater than in the fish of the other groups, this difference was caused by only one fish having VTG of 19,000 ng/g. The seven other males had VTG levels of ≤642 ng/g, similar to controls. In females, T and E2 levels were unaffected by exposure to effluent (Figure 3.4).

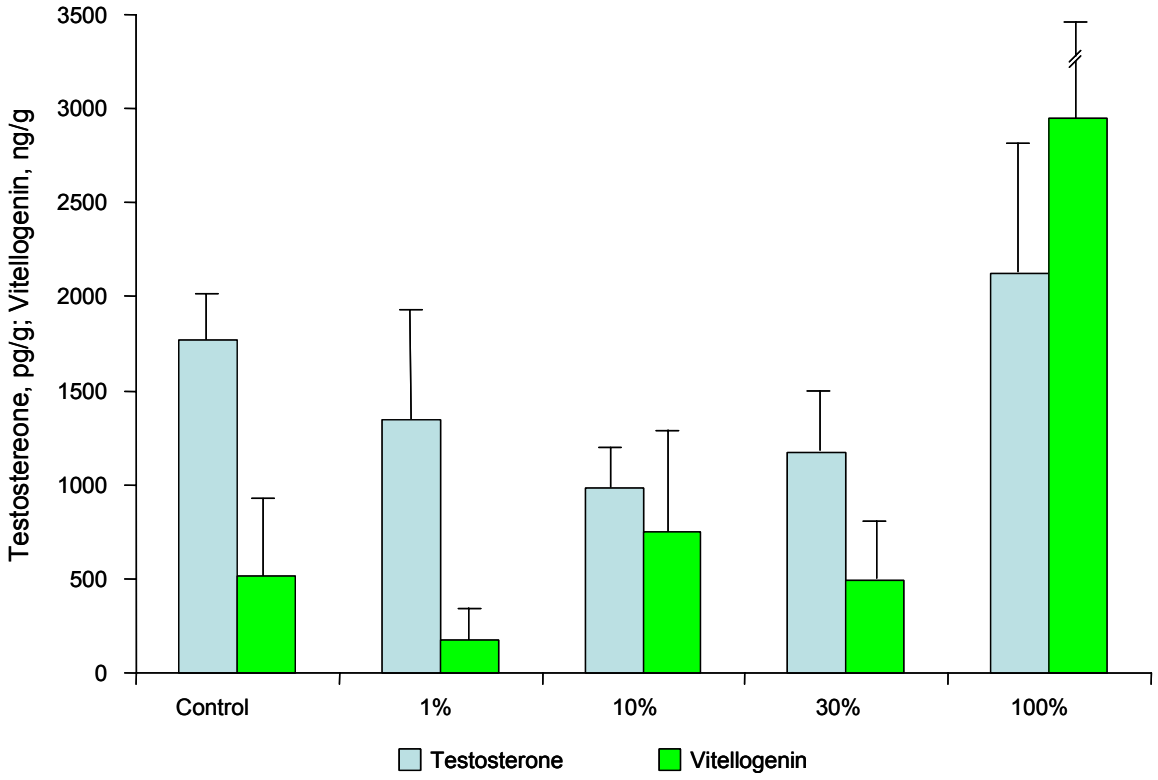

Figure 3.3. Medium-term test: Levels of testosterone and vitellogenin (mean and SE) in whole body homogenates of male fathead minnow exposed to the La Tuque mill effluent.

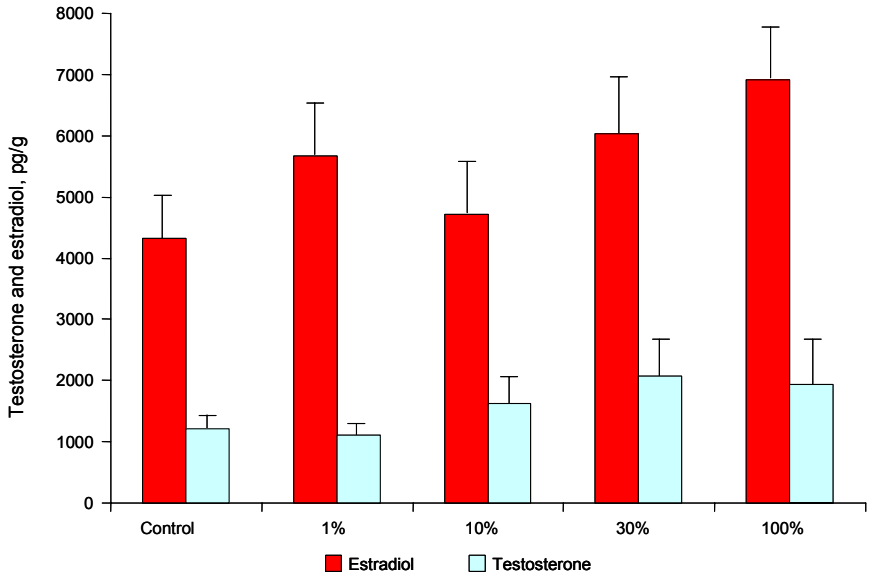

Figure 3.4. Medium-term test: Levels of estradiol and testosterone (mean and SE) in whole body homogenates of female fathead minnow exposed to the La Tuque mill effluent.

Short-term adult fathead minnow reproduction test

Just prior to the start of this project, work at Paprican with effluents from thermomechanical pulp (TMP) mills indicated that the duration of the medium-term test with fathead minnow could be substantially reduced without losing sensitivity, thereby making the test more suitable for IOC/IOS investigations. The results showed that the TMP mill effluent effects on egg production in particular were identical, irrespective of whether the fish were exposed to effluent for 21 d or only for ≤ 7 d (Kovacs et al., 2007). Based on the promising results with TMP effluents, it was considered worthwhile to include the short-term test with fathead minnow test as part of this project.

Except for egg fertilization, none of the endpoints (length/weight and secondary sexual characteristics, data not shown) were affected by the effluent in the short-term test of this project conducted during September 2006 (Table 3.4). The percentage of fertilized eggs was significantly lower in the groups exposed to 10% and 100% effluent, but not in the group exposed to 30% effluent. The inconsistent and concentration independent effect on egg fertilization may have been the consequence of the relatively short duration of the test. In our experience, when sexually naïve fathead minnow start to spawn, the fertilization and even hatching of the eggs produced during the first week of spawning can be quite variable. Because of this, the egg fertilization and egg hatching may not be appropriate endpoints for short-term tests with fathead minnow.

Table 3.4. Egg production, number of spawning events, percent fertilized eggs by fathead minnow in a short-term test with effluent from the La Tuque mill. Results are expressed as means (±SE). The numbers in brackets represent the range.

Treatment	Eggs/Female/Day	Number of Spawning Events	Fertilized Eggs, %
Control	48 (42) [45-53]	4 (0.5) [3-5]	90 (2) [86-94]
1% effluent	60 (13.5) [27-83]	4 (0.5) [2-5]	90 (2.5) [85-96]
10% effluent	73 (13) [50-105]	4 (0.25) [4-5]	58* (12) [23-78]
30% effluent	56 (9) [44-83]	5 (0.3) [4-5]	72 (10) [50-92]
100% effluent	53 (7) [37-70]	4 (0.3) [3-4]	68* (5) [56-78]

* Statistically significant difference by ANOVA and Dunnet's test ($p < 0.05$)

As occurred in the medium-term test, the egg production was increased, though not in a statistically significant manner, by effluent concentrations of 1% to 30% (Table 3.4 and Figure 3.5). However, in contrast to the medium-term test, the effluent in the short-term test did not cause a reduction of egg production at 100% concentration. This difference between the two tests could have occurred i) because the short-term test was less sensitive or ii) because there was a

change in effluent quality from May/June 2006 to September 2006. There is some evidence to suggest that a change in effluent quality was the more likely reason for the observed difference. First, the egg production data for the first five days of the medium-term test done in May/June 2006 was analyzed for effluent-related effect as this timeframe would correspond to the duration of the September 2006 short-term test. A simple t-test comparison of egg production by the control and 100% effluent-exposed fish indicated that 100% effluent decreased egg production in a statistically significant manner after only five days of exposure. This indicates that a shorter effluent exposure period did not make the test less sensitive. Second, chemical profiling of the effluents provides further insight for the possibility that effluent quality may have changed with time (see Section 7).

