Cycle 5 National Investigation of Cause Project
Final Report

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EXECUTIVE SUMMARY

The work described in this report is part of a national multi-agency project aimed at finding solutions for effects of pulp and paper mill effluents on fish reproduction. Entailed in this work is selecting the most appropriate laboratory tests for conducting Investigation of Cause (IOC) and Investigation of Solution (IOS) studies for the Environmental Effects Monitoring (EEM) Program.

The work described in this report represents studies conducted at two mills (Domtar bleached kraft mill at Espanola, ON; Lake Utopia Paper neutral sulphite semi-chemical mill at St. George, NB) between February 2007 and October 2009.

A long-term monitoring study at the Espanola mill provided insights into temporal effluent variability with respect to fish reproduction. Evaluations of laboratory tests were conducted to confirm hypotheses on the causes and sources of the variability. The laboratory tests ranged in duration from just a few days to several weeks and covered an assessment of reproductive endpoints in fish ranging from the biochemical level to egg production. The work also included an assessment of wild fish in the Spanish River which receives the mill effluent from Espanola. The results of the work with the Espanola mill effluent allowed the following conclusions to be made:

Contrary to previous EEM studies using white sucker, field collections in the Spanish River using silver redhorse and logperch determined that neither species exhibited reduced gonad sizes downstream of the Espanola mill.

- Depressions in the major biologically active reproductive steroids in both sexes of silver redhorse indicated the potential of the effluent to affect fish reproduction.
- Relative to upstream, there was evidence of enrichment in both species.
- Long-term monitoring of Domtar final effluent showed fathead minnow egg production tracked changes in effluent quality as it related to production upsets, mill restarts and conditions affecting biotreatment performance.
- Final effluent quality was characterized through measurement of a gas chromatographic (GC) Profiling Index, BOD and methyl-2-cyclopentenones, which all show promise as diagnostic tools for IOC/IOS work.
- Subsequent laboratory tests with five fish species confirmed the potential contribution of black liquor for causing final mill effluents related effects on fish reproduction.

Final effluent from the Lake Utopia Paper mill was used to evaluate laboratory tests with four species of fish. The laboratory tests ranged in duration from just a few days to several weeks and covered an assessment of reproductive endpoints in fish ranging from the biochemical level to egg production. The results of the work with the Lake Utopia mill effluent allowed the following conclusions to be made:

- In short-term tests (≤7d), reduced egg production in two fish species was found to be the most sensitive endpoint resulting from effluent exposure.

- In a medium-term test (~30d), reduced egg production was also found to be the most sensitive endpoint resulting from effluent exposure. The effluent-related effects in the medium-term test egg production occurred at lower concentrations than short-term tests.

Collectively, our work has identified egg production in short-term tests as having the most potential for future IOC/IOS studies. The exact relationship between effluent effects on egg production in short-term tests using laboratory fish species and gonad size in different species of wild fish is presently unclear. However it is recognized that effects on egg production are significant and directing IOC and IOS studies using such an endpoint would have environmental benefits. In our Cycle 5 work we have identified leads for future IOS studies that involve minimizing organics losses and upsets to biological treatment. Future IOS studies will need to include longer term tests incorporating egg production and gonad size measurements as part of the validation process.

INTRODUCTION

The national assessment of the first four 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 (see Hewitt et al., 2008) 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 the continued 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 a response pattern that is interpretable in the context of effects observed in wild fish during the EEM studies. The laboratory tests must also 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 tests or endpoints 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 two mill sites during Cycle 5.

MILLS SELECTED FOR SURVEY AND PROJECT OUTLINE

Mills Selected

The first mill selected for study was the Domtar bleached kraft pulp facility in Espanola, Ontario, producing about 1000 t/d of pulp and 225 t/d of specialty papers. This mill pulps softwoods (ODEoDnD bleaching) and hardwoods (OA(zd)EDnD bleaching). The softwood furnish is composed of 50% jack pine and 50% spruce. The hardwood furnish can vary between 100% maple, aspen or birch to a mixture of 75% maple and 25% birch. The effluent is treated in a settling basin before passage through an aerated lagoon with a 7-day hydraulic retention time and is discharged to the Spanish River.

The second mill selected was the Lake Utopia Paper mill in St.George, New Brunswick. This facility produces neutral sulphite semi-chemical pulp from hardwood chips. Recycled cardboard contributes 30% of the fibers used in the production of about 520 t/d of finished corrugated medium. Effluent treatment consists of a primary clarifier followed by two upflow anaerobic sludge blanket (USAB) reactors. Post-treatment is achieved with an aerated lagoon. The final effluent is discharged into the L'Etang Estuary.

Project Outline

At the Domtar kraft mill in Espanola, wild fish were sampled upstream and downstream from the effluent discharge during the fall of 2008 to determine the reproductive status of fish in the field. A long-term study was conducted between February 2007 and March 2009 to monitor effluent quality and evaluate the utility of the fathead minnow egg production test and effluent organic chemical profiling, in tracking effluent quality. The goal was to identify specific conditions that influence effluent quality in terms of the potential to affect fish reproduction. Testing included a battery of short-term and medium-term laboratory experiments. Black liquor spiking experiments were also completed to assess the sensitivity of the various laboratory test in distinguishing differences in effluent quality.

The work at the Lake Utopia Paper neutral sulphite semi-chemical mill was completed in October and November 2009. The effluents from this mill were used to evaluate the applicability of the battery of various short-term and medium-term laboratory tests for IOC and IOS work aimed at eliminating/reducing mill effluent-related effects on fish reproduction.

The responsibilities of the lead investigators/organizations are outlined in Table A.

Table A. Responsibilities of lead investigators.

Lead Investigators & Organization	Wild Fish	Fathead Minnow Medium-term Test	Fathead Minnow Short-term Test	Rainbow Trout Short-term Test	Mummichog Short-term Test	Zebrafish Short-term Test	Stickleback Short-term Test	Chemistry
McMaster NWRI	DE							
Parrott NWRI		DE LUP						
Kovacs/Martel FPInnovations			DE LUP	DE LUP				
MacLatchy WLU					DE LUP			
Van Der Kraak Uof Guelph						DE LUP		
van den Heuvel UPEI							DE	
O'Connor FPInnovations Hewitt NWRI								DE LUP

DE: Domtar Espanola mill effluent, LUP: Lake Utopia Paper mill effluent

The findings are presented in two Sections. Section 1 presents the field and laboratory data obtained with the Domtar mill effluents. Section 2 presents the results of laboratory tests conducted with the effluents from the Lake Utopia Paper mill.

FINDINGS

1. Evaluation of Reproductive Endpoints in Fish Exposed to a Bleached Kraft Mill Effluent

1.1 Field Studies at Espanola

The purpose of the wild fish collections were two-fold. The first objective was to conduct an EEM-like fish collection with fish exposed to the effluent that was also being shipped to the laboratories for IOC testing. This would ensure that reproductive effects in wild fish would correspond to the same effluent exposures as the laboratory fish tests. The second objective was to add additional reproductive endpoints in the wild fish studies that would correspond to the endpoints being used in the laboratory studies. These included measurement of circulating reproductive steroids, in vitro steroid hormone production, expression of 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. Two sites were sampled for fish; an upstream reference site above the dam in Espanola and an exposed site downstream of the effluent discharge. Previous EEM studies used the white sucker as the sentinel species so field collections were first attempted just prior to the spring spawning season to collect fish following complete gonadal development (May 4–15, 2009). Despite numerous fishing techniques used (trap nets and electrofishing boat) and many locations attempted (all spawning tributaries in the area as well as a large rapids on the river itself) very few white sucker were captured upstream of the effluent discharge above the dam in Espanola. Attempts were made downstream as well and although some fish were captured, they were not sampled due to the lack of reference fish for comparison. A second attempt to sample fish was then tried in the late summer/early fall of 2009. Boat electrofishing was the method of capture and white sucker were the species of choice when sampling started. Fishing took place from August 24 – September 3, 2009. Fishing started upstream of the effluent discharge due to the presence of the viral hemorrhagic septicemia virus (VHSV) that may be present downstream of the discharge due to its connection to the Great Lakes. Few white sucker were collected whereas Silver Redhorse (*Moxostoma anisurum*) were abundant upstream of the discharge. For this reason, this species was selected as the sentinel species. Logperch (*Percina caprodes*) were also present upstream of the discharge and were also selected as a second sentinel species.

Sampling Protocol

Silver redhorse were immobilized in a foam block and blood samples were taken from the caudal vessels using a syringe and heparinized vaccutainer. Blood was held on ice prior to separation of the plasma by centrifugation; plasma was immediately frozen in liquid nitrogen. Circulating levels of testosterone (both sexes), 17β-estradiol (females) and 11-ketotestosterone (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 tubercles 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 rated with respect to their 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 the Canada Center for Inland Waters for ethoxy-resorufin-o-dethylase (EROD) analysis (Parrott et al., 1999). During the sampling, a sub-sample of gonadal tissue was taken from 12 female silver redhorse, and placed in incubation media for *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 Davidson's solution for histological evaluation. Opercula were obtained from all fish for age analysis that was conducted at the National Water Research Institute in Burlington, ON.

Logperch were sacrificed by severing the spinal cord and then were preserved in Davidson's solution. Davidson's solution was prepared by mixing 200 mL of 37–40% formaldehyde, 100 mL of glycerol, 100 mL of glacial acetic acid and 300 mL absolute ethanol (Dietrich and Heiko, 2009). Greater than 10:1 fixative to sample was used to ensure adequate fixation of the fish. Upon returning to the laboratory, fish were transferred to 70% ethanol until sampled. The fish were removed from the ethanol and then the fork length (±0.1cm), and body weight (± 0.01g) were determined. The internal organs were removed and the gonads (± 0.01g) and liver (± 0.01g) were weighed. Gonadal tissue was placed into histological cassettes for analysis of stage of development for both ovarian and testicular tissue. Examination of the presence of the intersex condition (eggs in the testes) and for gonadal staging, the frequency of the different cell types as well as the size of the various cell types were determined.

Statistical Analysis

Analysis was conducted with sexes separated. 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), 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, and 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 12.0 statistical software (Wilkinson, 1990).

Results

Female silver redhorse collected downstream of the discharge in Espanola were shorter ($p=0.004$) but were of similar weight ($p=0.07$) to those collected upstream of the dam in Espanola (Table 1.1-1). Exposed females were also younger ($p<0.001$) and had an increased condition (were fatter; Figure 1.1-1) ($p=0.022$) relative to the upstream reference females. There were however, very few small fish at the upstream reference location so there was very little overlap of data at the lower end of the relationship. Fish growth was also evaluated relative to both fish weight and fish length. There was a significant site interaction between the relationships of fish weight and fish length with age of female silver redhorse ($p<0.002$). Examination of the data again demonstrates that most exposed females were younger and had an increased growth rate while reference females were older with a reduced growth rate (Figure 1.1-2). Female silver redhorse gonadal development when expressed relative to body weight demonstrated no significant site differences ($p=0.50$). There were no site differences in absolute fecundity numbers and relative to fish length and weight, no site differences exist ($p>0.39$). Female silver redhorse collected from the exposed site had significantly larger livers relative to the upstream reference females ($p=0.031$) (Figure 1.1-3). Internal fat stores were similar ($p=0.43$) and there were no differences in the expression of secondary sex characteristics ($p=0.22$) in female fish.

Male silver redhorse collected from the near field exposed site were similar in length ($p=0.18$) and weight ($p=0.36$) but were younger ($p<0.001$) than males collected upstream at the reference site (Table 1.1-1). There were no significant site differences in the condition of the fish ($p=0.54$) but examination of male growth identified significant site interactions similar to female fish with exposed males being younger and growing faster than the older upstream males ($p<0.005$) (Figure 1.1-4). Male silver redhorse gonadal development exhibited no significant site differences relative to the weight of the fish ($p=0.80$). Male silver redhorse liver weights also demonstrated no significant site differences in the relationship between liver weight and body weight ($p=0.75$). Examination of the internal fat stores in male silver redhorse demonstrated no site differences in storage of fat for energy ($p=0.16$). Expression of male secondary sexual characteristics also demonstrated no site differences with exposure ($p=0.17$).

Table 1.1-1. Silver redhorse collected from an upstream reference and a downstream exposed site on the Spanish River around Espanola, Ontario.

Sex	Parameter	Reference (Upstream)	Exposed (Downstream)
Female	Length (cm)	51.3 ± 0.5	48.8 ± 1.0*
	Weight (g)	2173.9 ± 54.7	1961.8 ± 98.5*
	K	1.60 ± 0.02	1.66 ± 0.03*
	Gonad Weight (g)	42.05 ± 3.39	32.16 ± 4.05
	Liver Weight (g)	19.50 ± 0.91	18.73 ± 1.38*
	Fecundity (eggs)	31962 ± 950	33907 ± 1585
	Fat Index	2.3 ± 0.3	2.5 ± 0.2
	Tubercle Index	0.4 ± 0.1	0.5 ± 0.1
	Age (yrs)	18.6 ± 1.3	10.8 ± 0.5*
	n	22	32
Male	Length (cm)	48.7 ± 0.5	47.4 ± 0.9
	Weight (g)	1905.2 ± 54.7	1834.9 ± 93.5
	K	1.64 ± 0.02	1.70 ± 0.03
	Gonad Weight (g)	36.92 ± 3.82	34.31 ± 3.25
	Liver Weight (g)	16.80 ± 0.73	16.43 ± 0.96
	Tubercle Index	1.7 ± 0.2	1.3 ± 0.1
	Fat Index	1.9 ± 0.2	2.3 ± 0.2
	Age (yrs)	19.3 ± 1.0	11.1 ± 0.8*
	n	20	22

* Significantly different than reference fish ($p<0.05$)

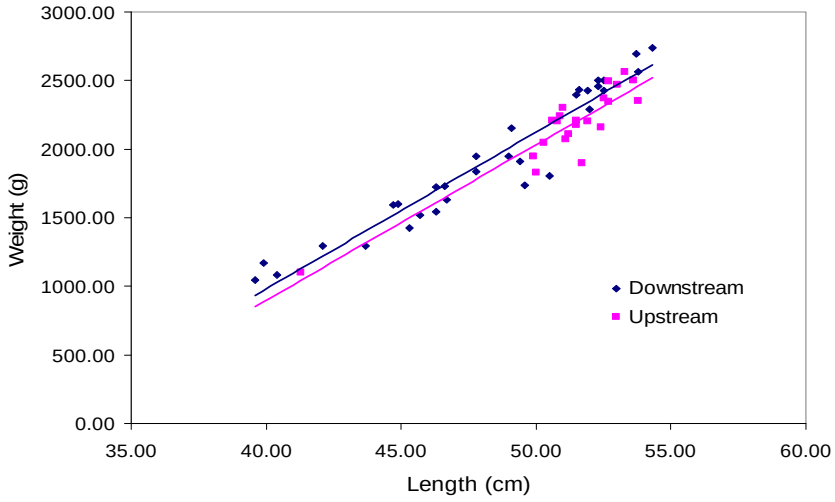

Figure 1.1-1. Condition of female silver redhorse (fish length vs fish weight) collected at a reference site and downstream of the effluent discharge.

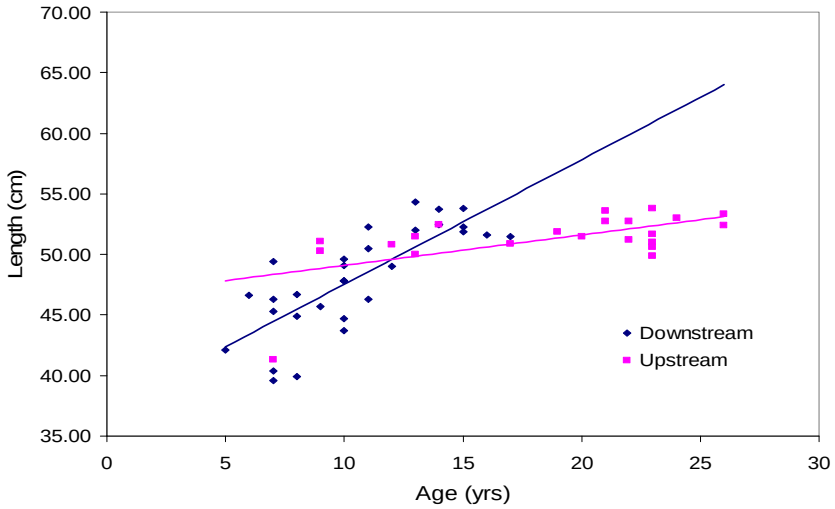

Figure 1.1-2. Female silver redhorse growth (fish length vs fish age) indicating that exposed fish were younger with an increased growth rate while reference fish were mostly older and had a reduced growth rate.

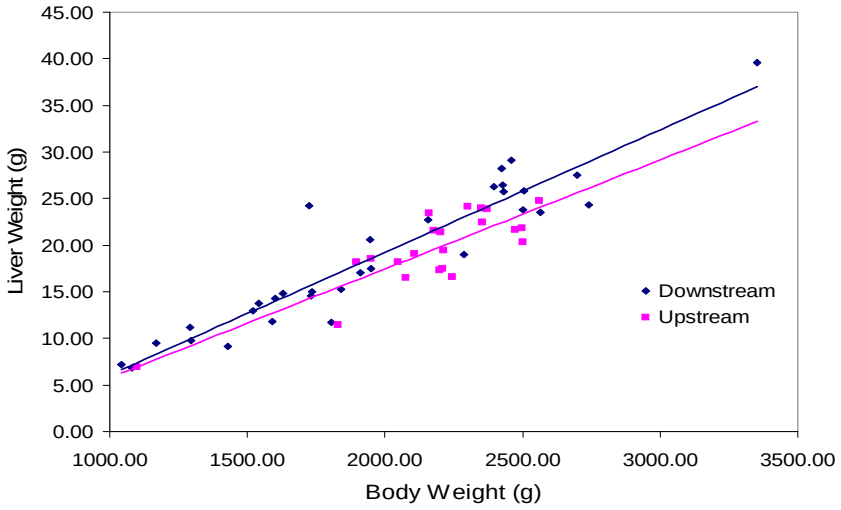

Figure 1.1-3. Female silver redhorse liver weight vs body weight indicating that exposed fish from downstream of the discharge had larger livers.

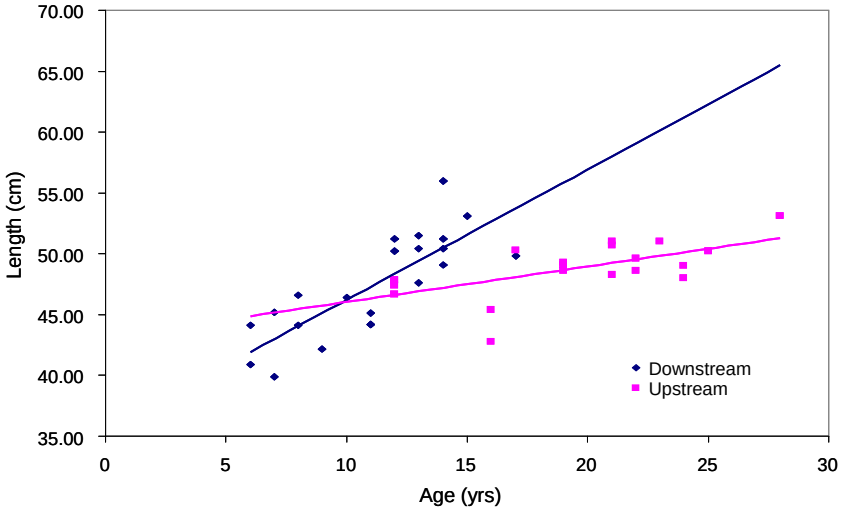

Figure 1.1-4. Male silver redhorse growth (fish length vs fish age) demonstrating increased growth of males at the near field exposed site.

Circulating and *In Vitro* Production of Steroid Hormones

Circulating levels of the two major biologically active reproductive steroids were measured in both the male and female silver redhorse. Circulating levels of both 17β-estradiol and testosterone were reduced in females collected downstream of the mill discharge on the Spanish River in Espanola ($p<0.05$) (Figure 1.1-5 and Table 1.1-2). Similar reductions were seen in circulating levels of 11-ketotestosterone levels in male silver redhorse ($p=0.001$), however no site differences were evident in circulating testosterone levels in males ($p=0.12$) (Table 1.1-2).

Follicles from female silver redhorse were incubated *in vitro* under either basal (nutrient media alone) or stimulated conditions (forskolin). Follicles from all sites responded in a positive fashion to forskolin stimulation in terms of both 17β-estradiol and testosterone production ($p<0.013$) (Table 1.1-3). Basal production of 17β-estradiol was similar between sites ($p=0.28$) however, stimulated 17β-estradiol production was greater in follicles collected from females downstream of the discharge ($p<0.001$). Basal production of testosterone was also similar between sites ($p=0.99$) however, stimulated production was again significantly higher in follicles collected from fish downstream of the effluent discharge ($p<0.001$).

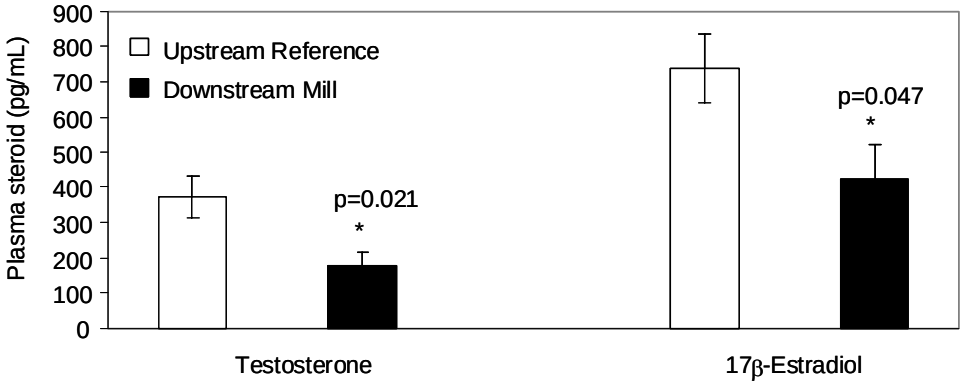

Figure 1.1-.5. Female silver redhorse circulating levels of testosterone and 17β-estradiol from an upstream reference site and a downstream effluent exposed site on the Spanish River around Espanola, Ontario.

Table 1.1-2. Circulating levels of testosterone (both sexes), 17β-estradiol (females) and 11-ketotestosterone (males) in plasma collected from silver redhorse from an upstream reference site and a downstream effluent exposed site on Spanish River around Espanola, Ontario.

Sex	Steroid (pg/ml)	Reference (Upstream)	Exposed (Downstream)
Female	17β-estradiol	738.4 ± 100.3	423.6 ± 98.4*
	Testosterone	371.2 ± 60.8	175.0 ± 40.4*
Male	11-KT	4412.0 ± 340.2	2645.9 ± 234.0*
	Testosterone	477.1 ± 39.1	382.5 ± 30.9

Table 1.1-3. *In vitro* steroid production by follicles collected from silver redhorse from an upstream reference site and a downstream effluent exposed site on the Spanish River around Espanola, Ontario. Production is either under basal incubation conditions or following stimulation with forskolin.

Steroid (pg/10 follicles)	Treatment	Reference (Upstream)	Exposed (Downstream)
17β-estradiol	Basal	156.1 ± 21.1	156.7 ± 10.0
	forskolin	189.1 ± 9.8	463.1 ± 24.9*
Testosterone	Basal	160.9 ± 14.4	148.2 ± 10.1
	forskolin	297.4 ± 31.0	473.6 ± 24.1*

Gonadal Histology

Gonadal histology was conducted on both male testicular tissue and female ovarian tissue in order to evaluate site differences in gonadal development. As no site differences on gonadal size development were present, it was unlikely that there would be large differences in the stage of gonadal development. Male testicular tissue was separated into four stages of development, spermatogonia, spermatocytes, spermatids and spermatozoa each representing a progressive stage further in testicular development. Figure 1.1-6 illustrates the percentage of cells in the various stages of development for individual fish from each site. As can be seen, fish were in the early stages of testicular development and there was a fair amount of variability between fish within each site. Overall, no site differences in testicular development were seen.

Female ovarian development was separated into follicles of primary development, cortical alveolar stages, vitellogenic stages and atretic follicles. Figure 1.1-7 illustrates the early stages of ovarian development as the majority of the follicles are of the primary stage, although there was considerable variability between fish within site. Overall, similar to male development, there were no site differences in the overall stage of ovarian development due to effluent exposure.

Logperch collected downstream of the effluent discharge were larger than the logperch collected upstream of the mill. Although exposed female logperch were both longer and heavier ($p<0.001$) than reference fish, they had similar condition factors ($p=0.10$) (Table 1.1-4). It is most likely these fish were also older than the ones collected upstream. Attempts are being made to remove the otoliths from these fish for age determinations. Although the fish were at the early stages of ovarian development in preparation for spawning in the spring, relative to the body size, female fish from the two sites show no differences in the amount of energy put into gonadal growth ($p=0.90$). Female fish exposed to effluent had significantly larger livers relative to body size compared to upstream reference females ($p=0.004$)(Figure 1.1-8). Examination of internal fat stores revealed no significant site differences ($p=0.38$).

Similar to female logperch, exposed males were longer and heavier ($p<0.001$) than upstream reference males (Table 1.1-4). Downstream male logperch were also significantly fatter (increased condition factor) relative to males collected upstream of the discharge (Figure 1.1-9). Corresponding to this increased condition, these fish also had increased internal fat stores (Table 1.1-4). Examination of gonadal development was rather limited (GSI~ 0.2%) and failed to show a significant regression with body weight ($p=0.20$) suggesting that this was very early in reproductive development for this spawning season. Male logperch showed no site differences in the size of the liver relative to the body size ($p=0.84$).

Histological examination revealed no significant site differences in females as all fish examined were in the very early stage of ovarian development (Figure 1.1-10). Testicular development was advanced in exposed male logperch relative to the upstream reference males (Figure 1.1-11), although this development was at the early stages of spermatogenesis for the next spawning season.

