EFFECTS OF WOOD LEACHATES DURING SHORT-TERM AND LIFE-CYCLE BIOASSAYS

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Abstract - Effluents from several types of mills including bleached and unbleached kraft, TMP and recycled fiber mills have been reported as having effects during both short-term and life-cycle laboratory bioassays, including effects on fish reproduction. Authors have suggested that naturally occurring constituents released during pulping may be the source of these effects. Leachates of wood chips from loblolly pine (*Pinus taeda*), western hemlock (*Tsuga heterophylla*), and Douglas fir (*Psuedotsuga menziesii*) were evaluated in the laboratory using: 1) 40 min marine echinoderm egg fertilization 2) 48-hr marine bivalve embryo/larval development, 3) 7-d *Ceriodaphnia dubia* survival and reproduction, 4) fathead minnow (*Pimephales promelas*) 7-d survival and growth, and 5) fathead minnow life-cycle tests to determine whether naturally occurring wood components are possible sources of effects noted during bioassays. Pine leachates caused effects in short-term tests but not in life-cycle tests. Concentrations of resin and fatty acids over 4 mg/L, may have been the cause of pine leachate effects. The concentrations of resin and fatty acids in the leachates are much higher than those found in modernized effluents, indicating resin and fatty acids are unlikely to cause effects on reproduction by mill effluents. Hemlock and fir leachate both caused effects during fish life-cycle tests, indicating that chemicals derived directly from wood furnish can cause effects on fish reproduction. However, effects of the leachate on fathead minnow and *C dubia* survival indicate that toxic components of the leachate are unlikely to be found in effluents at similar concentrations. Douglas fir leachate had the greatest effect during fathead minnow reproduction tests, but had intermediate to low concentrations of most of the measured effluent components. Similar chemical concentrations and life-cycle results have been measured in effluents from two mills with Douglas fir furnish. Thus, evaluations of hemlock and fir leachates indicate that effects of final mill effluents on fish reproduction may be from naturally occurring components derived from wood furnish.

Keywords: bioassays, reproduction, wood leachate, fathead minnow, *Ceriodaphnia,* marine

INTRODUCTION

Effects of pulp mill effluents on fish reproduction have been a focus of concern in recent years (1,2,3). Some researchers have suggested that effluents from mills without bleaching processes produce effects on fish reproduction or indicators of reproductive fitness that are similar to effluents from mills with bleaching (4,5). The observation of similar fish reproductive responses, using various mill effluent process types (bleached and unbleached) raises the question that some of the responses may be due to natural components of furnish used in the pulping process. This hypothesis is supported by information indicating that plants are known to produce a variety of secondary metabolites that are biologically active agents. These chemicals are known to work effectively against the consumption of plants by herbivores, including insects, and are also reported to have antifouling and anti-microbial properties (6). Plant secondary metabolites include toxicants such as pyrethrins, rotenone, atropine, strychnine, and aflatoxin (7). The number of known secondary metabolites produced by plants include over 7,500 terpenoids and 5,000 phenolic compounds. Numerous studies point to effects of water soluble secondary metabolites such as tannins on aquatic organisms, including effects on bacteria, phytoplankton, and a variety of aquatic plants (6). Adverse effects of tannins on fish have also been

reported, including gill damage to carp (*Cyprinus carpio*) (8), and sperm motility inhibition in walleye (*Stizostedion vitreum*) (9). Aspen leachate was acutely toxic to trout and *Daphnia* at 1 to 2% v/v (10)

The primary objective of the investigation reported in this paper was to determine if water soluble components of woods were possible sources of effects observed during bioassays commonly used to evaluate whole effluent toxicity in the USA, and to determine if these components could affect reproduction during fathead minnow (*Pimephales promelas*) life-cycle tests. Information is also provided about the amounts of chemical components found in the leachates. These are compared to chemical components measured in effluents during other life-cycle studies, as well as biological endpoints during these studies (5, 11-18).

The results of four studies with leachates from loblolly pine (*Pinus taeda)* (2 studies), hemlock (*Tsuga heterophylla*), and Douglas fir (*Psuedotsuga menziesii*) are described. A fathead minnow life-cycle test was completed during each study, accompanied by two or three short-term sub-lethal bioassays with *Ceriodaphnia dubia,* fathead minnows, echinoderms and bivalves. A summary of the results of these studies is presented. Additional descriptions of these studies may be found elsewhere (19-21)