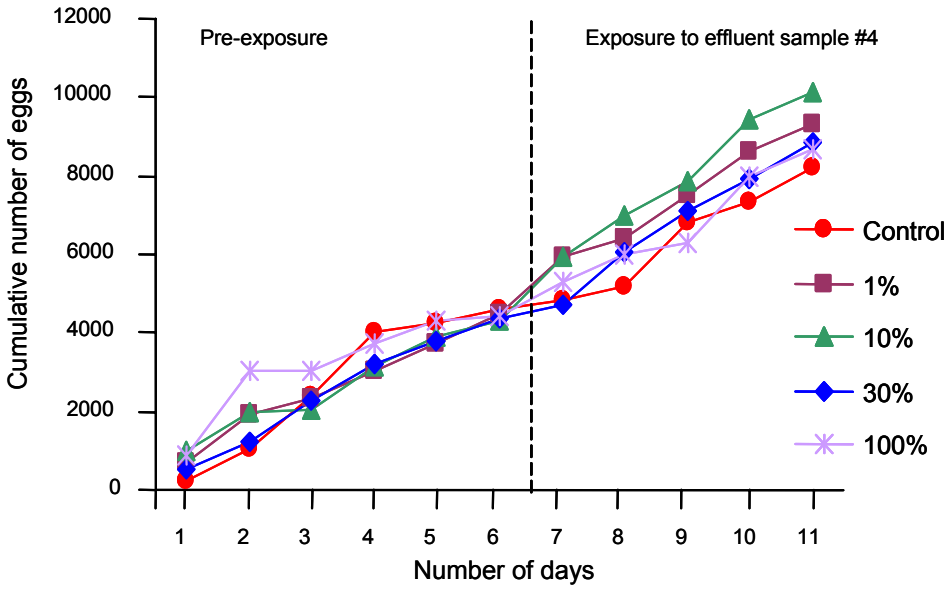

Figure 3.5. Cumulative egg production during the short-term test with effluent from the La Tuque mill during September 2006.

Vitellogenin levels in immature rainbow trout

The effluent had no statistically significant effect on VTG levels in the whole body homogenates of rainbow trout (Figure 3.6).

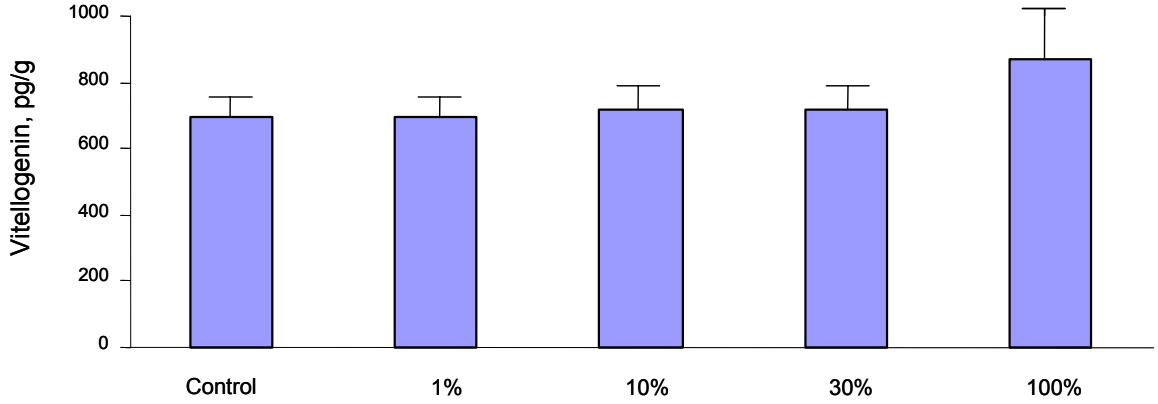

Figure 3.6: VTG (mean and SE) levels measured from whole body homogenates of immature rainbow trout exposed for 7 d to effluent from the La Tuque mill.

Conclusions

- The La Tuque mill effluent had only minimal (i.e., only at 100% concentration) or no effects on reproductive indicators of fathead minnow and rainbow trout evaluated in short- and medium-term tests. This is in general agreement with the findings of the wild fish survey as well as the findings of the life-cycle test with fathead minnow.
- Egg production appeared to be the most useful endpoint in the tests with fathead minnow. This endpoint:
  - Provided consistent concentration-dependent response patterns, i.e., slight increases at 1% to 30% concentrations in both short- and medium-term tests and significant reduction at 100% concentration in the medium-term test.
  - Detected differences in effluent quality, i.e., the effluent containing less extractable organics had no effect on egg production whereas effluent with greater amounts of organics affected egg production at 100% concentration (see Section 7).
- There is an indication that effluent effects on fathead minnow egg production can be just as reliably determined in short-term tests as in medium-term and life-cycle tests. The short-term test would be more applicable for IOC/IOS work.

4. Short- and Medium-term Tests with Mummichog

The purpose of the mummichog tests was to examine the effects of effluent in an estuarine species. Mummichog are endemic to the east coast of Canada and the USA and are an important monitoring species in field, artificial stream and laboratory settings for pulp and paper effluents (Leblanc et al., 1997; Dubé and MacLatchy, 2000; 2001). Many Canadian mills are located on the coast, and it is important to assess the effects of effluent in a saline environment as fish physiological processes may differ in these environments. Both the short-term and medium-term (reproductive) tests have been standardized (MacLatchy et al., 2003; Peters, 2005) by comparison to model endocrine disruptors.

Methods

Two sets of exposures of La Tuque final effluent were carried out on mummichog (*Fundulus heteroclitus*) at the University of New Brunswick, Saint John, NB. In the first set of exposures, adult male and female mummichog were exposed for seven days to determine effects on the reproductive endocrine system. In the second exposure, adult mummichog were exposed to effluent prior to and during an adult reproductive test; effects on offspring were also assessed during effluent exposure until the fall. The reference to the fall is not all that helpful. A duration would be more appropriate. In both experiments, mummichog were acclimated to flow-through test conditions for five days prior to effluent exposure. The concentrations for both tests were 0% (control), 1%, 10%, 30% and 100% effluent. Details of the exposure parameters can be found in Tables 4.1 and 4.2. Effluent was delivered once per week and was aerated and maintained at 4°C in insulated storage containers and brought to ambient temperature daily prior to use.

In the seven-day exposure, sampling occurred on 09 June 2006 as peak reproductive levels in mummichog coincide with the full moon (11 June 2006). Condition factor, GSI and LSI were calculated from body weight and length, gonad weight and liver weight measurements. Plasma T, 11-KT and E2 levels were measured using RIA .T and 11-KT were measured in males, T and E2 in females. Gonads were incubated in Medium 199 for *in vitro* steroid synthesis; livers were immediately frozen at -80^{o}C and kept for analysis of mixed function oxidase levels (analysis not completed). MacLatchy et al. (2003, 2005) include details of protocols for the exposures and endpoint analyses.