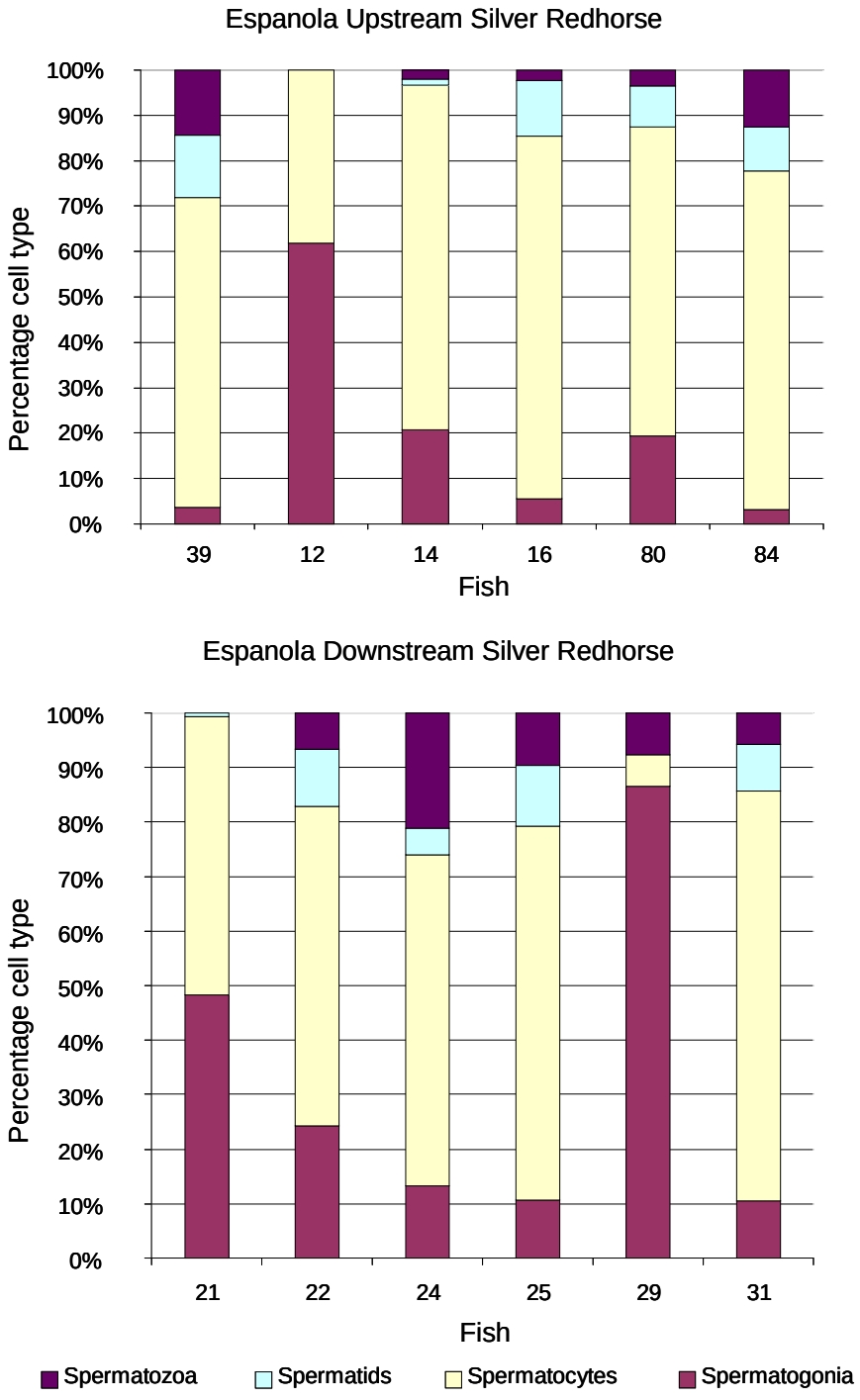

Figure 1.1-6. Male silver redhorse testicular development in individual fish collected from an upstream reference site and a downstream effluent exposed site in the Spanish River around Espanola, Ontario.

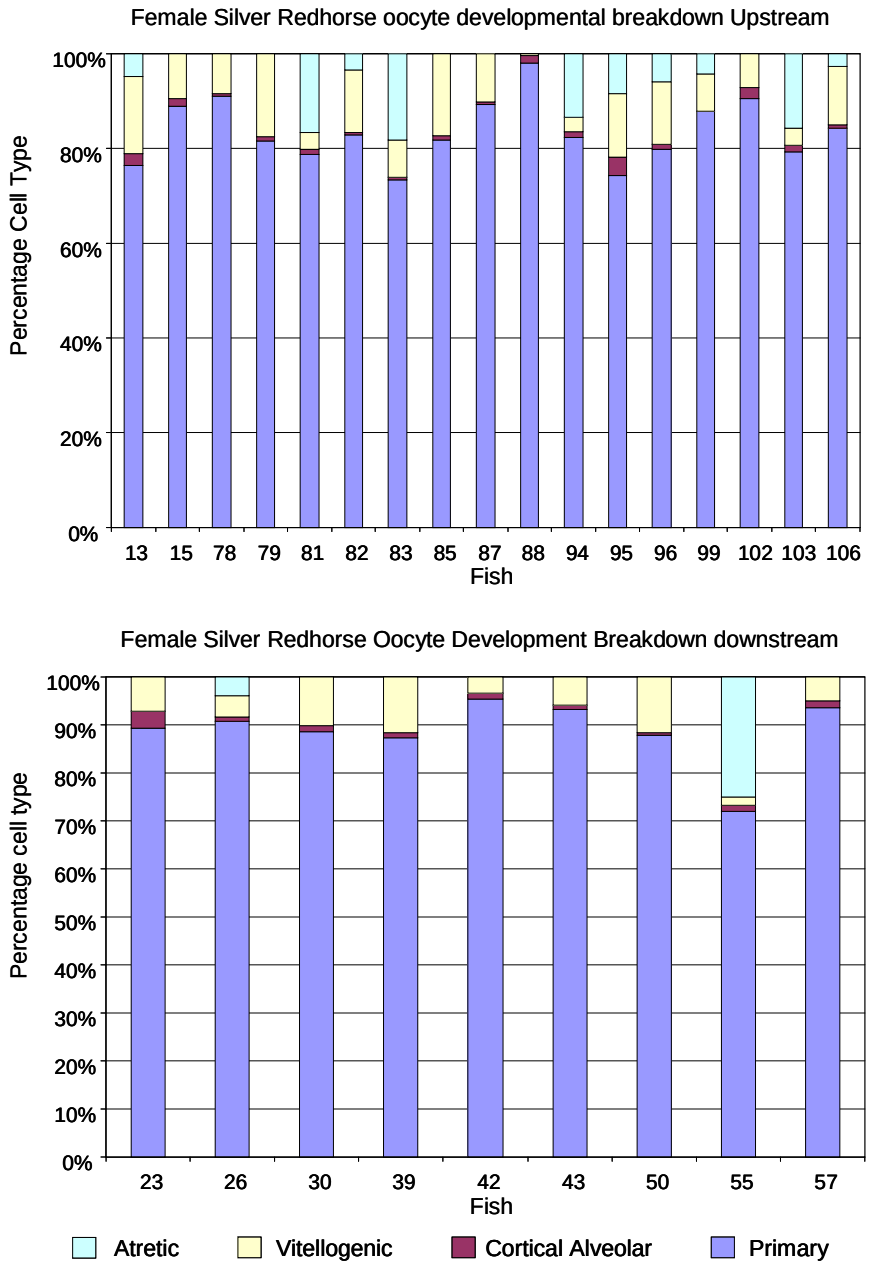

Figure 1.1-7. Female silver redhorse ovarian development in individual fish collected from an upstream reference site and a downstream effluent exposed site in the Spanish River around Espanola, Ontario.

Table 1.1-4. Logperch collected from an upstream reference and a downstream exposed site on the Spanish River around Espanola, Ontario.

Sex	Parameter	Reference (Upstream)	Exposed (Downstream)
Female	Length (mm)	68.8 ± 1.3	79.1 ± 0.8*
	Weight (g)	2.585 ± 0.182	4.001 ± 0.129*
	K	0.771 ± 0.010	0.804 ± 0.013
	Gonad Weight (g)	0.022 ± 0.003	0.022 ± 0.001
	Liver Weight (g)	0.021 ± 0.001	0.048 ± 0.003*
	Fat Index	2.6 ± 0.2	2.9 ± 0.3
	n	26	27
Male	Length (mm)	72.5 ± 1.4	83.9 ± 1.0*
	Weight (g)	2.926 ± 0.199	5.016 ± 0.198*
	K	0.751 ± 0.024	0.843 ± 0.020*
	Gonad Weight (g)	0.004 ± 0.001	0.009 ± 0.002
	Liver Weight (g)	0.027 ± 0.003	0.049 ± 0.004
	Fat Index	1.9 ± 0.2	3.1 ± 0.3*
	n	17	22

* Significantly different than reference fish ($p<0.05$)

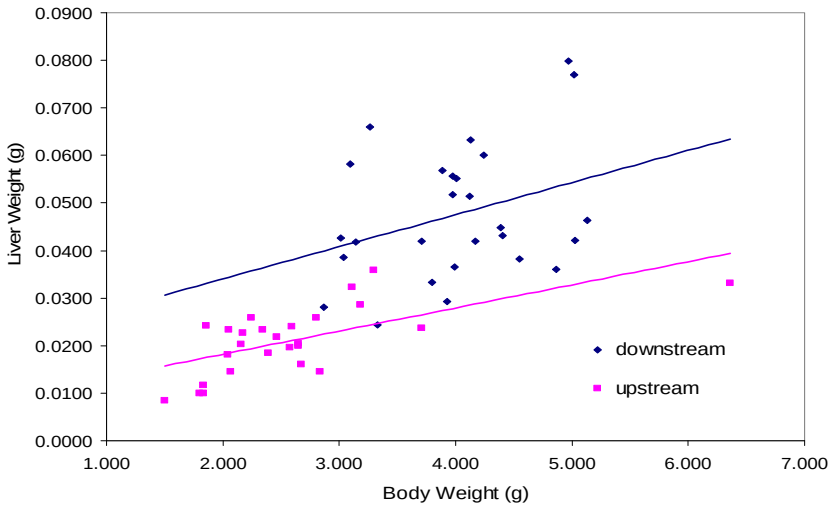

Figure 1.1-8. Female logperch liver weight vs body weight indicating that exposed fish from downstream of the discharge had larger livers.

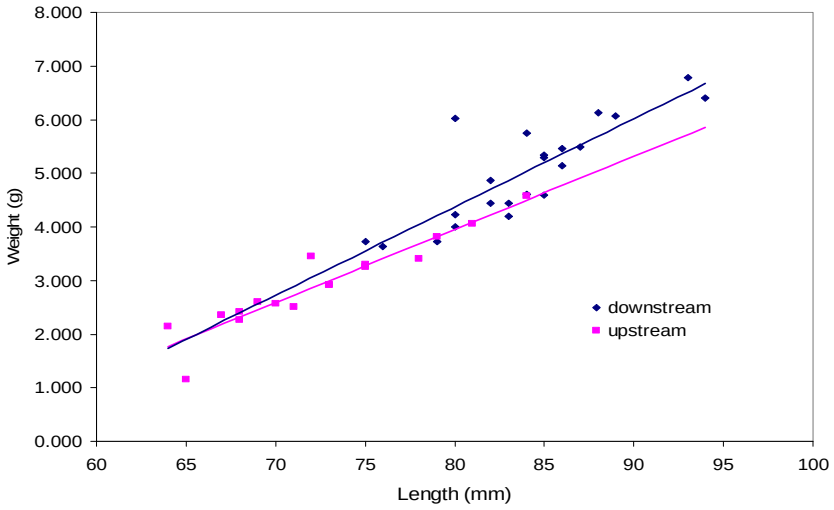

Figure 1.1-9. Male logperch condition (fish length vs fish weight) demonstrating increased condition in males collected downstream of the effluent discharge relative to upstream reference females.

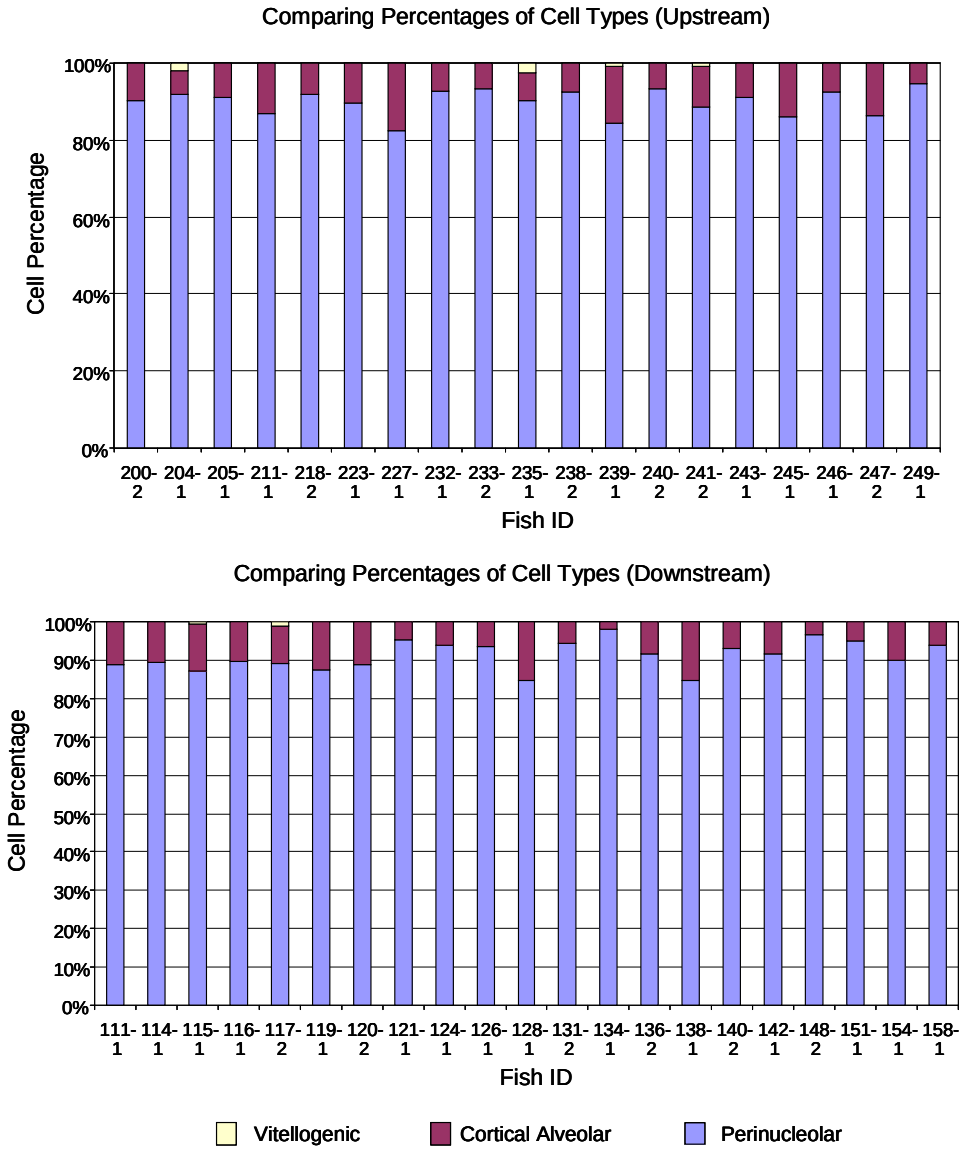

Figure 1.1-10. Female logperch ovarian staging demonstrating both the early stage of gonadal development for the upcoming spawning season and the lack of difference between the upstream reference and downstream effluent exposed site.

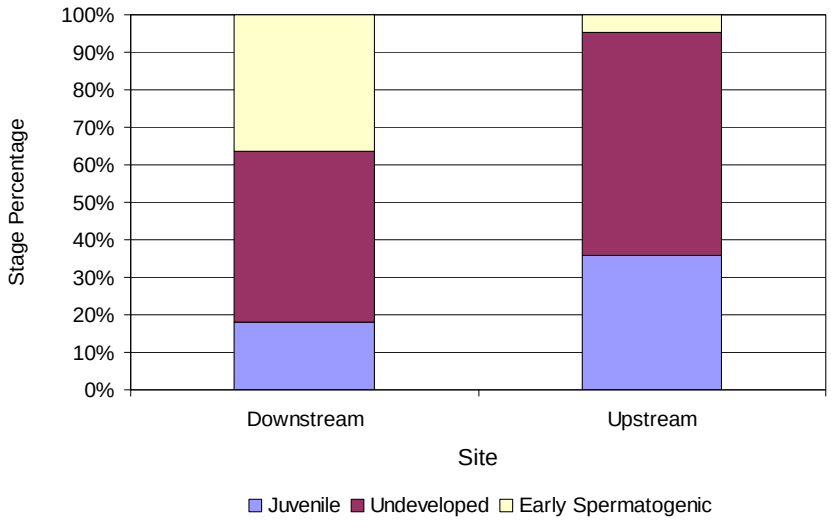

Figure 1.1-11. Male logperch testicular development showing the early stages of development for the upcoming spawning season and the slight increased rate of development in males collected downstream of the effluent discharge on the Spanish River in Espanola, Ontario.

Conclusions

Although previous EEM studies had identified reductions in gonadal development in white sucker downstream of the effluent discharge, very few white sucker were captured in this receiving environment. Silver redhorse were fairly abundant both upstream and downstream of the effluent discharge in the Spanish River as were the small-bodied logperch. Comparison of these two species upstream and downstream of the discharge revealed no effects on gonadal development in either species. Some effects of effluent exposure were evident however.

- Both male and female silver redhorse had reduced circulating levels of the major biologically active reproductive steroid hormones. Reductions in these steroids often correspond to decreased gonad size and other reproductive alterations.
- Silver redhorse populations are much younger downstream of the discharge and demonstrate increased growth compared to the older upstream fish.
- Female silver redhorse are also fatter and have larger livers compared to the upstream reference fish. This is often seen in fish downstream of effluent outfalls and is often associated with enrichment of the environment.
- Logperch females had increased liver size and males had increased condition factors and increase internal fat stores.

1.2 Long-term Monitoring of Biological Effects and Chemical Parameters

Introduction

Results of the Cycle 4 National Investigation of Cause Project found that effluent quality from a mill may be variable over time (Kovacs et al., 2007a). Specifically, effluent from a bleached kraft mill which did not affect egg production by the fathead minnow (as well as other species) in laboratory tests was found to cause a reduction in egg production by the fathead minnow (FHM) following a mill start up. Also, the organic loading of the effluent, as determined by the summation of peak areas in the gas chromatographic (GC) profile of solvent-extracted effluent, was found to be increased after the start up. Such variations could pose complications in identifying the causative agent(s) of effluent-related reproductive effects. On the other hand, understanding the causes for the variations in effluent quality could provide leads concerning effective remedial strategies. With this in mind, a study aimed at monitoring effluent variability at a mill site, the bleached kraft mill at Espanola, was initiated. The objectives were two-fold: i) to evaluate the utility of the FHM egg production test as well as effluent GC profiling in tracking effluent quality in relation to mill operational changes and thereby ii) attempt to identify specific mill operational conditions that influence effluent quality in terms of the potential to affect fish reproduction.

Materials and Methods

Experimental design

This monitoring study focused on the Domtar bleached kraft mill in Espanola Ontario. The mill process has been described earlier in detail in the "Mills selected for Survey and Project Outline" section of this report. The work in this section is based on the 21 biotreated effluent samples taken at the mill between February 2007 and May 2009. The effluent samples were taken by mill staff during normal mill operating conditions, during periods of upsets and during periods of operational upsets following mill start-up. Of these, eight effluents were tested for their effects on egg production, five in short-term tests (5-d exposure to effluent) and three in one medium-term test (21-d exposure to effluent) with fathead minnow. The relationship between egg production and effluent quality as described by chemical parameters was assessed by establishing a time line which includes normal operating conditions and three major events at the mill. The first event was an evaporator upset the night of February 6, 2007 which resulted in a loss of black liquor to the aerated lagoon. The second event was a mill scheduled shutdown between September 1 and 11, 2008. The third event was a mill scheduled shutdown between December 13 and 28, 2008.

Effluent Chemical Analyses

Chemical profiling

All samples received by our laboratory were subjected to solvent extraction, followed by gas chromatography/mass spectrometry (GC/MS) analyses. A volume of 250 mL of effluent was added to a separatory funnel with 25 µL of an internal standard solution containing 1 mg/mL

each of methyl heneicosanoate and tricosanoic acid. Samples were extracted with 2 × 100 mL volumes of HPLC grade methyl tert-butyl ether (MTBE). The MTBE extract was transferred to a 250 mL round bottom flask and reduced to approximately 1.5 mL with a vacuum rotary evaporator then brought to a final volume of 200 µL in a vial under a gentle stream of nitrogen. The extract was then methylated with diazomethane and 2 µl injected into the GC/MS. GC/MS analyses were performed with an Agilent 6890 Series GC coupled with a 5973 mass selective detector. The MTBE extract was injected for analysis in splitless mode at 250°C. Analytes were separated on a 30m x 0.25 µm i.d. Rxi-1MS capillary column, 0.25 µm film thickness, from Restek; helium carrier at 1.3 mL/min. GC oven program: 60°C for 10 minutes then ramped at 5°C/min to 290°C and held for 20 minutes. An attempt was made to capture the difference in the amounts of organics present in effluents, in a quantitative manner, by comparing the total peak area for each chromatogram. Since each effluent extracted contained a C21 standard it was easy to compare the relative areas of each. This subjective comparison provided a means of differentiating between effluents. In the remainder of this manuscript this type of comparison will be referred to as a GC Profiling Index. For the purpose of establishing a comparison point, the effluent collected on November 10, 2008, that did not affect egg production, was assigned a GC Profiling Index of 1.0.

The chromatograms for the 21 effluent samples analyzed were examined for the presence of 2,5-dimethyl 2-cyclopentenone and 2,3,4- and 2,3,5-trimethyl 2-cyclopentenone. The confirmation for these isomers was accomplished by comparison with standards that hade been synthesized in our laboratories (Voss, 1984). Methyl substituted 2-cyclopentenones are formed in the kraft process as a result of aqueous alkaline degradation reaction of pure cellulose. It was also demonstrated (Voss, 1984) that a number of these methyl substituted 2-cyclopentenones were resistant to degradation in aerated lagoons and this makes them good indicators of black liquor losses.

The eight effluent samples used in reproduction tests were also analyzed for conventional effluent parameters in our laboratory, such as biochemical oxygen demand over five days (BOD), chemical oxygen demand (COD) and total suspended solids (TSS) according to standard methods (APHA,AWWA,WPCF, 1998) as well as resin/fatty acids by a chromatographic procedure (Voss and Rapsomatiotis, 1985). The BOD, COD and TSS results for the thirteen other effluent samples not used in fathead minnow reproduction tests were obtained from the mill.

Adult fathead minnow reproduction tests

The adult fathead minnow test method is an adaptation of a test developed by Ankley et al. (2001) and has been used for previous work with mill effluents (Kovacs et al., 2007a, 2007b). The sexually mature fathead minnows were raised in laboratory well water (temperature 25 ± 1°C, pH ~8.2, hardness ~250 mg/L as CaCO3, alkalinity ~160 mg/L as CaCO3) until displaying first signs of sexual maturity and separated by sex until aged between 8 and 14 months. The pre-exposure phase of the tests was initiated by distributing adult fish in groups of two males and four females in each aquarium containing two spawning substrates. From these we selected groups of fish that demonstrated good reproductive performance (≥18 eggs/female/day) and these groups were randomly assigned to one of the four replicates for each

of the treatments. Detailed test conditions are described in Table 1.2-1. Five short-term tests (5-d effluent exposure) were conducted with one effluent sample each and one medium-term test (21-d effluent exposure) was conducted using a different effluent sample each week. Effluents were delivered to various replicates through a serial diluter and temperature, dissolved oxygen and pH were monitored daily. During the exposure phase, eggs were collected daily and counted. Egg production at the end of the exposure was calculated as the number of eggs per female per day in each replicate. Although only egg production data is considered in this report as the critical endpoint, several other endpoints were also recorded in each test and those are summarized in Table 1.2-2. At the end of the exposure, the fish were sacrificed and measured for body weight and length. Gonads were excised and weighed. For each test, egg production and morphologic parameters (e.g., weight and length) of the effluent-exposed fish was compared to the egg production events of the control fish by the t-test (at the $p<0.05$ significance level) if the data met assumptions of normality and homogeneity (either with or without log transformation) or the Mann-Whitney test if the data failed assumptions of normality/homogeneity even after log transformation.

Table 1.2-1. Summary of test conditions for short-term tests with fathead minnow.

Pre-exposure phase	7 to 14 d
Effluent exposure	5 to 7 d
Tank turnovers	4/d
Quantity of effluent used	200 L/d
Replicates	4 (2 males and 4 females in 12.5 L)
Loading density	0.33 to 0.40 g/L
Photoperiod	16 h light and 8 h dark
Feeding	*Ad libitum*; Freshly hatched *Artemia* (brine shrimp) three-times a day
Endpoints measured	Egg production, number of spawns, egg fertilization Gonad weight, body length and weight
pH	7.4 to 8.5
Dissolved oxygen	> 60% saturation
Temperature	24 to 26°C

Table 1.2-2. Summaries of additional endpoints in the reproduction tests used in the biological monitoring. Results are presented as means with standard error.