Materials and Methods

Facility Descriptions and Dilution Waters

Bioassays were performed at two facilities. All fathead minnow and *C.dubia* 7-d bioassays, and pine leachate fathead minnow life-cycle bioassays were performed at the NCASI Southeastern Aquatic Biology Facility near New Bern, North Carolina. All echinoderm and bivalve bioassays, and fathead minnow life-cycle bioassays with hemlock and fir leachates were completed at the NCASI Northwest Aquatic Biology Facility in Anacortes, Washington. Dilution systems at both labs were equipped with proportional solenoid valve diluters. Laboratory and dilution water temperatures were maintained at 25±2°C with one exception early in the hemlock leachate test when the temperature fell to 20°C. Dissolved oxygen in the exposure tank solutions was maintained above 6 mg/L by aeration. Lighting on the tanks was controlled between 80-100 ft-c by banks of broad-spectrum fluorescent lights. Electrical timers controlled the photoperiod to 16 hr of light and 8 hr of darkness.

Dilution water for the fathead minnow egg hatchability, 28-d, 56-d and life-cycle tests with pine leachate (pH 8.3±0.2, hardness 118±13 mg/L, conductivity 409 ±9 µmho/cm) was from a well located at the test site. Dilution water from the life-cycle studies with hemlock and fir was dechlorinated city drinking water with 2% seawater added for mineral content. During the hemlock life-cycle test the dilution water pH was 7.2±0.2, hardness was 137±11mg/L, and conductivity was 1318±111µmho/cm. The pH averaged 7.4±0.1, hardness averaged 124±5 mg/L, and conductivity averaged 1135±49 µmho/cm during the Douglas fir leachate test. Dilution water for the *C. dubia* and fathead minnow 7-d bioassays was synthetic, moderately hard water (pH 7.6±0.3 hardness was 73±4 mg/L, conductivity 289± 9 µmho/cm) (22). Dilution water used for the marine echinoderm and bivalve tests was reagent grade deionized water adjusted to full strength seawater with hypersaline brine (23).

Leachate Production and Characterization

Leachates for each of the four life-cycle tests and corresponding short-term bioassays were produced in a similar manner. Loblolly pine, western hemlock and Douglas fir wood chips were collected weekly or biweekly from mills near each test facility. Chips were placed in nylon mesh bags and placed in tanks of dilution water loaded with 62g of chips/liter of dilution water for the pine and hemlock studies, and at 100g/L for the fir study. After steeping for 5 days at ambient temperatures of approximately 20 – 25°C the leachate was pumped to head tanks for the bioassays. During the second test with pine chips the temperature was increased to 30°C±4°C and three foam fractionators were used in each leaching tank in an attempt to: a) reduce the concentration of resin acids in the leachate to levels found in effluents from mills that use pines as a furnish, 2) reduce the acute toxicity of the leachate to fathead minnows and 3) elevate polyphenol (tannin/lignin) and total organic carbon (TOC) concentrations to levels detected in pulp mill effluents during previous life-cycle studies.

The leachate was characterized for color, hardness, conductivity, biochemical oxygen demand (BOD), and TOC (24, 25). Samples were analyzed for resin acids, fatty acids, polyphenols, phytosterols and neutral semi-volatile compounds (26, 27,28). The polyphenol test is based on a folin phenol reagent reaction reaction and provides and approximation of tannin/lignin content as well as other materials containing aromatic hydroxyl groups.

Short-Term Bioassays

Ceriodaphnia dubia 7-d tests for survival and reproduction and the fathead minnow 7-d test for survival and growth followed standardized procedures (22). Minor modifications to the *C. dubia* and fathead minnow tests are described elsewhere (17). The marine echinoderm bioassay method was described in Cherr et al. (29) with modifications by Hall et al. (30) and NCASI (23). Sperm and eggs from either the purple sea urchin (*Strongylocentrotus purpuratus*) or sand dollar (*Dendraster excentricus*) were used for the tests described here. Marine bivalve development test methods were based on 48-h embryo-larval exposure of the blue mussel (*Mytilus edulis*). Hypersaline brine was added in appropriate amounts to each leachate dilution to achieve consistent 30-mg/kg full strength sea water solution salinity for both the echinoderm and bivalve tests. This method allowed a maximum of 70% v/v of leachate sample to be tested (31).

Fathead Minnow Life Cycle Test

Fathead minnow life-cycle testing was completed using proportional solenoid-valve diluters, modified with a solenoid switch to discharge the entire volume of the leachate and diluent mixture of each test concentration alternately into the A or B replicate test chamber. Flow rates to each test chamber averaged approximately 1L each 5 to 8 min cycle. Replicate A and B test chambers were randomly selected for exposure to leachate concentrations. Test concentrations were usually 0, 3, 6, 12, 24, 50, and 100% v/v of leachate. However during the first test with Pine leachate the highest concentration was 25% effluent because pre-test evaluations indicated that 32% of the leachate was acutely toxic to fathead minnows. The light intensity at the water surface of each chamber was maintained at approximately 100 ft-c using broad-spectrum fluorescent lights mounted over the tanks. Dissolved oxygen in each chamber was maintained above 6 mg/L by aeration.