In the mummichog reproductive test (Peters, 2005), male recrudescing adults (n=6/aquarium) were exposed in three tanks/treatment and female recrudescing adults (n=6/aquarium) in three separate tanks/treatment for 18 days (until 06 July 2006). The presence of skin flukes and sea lice prompted combination of the healthiest fish into 18 aquaria (n=6 controls; n=3 other treatments) containing three males and three females for the breeding exposure. During the spawning period, a mesh cage (made from aquaculture netting) was placed within each aquarium, 2-3cm above the bottom. Eggs fall to the bottom but adults are prevented from consuming them. Eggs were collected on a daily basis for seven days by dredging the tank with a fine dip-net. Exposure concentrations were maintained throughout the collection week. Collected eggs were placed into Petri dishes at a density of 40 eggs per dish and exposure concentrations replaced manually in each dish once a day until the eggs hatched. On 13 July 2006 adults were euthanized and exposure continued on the eggs.

Table 4.1. Mummichog seven-day adult exposure.	
Exposure Parameter	
Date(s) sampled	09 June 2006
Tank turnovers (#/day)	4
Replicates	6 (0%); 3 (1, 10, 30, 100%)
Loading densities	3.69 $\pm$ 0.014 g/L
Endpoints measured	K, GSI, LSI; plasma T, 11-KT and E2
Range of pH, DO, temperature	pH 7-8; >80%; t=19 $\pm$ 2 C

Table 4.2. Mummichog adult reproductive test.	
Exposure Parameter	
Date(s) sampled	06-13 July 2006; various July-Oct 2006
Tank turnovers (#/day)	4
Replicates	6 (0%); 3 (1, 10, 30, 100%)
	3.12 ± 0.40/3.73 ± 0.014 g/L (F/M isolated) 2.81 ± 0.38 g/L (F & M breeding)
Endpoints measured	Cumulative egg production; % fertilized eggs; time to hatch; size at hatch and 5 weeks; survival
Range of pH, DO, temperature	pH 7-8; >80%; t=19 $\pm$ 2 °C

Eggs were visually inspected daily for development of cleavage and any unfertilized eggs were removed. Test concentrations were maintained as per each treatment throughout development until the termination of the exposure; development through to the juvenile stage was monitored. Upon hatching, larvae were transferred to 50-mL beakers containing the appropriate exposure concentrations at a density of 10 larvae per beaker. Young fish were returned to the original test aquaria (containing 5 L of appropriate treatment) when the yolk sac was fully absorbed and swimming had commenced. Larvae were fed brine shrimp (*Artemia sp.* nauplii) twice daily and fry food once daily.

Offspring size was assessed at hatch and at five weeks by taking average length measurements from 25 fish in each tank. Fish survival was monitored until 17 October 2006. At the termination of the experiment, fish were over-anaesthetized to euthanize them.

Statistical analyses

Data were checked for normality and evaluated for homogeneity using the Levine's test prior to analysis; logarithmic transformations were used if data did not meet these assumptions. Body weights, plasma steroids, time to hatch, size at hatch, size at five weeks, % survival and % fertilization were tested with nested ANOVAs (Kruskal-Wallis if non-parametric) followed by Tukey's or Dunn's tests. ANCOVAs were used to assess condition, gonad size and liver size (as per Section 1, Wild Fish Survey). Cumulative egg production was assessed by a two-factor

ANOVA. Statistical analyses were carried out with SigmaStat 3 and Systat 11 (Systat Software Inc., San Jose, CA).

Results and Discussion

Mummichog seven-day adult exposure

There were no significant differences among the K, LSI or GSI of male and female fish except for significantly smaller livers in males at 100% (Table 4.3). There were also no differences in plasma steroid levels except for a reduction in 11-KT in males at 1% (Table 4.3).

Table 4.3. Condition factor (K), gonadosomatic index (GSI), liversomatic index (LSI) and plasma steroid levels in male and female mummichog exposed to La Tuque effluent for seven days in June 2006. All values are mean ± SE. Values with different letters are significantly different ($p<0.05$). Sample sizes (0% replicates = 6; 1-100% = 3).

Treatment	Weight (g)	K (%)	GSI (%)	LSI (%)	T (ng/L)	11-KT (ng/L)	E2 (ng/L)
Males							
0%	8.83 (0.68)	1.43 (0.065)	1.86 (0.19)	2.05a (0.40)	0.699 (0.29)	1.51a (0.21)	
1%	8.00 (0.63)	1.48 (0.11)	1.94 (0.18)	1.47ab (0.21)	0.223 (0.014)	0.602b (0.087)	
10%	8.21 (1.21)	1.32 (0.15)	2.13 (0.32)	2.55a (0.74)	0.388 (0.11)	0.969ab (0.26)	
30%	8.89 (1.0)	1.35 (0.097)	1.43 (0.22)	1.22ab (0.12)	0.338 (0.014)	0.733ab (0.11)	
100%	9.5 (0.81)	1.22 (0.079)	1.82 (0.25)	0.784b (0.13)	0.359 (0.011)	1.10ab (0.23)	
Females							
0%	9.43 (1.2)	1.17 (0.19)	5.87 (1.4)	2.60 (0.40)	0.710 (0.16)		3.44 (0.55)
1%	10.3 (1.1)	1.52 (0.11)	4.31 (1.0)	2.38 (0.35)	0.743 (0.16)		3.39 (0.51)
10%	11.1 (1.2)	1.28 (0.17)	3.98 (0.39)	1.90 (0.094)	0.520 (0.036)		3.95 (0.77)
30%	10.4 (0.64)	1.38 (0.069)	4.36 (0.52)	1.75 (0.23)	0.460 (0.035)		3.74 (0.38)
100%	9.57 (0.91)	1.40 (0.07)	3.81 (0.45)	1.68 (0.16)	0.484 (0.021)		3.12 (0.45)

Mummichog adult reproductive test

Fecundity (cumulative eggs laid/female) was significantly increased in fish exposed to 30 and 100% La Tuque effluent (Figure 4.1). Percent fertilization ranged from a low of 71.2 ± 13% (0%) to a high of 90.2 ± 0.76% (1%) but there were no significant differences among treatments (data not shown). There was no significant difference in time to hatch (Figure 4.2). At hatch, 1 and 100% effluents had larger larvae (Figure 4.3A); by week 5, fish at 10% were larger than controls but there were no differences among fish exposed to 1-100% effluents (Figure 4.3B). Survival to 17 October 2006 did not vary among treatments and ranged from 100% (1% effluent) to 74.7 ± 10% (100% effluent); survival in 0% effluent was 98.2 ± 2.0%.

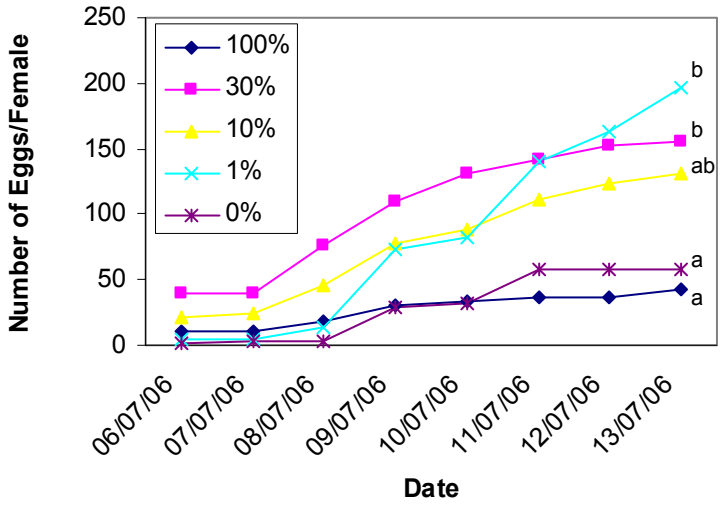

Figure 4.1. Cumulative eggs laid by female mummichog exposed to La Tuque final effluent between 07 June 2006 and 13 June 2006.