Effluent Sampling Dates	Parameter	Control		100% Effluent	
		Males	Females	Males	Females
Feb. 19, 2007	CF	1.6 (0.1)	1.3 (0.0)	1.5 (0.1)	1.3 (0.0)
	GSI, %	1.2 (0.1)	12 (0.7)	1.4 (0.2)	14 (0.8)
	BW, g	5.4 (0.3)	2.2 (0.1)	5.0 (0.2)	2.4 (0.2)
	BL, mm	70 (1.7)	55 (0.9)	69 (1.3)	57 (1.2)
Nov. 3, 10 and 17, 2008	CF	1.6 (0.04)	1.5 (0.04)	1.5 (0.02)	1.5 (0.07)
	GSI, %	1.1 (0.2)	13.9 (0.9)	1.0 (0.2)	13.5 (1.2)
	BW, g	5.4 (0.2)	2.6 (0.1)	5.4 (0.2)	2.5 (0.1)
	BL, mm	71 (1.2)	56 (0.6)	72 (0.9)	56 (0.8)
Jan. 13, 2009	CF	1.4 (0.02)	1.3 (0.03)	1.4 (0.04)	1.3 (0.02)
	GSI, %	0.9 (0.2)	13.4 (0.7)	1.3 (0.2)	13.6 (1.1)
	BW, g	4.7 (0.3)	2.3 (0.1)	4.2 (0.2)	2.3 (0.1)
	BL, mm	70 (1.4)	56 (0.6)	67 (1.1)	56 (0.7)
Feb. 16, 2009	CF	1.6 (0.03)	1.3 (0.03)	1.5 (0.04)	1.3 (0.0)
	GSI, %	1.2 (0.1)	11.3 (1.3)	1.4 (0.1)	13.8 (1.8)
	BW, g	3.8 (0.3)	1.8 (0.1)	3.2 (0.1)	1.8 (0.1)
	BL, mm	63 (1.4)	52 (0.6)	60 (0.8)	53 (0.4)
Mar. 18, 2009	CF	1.5 (0.04)	1.3 (0.03)	1.5 (0.04)	1.3 (0.03)
	GSI, %	1.5 (0.1)	13 (1.0)	2.1 (0.2)	13 (1.1)
	BW, g	4.1 (0.1)	1.9 (0.1)	4.2 (0.2)	1.9 (0.1)
	BL, mm	65 (1.1)	53 (0.6)	65 (1.3)	52 (0.7)
May 29, 2009	CF	1.4 (0.04)	1.3 (0.03)	1.4 (0.06)	1.3 (0.03)
	GSI, %	1.3 (0.1)	13 (1.0)	1.7 (0.1)	14 (1.2)
	BW, g	4.0 (0.2)	2.5 (0.1)	3.7 (0.1)	2.2 (0.1)
	BL, mm	66 (1.0)	58 (0.6)	65 (1.1)	56 (0.7)

CF: Condition factor; GSI: Gonad somatic index, BW: Body weight, BL: Body length

Results and Discussion

Mill event 1: Evaporator upset of February 6, 2007

The first fathead minnow reproduction test was completed in February 2007 shortly after upsets were experienced by the evaporators. This effluent was found to significantly reduce egg production by 91% (see Table 1.2-3). The concentrations of BOD and COD were 28 and 404 mg/L, respectively. The total methyl substituted 2-cyclopentenone concentration was 103.2 µg/L. The GC Profile Index was established at 1.75. This was the first egg production result that could be associated to a process difficulty where black escaped the recovery process and was accidentally released to the aerated lagoon. The presence of methyl substituted 2-cyclopentenones supported the hypothesis that black liquor was contributing organic material and could tentatively be associated to decreased egg production. However, more monitoring using the GC Profiling Index was necessary to determine the level of effluent quality necessary to prevent effects on egg production in fathead minnows.

Mill event 2: Mill shutdown between September 1 and 10, 2008

The extended chemical monitoring of effluents started on August 14 before a mill maintenance shutdown scheduled to occur between September 1 and 10, 2008. Five samples were analyzed for their GC Profile Index before the shutdown and these varied between 1.01 and 1.65 showing appreciable variation in organic content (Table 1.2-3) and are shown with BOD, COD and TSS data supplied by the mill in Table 1.2-3. Seven more effluent samples were analyzed starting two weeks after the mill start-up (September 14) in September and October. The first 4 samples taken between September 29 and October 9 showed GC Profile Indices varying between 1.47 and 2.53 suggesting that effluent quality was very variable. BOD values ranged from 26 to 43 mg/L but only trace amounts of 2-cyclopentenones were detected, suggesting that there were no obvious losses of black liquor but that the organic content of treated effluents was nonetheless elevated. From October 13 to 20, the GC Profile Indices stabilized between 0.74 and 1.17 were associated with BOD levels ranging from 14 to 31 mg/L. It is at this point that we proceeded with a 21 day effluent exposure during which three effluent samples were used in successive weeks. In Table 1.2-3 these samples are listed as November 3, 10 and 17. We chose to present the egg production data for controls and 100% exposed fish on a weekly basis as if they were tested independently for the purpose of extracting information illustrating the link with effluent characteristics. When considered on a weekly basis there was no statistically significant reduction in egg production during these three weeks. The same was true of the overall egg production over the 21 days of the exposure with mean (standard error) egg production for controls and 100% effluent exposed fish of 36 (4) and 36 (6), respectively. Only trace amounts of 2-cyclopentenones were detected on November 10 and 13, suggesting that black liquor losses were not a significant factor for these samples. Values for TSS and RFA were at their lowest levels between November 3 and 17, while COD were the highest so far between 389 and 415 mg/L. Together these results suggest that an effluent quality level illustrated by a GC Profile Index close to 1.00 and BOD levels below 20 mg/L would result in effluents with no significant effects on egg production.

Table 1.2-3. Summaries of egg production and chemical analysis results.

Effluent sample collection date	Mean egg production eggs/female/day (SEM)		GC Profile index	BOD mg/L	COD mg/L	TSS mg/L	RFA mg/L	Methyl substituted 2-cyclopentenones µg/L		
	100% effluent control							2,5-	2,3,4-	2,3,5-
Mill event 1: Evaporator upset February 6, 2007										
Feb. 19, 2007	7* (3)	81 (7)	1.75	28	404	23	0.10	34.3	37.3	31.6
Aug. 14, 2008			1.42	26[1]	277[1]	27[1]	0.04	nd	nd	nd
Aug. 18, 2008			1.01	19[1]	286[1]	27[1]	0.04	nd	nd	nd
Aug. 21, 2008			1.35	15[1]	248[1]	17[1]	0.04	nd	nd	nd
Aug. 25, 2008			1.62	15[1]	252[1]	30[1]	0.03	nd	nd	nd
Aug. 28, 2008			1.35	20[1]	266[1]	20[1]	0.03	nd	nd	nd
Mill event 2: Mill shutdown between September 1 and 10, 2008.										
Sep. 29, 2008			2.04	26[1]	268[1]	30[1]	0.17	nd	nd	nd
Oct. 2, 2008			1.95	43[1]	322[1]	43[1]	1.07	nd	nd	1.7
Oct. 7, 2008			2.53	31[1]	302[1]	30[1]	0.06	nd	nd	nd
Oct. 9, 2008			1.47	32[1]	316[1]	30[1]	0.03	nd	nd	nd
Oct. 13, 2008			0.74	16[1]	317[1]	27[1]	0.01	nd	nd	nd
Oct. 16, 2008			1.16	22[1]	323[1]	23[1]	0.06	nd	nd	nd
Oct. 20, 2008			1.17	31[1]	344[1]	20[1]	0.07	nd	nd	nd
Nov. 3, 2008	30 (7)	33 (4)	0.65	14	403	10	0.02	nd	nd	nd
Nov.10, 2008	28 (7)	40 (5)	1.00	19	415	5	0.03	nd	0.5	10.3
Nov. 17, 2008	49 (6)	34 (4)	0.90	17	389	15	0.04	nd	nd	4.1
Mill event 3: Mill shutdown between December 13 and 28, 2008 and difficulties with lagoon operation at startup.										
Jan. 13, 2009	4 * (4)	35 (9)	1.46	53	426	19	0.06	41.6	29.0	41.0
Feb. 3, 2009			1.43	32[1]		20[1]	0.12	15.3	30.5	33.4
Feb. 16, 2009	21 (4)	33 (4)	0.84	15	345	12	0.05	nd	nd	8.2
Mar. 18, 2009	18 (6)	33 (11)	1.15	18	307	27		28.5	32.4	27.1
May 29, 2009	15 (2)	19 (4)	0.75	16	301	14	0.04	nd	nd	nd

SEM: standard error of the mean; BOD: biochemical oxygen demand over five days; COD: chemical oxygen demand; TSS: total suspended solids; RFA: resin and fatty acids; [1]: BOD, COD and TSS data supplied by the mill; *: statistically significant ($p<0.05$) decrease in egg production by fish exposed to 100% effluent compared to those in controls; nd: methyl substituted 2-cyclopentanones are below the detection limit of 0.5 µg/L.

Mill event 3: Mill shutdown between December 13 and 28, followed by difficulties in the operation of the aerated lagoon

The second scheduled shutdown occurred between December 13 and 28, which provided the opportunity to monitor effluent quality soon after start-up when effluent quality may be variable and during recovery and stabilization of the process. Upon start-up of the mill, the ambient temperature in January was exceptionally low (~ -30°C). This made the start-up of the effluent biotreatment in the aerated lagoon particularly difficult. According to mill records, residual BOD levels for the first two weeks in January ranged between 76 and 98 mg/L. The third reproduction test was conducted with effluent collected January 13, 2009 which caused an 88% inhibition in the mean number of eggs produced per female per day. The concentrations of methyl substituted 2-cyclopentenones were found at their highest level in this study, totalling 111.6 µg/L and suggesting that black liquor was contributing organic substances. This was accompanied by a GC Profile Index of 1.46, of the same order as those recorded after the September 2008 shutdown and the highest BOD (53 mg/L) and COD (426 mg/L) values recorded for the twenty-one samples analyzed. The concentrations of RFA and TSS were not significantly different from those measured during the stabilized operation in October. A month after start-up (February 3) the presence of methyl substituted 2-cyclopentenones was still evident with a total of 79.2 µg/L. The GC Profiling Index and BOD were still elevated at 1.43 and 32 mg/L, respectively.

To monitor the recovery of the process and aerated lagoon to stabilized operations, the last three reproduction tests were conducted February 16, March 18 and May 29, 2009. The three samples had GC Profiling Indices between 0.75 and 1.15 and BOD concentrations between 15 and 18 mg/L. These samples did not cause statistically significant reductions in egg production, even though the sample of March 18 caused a reduction of 45% in egg production. The egg production by the 100% effluent-exposed fish is within the criteria for control as indicated by the control production during the test of the effluent sampled on May 29, 2009. Nevertheless, the 45% reduction of egg production by the fish exposed to the March 18 sample may be symptomatic of a decrease in effluent quality. The concentration of methyl substituted 2-cyclopentenones in the sample of March 18 was ~ 98 µg/L, which suggests weak black liquor losses.

Assessment of chemical and biological monitoring tools

The GC Profiling Index has the possibility to be a useful chemical means of comparing the effluent quality at different times. An illustration of the quality of the effluents on the basis of the GC Profiling index is provided in Figure 1.2-1. The chromatograms illustrate the GC/MS trace for two effluents that significantly affected egg production (February 19, 2007 and January 13, 2009) and the reference November 10, 2008 sample that did not affect egg production. From a visual inspection of the chromatograms, there is a lower number of individual peaks and an overall lower intensity of peaks for the effluent that did not affect egg production when compared to those that did affect fish reproduction. The greater number and intensity of peaks indicate the presence of a greater proportion of organic content. A plot of the GC Profiling Index and egg production results presented in Figure 1.2-2 shows that the GC Profiling Index describes fairly well the quality of the effluent samples in terms of effects on egg production, that is the higher the GC Profile Index, the greater the effect on egg production, yielding a strong correlation coefficient of -0.77575 for the eight data points available.

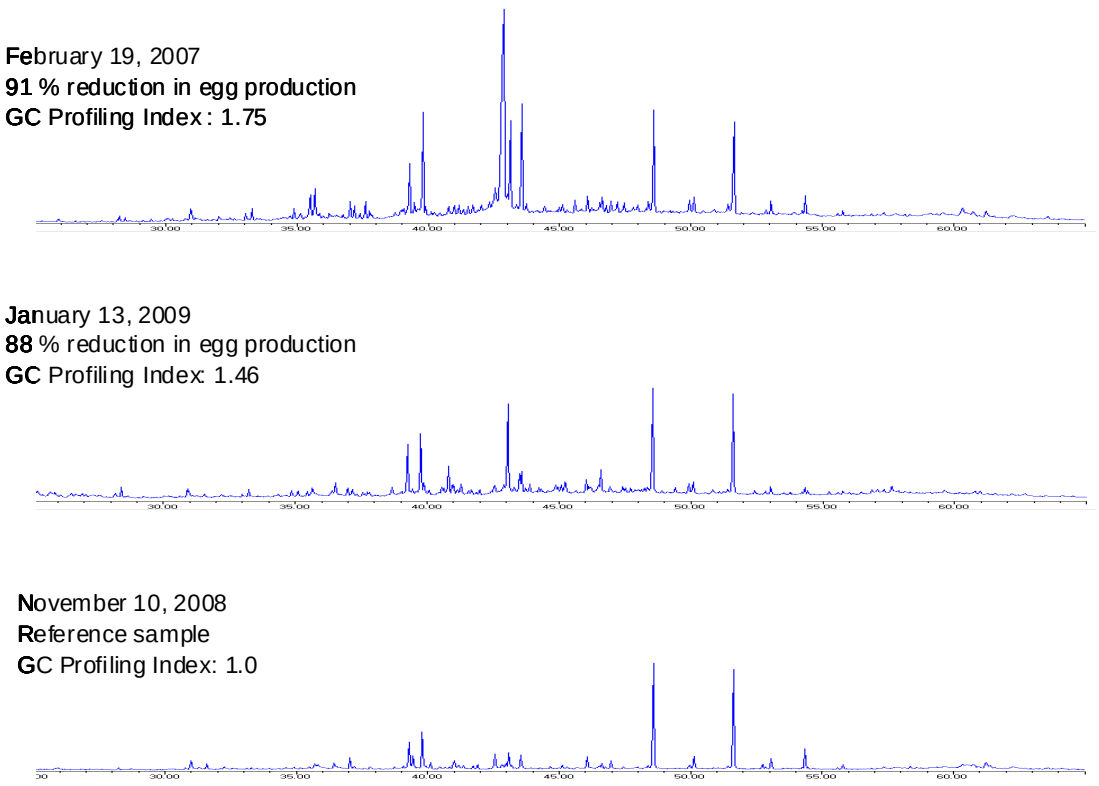

Figure 1.2-1. Illustration of chromatograms for the two samples found to cause a significant reduction in egg production in fathead minnows and the sample chosen as a reference for the Gas Chromatogram profiling Index.

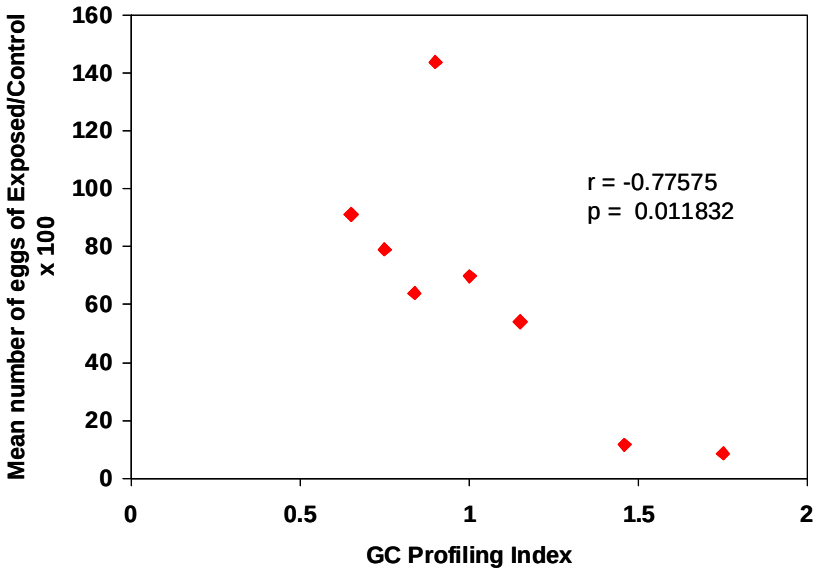

Figure 1.2-2. Relationship between total the GC Profiling Index and egg production (expressed as the ratio of the egg production of the exposed fish over that of the controls).

From the data compiled in Table 1.2-3 significant effects on egg production occurred in samples where BOD concentrations were above 19 mg/L. The relationship between egg production (expressed as the ratio of the egg production of the exposed fish over that of the controls) versus the BOD of the final treated effluents for the Domtar Espanola kraft mill is shown in Figure 1.2-3. The strength of a linear relationship between the two variables is moderate but significant (r = -0.66516 and p= 0.03593) and while more data points would be necessary to strengthen this comparison, a trend is apparent which suggests that the control of residual BOD of final treated effluents to approximately 20 mg/L or below, should produce effluents that will have a minimum impact on egg production in the adult fathead minnow test.

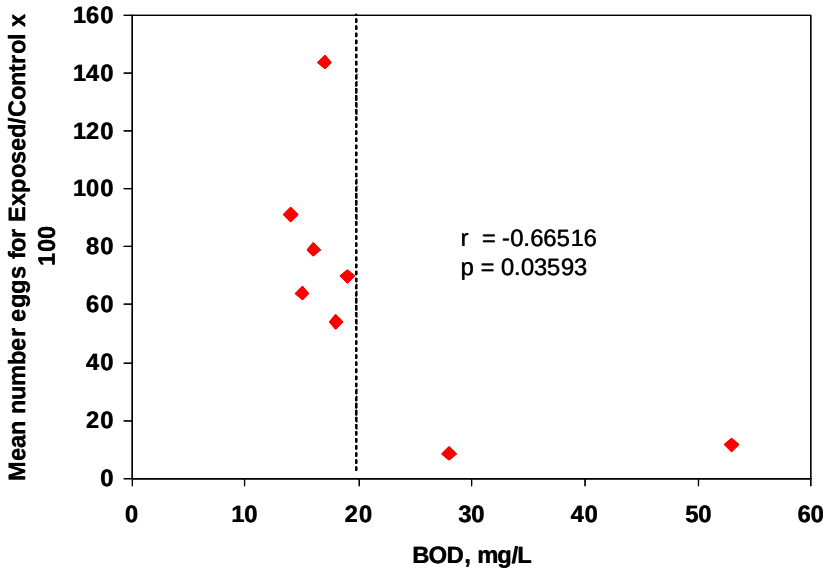

Figure 1.2-3. The relationship between BOD of the final treated effluents and egg production (expressed as the ratio of the egg production of the exposed fish over that of the controls).

The first and third mill events are associated with a significant reduction in egg production and both are linked to black liquor escaping the recovery process as witnessed by the presence of methyl substituted 2-cyclopentenones in concentrations > 100 µg/L. When the total concentration of methyl substituted 2-cyclopentenones is plotted against egg production a strong correlation is found (r = -0.7980 and p = 0.0088) which suggest that the presence of 2-cyclopentenones is associated with effects on egg production although no direct links are implied (see Figure 1.2-4). The third mill event is also related to the reduced efficiency of the aerated lagoon during a difficult start-up during a period of extreme cold. Mill records indicate that biotreated effluent BOD during this period was between 76 and 98 mg/L. The other measurements of biotreated effluent quality, COD, TSS and RFA, did not demonstrate a significant association with the egg production results. From this dataset it appears that these parameters do not provide a good indication of potential biological effects related to fish reproduction for the effluents we examined.

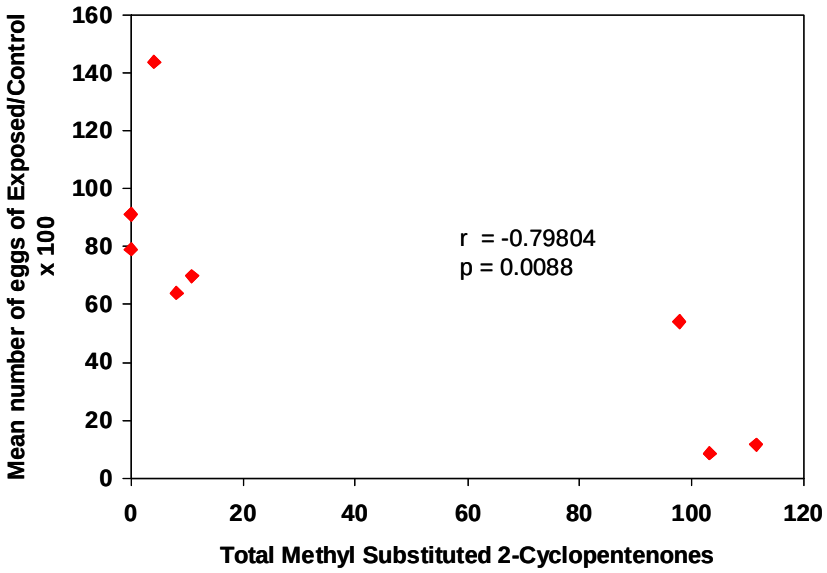

Figure 1.2-4. Relationship between total methyl substituted 2-cyclopentenones and egg production (expressed as the ratio of the egg production of the exposed fish over that of the controls).

Our ability to link mill events and chemical parameters to biological effects relied on the power of the fathead minnow reproduction test to discriminate between effluent samples of varying quality. To date, egg production appears as a reliable and sensitive endpoint for IOC and IOS work. Additional monitoring, over an extended period of time, would be necessary to further refine the level at which a GC Profiling Index, BOD or methyl substituted 2-cyclopentenones offer the best monitoring chemical monitoring approach to control the potential for kraft mill effluents to affect egg production.

Conclusions

The evidence to date suggests that variations in the quality of final effluents can be tracked by effects on egg production in fathead minnow, the GC Profiling Index, BOD and the presence of methyl substituted 2-cyclopentenones. These appear to be promising diagnostic tools for IOC and IOC work.

The events having negative effects on egg production were upsets of the recovery system and black liquor losses and conditions that reduce the performance of biotreatment (e.g., mill shutdown and low ambient temperatures).

1.3 Evaluation of Reproductive Effect Endpoints in Fish Exposed to a Simulated Black Liquor Spill

The long-term monitoring at the bleached kraft mill in Espanola suggested that there could be a link between black liquor losses and inhibition of egg production. To examine this further an experiment was conducted whereby a final treated effluent and water were spiked with a small amount of black liquor from the mill and tested with various short- and medium-term tests. While this experiment was not designed to accurately simulate the effect of black liquor losses at the mill, since the black liquor would normally be subject to degradation in the treatment system, it would examine whether there were active components of interest present in these streams.

1.3.1 Black Liquor Preparation

The following is a summary of the preparation of the diluted black liquor (BL) which was used for all the experiments described in this section. On May 6, 2008 approximately 20 L of BL was sampled by mill personnel of the Domtar bleached kraft mill in Espanola and shipped to FPInnovations laboratories in Pointe-Claire, QC. The BL was diluted with deionized water and the pH adjusted to 7.8 with 36–38% HCl. The final dilution of the BL stock solution was adjusted with deionized water to 10% v/v. The BL test concentrations used in the various bioassays were determined on the basis of a range finding reproduction test with fathead minnow at concentrations of 0.1%, 0.08%, 0.06% and 0.04% BL. The 0.1% BL was found to be acutely toxic and 0.04% was found not to inhibit egg production. On this basis, BL concentrations of 0.06% and 0.08% were recommended for testing. The 10% BL stock solution was divided into portions sufficient for each exposure and shipped to the laboratories of the Project team members.

1.3.2 Medium-term Test with Fathead Minnow

The medium test with fathead minnow exposes maturing fish to effluent for 30 days or more. The extended exposure during the maturation phase was thought to be a possible way to capture effects of the effluent on gonadal growth. The test can also measure reproductive capacity, depending on the age of the juveniles at the start of the exposures. Endpoints include growth, LSI, GSI, the development of secondary sexual characteristics, and spawning events, as well as biochemical indicators. The duration of the test is approximately one month.

The Espanola test was started on May 28, 2009, with fish that were 71 ± 3 days old (days post-hatch, or dph). The test ended June 27 and 28, 2009, after 30–31 days of exposure to effluent (100%) and black liquor (0.06 or 0.08%).

Methods

Effluent collection and storage

Effluent (2,000 L) was shipped weekly from Espanola to Burlington, ON. Shipping took 1 to 3 days. Diluted black liquor was shipped once. Effluent and black liquor was stored at 4°C until ready to use. Effluent flowed through a diluter which mixed it with Burlington laboratory water. Black liquor stock solution was pumped by peristaltic pumps into diluter mixing chambers. Flows were checked daily and recorded for confirmation of delivered volumes. Exposure concentrations were 100% effluent, 0.06% black liquor, 100% effluent + 0.06% black liquor, and 100% effluent + 0.08% black liquor. There were 4 replicates of each effluent concentration and 8 replicates of control (lab water) tanks. Effluent and lab dilution water flow was 25 mL/min to each aquarium, which provided about 3 solution turnovers per 24 h.

Fish exposures

Exposures were started with 71 day old (± 3 days) fathead minnows that had been hatched and reared in Burlington lab water. Fish care and methods are detailed in Parrott and Blunt (2005) and Parrott and Bennie (2009). Fish were immature at the start of the test. There were 15 fish added to each aquarium. After 30 d exposures, most of the fish were still immature, and dissections were difficult. Fish sex was assessed externally, internally (by assessment of the gonads), and by histological sectioning of gonadal tissues. Three breeding tiles were added to each aquarium to promote maturation and reproductive behaviours. There was no breeding during the exposures.