The fathead minnow life-cycle test was initiated when eggs spawned in laboratory cultures were placed in hatching chambers, suspended in exposure tanks. A total of 100 eggs per replicate with two replicates per concentration were added to the chambers. Hatching occurred by day 4 or 5, when the live larvae were counted and the percent hatch was calculated.

Fifty of the larvae were moved to grow-out chambers placed in the exposure tanks with exchange rate of 8 to 10 times daily. The larvae were fed newly hatched (<24h old) *Artemia* nauplii three times a day at the initial rate of 3 ml/chamber of a 1600/ml suspension. This amount was increased on a weekly basis to a maximum of 12 ml by day 23. All tanks and chambers were monitored twice daily, and cleaned every 7 days to remove extra food and waste. On day 28 the fish were not fed and the juveniles were individually weighed. The same procedures as outlined for the first 28 days continued through day 56. The food was supplemented with 2 ml of 250 g/L frozen brine shrimp slurry on day 29, and increased to 5 ml by day 55. On day 56 the fish were blotted dry, weighed to the nearest 0.1 mg, and moved to a second exposure system of larger aquaria for the reproduction portion of the life-cycle test.

The fathead minnows continued to be fed the frozen brine shrimp until day 75-80. When males began to show spawning coloration, one male and two females were placed in each of four spawning areas in each aquarium separated by stainless steel screens. Since each concentration had replicate aquaria, eight spawning groups were exposed to each concentration. Spawning fish were fed 3 ml/spawning group of the 250 g/L frozen brine shrimp slurry twice daily. An 8-cm half section of 10-cm stainless-steel pipe was placed in each chamber as spawning substrate. Spawning usually started between 80-90 days after hatching. The numbers of eggs and spawns, along with mortality, were recorded daily throughout the spawning period. The spawning continued for 6 weeks (12 weeks for hemlock leachate) before the test was terminated. The adult fish were measured, weighed, gonads and livers excised, and the sex confirmed.

Several spawns from each test concentration were analyzed for egg hatchability during the life cycle testing with pine leachates. Spawns were collected from as many different spawning chambers as possible and the collection of eggs continued throughout the reproduction period. The eggs were checked twice daily for viability and dead eggs were removed. The larvae were counted after the eggs hatched on day 4 or 5, and the percent hatch was computed.

Bioindicators

Egg size - Eggs collected at the same time of day $\pm$ 2 h during the reproduction period of the pine leachate tests were placed in 10% neutralized buffered formalin and later measured with an ocular micrometer. Fifty eggs from six or seven different spawns in each test concentration were measured. All egg measurements from each concentration were pooled for statistical evaluations.

Gonad/Somatic Index-The gonads from male and female fathead minnows were excised and weighed at the termination of life-cycle testing. The weight of the gonad was divided by the weight of the fish and the result was multiplied by 100 to calculate GSIs.

Liver Somatic Index - Livers of male and female fathead minnows from each test concentration were weighted at the termination of the life-cycle test. LSIs were calculated by dividing the weight of the liver by the total weight of the fish and multiplying the results by 100.

Condition Factor -The condition factor (K) was calculated for each test concentration at the termination of the life-cycle exposure for the male and female fathead minnows using the formula:

$$K=(100)(w)/L^3$$

where (w) is the weight in mg and L is the total length of the fish in mm.

Statistical Evaluations

IC25s were calculated for many of the short-term and life-cycle bioassays using a linear interpolation model (32). The IC25 is a point estimate of the concentration of leachate that causes a 25% reduction from the controls. The IC25 from the linear interpolation model is reported. This endpoint is usually between the no observed effect concentration and lowest observed effect concentration found using hypothesis testing with parametric and non-parametric tests (33). This endpoint is reported for all of the short-term bioassays and the endpoints of the fathead minnow life-cycle test except those described herein as bioindicators. Analyses for bioindicators were selected based upon whether they met assumptions for parametric testing. Biomarker data for egg size were subjected to ANOVA and Dunnett's Test, having met the criteria for parametric testing (34). The GSI, LSI, and condition factor data did not meet criteria for parametric testing; thus, the Kruskal-Wallis Test was used for comparisons (34).