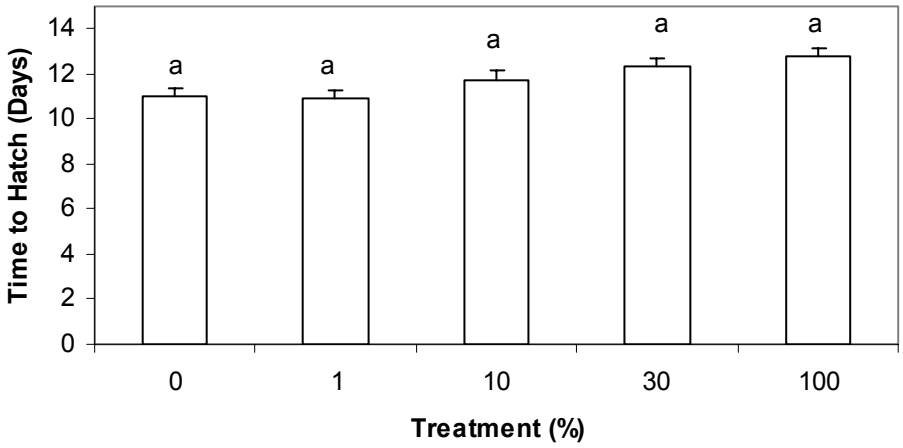

Figure 4.2. Average time to hatch of mummichog larvae from parents and eggs exposed to La Tuque effluent in June 2006.

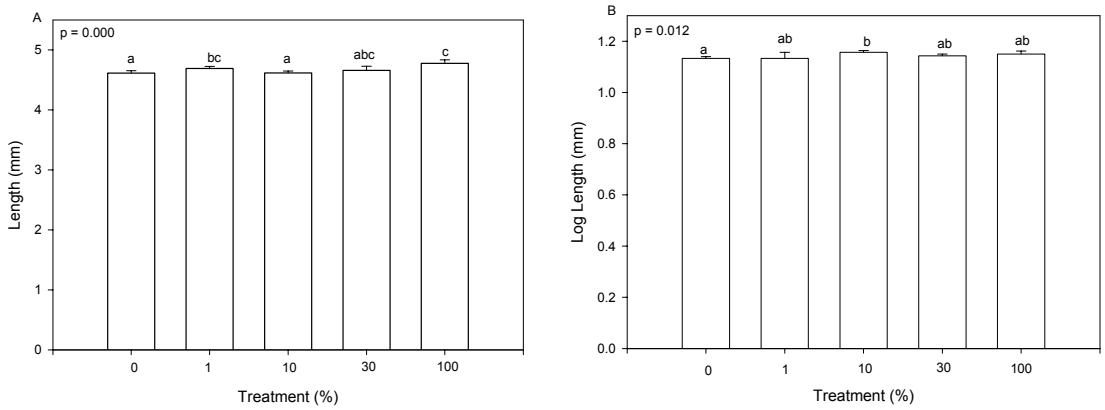

Figure 4.3. Length (A) at hatch and log length (B) at five weeks post-hatch for mummichog offspring exposed to LaTuque effluent.

Conclusions

The predominant effect of La Tuque effluent on mummichog is a general increase in cumulative egg production and size at medium effluent concentrations.

5. Zebrafish Tests

Zebrafish were used to evaluate the effects of pulp mill effluent on egg production and ovarian gene expression. Zebrafish were selected for these studies as they are sexually mature on a year round basis in the laboratory which may overcome the inherent problems of seasonal reproduction in some other test species. As well, tests to evaluate the effects of effluents on egg production may be conducted in relatively short time periods with limited volumes of effluent. Previous studies with zebrafish have shown that exposure to kraft mill chemical recovery condensates reduced egg production and the mRNA expression of selected genes that control steps in the steroid biosynthetic pathway (Ings, 2006).

Methods

Sexually mature adult zebrafish were exposed to different concentrations of effluent for seven days and effects on egg production were monitored (Table 5.1). Adult zebrafish were received from DAP International (Etobicoke, ON). Fish were transferred to the Hagen Aqualab at the University of Guelph where they were held in A-HAB units (Aquatic Habitats, Apopka, FL) at 28°C in an environmental chamber. Fish were maintained in recirculated well water with a 12 hour light and 12 hour dark photoperiod. Fish were fed to satiation two to three times per day with a combination of commercial salmon fry formulation (Martin Mills, Elmira ON) and frozen blood worms (Oregon Desert Brine Shrimp Co., Lakeview OR).

Sexually mature adult zebrafish were sexed and two male and two female zebrafish were housed in individual static breeding containers with 1 litre of Hagen Aqualab well water. These containers consisted of two plastic containers stacked together. The bottom of the top container was replaced with a mesh so that spawned eggs would fall through and be separated from the fish until collection. Each treatment contained six breeding containers which were placed in a random order in large water baths at 28°C. Water was changed daily and fish were held in experimental conditions for 2 days prior to the pre-exposure period which lasted for 7-days. Fish were then exposed for 7 days to 0, 1, 10, 30 and 65% pulp mill effluent diluted in Hagen Aqualab well water. Effluent was replaced on a daily basis.

Eggs were collected by siphoning the breeding containers with a Pasteur pipette and counted. At the end of the exposure, fish were over-dosed with MS-222 (Sigma, St. Louis MO) and weighed. Ovaries from fish in each breeding container were weighed and stored RNALater® (Ambion, Austin TX) at 4°C until separation into primary growth, pre-vitellogenic an vitellogenic follicle stages for RNA extraction. Bodies were snap frozen in liquid nitrogen and stored at -80°C prior to extraction of steroids.

RNA extraction, reverse transcription and Real-Time PCR followed the methods described by Ings and Van Der Kraak (2006). The genes evaluated included steroid acute regulatory protein (StAR), P450-aromatase A (P450-aromA) and 3β-hydroxy steroid dehydrogenase (3β-HSD).

Table 5.1. Test conditions for short-term tests with adult zebrafish.

Date(s)	July 11-26, 2006
Pre-exposure phase	2 d to confirm spawning 7 d collection of eggs for baseline
Effluent exposure, d	7 d for collection of egg for treatment effects
Tank Turnovers (#/d)	None static exposure , daily renewal
Quantity of effluent used	52 liters: 0, 1, 10, 30 and 65% effluent
Replicates	6 (2 males and 2 females in 1 L)
Loading density, g/L	1.2- 2.8 g/l
Feeding	*Ad libitum*; Salmon fry pellets two-times a day; fed blood worms every second day
Endpoints measured	• egg production, number of spawns, • weight, • expression of StAR, P450 arom, 3β-HSD in the three stages of ovarian follicles (primary growth, previtellogenic and vitellogenic follicles) • in males: whole body testosterone and vitellogenin • in females: whole body testosterone and estradiol
pH	7.0 to 8.0
Dissolved oxygen, % saturation	81-99
Temperature, °C	26 to 29

Results and Discussion

Cumulative egg production by zebrafish during the pre-exposure period and following exposure to varying concentrations of La Tuque effluent are shown in Figure 5.1. Interpretation of the effects of effluent on egg production was complicated by the high variation in egg production during the pre-exposure period. In this test, cumulative egg production in the control and the 30% effluent group varied by more than 2-fold. Consequently we determined egg production during the exposure period and showed reductions in egg production relative to the control in the 10 and 65% groups (Figure 5.2). Egg production in the 30% effluent group was similar to controls.

During the pre-exposure period, eggs were spawned in 71% of the tanks over the 7-day collection period (Figure 5.3). The spawning rate was reduced to 53% of the tanks for zebrafish following exposure to pulp mill effluent. The reduction in spawning rate was most pronounced for the fish exposed to 65% effluent when eggs were present on only 33% (14 of 42) of the collection days compared to 81% (34 of 42) of the collection days in the control group.