Table 1.3.2-1. Test conditions for 30-day test with fathead minnow.	
Date(s) effluent sampled	Weekly, May 25, 2009 to June 22, 2009
Pre-exposure phase	In lab, fish were 71 (± 3) d old at start of test
Effluent exposure	30–31 d
Tank turnovers	3 volumes/d
Quantity of effluent used	20,000 L (5 weeks)
Replicates	8 for control, 4 for each effluent concentration
Loading density	0.4 g/L/d
Feeding	Daily - frozen (and thawed) brine shrimp slurry
Endpoints measured	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	7.7 to 8.2 (see Table 1.3.2-1b)
Dissolved oxygen,	6.5 to 7.8 mg/L (see Table 1.3.2-1b)
Temperature	23.9 to 24.8°C (see Table 1.3.2-1b)

Fish sampling and grading of sex characteristics

After 30–31 days of exposure, fish were sampled as described in Parrott and Blunt (2005) with the following exceptions.

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 + 2 x large tubercles)/10 + banding score.

For female fish, ovipositor length and width were measured under a dissecting microscope, and triangular ovipositor area was calculated as length x width / 2.

Statistical analyses

Data were analyzed using Systat 11. Growth parameters of length (mm), weight (g), condition factor (CF), LSI, GSI, ovipositor area (mm^2), and male index, were assessed for differences

among treatments using ANOVA. Significant differences from controls were assessed using two sample t tests (separate variances) to determine levels of significance. P values were depicted in figures and tables as asterisks: * $p < 0.05$.

Results and Discussion

Water quality and black liquor exposure concentrations

Temperature was constant in exposure aquaria, but dissolved oxygen, pH and conductivity varied with effluent and black liquor treatment (Table 1.3.2-1b)

Table 1.3.2-1b. Mean (± standard deviation) temperature (°C), dissolved oxygen (mg/L), pH, and conductivity (µS/cm) in control water and in Espanola effluent and black liquor treatment combinations.

Treatment	Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Conductivity (µS/cm)
Control	24.2 ± 0.68	7.82 ± 0.45	7.72 ± 0.10	353 ± 18
100% Effluent	23.9 ± 0.72	7.36 ± 0.76	8.21 ± 0.12	1,627 ± 132
0.06% Black Liquor	24.7 ± 0.48	7.22 ± 0.51	7.82 ± 0.12	418 ± 18
Effluent + 0.06% Black Liquor	24.7 ± 0.39	6.77 ± 0.64	8.19 ± 0.11	1,727 ± 131
Effluent + 0.08% Black Liquor	24.8 ± 0.28	6.49 ± 0.83	8.17 ± 0.13	1,710 ± 93

Daily flow checks of peristaltic pumps were necessary to ensure delivery of accurate concentrations of black liquor and effluent + black liquor treatments. For the nominal '0.06% black liquor' the delivered volume resulted in an actual black liquor concentration of 0.055 ± 0.006% (mean and standard deviation, n = 26). For the nominal '100% effluent + 0.06% black liquor' the delivered volume resulted in an actual black liquor concentration of 0.055 ± 0.005% (n = 28). For the nominal '100% effluent + 0.08% black liquor' the delivered volume resulted in an actual black liquor concentration of 0.075 ± 0.006% (n = 27).

Fish maturation and growth

At the end of the 30–31 d exposure, fish were just beginning to mature (as they were about 100 days old). Only 31 of 359 fish (8.6%) had dorsal fin dots and dorsal fatpads, 22 fish had tubercles, and 3 fish had bands or a dark head. As a result, male index was very low for all groups, and ranged from 0.3 to 0.6 (Table 1.3.2-2). Female ovipositor area was also very low, ranging from 0.31 to 0.45 mm^2. 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). Because of their young age and lack of sex characteristics, fish did not breed in any of these 30 d exposures.

Exposure to Espanola effluent increased growth of fish. Increased weight of females was seen in all treatments that had effluent (100% Effluent, 100% Effluent + 0.06% Black Liquor, 100% Effluent + 0.08% Black Liquor). Condition was increased in male and female fathead minnows exposed to 100% Effluent + 0.06% Black Liquor and 100% Effluent + 0.08% Black Liquor.

Effluent and black liquor exposure also increased relative liver size (liver-somatic index or LSI) in male and female fathead minnows.

Gonad growth and ovipositor area was increased in females exposed to 100% Effluent + 0.08% Black Liquor.

Conclusions

- Exposure to Espanola effluent increased growth of fish.
- Effluent and black liquor exposure increased LSI.
- Gonad growth and ovipositor area was increased in females exposed to 100% Effluent + 0.08% Black Liquor.
- The pattern of effects seen with exposure to Espanola effluent and black liquor combinations was consistent with a eutrophication response (larger fish, larger livers).
- The 30 day fathead minnow juvenile to adult test was able to pick up this response pattern.

Table 1.3.2-2. Mean (± standard error) length, weight, condition factor (CF), liver-somatic index (LSI), gonadosomatic index (GSI), Male Index and Ovipositor Area (Ovi Area) for male and female juvenile fathead minnows exposed for 30–31 days to Espanola effluent (100%) and black liquor combinations. n is indicated in second column. Bold text with asterisk indicates significant difference ($p<0.05$) from controls, tested by two-sample t-test.

Males:	n	Length (mm)	Weight (g)	CF	LSI	GSI	Male Index
Control	52 – 53	43.5 ± 0.84	0.853 ± 0.055	0.986 ± 0.024	1.92 ± 0.086	0.514 ± 0.073	0.345 ± 0.11
Effluent 100%	29	42.5 ± 1.1	0.857 ± 0.09	1.03 ± 0.03	**2.81* ± 0.18**	0.437 ± 0.098	0.517 ± 0.26
Black Liquor 0.06%	38 – 39	43.0 ± 1.1	0.917 ± 0.08	1.04 ± 0.020	**2.59* ± 0.11**	0.537 ± 0.094	0.641 ± 0.20
Effluent + 0.06% Black Liquor	31 – 32	43.5 ± 1.1	0.937 ± 0.08	**1.06* ± 0.03**	2.72* ± 0.21	0.673 ± 0.12	0.619 ± 0.21
Effluent + 0.08% Black Liquor	33 – 34	43.0 ± 0.97	0.939 ± 0.082	**1.08* ± 0.03**	**3.29* ± 0.15**	0.601 ± 0.12	0.418 ± 0.18
Females:	n	Length (mm)	Weight (g)	CF	LSI	GSI	Ovi Area (mm^2)
Control	55	41.1 ± 0.47	0.684 ± 0.026	0.946 ± 0.02	2.28 ± 0.10	4.81 ± 0.51	0.306 ± 0.04
Effluent 100%	28	41.9 ± 0.51	**0.792* ± 0.04**	1.06 ± 0.020	**2.89* ± 0.19**	5.27 ± 0.79	0.328 ± 0.05
Black Liquor 0.06%	20	40.3 ± 0.69	0.661 ± 0.04	0.996 ± 0.03	**2.67* ± 0.12**	5.26 ± 1.2	0.311 ± 0.06
Effluent +0.06% Black Liquor	22 – 23	41.9 ± 0.74	**0.814* ± 0.05**	**1.08* ± 0.03**	2.51 ± 0.11	5.37 ± 1.2	0.373 ± 0.07
Effluent+ 0.08% Black Liquor	19 – 20	42.5 ± 0.62	**0.850* ± 0.05**	**1.12* ± 0.1**	**2.71* ± 0.26**	**7.43* ± 1.2**	**0.458* ± 0.06**

1.3.3 Short-term Test with Fathead Minnow and Rainbow Trout

The objective of this activity was to assess the applicability of short-term (<5 d) tests for IOC and IOS work aimed at eliminating/reducing mill effluent-related effects on fish reproduction. Based on observations made during the National IOC Project that black liquor spills may be linked to reproductive effects in fish during laboratory tests (see Section 1.2) we compared the response of fish exposed to mill effluent to which a small quantity of black liquor (BL) was added to simulate the impact of a spill. Two freshwater species, the fathead minnow (*Pimephales promelas*) and the rainbow trout (*Oncorhynchus mykiss*) were used in tests to compare the ability of various endpoints such as egg production and vitellogenin activity in tracking changes associated with the simulated spill. The expectation was that the addition of BL to the biotreated effluent should increase the effluent potency on various endpoints related to reproduction.

Methods

Short-term test with adult fathead minnow

The adult (11 month old) fathead minnow was used to assess overall reproductive performance. The fish, separated by sex, were raised in laboratory well water (also used for making effluent dilutions) as described in Kovacs et al. (2007b). The short-term test (seven days pre-exposure and five days effluent exposure) is adaptations of a test developed by Ankley et al. (2001) and has been used at FPInnovations for previous work with mill effluents (Kovacs et al., 2007b). 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 tests was initiated by selecting groups of fish that demonstrated good reproductive performance (≥18 eggs/female/day, ≥3 spawning events over 7 to 8 days) during the pre-exposure phase and these groups were randomly assigned to one of the four replicates for each of the treatments. The effluent-exposure phase occurred in two separate experiments initiated 1 day apart. In Experiment I minnows were exposed to 0%, effluent (control water), 100%, effluent + 0.06% BL and 100% effluent + 0.08% BL. In Experiment II minnows were exposed to 0% effluent (control) and water to which 0.06% BL was added. The detailed test conditions are given in Table 1.3.2-1.

In both experiments the fish were monitored for the number of spawns, egg production and egg fertilization. At the end of the tests, after anaesthesia each fish was weighed and measured for length (condition factors or weight/length^3 were calculated). Plasma was collected by caudal puncture and assayed for testosterone (T) (males and females), estradiol (E2) (females) and vitellogenin activity (males) as described previously (Kovacs et al., 2007b). The gonads were excised and weighed to calculate gonad somatic indices (gonad weight/body weight x 100).

Short-term test with immature rainbow trout

Immature rainbow trout were used in a 7 d test to specifically measure levels of plasma vitellogenin (VTG) and hepatic mixed function oxidase (MFO) enzyme activity. Vitellogenin is a protein normally produced only by mature females during oogenesis. Hepatic MFO activity is a marker of exposure to substances inducing a family of enzymes involved in detoxification. MFO enzyme activity is measured on an artificial substrate as ethoxyrsorufin-*O*-deethylase (EROD)

activity. The fish were exposed in 40 L containers for seven days with daily effluent solution renewals at concentrations of 0% (control water), 100% effluent, 100% effluent + 0.06% BL, 100% effluent + 0.08% BL and water + 0.06% BL. The conditions for the test are given in Table 1.3.2-2.

Table 1.3.3-1. Test conditions for short-term tests with fathead minnow.	
Date effluent sampled	May 26, 2009
Pre-exposure phase	7 d
Effluent exposure	5 d
Tank turnovers	4/d
Quantity of effluent used	5000 L
Replicates	4 (2 males and 4 females in 12.5 L)
Loading density	0.24 to 0.37 g/L
Feeding	*Ad libitum*; Freshly hatched *Artemia* (brine shrimp) three-times a day
Endpoints measured	Egg production, number of spawns, Egg fertilization, Length, weight, In males: testosterone and vitellogenin In females: testosterone and estradiol
pH	8.1 to 8.7
Dissolved oxygen	70 to 94% saturation
Temperature	24 to 26°C

Table 1.3.3-2. Test conditions for short-term test with immature rainbow trout.	
Date effluent sampled	May 26, 2009
Effluent exposure	7 d
Tank turnovers	1/d
Quantity of effluent used	1392 L
Replicates	2 (3 fish in 40 L)
Loading density	0.29 to 0.59 g/L
Feeding	None
Endpoint measured	Vitellogenin Ethoxyresorufin -*O*- deethylase
pH	7.9 to 8.6
Dissolved oxygen	75 to 100% saturation
Temperature	11 to 14°C

At the end of the exposure period, the trout were anesthetized and weighed. Blood was collected by caudal puncture, pooled and centrifuged at 26000 g for 3 minutes. The plasma was collected

and 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. The livers of fish in a replicate were removed, rinsed and pooled before homogenization in Tris-KCl pH 7.4 buffer at 4°C and centrifugation at 10 000 g for 25 minutes. The resulting supernatants were stored at -85°C until analyzed for EROD activity following the method described in Martel et al. (1995).

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. When necessary, the data were log transformed to meet assumptions of normality and homogeneity. When the data met these assumptions, statistically significant differences were determined using parametric analysis of variance (ANOVA) or the two-sample t-test. In cases when the ANOVA indicated a significant effluent-related effect, the Dunnett's test was used to identify the specific effluent concentrations that had a statistically significant difference from the control. For data that failed to meet the assumptions of normality or homogeneity, the non-parametric Kruskal-Wallis and the two-sample Mann-Whitney tests were used. When the Kruskal-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 statistical difference from the control. All statistical comparisons were made at the 5% significance level ($p<0.05$). For all test endpoints, the aquarium was the experimental unit of replication. Measurements collected from individual fish (i.e., full body weight, length, gonad weight) were analyzed using a nested factor and a covariate (i.e., gonad weight a covariate of body weight and for fish weight a covariate of length). Pooled and averaged data (i.e., the mean eggs produced per female per day, number of spawns, % fertilization, % hatching from fertilized eggs, steroids, vitellogenin, EROD) were analyzed without nesting or covariates.

Results and Discussion

Short-term adult fathead minnow reproduction test

Egg production and spawning: In Experiment I we compared the response of minnows exposed to effluent and effluent spiked with two concentrations of BL. The concentrations of BL were chosen to be representative of a spill but without causing acute toxicity and mortality. The results of this testing is provided in Table 1.3.3-3. Egg production, number of spawns and egg fertilization in fish exposed to 100% effluent was not different from controls. In contrast, adding 0.06% and 0.08% BL to effluent caused a reduction of egg production to 1 and 2 eggs/female/day respectively. The mean number of spawning events in these treatments was not significantly reduced compared to controls. The effluent did not affect the fertilization rate in treatments where egg production was normal but could not be calculated in the other treatments because of the small number of eggs produced. Overall, Experiment I showed that egg production was the most sensitive endpoint and this endpoint was able to detect the worsening effluent quality resulting from the addition of BL. The experiment also conclusively confirmed that black liquor losses could influence final mill effluent quality as suggested in Section 1.2.

In Experiment II we examined the effect of BL alone. The addition of 0.06% BL to water alone was sufficient to virtually stop spawning and egg production. It is the first time that BL alone was tested and found to have an effect on egg production. This again showed that the role of BL in causing effects and that egg production was a sensitive endpoint.

Table 1.3.3-3. Egg production, number of spawning events and egg fertilization for fathead minnows exposed to mill effluent, effluent in combination to black liquor (BL) and BL alone. Results are expressed as means (standard error).

Treatment	Number of eggs/female/day	Number of spawning events	Egg fertilization rate, %
Experiment I			
Control	19 (4)	3 (1)	90 (3.7)
100% effluent	15 (2)	3 (0)	99 (0.9)
100% effluent + 0.06% BL	1* (0)	2 (0)	‡
100% effluent + 0.08% BL	2* (2)	1 (0)	‡
Experiment II			
Control	41 (5)	4 (1)	92 (2)
0.06% BL in water	0† (0)	1† (0)	‡

* statistically significant difference from control, $p < 0.05$ (Anova and Dunnett's)
† statistically significant difference from control, $p < 0.05$ (t-test)
‡ insufficient number of eggs to calculate fertility

Morphometric parameters: In the EEM Program morphometric parameters in wild fish such as body weight, body length, liver weight and gonad weight are used to establish differences between populations from control and effluent impacted areas. In short-term tests the parameters are also measured but because of the very short duration of exposure their usefulness is more in establishing that the fish used in controls and treatments are comparable. In all treatments of Experiments I and II, the GSI of males and females were not statistically different from controls (Table 1.3.3-4). Similarly for the morphological parameters of males and females there were no differences in weights and lengths and condition factors.

Steroid hormones and vitellogenin: Plasma steroid hormones levels are used in the field and in laboratory studies to compare the physiological status of fish during periods of sexual maturation or during reproduction. Previous studies have shown that such indicators can be affected by mill effluents (reviewed in Hewitt et al., 2008). Vitellogenin measured in males is used as a marker of exposition to potentially estrogenic substances. In Experiments I and II, plasma testosterone and vitellogenin levels in males were not significantly affected by 100% effluent or 100% effluent spiked with BL or BL spiked in water (Table 1.3.3-5). In females, estradiol levels were not affected in fish exposed to 100% effluent, or 100% effluent spiked with 0.06% and 0.08% BL but were reduced by 1.8-fold in females exposed to 0.06% BL in water. Testosterone levels in females were not affected by 100% effluent, 100% effluent spiked with 0.06% BL or 0.06% BL spiked in water but were significantly elevated 2.7-fold in females exposed 100% spiked with

0.08% BL. These endpoints provided no consistent response to BL and appeared to be less sensitive that the egg production endpoint in these tests.

Table 1.3.3-4. Morphometric parameters and gonad somatic index for fathead minnows exposed to mill effluent, effluent in combination to black liquor (BL) and BL alone. Results are expressed as means (standard error).

Treatment		Fork Length, mm	Body weight, g	Condition factor	Gonad weight, mg	Gonad somatic index, %
Experiment I						
Control	Females	58 (0.6)	2.5 (0.1)	1.3 (0.03)	327 (26)	13 (1.0)
	Males	66 (1.0)	4.0 (0.2)	1.4 (0.04)	53 (4.2)	1.3 (0.1)
100% Effluent	Females	56 (0.7)	2.2 (0.1)	1.3 (0.03)	314 (37)	14 (1.2)
	Males	65 (1.1)	3.7 (0.1)	1.4 (0.06)	63 (5.2)	1.7 (0.1)
100% Effluent + 0.06% BL	Females	56 (0.9)	2.3 (0.1)	1.3 (0.02)	317 (24)	14 (1.0)
	Males	65 (1.0)	3.9 (0.2)	1.4 (0.05)	53 (4.1)	1.4 (0.1)
100% Effluent + 0.08% BL	Females	57 (0.7)	2.5 (0.1)	1.3 (0.02)	377 (34)	15 (1.0)
	Males	67 (1.4)	4.0 (0.2)	1.3 (0.04)	64 (8.2)	1.6 (0.2)
Experiment II						
Control	Females	57 (0.5)	2.3 (0.1)	1.3 (0.02)	270 (20)	12 (0.9)
	Males	67 (1.5)	4.2 (0.2)	1.4 (0.03)	37 (2.9)	0.9 (0.1)
Water + 0.06% BL	Females	54 (0.9)	2.2 (0.1)	1.3 (0.05)	309 (32)	14 (1.1)
	Males	67 (0.8)	4.0 (0.2)	1.3 (0.03)	41* (5.5)	1.0 (0.1)

* statistically significant difference from control, $p < 0.05$ (t-test)

Table 1.3.3-5. Plasma estradiol, testosterone and vitellogenin for fathead minnows exposed to mill effluent, effluent in combination to black liquor (BL) and black liquor alone. Results are expressed as means (standard error).

Treatment	Sex	Estradiol, pg/ml	Testosterone, pg/ml	Vitellogenin, ng/ml
Experiment I				
Control	Males		2230 (654)	177000 (99200)
	Females	14000 (654)	2080 (418)	
100% Effluent	Males		2760 (625)	255000 (113000)
	Females	11900 (2650)	2260 (286)	
100% Effluent + 0.06% BL	Males		2620 (659)	858000 (367000)
	Females	10600 (1150)	3320 (704)	
100% Effluent + 0.08% BL	Males		3880 (1220)	144000 (118000)
	Females	11000 (3270)	5650* (1410)	
Experiment II				
Control	Males		2860 (1150)	62200 (11200)
	Females	14300 (418)	2250 (402)	
0.06% BL in water	Males		2740 (442)	45200 (30200)
	Females	7840* (1100)	2360 (257)	

* Statistically significant difference from the control, $p < 0.05$ (Anova and Dunnett's).

Short-term test with immature rainbow trout

Vitellogenin levels and EROD activity: The immature rainbow trout were used to measure the potential of effluents to increase hepatic EROD activity and plasma vitellogenin levels. Plasma vitellogenin levels were significantly increased in 100% effluent, 100% effluent spiked with 0.08% BL and water spiked with 0.06% BL. However, trout exposed to 100% effluent spiked with 0.06% BL was not sufficiently elevated to be statistically significant (Figure 1.3.3-1). EROD activity was significantly induced in the liver of trout exposed to the 100% effluent and 100% effluent spiked with 0.06% and 0.08% BL. Although elevated EROD activity was also observed in trout exposed to water spiked with 0.06% BL, this was not statistically significant. (Figure 1.3.3-2).

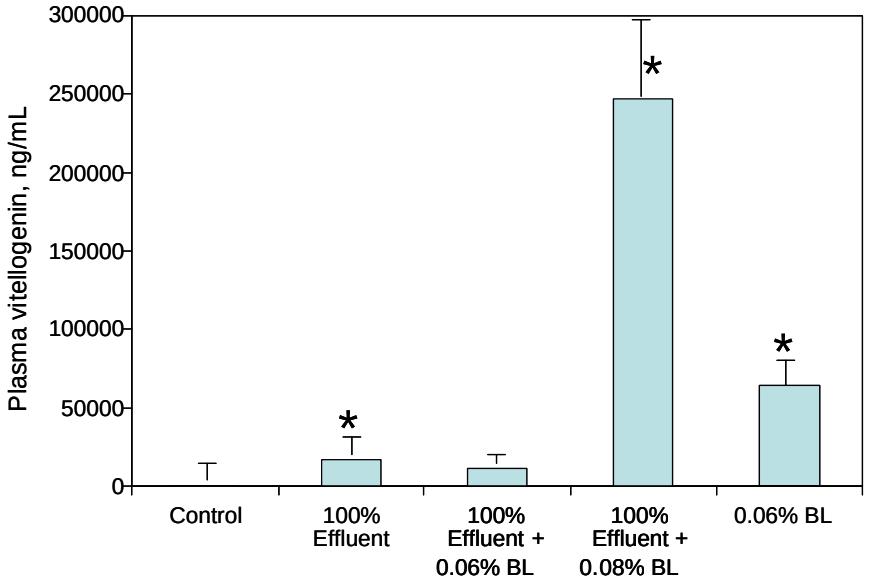

Figure 1.3.3-1. Plasma vitellogenin levels in rainbow trout exposed to mill effluent, effluent in combination to black liquor (BL) and BL alone. * denotes 5% significance level ($p<0.05$).

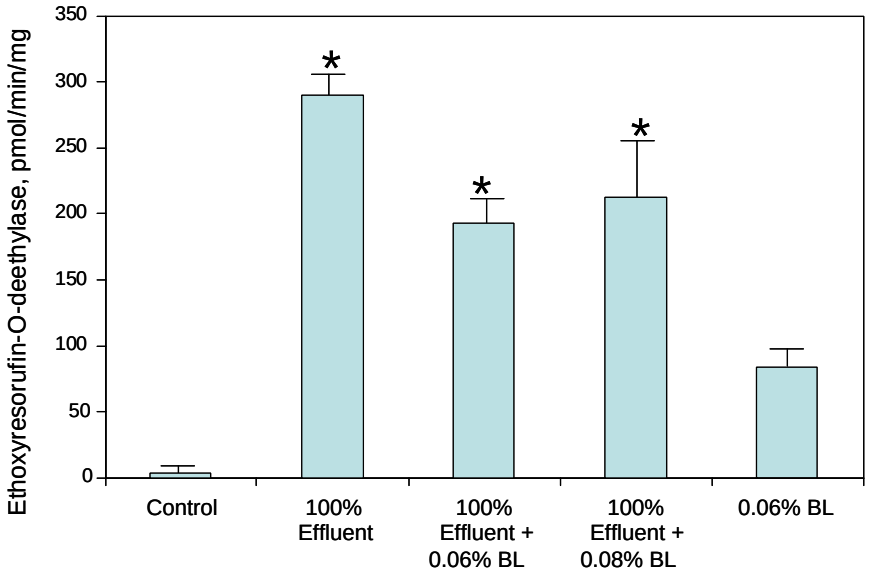

Figure 1.3.3-2. Hepatic ethoxyresorufin-O-deethylase activity in rainbow trout exposed to mill effluent, effluent in combination to black liquor (BL) and BL alone.* denotes 5% significance level ($p<0.05$).

The experiments with rainbow trout indicated that endpoints such as vitellogenin levels and EROD activity are both affected by the mill effluent. However, the EROD response was fairly consistent irrespective of the addition of BL. The vitellogenin response was better able to discriminate between 100% effluent exposure and exposure to 100% effluent spiked with BL. For example, the vitellogenin response in fish exposed to effluent spiked with 0.08% BL was 3.8-fold higher than in trout exposed to effluent alone. On the other hand, the effluent spiked with 0.06% BL did not cause a statistically significant increase in vitellogenin activity. Because of such inconsistencies, it may be necessary to design additional tests where the number of replicates is increased as well as the size of the fish. The larger fish should provide sufficient blood volumes such that individual fish could be analyzed for plasma vitellogenin. Such actions may improve the statistical power and consistency of the results.

Overall, the vitelogenin and EROD responses appeared to be more sensitive than endpoints examined in the test with fathead minnow. However, the ability of the endpoints in the trout test to discriminate between the different effluents was lower than the egg production test with the fathead minnow. As such, without further refinements, such tests may be less useful for IOC/IOS work. In addition, the vitellogenin and EROD responses were not directly related to egg production in the fathead minnow making it difficult to use the vitellogenin and EROD endpoints as surrogates for egg production.

Summary and Conclusions

The short-term test with fathead minnow was able to discriminate between an effluent causing no effect on reproduction and the same effluent spiked with BL that inhibited reproduction. This supports earlier evidence (Kovacs et al., 2009), that the short-term test can discriminate between effluents of different potency. The short-term test was not sensitive enough to detect interpretable effects on plasma steroid hormones, vitellogenin or gonad size.

The short-term test with rainbow trout responded to effluent and BL addition by showing increased levels of vitellognin and induction of EROD activity. However, to increase the statistical power of this test it is recommended that plasma be collected from fish of larger size to allow measurements from individual specimen rather than from plasma pooled from several fish.

The shutdown of egg production in fathead minnow caused by the addition of 0.06% or 0.08% BL supports observations made in Section 1.2 those black liquor losses during mill operation could play a role in inhibiting egg production in short-term tests.