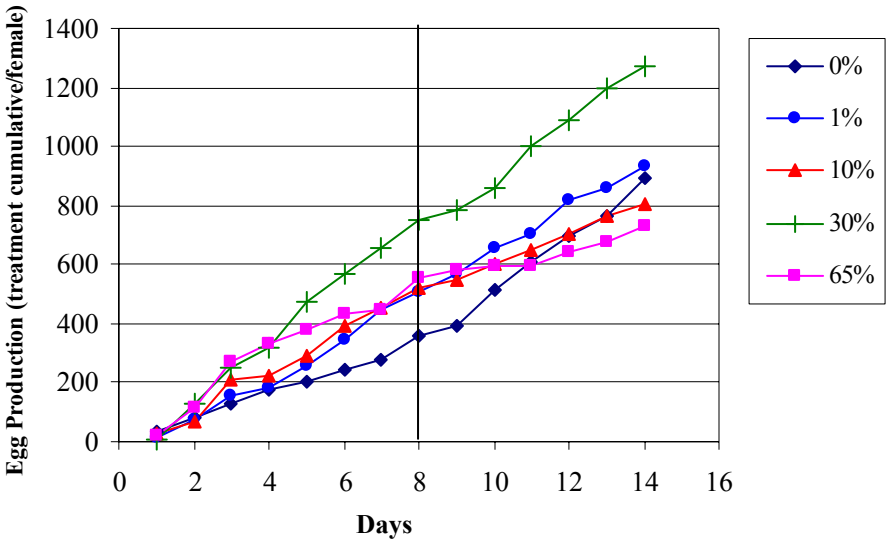

Figure 5.1. Cumulative egg production of female zebrafish during a pre-exposure period and following seven days of exposure to varying concentrations of La Tuque effluent. The vertical line on day 8 indicates the start of the exposure period.

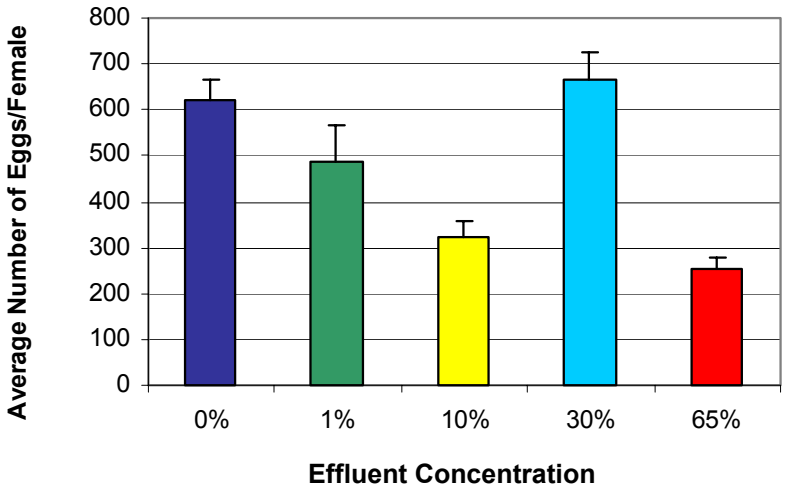

Figure 5.2. Egg production (Mean ± SE; n=6 tanks) of female zebrafish following seven days of exposure to varying concentrations of La Tuque effluent.

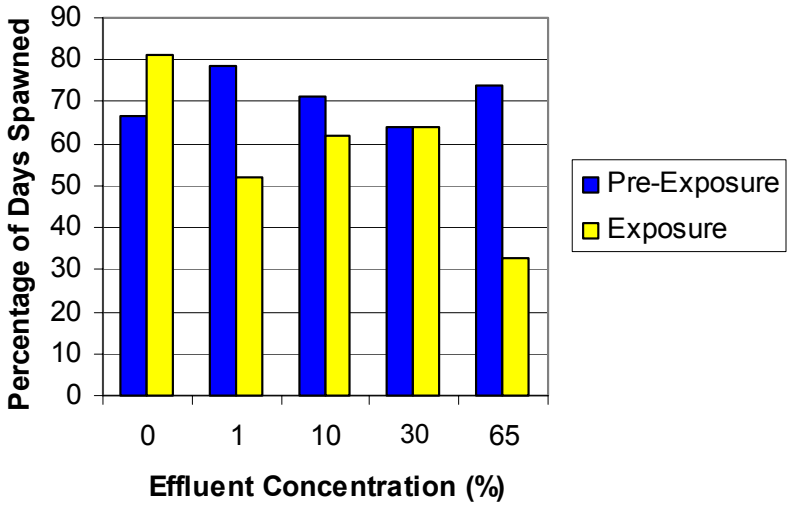

Figure 5.3. Spawning events in adult zebrafish during the pre-exposure period and following exposure to varying amounts of pulp mill effluent. The percentage of days spawned was determined from the number of days when eggs were present in the tanks relative to the total number of days the tanks were sampled.

Exposure to 10 and 65% effluent had no effect on the expression of 3β–HSD, P450aromA and StAR (Figure 5.4). While the data were quite variable there was more than a 50% reduction in the average expression of StAR in the high effluent group. The analyses of whole body steroid and VTG levels are still pending.

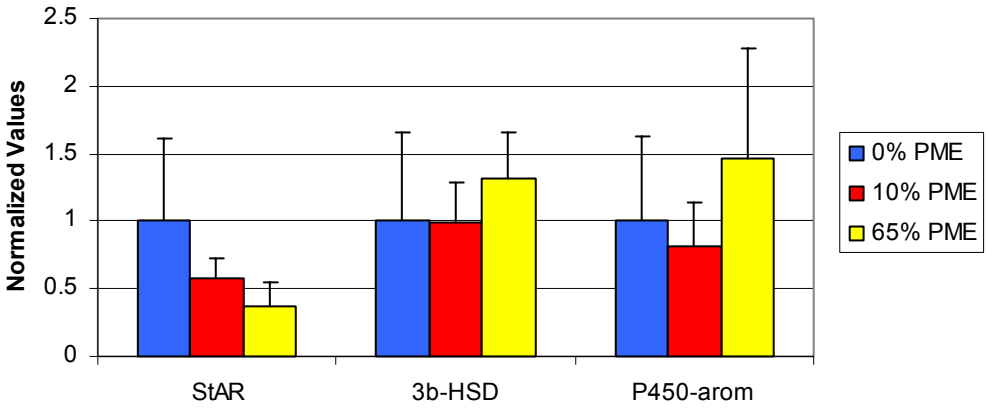

Figure 5.4. Expression of StAR, 3β-HSD and P450-aromA in vitellogenic ovarian follicles from zebrafish exposed to 0, 10 and 65% pulp mill effluent. Expression on the genes in normalized to β-actin. n=9-10 fish per treatment.

Conclusions

These studies have shown that the La Tuque effluent when tested at a final concentration of 65% caused a significant reduction in egg production and a marked decrease in the number of spawning events. There were no significant effects of the effluent on the expression of genes involved with steroid biosynthesis when examined in vitellogenic ovarian follicles.

6. Determination of the Androgenic Potency of Pulp Mill Effluent Using Three-Spine Stickleback (*Gasterosteus aculeatus*)

The purpose of the stickleback studies was to provide and in vivo endpoint for androgenic effects parallel to estrogenic endpoints provided by other methods used in the study. Androgenic effects in fishes have been documented after exposure to pulp and paper effluents including mosquitofish masculinization (Ellis et al., 2003) and induction of the androgen dependent gene for spiggin, a glue-like protein produced by male stickleback (Katsiadaki et al., 2002). In addition to the primary androgenic endpoint developed, estrogenic and steroid synthesis measures are included within the same stickleback bioassays.