1.3.4 Short-term and Medium-term Tests with Zebrafish

Introduction

The primary objective of this activity was to assess the effects of exposure to 100% effluent from the mill at Espanola alone and in combination with black liquor (0.06 or 0.08%) on reproduction in the zebrafish (*Danio rerio*). This included 7 day exposure studies with adult fish that examined egg production, ovarian size and selected ovarian and hepatic gene expression. As well, a longer term test (21 days) was conducted with juvenile fish to examine the effects of effluent and black liquor on gonadal development during the period of active gonadal recrudescence. This test evaluated gonadal size and included a histological assessment of gonadal development.

Materials and Methods

Short-term tests with adult zebrafish

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 h light and 12 h dark photoperiod. Fish were fed to satiation two 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).

The spawning tests were conducted in 4 L glass beakers containing 3.5 L of water or effluent. There were 6 females and 3 males in each breeding tank. The pre-exposure period was 9 days followed by an exposure period of 7 days. These tests included a control, 100% effluent, 100% effluent + 0.06% weak black liquor (BL), 100% effluent + 0.08% BL and 0.06% BL alone. There were 6 replicate tanks per treatment. Test conditions are detailed in Table 1.3.4-1.

At the end of the exposure, fish were overdosed with MS-222 (Sigma, St. Louis MO) and weighed. The gonad was excised and weighed for the calculation of gonadosomatic index. Ovaries were immediately snap frozen in liquid nitrogen before processing. A portion of ovarian tissue was used for the measurement of steroid content. Additionally, the intestine and liver were dissected and placed in RNAlater. The livers were subsequently dissected from the intestine and stored at -80°C for future measurement of vitellogenin and cytochrome P450 (P450-1a1) mRNA expression.

RNA extraction, reverse transcription and Real-Time PCR followed the methods described by Ings and Van Der Kraak (2006). The ovarian genes evaluated included StAR and P450-arom. The expression of vitellogenin and P450-1a1 was measured in the liver. The primers used for the measurement of P450-1a1were Forward CTGGACGAAAACTCCAACCTG; and Reverse GATAGTGTCGAAACCGGCTCC (based on Accession number AY398333.1). The primers used for vitellogenin were Forward TGCGTGAAGTTGTCATGCTTG; Reverse GATCTCGTGGATGGGCCTG (based on NM_170767.1).

Table 1.3.4-1. Test conditions for adult zebrafish exposed to pulp mill effluent and black liquor.	
Date(s) effluent sampled	May 25, 2009
Pre-exposure phase	Start May 19, 2009, for 9 d
Effluent exposure	Start May 28, 2009, for 7 d
Tank turnovers	Static, complete replacement once per day (3.5 L)
Quantity of effluent used	588 L of pulp mill effluent 2.94 L of black liquor
Replicates	6 tanks per treatment (6 females and 3 males)
Loading density	0.89 g/L/d ± 0.02
Feeding	Fed twice/d (salmon-fry pellets in the morning and bloodworms in the afternoon)
Endpoints measured	Egg production Gonadosomatic index Gene expression – vitellogenic follicles (StAR and aromatase) and liver (CYP26 1a1 and vitellogenin)
pH	8.51 ± 0.01
Dissolved oxygen	> 90% saturation
Temperature	27.5 ± 0.09°C

For steroid measurement, ovarian tissue was extracted with methanol, and the extracted were purified on a Amprep C-18 mini columns according to the methods described by Lister and Van Der Kraak, 2008. The amounts of 17β-estradiol and testosterone were measured by enzyme immuno assay using kits from Cayman Chemical (Ann Arbor, Michigan) according to the methods described by Lister and Van Der Kraak, 2008.

Mid-term tests with juvenile zebrafish

Juvenile 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 h light and 12 h 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).

The grow-out tests were conducted in 20 L glass aquaria containing 15 L of water or effluent. There were 25 individuals per tank. The pre-exposure period was 7 days followed by an exposure period of 21 days. These tests included a control, 100% effluent, 100% effluent + 0.06% black liquor (BL), and 0.06% BL alone. Test conditions are detailed in Table 1.3.4-2.

Table 1.3.4-2. Test conditions for juvenile zebrafish exposed to pulp mill effluent and black liquor.

Date(s) effluent sampled	May 25, 2009
Pre-exposure phase	Start May 20, 2009, for 7 days
Effluent exposure	Start May 28, 2009, for 21 days
Tank turnovers	Static, complete replacement once every 4–5 days (15 L)
Quantity of effluent used	126 L of pulp mill effluent 0.756 L of black liquor
Replicates	25 individuals per 20 L tank, 1 tank per treatment
Loading density	0.26 g/L/d ± 0.01
Feeding	Fed twice/d (salmon-fry pellets in the morning and bloodworms in the afternoon)
Endpoints measured	Gonadosomatic index Histology
pH	8.57 ± 0.02
Dissolved oxygen	> 90% saturation
Temperature	27.1 ± 0.16°C

At the end of the exposure, fish were overdosed with MS-222 (Sigma, St. Louis MO) and weighed. The gonad was excised and weighed for the calculation of gonadosomatic index. Ovaries were fixed in 10% buffered formalin and subsequently hematoxylin and eosin stained.

Results

Short-term tests with adult zebrafish

There was a significant reduction in egg production for fish exposed to 100% effluent and 100% effluent + 0.08% BL. There was approximately 50% reduction in egg production for those groups exposed to 100% effluent + 0.06% BL and 0.06% BL alone (Figure 1.3.4-1). There was no change in gonadosomatic index (GSI) following exposure to effluent ± BL (data not shown). There were no changes in the expression of ovarian StAR and P450-arom or hepatic vitellogenin (Figure 1.3.4-2). There was a significant induction on P450-1a1 expression in the liver samples from fish exposed to 100% effluent, whereas there were no changes for any of the other treatments (Figure 1.3.4-3).

Mid-term tests with juvenile zebrafish

Exposure to effluent ± BL for 21 days had no effect on either body weight (Figure 1.3.4-4) or GSI (Figure 1.3.4-5) of juvenile zebrafish. There were no obvious changes in gonadal development as determined by histological assessment.

Conclusions

Exposure to Espanola effluent alone and in combination with BL for 7 days contributed to a significant decline in the numbers of eggs spawned by adult zebrafish.

Biomarker responses (expression of StAR and aromatasegene expression) were not affected by effluent exposure.

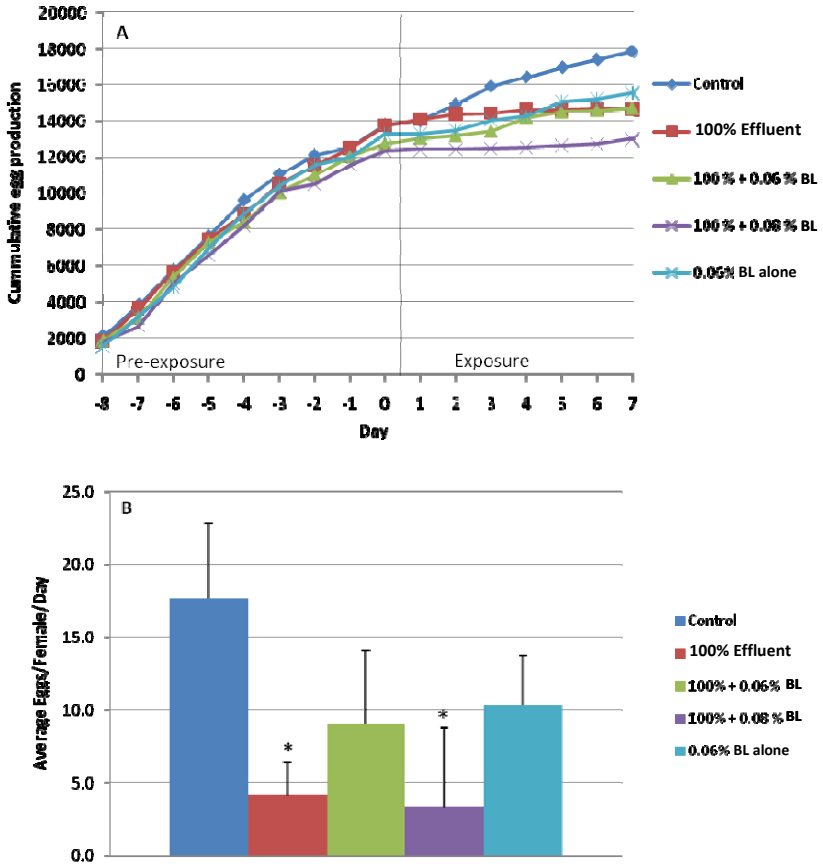

Figure 1.3.4-1. Cumulative egg production (A) of female zebrafish during a pre-exposure (9 days) period and following seven days of exposure to varying concentrations of pulp mill effluent and black liquor. Average number of eggs per female per day (B) during 7 days of exposure to varying concentrations of pulp mill effluent and black liquor.

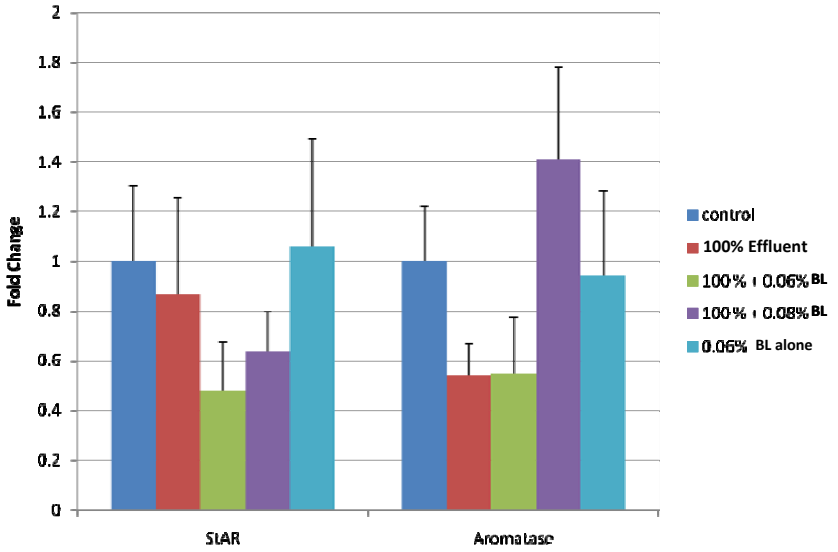

Figure 1.3.4-2. Expression of Aromatase and StAR in the ovary of adult zebrafish following seven days of exposure to varying concentrations of pulp mill effluent and black liquor. Expression of the genes is normalized to β-actin.

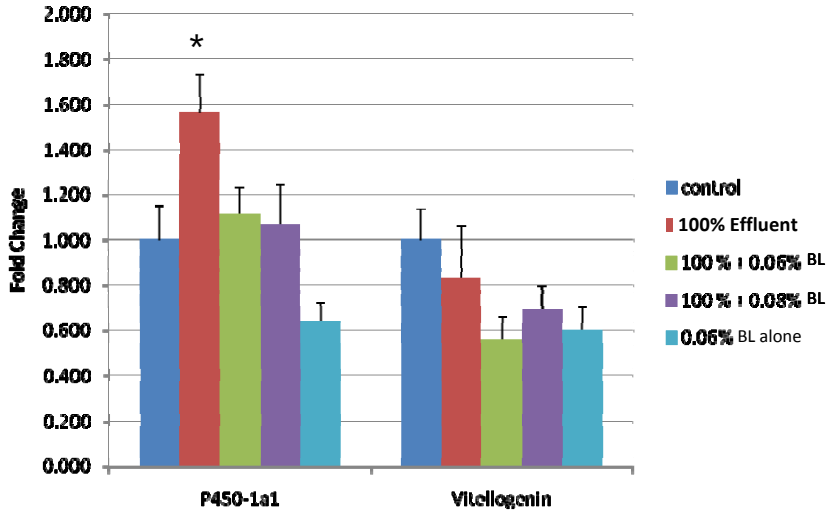

Figure 1.3.4-3. Expression of P450-1a1 and vitellogenin (female) in the livers of adult zebrafish following seven days of exposure to varying concentrations of pulp mill effluent and black liquor. Expression of the genes is normalized to β-actin.

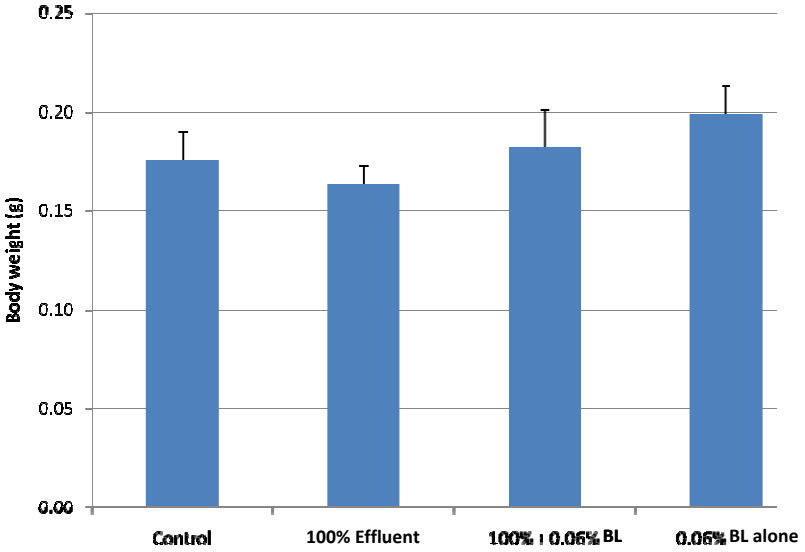

Figure 1.3.4-4. Body weight of juvenile zebrafish following 21 days of exposure to varying concentrations of pulp mill effluent and black liquor.

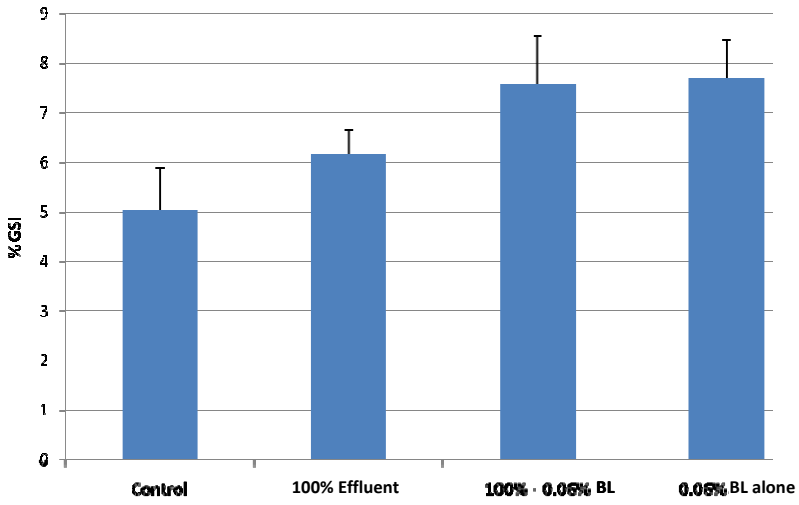

Figure 1.3.4-5. Gonadosomatic index of juvenile zebrafish following 21 days of exposure to varying concentrations of pulp mill effluent and black liquor.

1.3.5 Short-term Test with Threespine Stickleback

The purpose of the short-term test threespine stickleback was to determine exposure to both androgens and estrogens in a short 5 days exposure. To establish the relationship of this exposure to reproduction, a biochemical reproductive endpoint, in vitro gonadal sex steroid hormone production was measured. Stickleback are endemic to estuarine waters and associated streams on both coasts of Canada and have become a model species for both toxicology and ecology.

Methods

Effluent collection and storage

Effluent (1,000 L) was shipped from Espanola to Charlottetown, PEI in 7 days (collected May 25th, arrived June 1, 2009). Black liquor (WBL) was diluted at FPInnovations and shipped to PEI. Black liquor was stored at 4°C until ready to use and Espanola effluent was used 2 days after arrival and was kept at approximately 12°C during the 2 d pre-exposure and 5 d exposure. Effluent and black liquor was diluted to the appropriate concentrations with UPEI laboratory well water in 50 L glass reservoirs every 12 h. Pre-mixed solutions were pumped into flow-through 30 L aquaria using a peristaltic pump. Exposure concentrations were 100% effluent, 0.06% black liquor, 100% effluent + 0.06% black liquor, and 100% effluent + 0.08% black liquor. Effluent and lab dilution water flow was 41 mL/min to each aquarium, which provided about 2 solution turnovers per 24 h.

Fish and exposures

Sexually mature (~1 g) threespine stickleback were captured in the Stanley Estuary (New London Bay) Prince Edward Island approximately two weeks prior to experimental exposure. Stickleback were held in 200 L of laboratory water initially at 15 ppt salinity and 5°C. Static water changes (30%) were made with fresh laboratory well water in order to gradually move the stickleback to freshwater and temperature was adjusted up at 1°C per day until it reached 15ºC. Laboratory light was set at 16 h daylight, 8 h dark. The pre-exposure acclimation period was approximately 3 weeks. Exposures were started by adding stickleback directly to pre-mixed concentrations in the exposure aquaria. After 5 days of exposure, stickleback were sacrificed and samples collected.

Fish sampling and grading of sex characteristics

Animals were stunned by a blow to the head and sacrificed by decapitation and the weight and length of each fish was recorded. The gonads, kidneys and liver were excised and weighed. The threespine stickleback has a discrete posterior kidney that can be removed intact in the majority of cases through careful dissection with fine forceps. The occasional incidence of *Glugea sp.* parasites was observed and fish containing the parasite were excluded from any subsequent analysis. The kidney and liver were placed in 0.5 mL RNA later solution (Sigma) and were stored at 4ºC overnight and then at −20ºC until RNA extraction. Male fish secondary sex characteristics were assessed by examination of the presence or absence of the red male colouration pattern.

Table 1.3.5-1. Test conditions for 5-d test with threespine stickleback.	
Date(s) effluent sampled	May 25, 2009
Pre-exposure phase	Lab well water only
Effluent exposure	5 d, Start June 3, 2009
Tank turnovers	2 volumes/d
Quantity of effluent used	1,000 L
Replicates	1 for each effluent concentration
Loading density	0.6 g/L/d
Feeding	Bloodworms and flake food
Endpoints measured	Length, weight, condition factor of adults Liver-somatic index, gonadosomatic index of adults Secondary sexual characteristics In males: testosterone and 11-ketotestosterone production by testes, vitellogenin mRNA production in liver In females: testosterone and estradiol production by ovaries, spiggin production
pH	7.5 to 7.8
Dissolved oxygen	> 90% saturation
Temperature	14.9 to 15.3°C

RT-PCR and in vitro *steroidogenesis*

A nominal size of twelve males and twelve females were sampled per treatment. Endpoints presented here are in vitro steroidogenesis, posterior kidney spiggin mRNA production in females, and liver VTG mRNA abundance in males. Methods for steroidogenesis and mRNA measurements have been previously described (Hogan et al., 2008).

Statistical analyses

Data were checked for homogeneity of variance using the Levine and Browne-Forsythe test and normality was assessed visually using categorized normal probability plots prior to analysis. Logarithmic transformations were used if data did not meet the test assumptions. RT-PCR endpoints and *in vitro* steroid levels were tested with analyses of variance (ANOVA) followed by Dunnett's post-hoc test to examine for differences between treatments and controls. All statistics were conducted with STATISTICA v.8.

Results and Discussion

Males

There were no mortalities in male stickleback during the exposure. Male threespine stickleback showed a consistent (100% of individuals) and dramatic increase in breeding colouration in all effluent exposure concentrations. There was no evidence of breeding colouration in the control males. The time of year at which the exposures were conducted is just prior to the peak breeding season and either the dark colouration in the tanks, or some chemical cue in the effluent appeared to induce male colouration. There was also a general increasing trend in vitro steroid hormone production for both testosterone and 11-ketotestosterone (Figure 1.3.5-1). However, the only statistically significant increase was for testosterone at the 100% effluent plus 0.08% black liquor concentration. Male stickleback had significantly elevated liver vtg mRNA at the effluent plus 0.08% black liquor concentration only (Figure 1.3.5-2).

In male stickleback, there appears to have been a mild stimulation in gonadal steroid hormone production due to the effluent and black liquor. Exposure to estrogens was also indicated by induced vtg mRNA and this may have been related to the stimulation of steroid production in the same treatment. These results were not consistent with the increased sexual colouration that appeared to occur to a similar degree in all of the effluent and black liquor treatments.

Females

There were no mortalities in female stickleback during the exposure. As with male stickleback, there was also a statistically significant increase in gonadal testosterone production but there were no significant changes in in vitro estradiol production (Figure 1.3.5-3). Female stickleback did not show increases in spiggin mRNA indicating that there was no detectable exposure to androgens due to exposure to Espanola effluent/black liquor (Figure 1.3.5-3).

The overall response of males and females appear to be one of exposure to low-levels of estrogens, likely derived from the black liquor. This results was the opposite of a recent study using the same techniques where exposure to androgens was indicated by elevated spiggin mRNA in female stickleback (Wartman et al., 2009). The weak estrogenic exposure or other chemical factors could have caused the increase in steroid hormone production observed in both males and females. However, clearly those compounds in black liquor are unlikely to reach the receiving environment with the Espanola effluent that is well-treated. At the time of the year that experiment was conducted, stickleback are primed to start spawning. Thus the steroidal response, or the interesting and very rapid onset of male sexual colouration could have been induced by a number of effluent-related factors, the most obvious of which is the dark colouration of the effluent, that the stickleback appear to prefer.

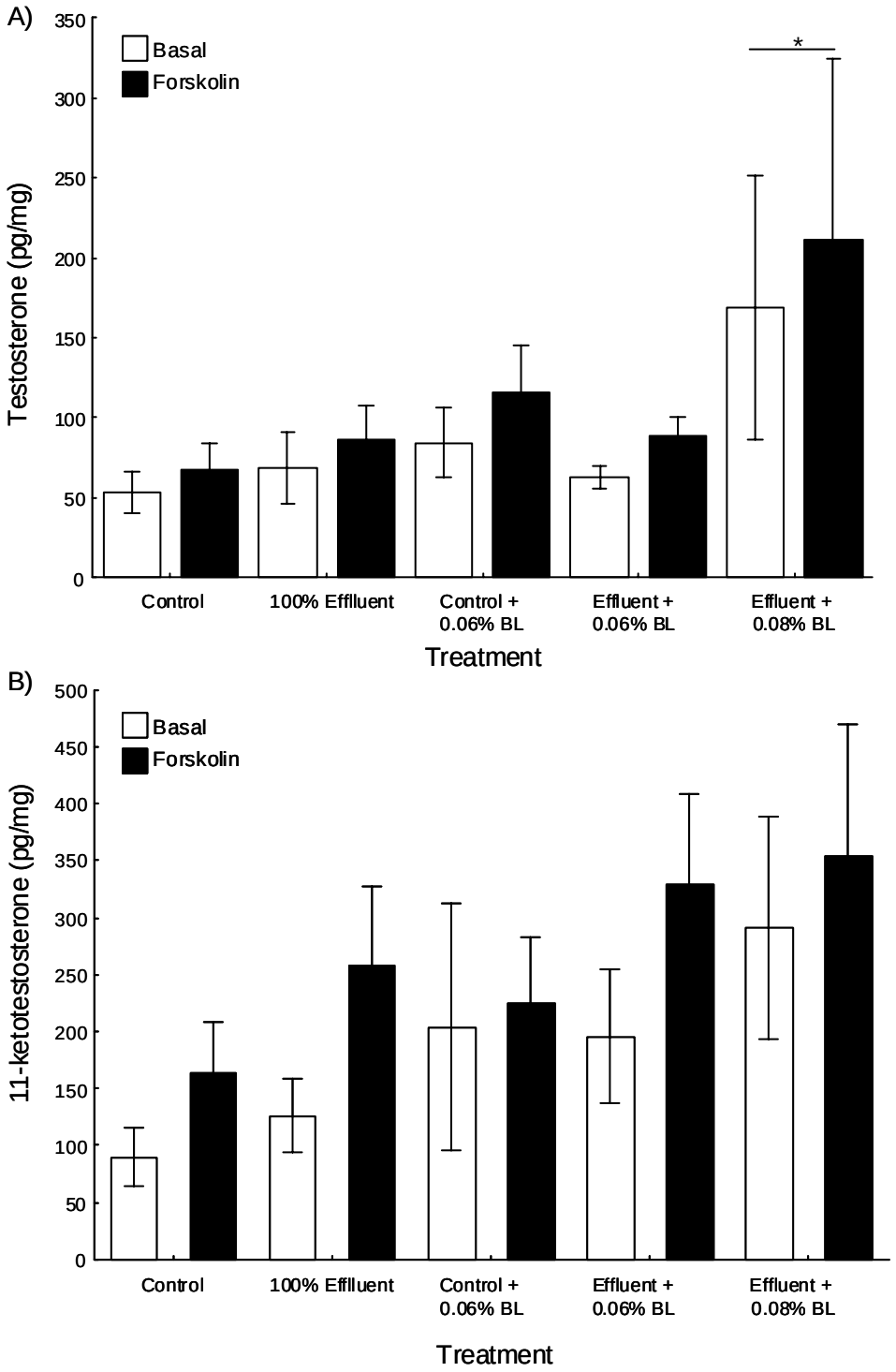

Figure 1.3.5-1. Mean in vitro testicular A) testosterone, and B) 11-ketotestostenone production in threespine stickleback males. Error bars indicate the standard error of the mean. Asterisk indicates statistical significance, $p < 0.05$.