Materials and Methods

***Experimental design*:** Fish were exposed to La Tuque effluent at 0, 1, 10 and 100% in a flow-through system (50% total replacement over 24 hours) for 7 and 21 days in duplicate tanks using brackish (5 ppt) water as the diluent. Exposure temperature was maintained at 15 ± 0.5°C by control of ambient room temperature. Effluent was also adjusted to 5 ppt salininty prior to the experiment. Static-renewal exposures to positive (methyltestosterone) and negative (E2) controls were conducted concurrently with the effluent experiment. Eight to ten males and females were sampled per treatment per period. Design and development of a quantitative real-time PCR method for measurement of spiggin (posterior kidney in females) and VTG (liver in males) mRNA is ongoing in order to simultaneously examine androgenic and estrogenic endpoints. Other endpoints examined include *in vitro* ovarian steroid production, secondary sexual characteristics such as male specific colouration (an androgenic endpoint), kidney epithelial cell height and histological examination of glycoproteins in kidney as alternative indicators of spiggin production.

***Stickleback*:** Three-spine stickleback were captured by seine netting in the Flat River estuary, PEI in December 2006. The Flat River basin is relatively unimpacted with low agricultural land-use only. Stickleback were transported to the laboratory at UPEI and transferred to aquaria held at 5°C and 15 ppt salinity. Water was gradually changed to 5 ppt salinity and 15°C over a period of one week.

***In vitro steroid production*:** For each fish, in quadruplicate, ~ 25mg sections of ovary were added to 1 mL of Medium 199 buffer in 24 well plates. Two of the wells were stimulated with forskolin and the additional two wells were dosed with ethanol carrier as a measure of basal steroid production. Media were removed and frozen pending analysis after an incubation of 18 h at 18^{o}C.

***Kidney epithelial cell height*:** Kidneys were removed from fish and placed in 10% neutral buffered formalin. These samples were dehydrated stepwise in ethanol, cleared in xylene and embedded in wax. Histological sections of the kidney taken (4 μm) were cut and stained with either haemotoxylin and eosin (HE) or peroxic acid/Schiff's reagent and aniline blue (PAS). The height of epithelial cells was digitally estimated in circular tubules.

***Effluent androgens*:** Effluent fractions were isolated using liquid-liquid and solid phase extraction methods. Fractions will be analysed for estrogenic and androgenic components using receptor binding and enzyme inhibition endpoints.

Results

La Tuque effluent exposures were completed with only one mortality recorded in the 1% exposure concentration. There was no evidence of colouration typically found in breeding males in female stickleback during either 7 or 21 d of exposure. Gonad size, liver size and condition factor were examined and no statistical differences found at either sampling time or in either sex (data not shown). Kidney size in females was examined as a potential indicator of androgenicity (as kidney cells hypertrophy when spiggin is produced). Though kidney size was slightly elevated at 21 d exposure to 100% effluent, the change was not statistically significant (Figure 6.1).

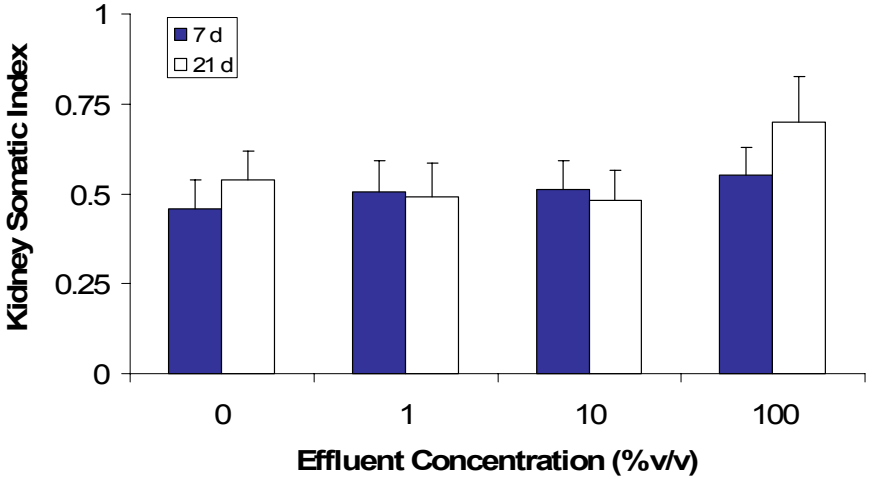

Figure 6.1. Kidney somatic index in three-spine stickleback exposure to La Tuque pulp mill effluent for 7 and 21 d.

Kidney histology sections are currently prepared and histological analysis is proceeding. Effluent extracts have been prepared and are awaiting analysis. Spiggin mRNA analysis is currently under development.

Conclusions

Survival data suggests that La Tuque effluent treatment is highly efficient at removing any acute lethality. While analysis is still underway, kidney size data is suggestive that induction of androgenic endpoints may be occurring at 100% effluent.

7. Effluent Chemistry and *In Vitro* Tests for Hormone Analogs

The objective of performing measurements of effluent chemistry is to provide an indication of effluent quality over the course of the Cycle 4 IOC studies conducted over several months with La Tuque effluent. These measures would then be able to facilitate the interpretation of bioassay results from different laboratories conducted at different times.

The objective of *in vitro* tests for hormone analogs is to determine the potential of these tests for IOC and IOS; they can be conducted rapidly and economically but their ability to predict gonad size reductions is unknown. The *in vitro* tests for hormone analogs in effluents and fish tissues was conducted according to established protocols (Hewitt et al., 2000) and samples are awaiting analysis at this time (estrogens and androgens in Figure 7.1).

For effluent quality monitoring, effluent samples were collected weekly from May 30, 2006 – January 24, 2007 inclusive. A schematic breakdown of the samples collected and the analyses performed is shown in Figure 7.1.

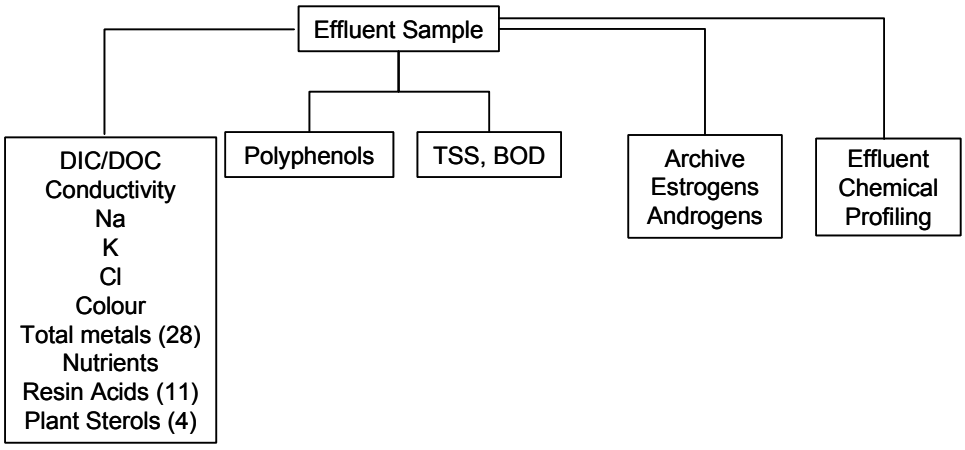

Figure 7.1. Schematic of effluent sampling from the La Tuque mill.

Dissolved Inorganic Carbon (DIC)/Dissolved Organic Carbon (DOC), major ions (Na, K, Cl), colour, total metals, nutrients, resin acids and plant sterols were conducted according to protocols at Environment Canada's National Laboratory for Environmental Testing Laboratory in Burlington ON (National Laboratory for Environmental Testing, 2007).

Total suspended solids (TSS) and carbonaceous biological oxygen demand (BOD) analyses were conducted by Environment Canada's Wastewater Technology Centre according to established protocols (Method INW3 - Determination of Biochemical Oxygen Demand in Water, Method 2540D - Total Suspended Solids).