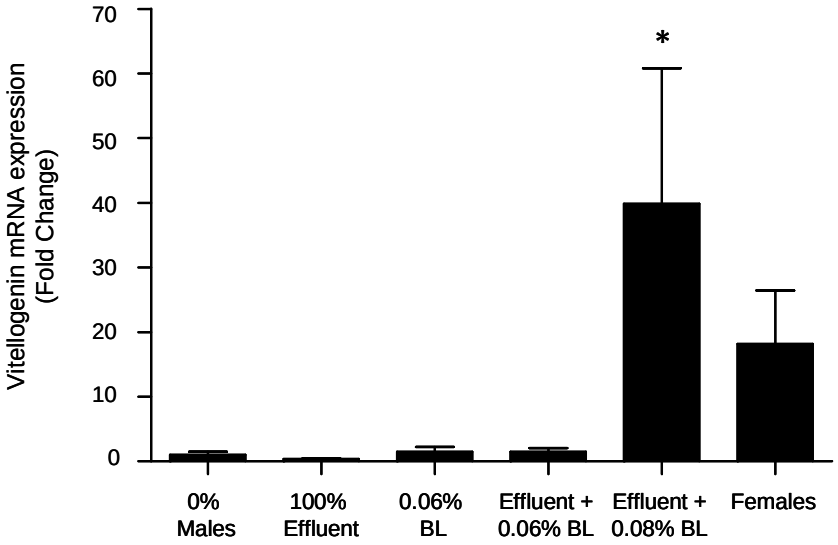

Figure 1.3.5-2. Mean hepatic vitellogenin mRNA production in male stickleback as measured by RT-PCR. All values are normalized to the beta-actin control gene. Error bars indicate the standard error of the mean. Asterisk indicates statistical significance, $p < 0.05$.

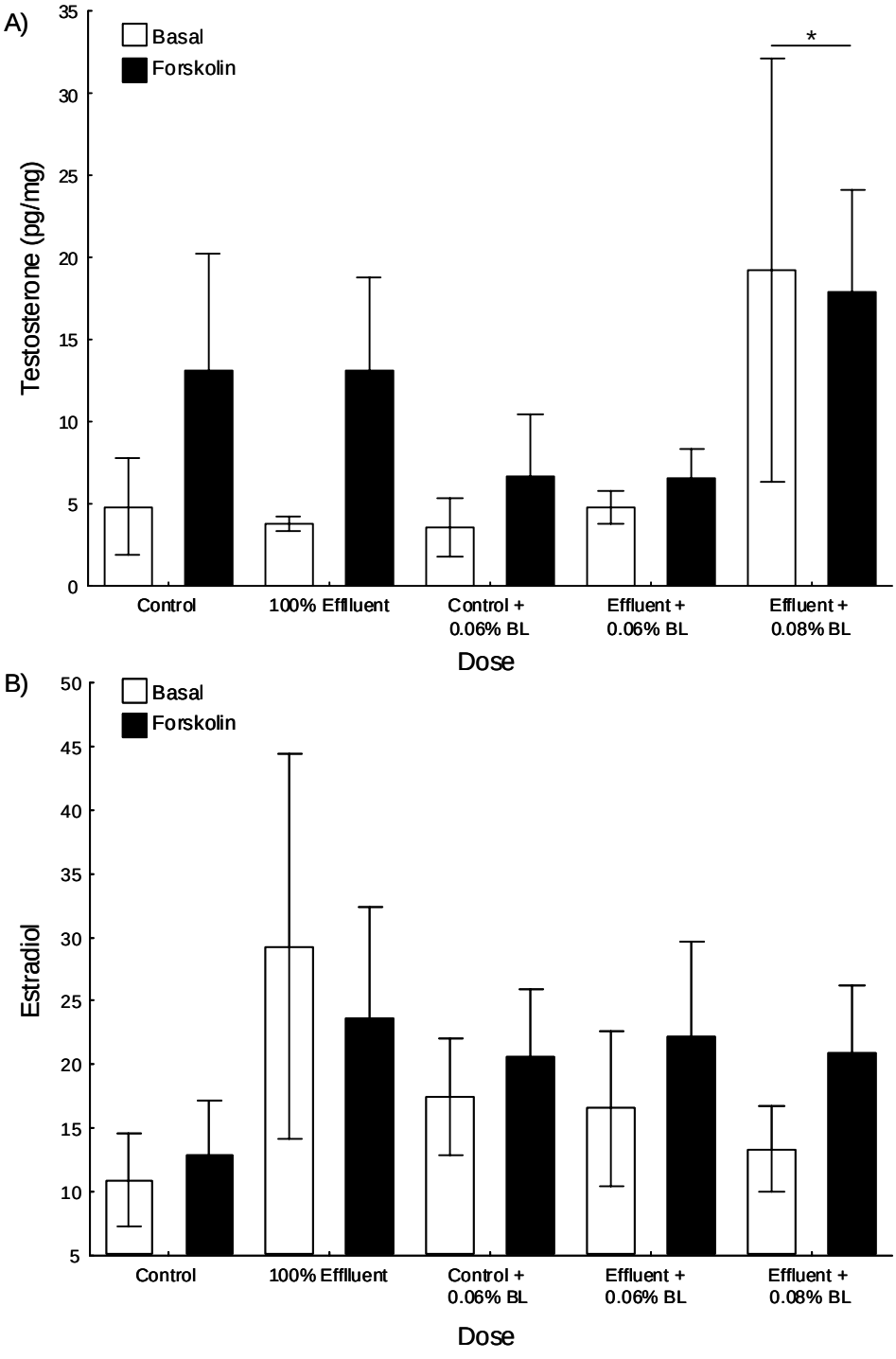

Figure 1.3.5-3. Mean in vitro ovarian A) testosterone, and B) estradiol production in threespine stickleback males. Error bars indicate the standard error of the mean. Asterisk indicates statistical significance, $p < 0.05$.

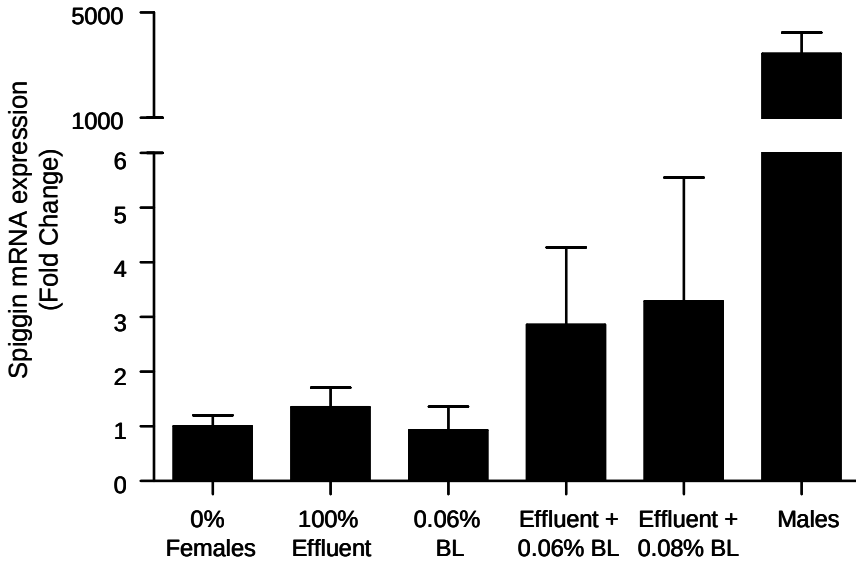

Figure 1.3.5-4. Mean hepatic vitellogenin mRNA production in female stickleback as measured by RT-PCR. All values are normalized to the beta-actin control gene.

1.3.6 Short-term Test with Mummichog

The purpose of the short-term (7-day) mummichog test was to examine the effects of effluent in an estuarine species. Mummichog, an endemic estuarine species of the Canadian and American Atlantic coasts, have previously been used to undertake EEM wild fish studies (e.g., Cycles 3 and 4, Lake Utopia Paper, St. George, NB), EEM artifical stream studies (e.g., Cycle 2, Irving Paper, Saint John, NB) and EEM IOC studies (e.g., Cycle 4, Irving Pulp and Paper, Saint John, NB). Because many Canadian mills are located on the coast, it is important to assess the effects of effluent in a saline environment as fish physiological processes may differ in these environments. The short-term endocrine bioassay used here has been standardized for use with both model endocrine disrupting contaminants (MacLatchy et al., 2003; Sharpe et al., 2004) as well as pulp mill waste streams (Shaughnessy et al., 2007; Bosker et al., 2010a, 2010b).

Methods

Espanola effluent collection and storage

Effluent from the Espanola mill and 10 times diluted black liquor (BL; provided by FPInnovations-Paprican) was shipped to the Hagen Aqualab, University of Guelph in May 2009. Effluent was stored outside until being warmed to room temperature prior to use. The BL was stored at 4ºC.

Fish exposure

Adult male and female mummichog (*Fundulus heteroclitus*) were exposed for 7 days to various treatments of effluent and/or BL to determine the potential effects on the reproductive endocrine system. The fish were acclimated in aerated, 20L glass tanks containing Hagen Aqualab well water that was salted with Crystal Sea® to approximately 16 ppt. A flow-through system was used in which treatments were pre-mixed in large aerated reservoirs and pumped continuously to the tanks at a flow rate of 1.7L/h, which provided approximately 2 tank turnovers per 24 h.

There were 5 treatments in the experiment: 1) 0% (control; well water only), 2) 100% effluent, 3) 100% effluent containing 0.06% BL, 4) 100% effluent containing 0.08% BL, and 5) 0.06% BL diluted in control water. Salinity concentrations were maintained at approximately 16 ppt for every treatment. There were 5 replicate tanks for each treatment; each tank contained 4 males and 3–4 females. The exposure was conducted at ambient temperatures (18.0 ± 1ºC) with 16 h light: 8 h darkness. Other exposure parameters can be found in Table 1.3.6-1. Fish were fed twice daily.

After 7 days of exposure, fish were anaesthetized and blood was taken from the caudal vein. Fish were then killed by cervical transection. Fish length, body weight, and weights of the gonads and liver were recorded for each fish for the assessment of condition factor (K) and gonadosomatic (GSI) and liversomatic indices (LSI). Blood was spun at 5000 rpm for 20 min for the collection of plasma, which was analyzed for sex steroid levels by radioimmunoassay. In males, plasma levels of testosterone (T), 11-ketotestosterone (11-KT), and 17β-estradiol (E2) were analyzed. E2 and T were analyzed in females. *In vitro* production of steroids (T and 11-KT for males; T

and E2 for females) was assessed by incubating gonadal tissue (20 ± 2 mg/well) in Medium 199 for 18 h Protocols for the fish exposures and endpoint analyses can be found in MacLatchy et al. (2003, 2005).

Table 1.3.6-1. Test conditions for mummichog exposed to Espanola pulp mill effluent and black liquor for 7 days.

Date(s) effluent sampled	May 25, 2009
Pre-exposure phase	Start May 21, 2009, for 7 d
Effluent exposure	Start May 28, 2009, for 7 d
Tank turnovers	Two volumes/day (40L/tank/d)
Quantity of effluent used	4200 L of pulp mill effluent 44.8 L of 10X diluted black liquor
Replicates	5 tanks per treatment (3–4 females, 4 males/tank)
Loading density	2.39 ± 0.22 g/L/d
Feeding	fed twice/d (commercial trout chow)
Endpoints measured	Gonadosomatic index Liversomatic index Condition factor Plasma T, 11-KT, E2 in males Plasma T, E2 in females In vitro T, 11-KT by male testis pieces In vitro T, E2 by female ovarian pieces
pH	8.0 – 8.5 (measured in each tank daily)
Dissolved oxygen	> 90% saturation (measured in each tank daily)
Temperature	18.0 ± 1°C (measured in each tank daily)

Statistical Analyses

Data were checked for homogeneity of variance using the Levine's test prior to analysis; logarithmic transformations were used if data did not meet the test criteria. Body weight and plasma and *in vitro* steroid levels were tested with analyses of variance (ANOVA) (Kruskal Wallis, if non-parametric) followed by Tukey's post-hoc test ($p<0.05$). Analyses of covariance (ANCOVA) were used to analyze gonad and liver weight relative to body weight, and body weight relative to body length across treatments after checking the homogeneity of regression (slopes). ANCOVA results are presented with the associated means of K, GSI, and LSI. Data were analyzed using PASW (SPSS) v17.0.

Results and Discussion

Males

The body weight of male fish was found to be significantly decreased in the 0.06% BL in effluent group compared with the control fish (0% group) (Table 1.3.6-2). However, when body length was used as a covariant in the ANCOVA, no significant differences in body weight were found (Table 1.3.6-2). Testis and liver weights as a function of body weight were not significantly different among treatments (ANCOVA; Table 1.3.6-2).

Table 1.3.6-2. Body weight (g), condition factor (K), gonadosomatic index (GSI), liversomatic index (LSI), and plasma testosterone (T), 11-ketotestosterone (11-KT), and 17β-estradiol (E2) levels in male and female mummichog exposed to Espanola effluent with or without the addition of black liquor (BL) for 7 days (May 28–June 4, 2009). All values are Mean ± S.E.M. Values with different letters are significantly different ($p<0.05$; Tukey's). There were 5 replicate tanks per treatment; each tank contained 4 males and 3–4 females.

Treatment	Weight (g)	K (%)	GSI (%)	LSI (%)	T (ng/mL)	11-KT (ng/mL)	E2 (ng/mL)
Males							
0%	7.49 [a]	1.12	2.36	4.10	3.87	1.39	4.64
	(0.23)	(0.026)	(0.19)	(0.25)	(0.50)	(0.33)	(0.79)
100% effluent	5.24 [ab]	1.06	1.86	4.072	4.24	2.12	5.42
	(0.76)	(0.007)	(0.24)	(0.28)	(1.41)	(0.50)	(1.26)
0.06% BL	5.56 [ab]	1.06	2.40	4.04	3.75	1.34	4.96
	(0.68)	(0.015)	(0.11)	(0.37)	(0.85)	(0.39)	(0.97)
0.06% BL in effluent	4.57 [b]	1.03	2.36	3.86	4.04	1.53	4.44
	(0.30)	(0.008)	(0.30)	(0.20)	(1.08)	(0.25)	(0.97)
0.08% BL in effluent	6.11 [ab]	1.12	2.04	3.53	3.90	0.93	3.94
	(0.60)	(0.017)	(0.071)	(0.19)	(1.24)	(0.13)	(0.96)
Females							
0%	8.32	1.12	4.81	4.22	2.99 [ab]		8.39 [a]
	(0.93)	(0.022)	(0.59)	(0.48)	(0.60)		(1.79)
100% effluent	6.342	1.06	3.93	4.676	2.64 [ab]		4.56 [ab]
	(0.49)	(0.033)	(0.37)	(0.52)	(0.51)		(0.90)
0.06% BL	6.80	1.06	3.60	4.26	3.79 [a]		6.83 [ab]
	(0.81)	(0.032)	(0.49)	(0.63)	(0.91)		(0.74)
0.06% BL in effluent	7.33	1.10	3.95	4.68	1.29 [b]		3.25 [b]
	(0.84)	(0.028)	(0.66)	(0.42)	(0.36)		(0.86)
0.08% BL in effluent	7.642	1.07	4.34	3.96	1.61 [ab]		4.97 [ab]
	(0.33)	(0.031)	(0.60)	(0.26)	(0.27)		(0.88)

The levels of T produced *in vitro* of fish exposed to 0.06% BL alone were significantly lower than the 100% effluent group (Table 1.3.6-3). There were no significant differences between the levels of plasma (Table 1.3.6-2) or *in vitro* (Table 1.3.6-3) steroids produced by the effluent or BL treated fish compared with the control group.

Table 1.3.6-3. Testosterone (T), 11-ketotestosterone (11-KT), and 17β-estradiol (E2) levels (pg/mL) produced by gonadal tissue *in vitro* of male and female mummichog exposed to Espanola effluent with or without the addition of black liquor (BL) for 7 days (May 28–June 4, 2009). All values are Mean ± S.E.M. Values with different letters are significantly different ($p<0.05$; Tukey's). There were 5 replicate tanks per treatment; each tank contained 4 males and 3–4 females.

Treatment	T (pg/mL)	11-KT (pg/mL)	E2 (pg/mL)
Males			
0%	147 [ab] (9.44)	155 (10.7)	
100% effluent	197 [a] (23.7)	260 (44.2)	
0.06% BL	134 [b] (9.35)	157 (12.2)	
0.06% BL in effluent	142 [ab] (11.9)	194 (32.6)	
0.08% BL in effluent	165 [ab] (7.86)	236 (60.5)	
Females			
0%	162 (8.83)		444 [a] (34.7)
100% effluent	190 (24.2)		376 [a] (48.9)
0.06% BL	153 (19.0)		331 [ab] (24.7)
0.06% BL in effluent	152 (11.9)		322 [ab] (28.3)
0.08% BL in effluent	133 (11.5)		218 [b] (20.2)

Females

There were no significant differences found in female body weight (ANOVA) or ovarian and liver weights (as a function of body weight; ANCOVA) or condition among treatments (Table 1.3.6-2).

Female plasma levels of E2 were significantly decreased in fish exposed 0.06% BL in effluent compared with the control fish (0% group) (Table 1.3.6-2). *In vitro* E2 production by ovarian tissue of fish exposed to 0.08% BL in effluent were decreased significantly compared with the controls and 100% effluent groups (Table 1.3.6-3). No differences were found in plasma T levels

between the controls and any of the treatments, but the levels of T were significantly decreased in fish exposed to effluent containing 0.06% BL compared with the 0.06% BL alone group (Table 1.3.6-2).

Conclusions

There were limited effects of effluent (with or without black liquor addition) on mummichog body metrics or reproductive endocrine status. There is some indication that black liquor has the potential (in isolation or combined with effluent) to cause alterations in reproductive endocrine status, primarily in female mummichog; however, further work is required to confirm this and to establish if a consistent pattern exists.

1.3.7 Effluent Chemistry

The objective of conducting measurements of effluent chemistry was to provide an indication of effluent quality over the course of the Cycle 5 IOC studies. These measures would then be able to facilitate the interpretation of bioassay results from different laboratories conducted at different times.

For effluent quality monitoring, effluent samples from the Espanola mill were collected directly from shipping totes during a preliminary sampling that occurred over 3 weeks August 19 – September 4, 2008. During the simulated mill upset studies, effluents were sampled directly from fish exposure aquaria (fathead minnow juvenile gonadal growth test) from weekly from June 5 – 26, 2009. A schematic breakdown of the samples collected and the analyses performed is shown in Figure 1.3.7-1.

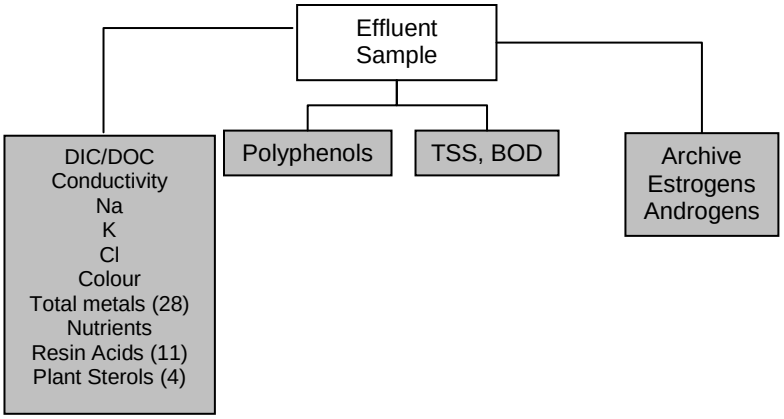

Figure 1.3.7-1. Schematic of effluent sampling from the Espanola mill.

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.

Results

The black liquor dilutions were calculated by FPInnovations Paprican and a stock solution distributed to participating laboratories.For the purposes of this section of the report, EFF+0.06% BL is 100% effluent spiked with 0.06% (v/v) black liquor. Similarly, EFF+0.08% BL is effluent spiked with 0.08% (v/v) black liquor and CON+ 0.06% BL is control water spiked with 0.06% (v/v) black liquor.

Basic effluent parameters are provided below for both of the Espanola effluents studied (Table 1.3.7-1). Generally, the effluent samples from 2008 and 2009 were very similar in all parameters measured. Among the treatments of the 2009 study, the control water spiked with black liquor was different from other treatments involving effluent and the control water itself.

Table 1.3.7-1: Summary of physical parameters measured in Espanola effluents and associated treatments in 2008 and 2009.

Parameter	2008		2009									
	Effluent		Control		Effluent		Effluent + 0.06% BL		Effluent + 0.08% BL		Control + 0.06%BL	
	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
NH_3 (mg/L)	2.8	0.70	0.2	0.26	0.9	1.10	1.2	0.87	1.9	0.92	0.1	0.05
Conductivity (µS/cm)	1602.0	71.94	345.8	17.11	1755.0	158.64	1822.5	151.30	1910.0	229.64	421.8	12.04
pH	8.2	0.11	8.1	0.09	8.3	0.04	8.2	0.06	8.3	0.16	8.0	0.06
Alkalinity	405.0	8.94	83.2	3.29	334.5	7.59	349.3	4.27	353.5	9.00	95.5	2.05
Cl (mg/L)	138.2	3.25	29.9	3.12	153.8	4.57	165.5	5.00	169.5	5.51	40.5	3.44
SO_4 (mg/L)	225.4	31.44	35.6	1.41	305.8	74.13	312.0	71.97	344.5	99.87	42.9	1.97
Colour (CU)	649.8	97.99	1.0	0.46	443.5	229.91	674.8	32.72	711.5	26.45	207.7	220.87
DOC (mg/L)	88.4	6.10	1.5	0.25	73.8	7.41	86.6	7.97	88.6	7.91	12.7	2.81
DIC (mg/L)	90.0	3.19	19.7	0.78	73.0	2.09	79.0	1.31	80.5	0.63	22.8	0.49
Ca (mg/L)	59.8	3.05	35.6	0.64	49.0	3.77	48.8	3.30	48.8	4.85	35.9	0.88
Mg (mg/L)	7.1	0.07	9.0	0.22	6.6	0.55	6.6	0.58	6.5	0.70	9.1	0.16
Na (mg/L)	280.4	34.06	16.8	1.52	327.3	41.55	328.5	34.58	368.3	52.70	34.5	1.20
K (mg/L)	15.3	0.78	1.8	0.13	22.7	6.52	25.1	6.43	28.2	9.01	3.8	0.18
NO_2 (mg/L)	0.1	0.01	0.3	0.33	0.4	0.53	0.3	0.30	0.6	1.14	0.0	0.01

The majority of the suite of metals measured showed little differences throughout the sampling periods of testing with Espanola effluent. Table 1.3.7-2 shows the concentrations of metal measured that were <10 ug/L. All 2009 measurements were performed on samples collected from exposure aquaria.

Table 1.3.7-2. Effluent levels of metals detected in Espanola effluents that were <10 µg/L.

Metal	2008		2009									
	Effluent		Control		Effluent		Effluent + 0.06% BL		Effluent + 0.08% BL		Control + 0.06% BL	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Silver	0.04	0.03	0.00	0.00	0.04	0.04	0.04	0.03	0.03	0.03	0.00	0.00
Arsenic	0.88	0.08	1.04	0.52	0.83	0.09	0.95	0.18	1.14	0.48	0.77	0.12
Arsenic	0.88	0.08	1.04	0.52	0.83	0.09	0.95	0.18	1.14	0.48	0.77	0.12
Beryllium	0.02	0.00	0.00	0.00	0.03	0.00	0.03	0.01	0.03	0.01	0.00	0.00
Bismuth	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00
Cadmium	1.04	0.13	0.01	0.00	1.05	0.17	1.14	0.16	1.18	0.22	0.03	0.01
Cobalt	0.55	0.11	0.05	0.01	0.60	0.05	0.63	0.17	0.58	0.18	0.08	0.02
Chromium	3.10	0.30	0.27	0.05	2.96	0.30	3.05	0.23	2.99	0.24	0.29	0.06
Copper	3.92	1.01	1.34	1.01	4.53	1.16	5.07	1.13	6.27	2.29	3.93	6.37
Gallium	0.27	0.03	0.18	0.01	0.30	0.03	0.33	0.02	0.32	0.02	0.18	0.02
Lanthanum	0.87	0.26	0.01	0.00	0.94	0.07	1.07	0.14	0.95	0.20	0.03	0.01
Lithium	7.45	5.92	2.46	0.18	8.14	4.59	8.28	4.35	8.62	4.25	2.60	0.14
Molybdenum	0.87	0.42	1.73	0.17	1.61	0.51	1.20	0.64	1.03	0.77	1.48	0.38
Lead	1.29	0.24	0.10	0.06	1.55	0.36	1.80	0.25	1.83	0.20	0.18	0.10
Antimony	0.07	0.03	0.16	0.01	0.12	0.03	0.09	0.02	0.10	0.04	0.14	0.04
Selenium	0.26	0.05	0.35	0.15	0.31	0.09	0.35	0.10	0.38	0.17	0.27	0.10
Thallium	0.03	0.01	0.02	0.02	0.05	0.02	0.05	0.02	0.05	0.01	0.02	0.01
Uranium	0.37	0.13	0.05	0.02	0.39	0.09	0.38	0.09	0.38	0.10	0.06	0.02

Other metals were measured at higher concentrations and are provided in Figure 1.3.7-2. It is interesting to note that the concentrations of all metals were consistent between the 2008 and 2009 sampling periods. Metals were also consistent between the 2009 treatments, with the exception of black liquor spiked into control water; demonstrating that there was negligible metal content associated with black liquor; this further suggests that chemicals affecting fish reproduction in black liquor are organics, not metals.

The overall trend in effluent similarities between years and the distinctness of the control water spiked with black liquor was also consistent in BOD and TSS data (Figure 1.3.7-3), and in the content of polyphenols (Figure 1.3.7-4).

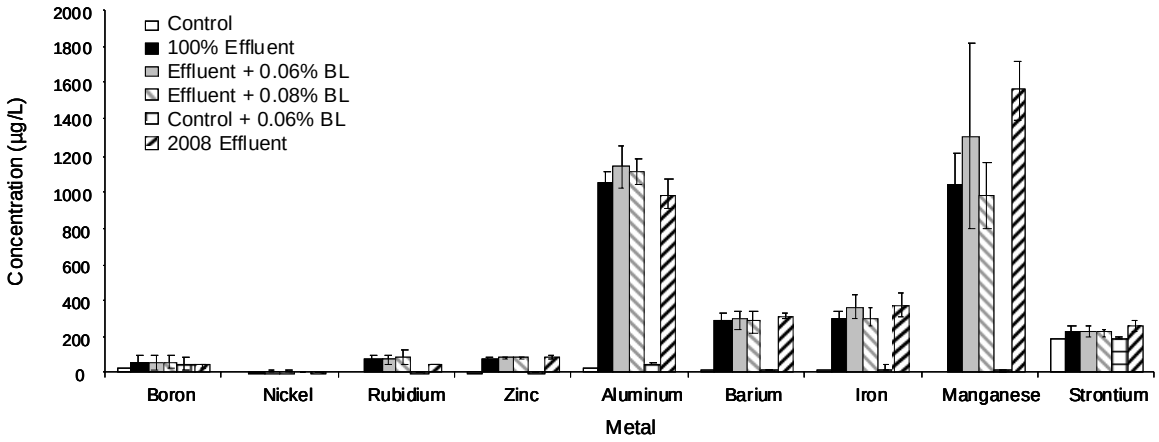

Figure 1.3.7-2. Levels of metals in Espanola effluent samples and associated treatments that were >10 µg/L.

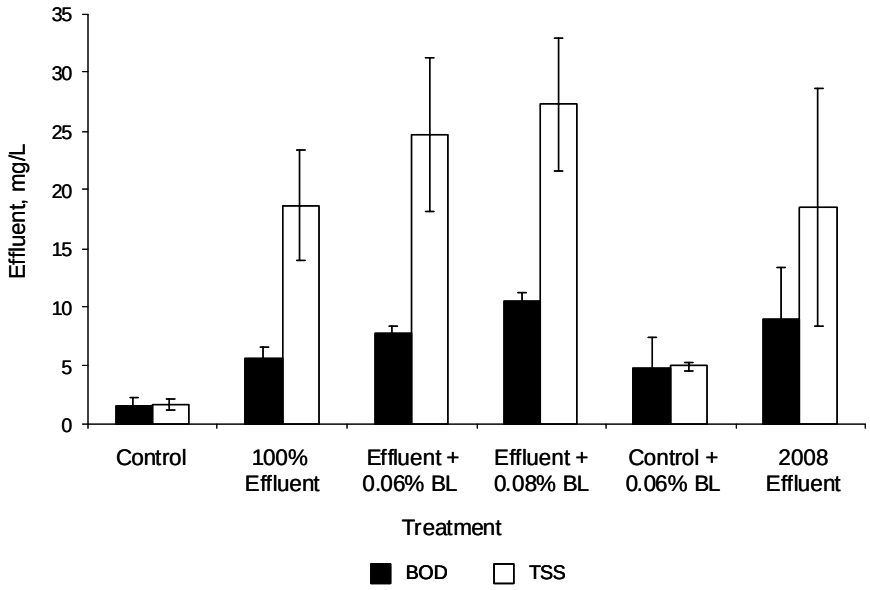

Figure 1.3.7-3. Monthly averages of total suspended solids and biological oxygen demand of weekly samples of Espanola effluent and associated treatments from 2008 and 2009 studies. Average detection limits were 2.48 mg/L (BOD) and 4.22 mg/L (TSS).

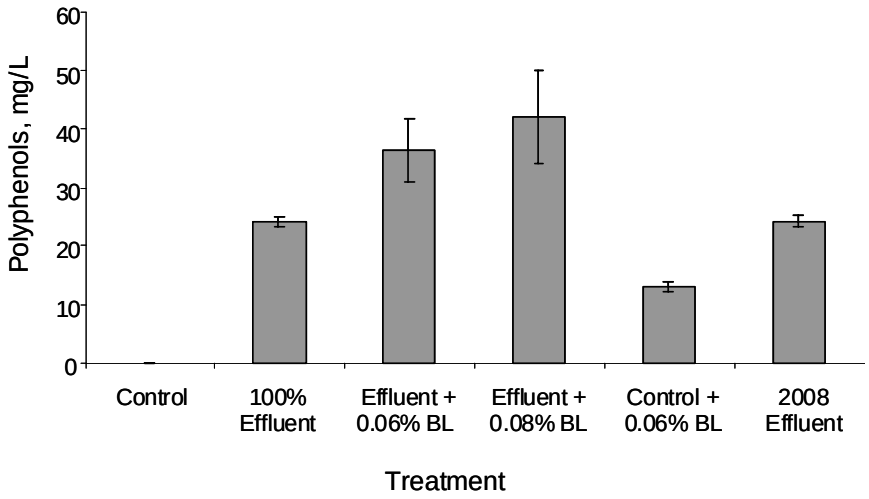

Figure 1.3.7-4. Monthly averages of polyphenol contents of weekly samples of Espanola effluent and associated treatments from 2008 and 2009 studies.

2. Evaluation of Reproductive Effects in Fish Exposed to a Neutral Sulphite Semi-Chemical (NSSC) Corrugated Medium Mill Effluent

The objective of this activity was to assess the applicability of laboratory tests for IOC and IOS work aimed at eliminating/reducing mill effluent-related effects on fish reproduction. For this purpose, the fish were exposed to a treated effluent from a neutral sulphite semi-chemical (NSSC) pulp mill effluent from the Lake Utopia Paper mill which has been shown to affect the gonad size of male mummichog (*Fundulus heteroclitus*) in the Cycle 4 regulatory Environmental Effects Monitoring (EEM) study.

2.1 Medium-term Test with Fathead Minnow

The medium-term test with fathead minnow exposes maturing fish to effluent for 30 days or more. The extended exposure during the maturation phase was thought to be a possible way to capture effects of the effluent on gonadal growth. The test can also measure reproductive capacity, if the fish are over 90 days old at the start of the exposures. Endpoints include growth, LSI, GSI, the development of secondary sexual characteristics, and spawning events, as well as biochemical indicators. The duration of the test is approximately one month.

The test with the Lake Utopia Paper mill effluent was started on October 3, 2009, with fish that were 105 ± 3 days old (days post-hatch, or dph). The test ended November 2 to 4, 2009, after 30–32 days of exposure to Lake Utopia effluent (0% to 100%).

Methods

Effluent collection and storage

Effluent (2,000 L) was shipped weekly from Lake Utopia to Burlington, ON. Shipping usually took 3 to 4 days, but on one occasion it took 1 week to receive the effluent. Effluent was stored at 4°C until ready to use. Effluent flowed through a diluter which mixed it with Burlington laboratory water. Flows were checked and adjusted daily. 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 25 mL/min to each 12 L aquarium, which provided about 3 solution turnovers per 24 h.

Fish exposures

Exposures were started with 105 day old (± 3 days) fathead minnows that had been hatched and reared in Burlington lab water. Fish care and methods are detailed in Parrott and Blunt (2005) and Parrott and Bennie (2009), with the exception that as fish matured and could be sexed externally, females and males were removed to and reared in separate tanks. Fish were mature at the start of the test, so 3 males and 4 females were added to each aquarium. Breeding tiles were added (3 per tank) and breeding began immediately in many aquaria.

Fish sampling and grading of sex characteristics

After 30–33 days of exposure fish were sampled as described in Parrott and Blunt (2005) with the following exceptions.

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 1 to 3 for banding. Male Index was calculated as sum of fin dot score + dorsal fatpad score + (tubercles + 2 x large tubercles)/10 + banding score.

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.

Table 2.1-1. Test conditions for 30-day test with fathead minnow.

Date(s) effluent sampled	Weekly, Sept 28, 2009 to Oct 26, 2009
Pre-exposure phase	In lab, fish were 105 (± 3) days old at start of test
Effluent exposure	30–33 d
Tank turnovers	3 volumes/d
Quantity of effluent used	10,000 L (5 weeks)
Replicates	8 for control, 4 for each effluent concentration
Loading density	0.5 g/L/d
Feeding	Daily - frozen (and thawed) brine shrimp slurry
Endpoints measured	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
pH	7.8 to 8.4 (see Table 2.1-1b)
Dissolved oxygen,	8.0 to 5.1 mg/L (see Table 2.1-1b)
Temperature	22.5 to 22.9°C (see Table 2.1-1b)

Statistical analyses

Data were analyzed using Systat 11. Growth parameters of length (mm), weight (g), condition factor (CF), LSI, GSI, ovipositor area (mm^2), and male index, were assessed for differences among treatments using ANOVA. Significant differences from controls were assessed using two

sample t tests (Bonferroni's adjusted p values, separate variances) to determine levels of significance. P values were depicted in figures and tables as asterisks: * $p < 0.05$.

Results and Discussion

Water quality

Temperature was constant in exposure aquaria, but dissolved oxygen, pH and conductivity varied with effluent treatment (Table 2.1-1b)

Table 2.1-1b. Mean (± standard deviation) temperature (°C), dissolved oxygen (mg/L), pH, and conductivity (µS/cm) in control water and in Lake Utopia effluent (1% to 100%).

Treatment	Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Conductivity (µS/cm)
0	22.8 ± 0.39	7.97 ± 0.37	7.77 ± 0.089	333 ± 6.3
1%	22.9 ± 0.42	8.04 ± 0.32	7.81 ± 0.18	340 ± 8.5
3%	22.9 ± 0.39	7.80 ± 0.45	7.84 ± 0.10	374 ± 8.9
10%	22.8 ± 0.32	7.46 ± 0.63	7.91 ± 0.15	451 ± 19
30%	22.7 ± 0.33	6.39 ± 1.4	8.02 ± 0.30	751 ± 53
100%	22.5 ± 0.34	5.12 ± 1.6	8.42 ± 0.16	1,739 ± 177

Fish maturation and sex characteristics

At the end of the 30–33 d exposure all fish were mature (as they were 135 days post-hatch, dph). All 75 male fish had dorsal fin dots and 74 had dorsal fatpads, 71 fish had tubercles, and 62 male fish had bands or a dark head. As a result, male index was calculates for all groups, and ranged from 7 to 9 (Table 2.1-2). This was normal for male fathead minnows, and compared well to male indices of previous tests (which were 7.1 to 7.8 for 140–143 dph fish, Parrott and Bennie, (2009)). Female ovipositor area was normal, and ranged from 1.1 to 1.4 mm^2. 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)). Because these fathead minnows were sexually mature at the start of the tests, breeding began immediately in most aquaria.

Egg production

Egg production was decreased in a dose-responsive manner with exposure to Lake Utopia effluent. Fish exposed to 100% effluent laid no eggs, on those exposed to 30% effluent for 30 days laid only 5% of the eggs laid by control fish. Exposure to 10% effluent resulted in production of 60% of control eggs, but this was not significant ($p=0.087$) for total eggs and eggs per female (Figure 2.2-1). The number of egg clutches per tank was reduced for fish exposed to

10, 30 and 100%, and 10% effluent decreased the clutch numbers significantly (p = 0.044) from 20 in controls to 15 (Figure 2.1-2). Most treatments produced eggs within the first 1–4 days of the exposure, except for fish from 30% effluent which produced no eggs until 20 days into the exposure period, and 100% effluent which produced no eggs.

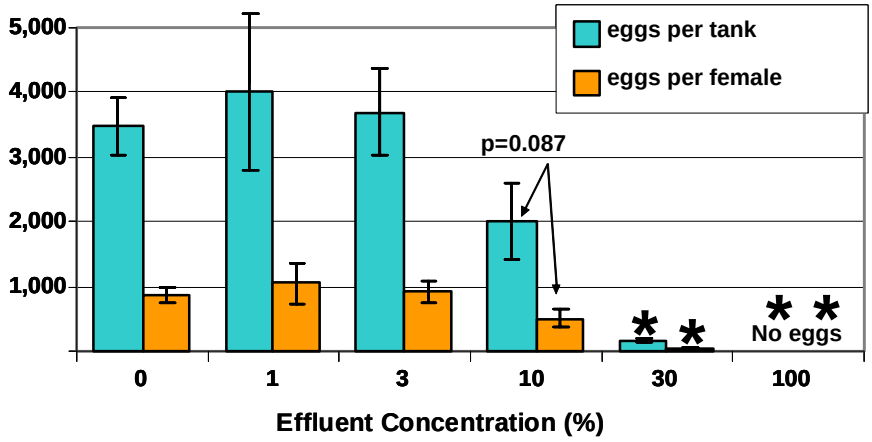

Figure 2.1-1. Mean (± standard error) number of eggs per tank and number of eggs per female for fathead minnows exposed to lab water (0%) or to Lake Utopia effluent (1 to 100%).

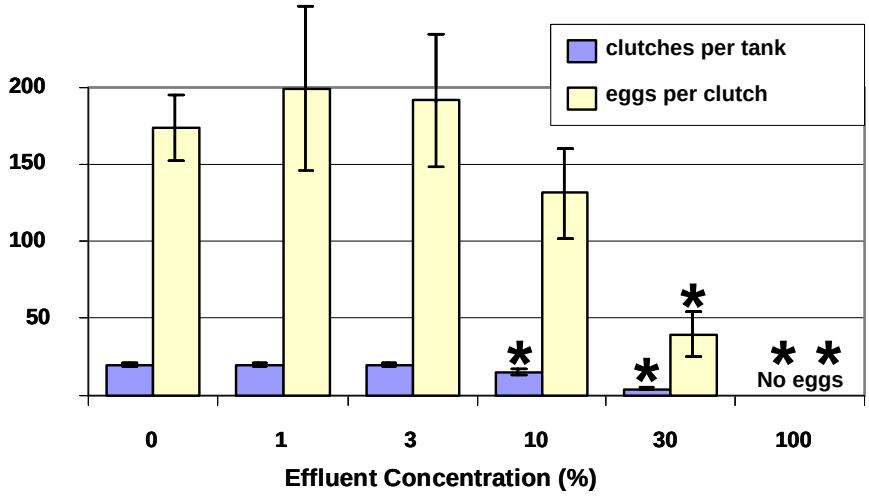

Figure 2.1-2. Mean (± standard error) number of clutches per tank and number of eggs per clutch for fathead minnows exposed to lab water (0%) or to Lake Utopia effluent (1 to 100%).

Fish growth

Exposure to Lake Utopia 30% and 100% effluent increased growth of female fish. Female condition was increased with exposure to 30% ($p=0.017$) and 100% effluent ($p = 0.41$).

Gonad and liver growth were also increase with high effluent exposures. GSI was significantly increased in male fathead minnows exposed to 30% () and 100% effluent ($p = 0.49$ and $p<0.001$, respectively). Female LSI was enlarged significantly with exposure to 30% effluent ($p=0.011$), but decreased significantly with exposure to 100% effluent ($p = 0.014$), probably due to toxicity of the 100% effluent. Gonadosomatic index was increased in females exposed to 30% ($p = 0.004$) effluent.

Conclusions

- Exposure to 30 and 100%.Lake Utopia effluent increased growth of fish..
- Effluent exposure (30% and 100%) increased GSI.
- LSI was increased in females exposed to 30% effluent
- Egg production was reduced with exposure to 10%, 30% and 100% Lake Utopia effluent
- Reproduction was reduced with no reduction in gonadal growth
- The 30 day fathead minnow juvenile to adult test was able to pick up slight growth enhancement, and reduced reproduction.

Table 2.1-2. Mean (± standard error) length, weight, condition factor (CF), liver-somatic index (LSI), gonadosomatic index (GSI), male index and ovipositor area (Ovi Area) for male and female adult fathead minnows exposed for 30–32 days to lab control water (0%) or to Lake Utopia effluent (1% to 100%). N is indicated in second column. Bold text with asterisk indicates significant difference ($p<0.05$) from controls, tested by two-sample t-test.

Males	n	Length (mm)	Weight (g)	CF	LSI	GSI	Male Index
0	23 – 24	56.2 ± 0.67	2.52 ± 0.089	1.41 ± 0.029	3.07 ± 0.13	1.21 ± 0.060	8.80 ± 0.68
1%	11 – 12	54.5 ± 1.1	2.25 ± 0.13	1.41 ± 0.060	3.05 ± 0.21	1.08 ± 0.062	7.01 ± 1.2
3%	12	57.0 ± 1.3	2.58 ± 0.12	1.40 ± 0.062	3.38 ± 0.25	1.20 ± 0.062	8.14 ± 0.88
10%	12	56.2 ± 0.51	2.45 ± 0.074	1.38 ± 0.029	3.34 ± 0.12	1.13 ± 0.068	8.16 ± 0.87
30%	12	57.2 ± 0.71	2.49 ± 0.076	1.33 ± 0.025	3.04 ± 0.17	1.40* ± 0.072	9.01 ± 0.51
100%	3	56.0 ± 2.5	2.25 ± 0.27	1.27 ± 0.052	3.79 ± 0.38	1.62* ± 0.043	7.10 ± 0.78

Females	n	Length (mm)	Weight (g)	CF	LSI	GSI	Ovi Area (mm2)
0	31	46.9 ± 0.61	1.20 ± 0.043	1.15 ± 0.017	4.35 ± 0.22	14.8 ± 0.46	1.05 ± 0.086
1%	16	48.4 ± 0.80	1.37 ± 0.075	1.19 ± 0.037	4.77 ± 0.23	14.9 ± 0.76	1.25 ± 0.11
3%	16	47.1 ± 0.76	1.23 ± 0.059	1.17 ± 0.019	4.57 ± 0.21	15.3 ± 0.61	1.33 ± 0.14
10%	16	47.1 ± 0.68	1.26 ± 0.064	1.19 ± 0.018	4.95 ± 0.28	14.9 ± 0.86	1.15 ± 0.13
30%	16	48.1 ± 0.87	1.37 ± 0.053	1.23* ± 0.027	5.17* ± 0.21	17.3* ± 0.65	1.40 ± 0.12
100%	10	46.2 ± 1.2	1.28 ± 0.076	1.30* ± 0.063	3.52* ± 0.23	17.2 ± 1.8	1.07 ± 0.18

2.2 Short-term Tests with Fathead Minnow and Rainbow Trout

The objective of this activity was to assess the applicability of short-term (<7 d) tests with the fathead minnow (*Pimephales promelas*) and the rainbow trout (*Oncorhynchus mykiss*) for IOC and IOS work aimed at eliminating/reducing mill effluent-related effects on fish reproduction. For this purpose, the fish were exposed to a treated effluent from a neutral sulphite semi-chemical (NSSC) Lake Utopia pulp mill which has been shown to affect the gonad size of male mummichog (*Fundulus heteroclitus*) in the Cycle 4 regulatory Environmental Effects Monitoring (EEM) study.

Methods

Test with adult fathead minnow

The adult (9 month old) fathead minnow was used. The fish, separated by sex, were raised in laboratory well water (also used for making effluent dilutions) as described in Kovacs et al. (2007b). The short-term test (seven days pre-exposure and five days effluent exposure) is our adaptation of a test developed by Ankley et al. (2001) and has been used at FPInnovations for previous work with mill effluents (Kovacs et al., 2007b). 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 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 (0%, 1%, 10% 30% and 100% effluent). The detailed test conditions are given in Table 2.2-1.

The fish were monitored for the number of spawns, egg production and egg fertilization. At the end of the tests, each fish was weighed and measured for length (condition factors or weight/length3 were calculated). At the completion of the exposure, the fish were anesthetized and plasma collected by caudal puncture and assayed for testosterone (males and females), estradiol (females) and vitellogenin activity (males) as described previously (Kovacs et al., 2007a). The gonads were excised and weighed to calculate gonad somatic indices (gonad weight/body weight x 100).

Test with immature rainbow trout

Immature rainbow trout were used in a 7 d test to measure levels of plasma vitellogenin (VTG) and hepatic mixed function oxidase (MFO) enzyme activity. Vitellogenin is a protein normally produced only by mature females during oogenesis. Hepatic MFO activity is a marker of exposure to substances inducing a family of enzymes involved in detoxication metabolism. MFO enzyme activity is measured on an artificial substrate as ethoxyrsorufin-O-deethylase (EROD) activity. The fish were exposed in 40 L containers for seven days with daily effluent solution renewals at concentrations of 0%, 1%, 10% 30% and 100% effluent. The conditions for the test are given in Table 2.2-2.

At the end of the exposure period, the trout were anesthetized and weighed. Blood was collected by caudal puncture, centrifuged at 26000 g for 3 minutes. The plasma was collected and 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. The liver was removed, rinsed and homogenized in Tris KCl pH 7.4 buffer at 4ºC and centrifuged at 10 000 g for 25 minutes. The resulting supernatants were stored at -85 C until analyzed for EROD activity following the method described in Martel et al. (1995).

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 plasma 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.

Table 2.2-1. Test conditions for short-term tests with fathead minnow	
Date effluent sampled	October 12, 2009
Pre-exposure phase	7 d
Effluent exposure	5 d
Tank turnovers	6/d
Quantity of effluent used	2203 L
Replicates	4 (2 males and 4 females in 12.5 L)
Loading density	0.14 to 0.20 g/L
Feeding	Ad libitum; freshly hatched Artemia (brine shrimp) three-times a day
Endpoints measured	Egg production, number of spawns, Egg fertilization, Length, weight, In males: testosterone and vitellogenin In females: testosterone and estradiol
pH	8.2 to 8.4
Dissolved oxygen	72 to 93% saturation
Temperature	24 to 25°C

Table 2.2-2. Test conditions for short-term test with immature rainbow trout.	
Date effluent sampled	October 12, 2009
Effluent exposure	7 d
Tank turnovers	1/d
Quantity of effluent used	655 L
Replicates	2 (3 fish in 40 L)
Loading density	0.51 to 0.67 g/L
Feeding	None
Endpoint measured	Vitellogenin Ethoxyresorufin -O- deethylase
pH	8.0 to 8.7
Dissolved oxygen	71 to 105% saturation
Temperature	11 to 13°C

Results and Discussion

Short-term adult fathead minnow reproduction test

Egg production and spawning: The egg production of fathead minnow was not significantly affected by exposure to the NSSC effluent up to 30% concentration. The egg production of fish exposed to the 100% concentration was severely inhibited and resulted in an average 3 eggs per female per day compared to controls which produced 31 eggs per female per day (Table 2.2-3). In the 100% effluent treatment there were not enough eggs produced to calculate a reliable fertility rate.

Table 2.2-3. Egg production, number of spawning, and egg fertilization rate for fathead minnow exposed to a final biotreated neutral sulphite semi-chemical pulp mill effluent. Results are expressed as means (standard error).

Treatment	Eggs/female/day	Number of spawning events	Fertility, %
Control	31 (6)	4 (1)	80 (11)
1%	48 (9)	4 (0)	90 (5)
10%	30 (11)	4 (0)	97 (1)
30%	19 (7)	3 (1)	73 (24)
100%	3* (3)	1 (0)	‡

* statistically significant difference from control, $p < 0.05$ (Anova and Dunnett's)
‡ insufficient number of eggs to calculate fertility

Morphometric parameters: The gonad somatic index of male and female minnow was not significantly affected by exposure to effluents in all treatments. Similarly we observed no significant differences in condition factor, body length and weight (Table 2.2-4).

Steroid hormones and vitellogenin: There was no significant difference between controls and all treatments for plasma estradiol and testosterone concentrations in females (Figure 2.2-1). Similarly there was no significant difference in plasma vitellogenin and testosterone concentrations in males for all treatments compared to controls (Figure 2.2-2).

Overall, this effluent caused an inhibition of reproduction in fathead minnow but no significant was observed on steroid hormones and vitellogenin, once again supporting the observation that egg production is the most sensitive endpoint in this test.

Table 2.2-4. Morphological parameters and gonad somatic index for male and female fathead minnow exposed to a final, biotreated neutral sulphite semi-chemical pulp mill effluent. Results are expressed as means (standard error).

Treatment		Fork length, mm	Body weight, g	Condition factor	Gonad weight, mg	Gonad somatic index, %
Control	Females	50 (0.7)	1.6 (0.05)	1.3 (0.03)	231 (24)	14 (1.0)
	Males	61 (1.1)	3.4 (0.1)	1.5 (0.04)	51 (7.2)	1.5 (0.2)
1%	Females	50 (0.7)	1.6 (0.08)	1.3 (0.03)	240 (43)	14 (1.6)
	Males	61 (1.0)	3.2 (0.1)	1.4 (0.05)	39 (3.9)	1.2 (0.1)
10%	Females	50 (0.7)	1.6 (0.07)	1.3 (0.03)	289 (40)	17 (1.7)
	Males	59 (0.8)	3.0 (0.1)	1.5 (0.04)	35 (4.4)	1.1 (0.1)
30%	Females	50 (0.5)	1.7 (0.05)	1.4 (0.02)	223 (16)	14 (1.0)
	Males	62 (1.2)	3.3 (0.1)	1.5 (0.09)	49 (3.6)	1.4 (0.05)
100%	Females	49 (0.7)	1.7 (0.08)	1.4 (0.04)	295 (38)	17 (1.7)
	Males	61 (1.2)	3.3 (0.2)	1.5 (0.03)	40 (5.3)	1.2 (0.1)

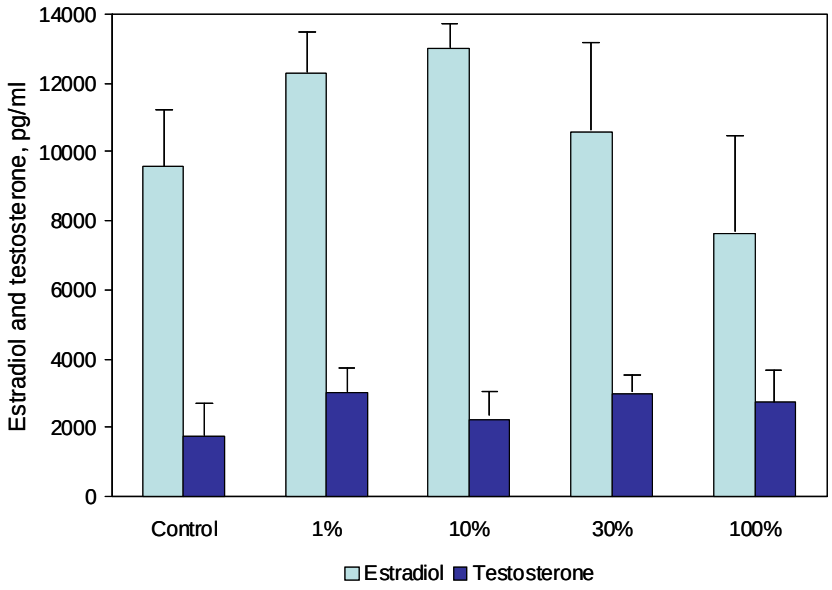

Figure 2.2-1. Plasma estradiol and testosterone in female fathead minnow exposed to a biotreated neutral sulphite semi-chemical pulp mill effluent.