Polyphenol analysis followed the Hach Method 8193 for tannin and lignin and was conducted by the Northwest Aquatic Biology Facility of the National Council for Air and Stream Improvement Inc. in Ancartes WA. The method involves the reaction of Folin phenol reagent with the

aromatic hydroxyl groups of lignin and consequently the term "polyphenols" is used to describe the quantified material.

Effluent chemical profiling

Effluent samples collected for the short and medium term fathead minnow reproduction tests (see Table 3.1) were qualitatively characterized by solid phase microextraction (SPME) using a commercially available apparatus (Supelco) at Paprican. Samples (10 mL) of each effluent were transferred to inert Teflon vessels and stirred at a constant rate with Teflon coated micro stir bars. The SPME fiber with a 100 µm polydimethylsiloxane coating was then immersed in the effluent sample for 60 min at room temperature (approx. 22°C). The loaded fibers were analyzed by gas chromatography/mass spectrometry (GC/MS). The GC/MS analyses of the extracts were done with an Agilent 6890 Series GC coupled with a 5973 mass selective detector. The samples were desorbed in the injection port in splitless mode for 1.5 minutes at 250°C. The chemical components were separated on a 30m x 0.25 mm (0.25 µm film thickness) DB-5MS capillary column (J&W Scientific) using a helium carrier flow rate of 1.3 mL/min. The oven temperature program began at a temperature of 50°C for 5 minutes followed by a 5°C/min increase to 260°C and a final hold of 13 minutes.

Results and Discussion

Conventional effluent parameters (eg. BOD, major ions) showed some variation throughout the sampling period of bioassay testing. The typical fluctuations for BOD, total suspended solids (TSS) and polyphenols are shown in Figures 7.2 and 7.3. For these three parameters, TSS showed the greatest fluctuation.

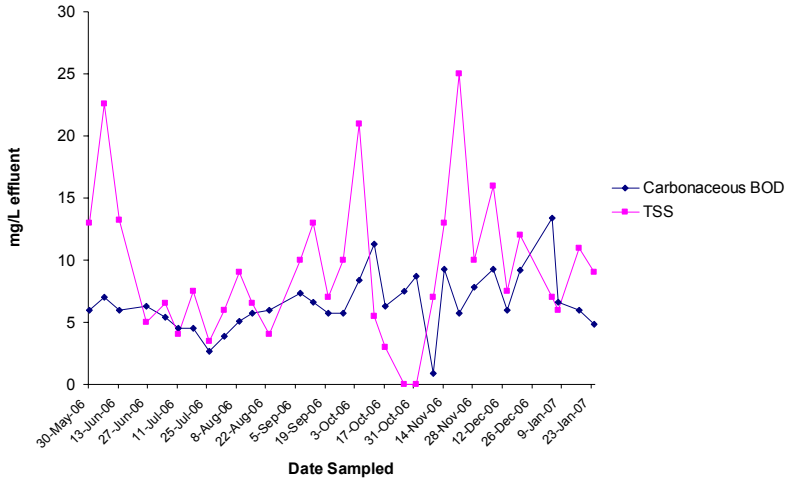

Figure 7.2. Total suspended solids and biological oxygen demand of La Tuque effluent from weekly samples collected from May 30, 2006 – January 24, 2007 (analyses complete). Average detection limits were 2.48 mg/L (BOD) and 4.22 mg/L (TSS).

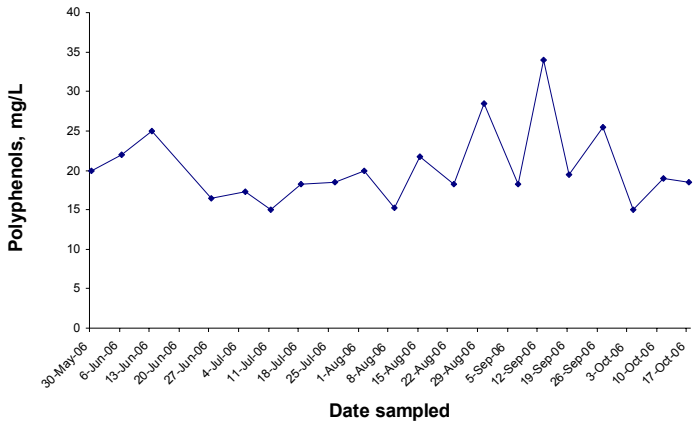

Figure 7.3. Polyphenol content of La Tuque effluent from weekly samples collected from May 30 – October 17, 2006. Balance of analysis to January 24, 2007 is pending.

The balance of conventional effluent parameters, also indicating moderate variability, is summarized in Table 7.1 and the metals data is given in Table 7.2.

Table 7.1. Summary of conventional effluent parameters measured in La Tuque effluent. Shown are data from weekly samples collected from May 30 - November 9, 2006. Balance of analysis to January 24, 2007 is pending.

Parameter Concentration (mg/L)	Mean ± SD (n=24 weeks)	Parameter Concentration (mg/L)	Mean ± SD (n=24 weeks)
NO_3/NO_2	0.07 ± 0.06	DIC	64.6 ± 20.5
NH_3	1.25 ± 1.48	Ca	39.5 ± 8.7
Conductivity (µS/cm)	1387 ± 313	Mg	2.8 ± 0.4
pH	7.20 ± 0.25	Na	252 ± 100
Alkalinity	281 ± 67	K	9.9 ± 3.8
Cl	130 ± 14	NO_2	0.03 ± 0.01
SO_4	212 ± 120		
Colour (PT-CO)	346 ± 60		
DOC	76.9 ± 19.3		

Table 7.2. Summary of metals concentrations measured in La Tuque effluent. Shown are data from weekly samples were collected from May 19 – October 4, 2006. Balance of analysis to January 24, 2007 is pending.

Metal	Mean ± SD (n=16 weeks)	Metal	Mean ± SD (n=16 weeks)
Silver	0.059 ± 0.081	Lanthanum	0.09 ± 0.04
Arsenic	0.52 ± 0.30	Lithium	1.34 ± 0.24
Boron	38.2 ± 17.9	Molybdenum	3.8 ± 2.7
Beryllium	0.026 ± 0.013	Nickel	4.3 ± 1.9
Bismuth	0.003 ± 0.002	Lead	2.2 ± 2.7
Cadmium	0.30 ± 0.19	Rubidium	28.0 ± 20.0
Cobalt	0.34 ± 0.15	Antimony	1.8 ± 1.5
Cromium	1.9 ± 1.0	Selenium	0.08 ± 0.02
Copper	19.1 ± 36.9	Strontium	151 ± 68
Iron	574 ± 210	Thallium	0.03 ± 0.01
Gallium	0.19 ± 0.11	Vanadium	28.0 ± 20.3
		Zinc	87.9 ± 34.0

Samples collected in June showed increases (in some cases dramatic) in some metals relative to previous concentrations. Three metals in particular, aluminum, manganese and barium, showed increases in mid June 2006, which did not return to their previously measured concentrations for the data obtained thus far (Figure 7.4). These increases occurred in parallel with qualitative profiles of effluent organics obtained by SPME (see next section).

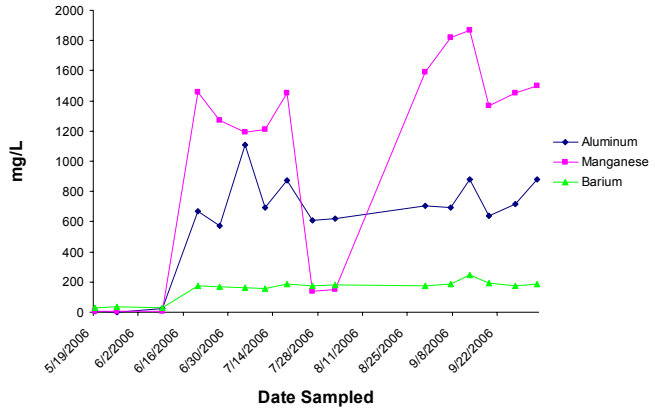

Figure 7.4. Selected metals exhibiting increases in concentrations during June 2006 La Tuque effluent collections. Balance of analysis to January 24, 2007 is pending.