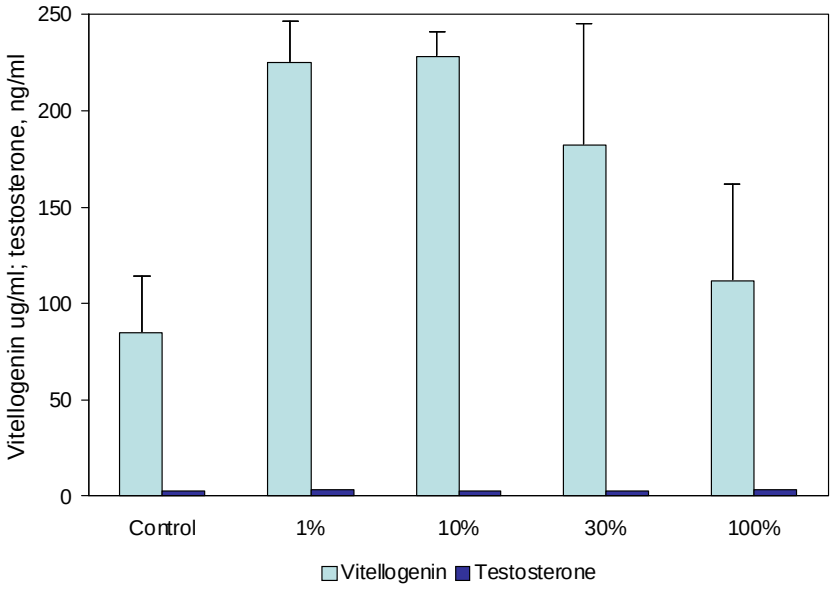

Figure 2.2-2. Plasma vitellogenin and testosterone of male fathead minnow exposed to biotreated neutral sulphite semi-chemical pulp mill effluent.

Short-term with immature rainbow trout

Vitellogenin and EROD: Plasma concentrations of vitellogenin were not significantly different from controls despite an apparent trend towards lower levels in the 100% exposed fish (Figure 2.2-3). Hepatic ethoxyresorufin-O-deethylase activity was induced 3-fold in fish exposed to 100% effluent and this induction was marginally significant ($p = 0.054$). As observed in Section 1.3.3 the statistical power and sensitivity of this test could be improved by measuring these endpoints in individual fish by using fish of a larger size.

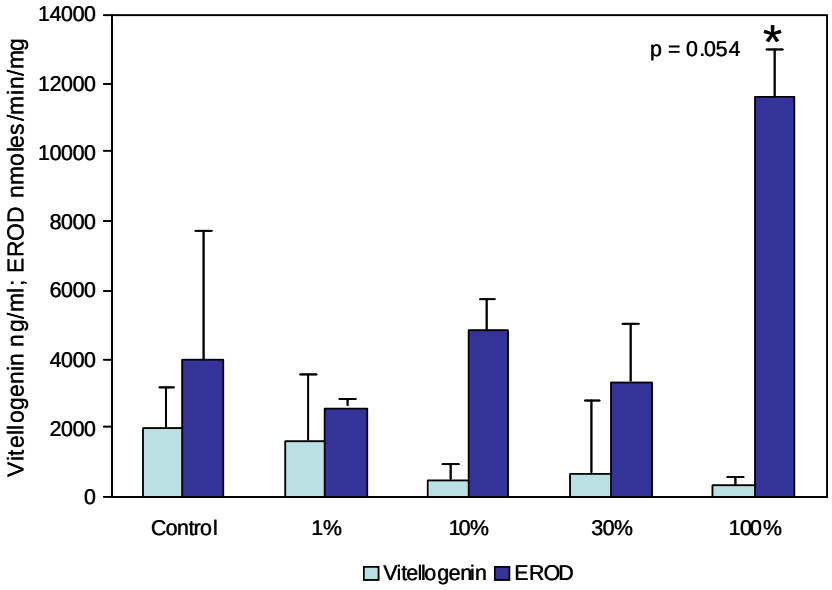

Figure 2.2-3. Plasma vitellogenin concentrations and hepathic etoxyresorufin-O-deethylase activity in immature rainbow trout exposed to biotreated sulphite semi-chemical pulp mill effluents. * marginally significant difference (p = 0.054).

Conclusions

- In fathead minnow the only significant effect measured was inhibition of egg production in the 100% effluent treatment. No effect on gonad size was detected.
- Immature rainbow showed a statistically marginal induction of EROD activity in response to exposure to 100% of this effluent.
- The results of these two short-term test showed that the effect on fathead minnow egg production was more pronounced and unequivocal than EROD induction in trout.

2.3 Short-term Tests with Adult Zebrafish

Introduction

The primary objective of this activity was to assess the effects of exposure to graded amounts of effluent from the mill at Lake Utopia on reproduction in the zebrafish (*Danio rerio*). This included 7 day exposure studies with adult fish that examined egg production, ovarian size and selected ovarian and hepatic gene expression.

Materials and Methods

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 h light and 12 h 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).

The spawning tests were conducted in 4 L glass beakers containing 3.5 L of water or effluent. There were 6 females and 3 males in each breeding tank. The pre-exposure period was 4 days followed by an exposure period of 7 days. These tests included a control, 1, 10, 30 and 100% effluent. There were 6 replicate tanks per treatment. Test conditions are detailed in Table 2.3-1.

Table 2.3-1. Test conditions for adult zebrafish exposure.

Date(s) effluent sampled	October 13, 2009
Pre-exposure phase	Start October 16, 2009, for 4 d
Effluent exposure	Start October 20, 2009, for 7 d
Tank turnovers	Static, complete replacement once per day (3.5 L)
Quantity of effluent used	207 L
Replicates	6 tanks per treatment (6 females and 3 males)
Loading density	1.15 g/L/d ± 0.02
Feeding	Fed twice (salmon-fry pellets in the morning and bloodworms in the afternoon)
Endpoints measured	Egg production Gonadosomatic index Gene expression –StAR and aromatase in vitellogenic follicles and CYP450 1a1 and vitellogenin in the liver
pH	8.33 ± 0.03
Dissolved oxygen	83 ± 0.79% saturation
Temperature	26.7 ± 0.44°C

At the end of the exposure, fish were overdosed with MS-222 (Sigma, St. Louis MO) and weighed. Ovaries were excised and weighed for the calculation of gonadosomatic index (GSI) and then immediately snap frozen in liquid nitrogen before processing. A portion of ovarian tissue was used for the measurement of steroid content. Additionally, the intestine and liver were dissected and placed in RNAlater. The livers were subsequently dissected from the intestine and stored at -80°C for future measurement of vitellogenin and cytochrome P450 1a1 (P450-1a1) mRNA expression.

RNA extraction, reverse transcription and Real-Time PCR followed the methods described by Ings and Van Der Kraak (2006). The ovarian genes evaluated included StAR and P450-arom. The expression of vitellogenin and P450-1a1 was measured in the liver. The primers used for the measurement of P450-1a1were Forward CTGGACGAAAACTCCAACCTG; and Reverse GATAGTGTCGAAACCGGCTCC (based on Accession number AY398333.1). The primers used for vitellogenin were Forward TGCGTGAAGTTGTCATGCTTG; Reverse GATCTCGTGGATGGGCCTG (based on NM_170767.1).

For steroid measurement, ovarian tissue was extracted with methanol, and the extracted were purified on a Amprep C-18 mini columns according to the methods described by Lister and Van Der Kraak, 2008. The amounts of 17β-estradiol and testosterone were measured by enzyme immuno assay using kits from Cayman Chemical (Ann Arbor, Michigan) according to the methods described by Lister and Van Der Kraak, 2008.

Results

There was a significant reduction in egg production for fish exposed to 100% effluent (Figure 2.3-1). There was no change in GSI following exposure to effluent (data not shown). Similarly, there were no differences in the ovarian expression of StAR and P450-arom (Figure 2.3-2).

Conclusions

- Exposure to Lake Utopia effluent contributed to a significant dose related decline in the numbers of eggs spawned by adult zebrafish.
- Exposure to effluent or BL had no effects on growth or ovarian development in juvenile Zebrafish.
- Biomarker responses (steroids, vitellogenin, P4501A) were not always predictive of egg production.

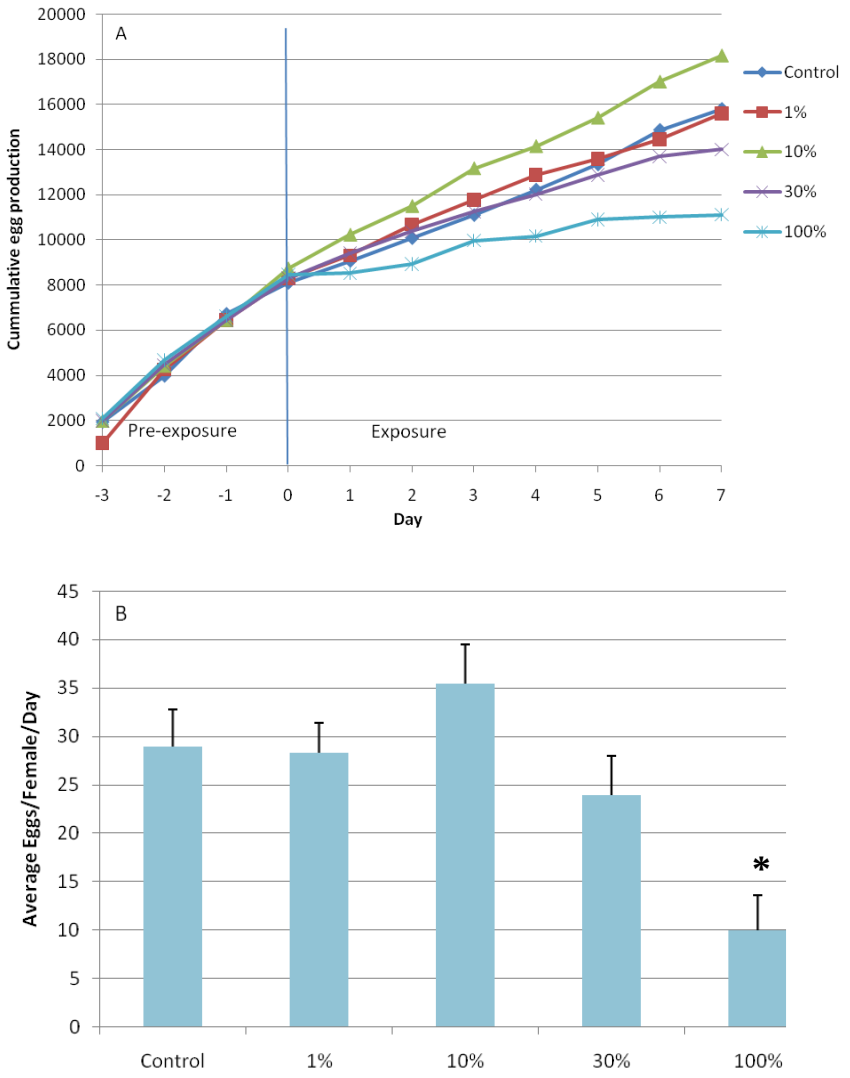

Figure 2.3-1. Cumulative egg production (A) of female zebrafish during a pre-exposure (4 days) period and following seven days of exposure to varying concentrations of pulp mill effluent. Average number of eggs per female per day (B) during 7 days of exposure to varying concentrations of pulp mill effluent.

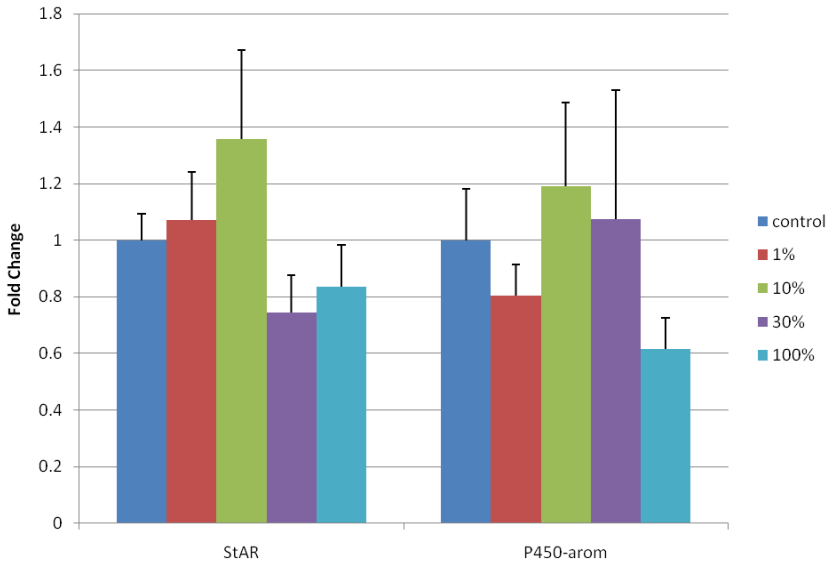

Figure 2.3-2 Expression of P450-aromA and StAR in the ovary of adult zebrafish following seven days of exposure to varying concentrations of pulp mill effluent. Expression of the genes is normalized to β-actin.

2.4 Medium-term Test with Mummichog

The purpose of the medium-term (30-day) mummichog gonadal growth test was to determine the effect of effluent exposure during gonadal maturation in this estuarine, asynchronous spawner. It has previously been established that Lake Utopia Paper final effluent affects mummichog energy balance in wild fish (EEM Cycles 3 and 4, Lake Utopia Paper, St. George, NB) as well as egg production in the standardized adult mummichog reproductive test (EEM Cycle 5, Lake Utopia Paper, St. George, NB; Bosker et al., 2010a). The medium-term gonadal growth bioassay exposes fish during the period of gonadal recrudescence (gonadal maturation) in adult fish; final endpoints include those standardized in the endocrine bioassay in mummichog (see section 1.3.6). This was the first attempt to assess effluent exposure during a 30-day period of gonadal maturation.

Methods

Lake Utopia effluent collection and storage

Effluent was shipped from the Lake Utopia mill to the Hagen Aqualab, University of Guelph in October (5000 L) and November (3000 L), 2009. The effluent was stored outside and used directly in the exposure or it was warmed to room temperature prior to use depending on the target temperatures within the fish tanks.

Fish exposure

Adult male and female mummichog were exposed for 31 days to increasing concentrations of effluent to determine the potential effects on the reproductive endocrine system. The fish were acclimated in aerated, 20L glass tanks containing Hagen Aqualab well water that was salted with Crystal Sea® to approximately 16 ppt at a temperature of 4–6ºC. A flow-through system was used in which treatments were pre-mixed in large aerated reservoirs and pumped continuously to the tanks at a target flow rate of 1.7 L/h, which provided approximately 2 tank turnovers per 24 h. Flow rates were checked daily and adjustments made when necessary.

The fish were exposed to well water only (0% control), or to increasing concentrations of effluent (1, 10, 30, and 100%). Salinity concentrations were maintained at approximately 16 ppt for every treatment. There were 5 replicate tanks for each treatment; each tank contained 3 males and 3 females. Water temperatures of the tanks were regulated by water baths throughout the experiment. At the beginning of the exposure period, all tanks were 4–6ºC and increased in temperature by approximately 1ºC per day until a temperature of 17 ± 1ºC was obtained on the 18th day of exposure. In an attempt to maximize gonadal growth during the exposure, the light cycle was set at 16 h light: 8 h darkness. Other exposure parameters can be found in Table 2.4-1.

After 31 days of exposure, fish were anaesthetized and killed by cervical transection. Fish length, body weight, and weights of the gonads and liver were recorded for each fish for the assessment of condition factor (K) and gonadosomatic (GSI) and liversomatic indices (LSI).

Table 2.4-1. Test conditions for mummichog exposed to Lake Utopia effluent for 31 days.	
Date(s) effluent sampled	October 13 and November 9, 2009
Pre-exposure phase	Start October 26, 2009, for 4 d
Effluent exposure	Start October 30, 2009, for 31 d
Tank turnovers	About two volumes/day (~ 40L/tank/d)
Quantity of effluent used	8742 L
Replicates	5 tanks per treatment (3 females, 3 males/tank)
Loading density	1.91 ± 0.05 g/L/d
Feeding	Week 1: every other day; Week 2–4: once a day
Endpoints measured	Gonadosomatic index Liversomatic index Condition factor
pH	8.2 – 8.7 (n=9 for each tank)
Dissolved oxygen	> 80% saturation (monitored in each tank daily)
Temperature	Regime: Fish at 4ºC prior to experiment. During exposure period, temperature increased approximately 1ºC per day until 17 ± 1ºC was reached (monitored in each tank daily).

Statistical Analyses

Data were checked for homogeneity of variance using the Levine's test prior to analysis; logarithmic transformations were used if data did not meet the test criteria. Body weight was tested with analyses of variance (ANOVA) (Kruskal Wallis, if non-parametric) followed by Tukey's post-hoc test ($p<0.05$). Analyses of covariance (ANCOVA) were used to analyze gonad and liver weight relative to body weight, and body weight relative to body length across treatments after checking the homogeneity of regression (slopes). ANCOVA results are presented with the associated means of K, GSI, and LSI. Data were analyzed using PASW (SPSS) v17.0.

Results and Discussion

Males

Body weights (ANOVA), testis and liver weights as a function of body weight (ANCOVA), and condition of the control fish did not differ significantly from the effluent treatments (Table 2.4-2).

Table 2.4-2. Body weight (g), condition factor (K), gonadosomatic index (GSI) and liversomatic index (LSI) of male and female mummichog exposed to Lake Utopia Paper effluent for 31 days (Oct 30–Nov 30, 2009). All values are Mean ± S.E.M. Values with different letters are significantly different ($p<0.05$; Tukey's). There were 5 replicate tanks per treatment; each tank contained 3 males and 3 females.

Treatment	Weight (g)	K (%)	GSI (%)	LSI (%)
Males				
0%	5.51 [ab] (0.25)	1.05 (0.02)	1.62 (0.34)	4.37 (0.17)
1% effluent	5.63 [ab] (0.23)	1.11 (0.02)	1.45 (0.18)	3.95 (0.14)
10% effluent	4.72 [a] (0.32)	1.08 (0.02)	2.35 (0.26)	4.62 (0.08)
30% effluent	5.09 [ab] (0.31)	1.11 (0.04)	2.42 (0.27)	4.22 (0.22)
100% effluent	5.97 [b] (0.26)	1.04 (0.01)	1.26 (0.21)	4.09 (0.20)
Females				
0%	7.85 (0.64)	1.11 (0.02)	3.95 (0.86)	5.15 (0.30)
1% effluent	7.31 (0.51)	1.15 (0.03)	4.37 (1.05)	5.27 (0.44)
10% effluent	7.90 (0.51)	1.10 (0.01)	5.81 (0.75)	5.47 (0.32)
30% effluent	8.48 (0.66)	1.14 (0.02)	5.07 (0.91)	5.75 (0.10)
100% effluent	6.57 (0.86)	1.03 (0.04)	3.39 (0.63)	4.94 (0.39)

Females

There were no statistically significant differences found in body weights (ANOVA), ovarian and liver weights as a function of body weight (ANCOVA), and condition of the control fish from the effluent treatments (Table 2.4-2).

Conclusions

There were no effects of final Lake Utopia Paper effluent on condition, gonadal size or liver size in a 31-day exposure to recrudescing (maturing) mummichog.

2.5 Effluent Chemistry

For effluent quality monitoring, samples from the Lake Utopia Paper mill were collected directly from shipping totes used for the fathead minnow juvenile gonadal growth test that occurred over 5 weeks from October 2 – November 2, 2009. The objectives and methods of this work are as described above in Section 1.3.7 for the Espanola studies.

Results

While not a bleached kraft mill effluent which has been studied previously, the general parameters measured from the mill effluent from Lake Utopia were within regulatory limits. Some differences between this mill effluent and those studied earlier with Espanola included a 10-fold higher value for colour (Table 2.5-1). However a higher value for colour is expected in this case as effluent biotreatment involves a sequential anaerobic/aerobic treatment which creates colour (Milestone et al., 2004). The variability for BOD can be attributed to the first sample measured, which was nearly 200 mg/L; all other values were close to 45mg/L. Metals levels in the effluent samples collected are presented in Table 2.5-2 and are again indicative of a different profile that that previously encountered with bleached kraft effluents.

Table 2.5-1. Summary of physical parameters measured in Lake Utopia effluent sampled during the weeks October 2–November 2, 2009. Data presented are means and standard deviations (SD) of weekly samples (n=5).

Parameter	Mean ± SD
pH	7.96 ± 0.07
Total Alkalinity	904 ± 128 mg/L $CaCO_3$
Ammonia as N by CFA	0.99 ± 0.75 mg/L as N
Carbonaceous BOD	82.5 ± 77.0 mg/L
Chloride by IC	20.1 ± 1.7 mg/L
NO_3 as N by IC	0.61 ± 0.08 mg/L
SO_4 by IC	85.6 ± 12.2 mg/L
Total Inorganic Carbon in Water	183 ± 30 mg/L
Total Organic Carbon	357 ± 44 mg/L
Total Suspended Solids	61.5 ± 23.7 mg/L
Colour	6600 ± 657 CU
Polyphenols	205.7 ± 24.1 mg/L

Table 2.5-2. Summary of metals measured in Lake Utopia effluent sampled during the weeks October 2–November 2, 2009. Data presented are means and standard deviations (SD) of weekly samples (n = 5).

Metal (μg/L)	Mean ± SD
Aluminum	1714 ± 104
Arsenic	2.1 ± 0.2
Barium	176 ± 45
Beryllium	0.16 ± 0.07
Boron	604 ± 200
Calcium (mg/L)	28.5 ± 4.6
Cadmium	0.36 ± 0.18
Cobalt	4.2 ± 0.9
Chromium	6.6 ± 1.2
Copper	55.3 ± 7.2
Iron	2992 ± 1739
Gallium	10.5 ± 3.4
Potassium (mg/L)	26.1 ±1.3
Lanthanum	0.53 ± 0.05
Lithium	15.8 ± 12.2
Magnesium	5,328 ± 152
Manganese	568 ±126
Molybdenum	8.0 ± 4.6
Sodium (mg/L)	456.4 ± 51.5
Nickel	10.7 ± 2.0
Lead	3.6 ± 0.6
Rubidium	69.6 ± 12.8
Antimony	0.7 ± 0.1
Selenium	2.5 ± 0.7
Strontium	137 ± 24
Uranium	0.67 ± 0.49
Vanadium	8.1 ± 0.8
Zinc	48.5 ±21.1

OVERALL SUMMARY AND CONCLUSIONS

The work described in this report is part of a national multi-agency project aimed at finding solutions for effects of pulp and paper mill effluents on fish reproduction. Entailed in this work is selecting the most appropriate laboratory tests for conducting Investigation of Cause (IOC) and Investigation of Solution (IOS) studies for the Environmental Effects Monitoring (EEM) Program.

The work described in this report represents work conducted at two mills (Domtar bleached kraft mill at Espanola, ON; Lake Utopia Paper neutral sulphite semi-chemical Lake Utopia mill at St. George, NB) between February 2007 and October 2009.

A long-term monitoring study at the Espanola mill provided insights into temporal effluent variability with respect to fish reproduction. Evaluations of laboratory tests were conducted to confirm hypotheses on the causes and sources of the variability. The laboratory tests ranged in duration from just a few days to several weeks and covered an assessment of reproductive endpoints in fish ranging from the biochemical level to egg production. The work also included an assessment of wild fish in the Spanish River receiving the mill effluent from Espanola. The results of the work with the Espanola mill effluent allowed the following conclusions to be made:

Contrary to previous EEM studies using white sucker, field collections in the Spanish River using silver redhorse and logperch determined that neither species exhibited reduced gonad sizes downstream of the Espanola mill.

- Depressions in the major biologically active reproductive steroids in both sexes of silver redhorse indicated the potential of the effluent to affect fish reproduction.
- Relative to upstream, there was evidence of enrichment in both species.
- Long term monitoring of Espanola final effluent showed fathead minnow egg production tracked changes in effluent quality as it related to production upsets, mill restarts and conditions affecting biotreatment performance.
- Final effluent quality variations were further tracked by a gas chromatographic (GC) Profiling Index, BOD and methyl-2-cyclopentenones, which all show promise as diagnostic tools for IOC/IOS work.
- Subsequent laboratory tests with five fish species confirmed the potential contribution of black liquor for causing final mill effluents related effects on fish reproduction.

Final effluent from the Lake Utopia Paper mill was used to evaluate laboratory tests with four species of fish. The laboratory tests ranged in duration from just a few days to several weeks and covered an assessment of reproductive endpoints in fish ranging from the biochemical level to egg production. The results of the work with the Lake Utopia mill effluent allowed the following conclusions to be made:

- In short-term tests ($\leq$7d), reduced egg production in two fish species was found to be the most sensitive endpoint resulting from effluent exposure.

- In medium-term test (~30d) reduced egg production was also found to be the most sensitive endpoint resulting from effluent exposure. The effluent-related effects in the medium-term test egg production occurred at lower concentrations than short-term tests.

Collectively, our work has identified egg production in short-term tests as showing the most potential for future IOC/IOS studies. We have identified leads for future IOS studies that involve minimizing organics losses and upsets to biological treatment. Future IOS studies will need to include longer term tests as part of the validation of solutions.

ACKNOWLEDGEMENTS

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