Effluent chemical profiling

The chemical profiles shown in Figure 7.5 illustrate that the final effluent quality changed between May and September 2006. Essentially, the amount of extractable organics by the SPME method was less in the sample taken on September 4th 2006 than in the three samples taken during May and June 2006. This indicates a general improvement of effluent quality with time. The effluent samples in May/June 2006 were taken about a month after a mill shutdown and the greater extractable organics at that time may have been associated with the startup of the mill when the mill operating conditions can be variable and atypical. Thus, the sample taken in September 2006 would be more reflective of normal mill operating conditions. This would confirm that improved effluent quality as indicated by chemical profiling, rather than lesser sensitivity of the short-term test, can best explain the lack of effluent effect on egg production in the September test. The origin of the change in profile is not known at this time.

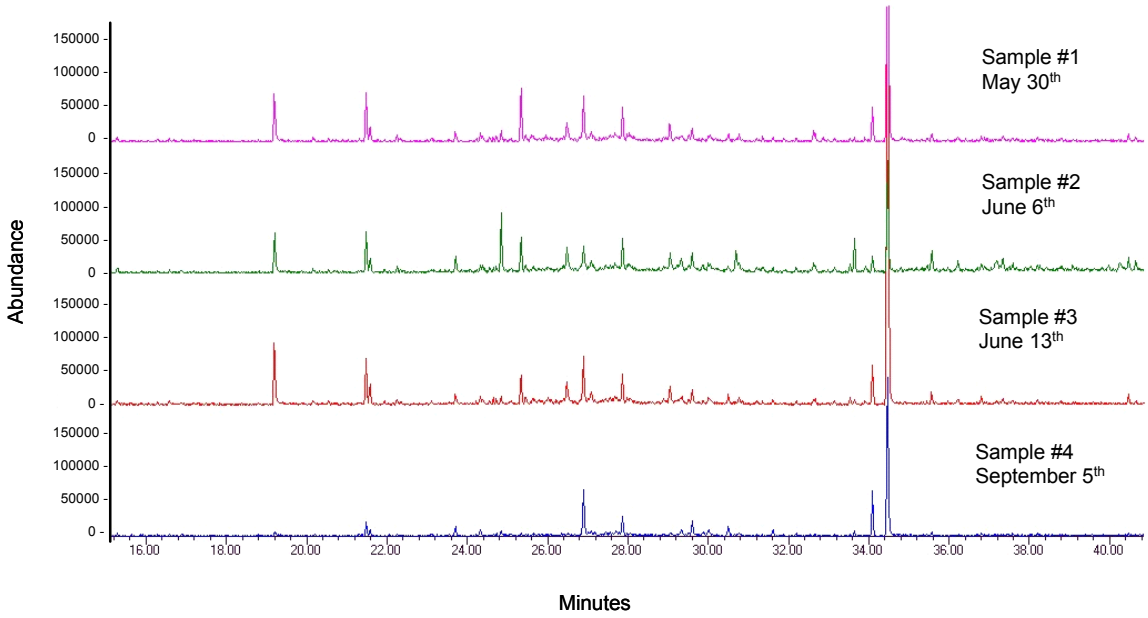

Figure 7.5. Chemical profiles obtained by solid phase microextractions followed by elution through a gas chromatograph/mass spectrometer. Samples #1, #2 and #3 were used in the medium-term test and Sample #4 was used in the short-term test.

Conclusions

- The data obtained to date indicated minor and moderate variability for most conventional effluent parameters such as BOD and major ions as well as metals throughout the study period.
- Increases in aluminum, barium and manganese concentrations that occurred after mid-June 2006, coinciding with a decrease in the amount of extractable organic material obtained by passive sampling during the summer of 2006, suggest a change in effluent quality that was also noted with the short- and medium-term fathead minnow tests (see Section 3).

Overall Summary and Conclusions

The work described in this report is the first part of a project aimed at selecting the most appropriate laboratory tests for conducting Investigation of Cause (IOC) and Investigation of Solution (IOS) studies meant to eliminate effluent-related effects on fish reproduction. The full project calls for similar investigations at two other mill sites followed by the use of the selected laboratory tests with effluents from five mills before and after biotreatment.

The work described in this report represents an unprecedented volume of work done on one mill (Smurfit-Stone bleached kraft mill at La Tuque, QC) effluent in a relatively short timeframe (May 2006 to January 2007). The work included an assessment of wild fish in the actual river receiving the mill effluent as well as a series of laboratory tests with four species of fish, coupled with extensive chemical analyses of the effluents tested. The laboratory tests ranged in duration from just a few days to over six months and covered an assessment of reproductive indicators in fish ranging from the biochemical level to egg production. The results of the work with the LaTuque mill effluent allowed the following conclusions to be made:

- Field work and recently submitted Cycle 4 EEM data showed that the La Tuque mill effluent did not cause reduced gonad size in wild fish as was the case in previous EEM cycles.

- The minimal responses of fish in all the laboratory tests evaluated in this study were consistent with the observations on wild fish.
  - The lifecycle test with fathead minnow found that the overall condition of the fish exposed to the La Tuque mill effluent was similar to the condition of wild fish living downstream from the mill and the various medium- and short-term tests with three different species provided accurate assessment of the reproductive status of fish exposed to the mill effluent.

- Despite the minimal responses observed in laboratory fish, the effects on some reproductive endpoints at high concentrations of the La Tuque mill effluent illustrated the potential value of these endpoints for IOC/IOS work.

- Based on the results of this study, the egg production endpoint in laboratory tests appears to have the greatest potential for assessing an effluent's ability to affect fish reproduction as effluent-related effects were found to be similar (i.e., slight stimulation at low and intermediate effluent concentrations and reduced egg production at 65% and 100% effluent concentrations) in three species of fish.

- There were indications that the effect of a mill effluent on egg production can be reliably assessed in a shorter timeframe than previously thought and, if confirmed in future studies, this would make tests using the egg production endpoint (possibly using a variety of species) as one of the tools for IOC/IOS work.

- Chemical fingerprinting and differential responses of fathead minnow in tests done during the course of the project suggested that effluent quality from a mill may be somewhat variable indicating the necessity for continued chemical characterization of effluents and the complexity of the task to identify causative agent(s).

Path Forward

The selection of the appropriate diagnostic tools is the critical first step for successful IOC/IOS work aimed at mitigating mill effluent-related effects on fish reproduction. While very useful information was obtained from the work done at the La Tuque mill site, it is essential to complete the full project workplan in order to complete tool selection. This calls for i) the detailed tool assessment described in this report at two additional mill sites followed by ii) an assessment of effluents before and after biotreatment at five mill sites using the laboratory tests selected on the basis of the work from i).

The mill sites for future work include another kraft mill and a TMP newsprint mill for the three-mill work and the same three mills as well as two additional mill sites for the five-mill work. It will be important to ensure that some of the effluents for future work be more potent than the La Tuque mill effluent and, because of this, a pre-screening survey of effluents from several mills may be necessary.

Additionally, continuous refinements of the tests under evaluation will be needed to ensure that the final tests selected for IOC/IOS work will have the ability to identify successful mitigation strategies. Finally, as the tasks are complex, attempts will also continue to foster new collaborations with other experts in the field to maximize the probability for success.

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