RESULTS

Leachate Chemical Components

Chemical characteristics of leachate samples used in short-term *C. dubia*, fathead minnow, echinoderm and bivalve bioassays are shown in Table 1 and averages of characteristic measured in leachate samples taken during fathead minnow life-cycle tests are shown in Table 2. Samples from pine leachate had much higher concentrations of fatty acids, resin acids and neutral semi-volatiles than the hemlock and fir leachates. Fatty Acids were primarily oleic and linoleic acids, and resin acids were usually about 50% dehydroabietic acid with lesser amounts of abietic, palustric, pimeric and isopimeric acids. Neutral semi-volatiles were predominately camphor, terpinen-4-ol, and fenchyl alcohol. The amounts of resin acids in pine leachate samples varied substantially over time from a high of 8900 µg/L in the first leachate sample taken in January, to a low of only 70 µg/L taken in a late May sample. A screening bioassay of the first sample was acutely toxic to juvenile fathead minnows at 35% effluent and because of this, 25% leachate was used in the first test. However, the first sample was not typical and although later samples had over 4000 ug/L of resin acids, none were as high as the first pine leachate sample. The pine leachates also had higher average amounts of phytosterols, but were only about 40% greater than concentrations in the hemlock leachates. Although the mean resin acid concentration was higher during the second life-cycle test with pine leachate (Table 2, Pine1 and Pine 2), the variability was reduced to the extent that 100% effluent could be used in the short-term bioassays and life-cycle test. Other effluent components such as TOC, BOD, COD, color, and polyphenols were increased by 20 to 90% in the second test with pine leachate. These increases, in addition to the change from 25% to 100% leachate, indicate that the fish were exposed to substantially greater concentrations of chemical components during the second exposure to pine leachate (Table 1 and Table 2, Pine 2).

Hemlock and Douglas fir leachates had higher levels of hardness and conductivity than pine leachate. However, this is due to dilution water since the contribution of the leachates to these water characteristics is minimal. The concentration of polyphenols was the only leachate characteristic that was substantially higher in the hemlock leachate, being nearly three times greater than in the fir leachate and about eight times as much as pine. TOC and COD were also highest in hemlock leachate, but the increase was only about 50%. Douglas fir leachate was highest in color, but only about 28% greater than the color of hemlock and twice the amount as the Pine 2 leachate.

Table 1 Chemical characteristics of leachate samples used in 7-d *C. dubia*, 7-d fathead minnow 40 min. echinoderm, and 48-h bivalve bioassays.

Compound or Parameter	Units	Pine 1 Test 1	Pine 1 Test 2	Pine 2 Test 1	Pine 2 Test 2	Pine 2 Test 3	Hemlock Test 1	Hemlock Test 2	Douglas Fir Test 1	Douglas Fir Test 2
TOC	mg/L	57	67	84	73	93	90	NA[a]	52	NA
pH	pH units	7.8	7.6	7.7	7.4	7.2	6.8	6.6	7.7	7.7
Hardness	mg/L	98	120	88	76	75	132	158	110	92
BOD	mg/L			46	57	150	134	NA	42	31
COD	mg/L	144	165	232	181	378	272	293	185	250
Color	mg/L	68	44	65	28	256	153	181	158	161
Conductivity	μmhos/cm	304	305	340	343	330	1340	1528	1147	1101
Polyphenols	mg/L	5.6	7.8	4.2	4.5	13	72	36	14	31
TSS	mg/L	14	NA	31	32	40	NA	NA	8.2	3.5
NSVs[b]	μg/L	NA	NA	299	36	1036	4	NA	ND	157
Fatty acids[c]	μg/L	18	17	17	4	105	ND	NA	22	8
Resin acids[d]	μg/L	416	2216	795	2508	3212	1.9	NA	5	149
Phytosterols[g]	μg/L	38	35	106	13.5	NA	25	NA	18	13
Arom. Acids[h]	μg/L	9	12	NA	NA	NA	0.8	NA	NA	NA

[a]Not analyzed
[b] total of 14 neutral semi volatiles: 2,3-dimethylcyclopentenone, acetophenone, 2-acetylthiophene, 3-acetylthiophene, 2,3,4,5-tetramethylcyclopentenone, fenchyl alcohol, camphor, terpinen-4-ol, p-cymene-8-ol, verbenone, dichlorodimethyl sulfone, trimethylcyclopentenone, 2-propionylthiophene
[c] total of 3 fatty acids: oleic, linoleic and dichlorosteric acids
[d] total of 7 resin acids: pimaric, sandracopimaric, isopimaric, palustric, dehydroabietic, abietic and neoabietic acids
[e] total of 3 chlorinated resin acids:14-chlorodehydroabietic, 12-chlorodehydroabietic, dichlorodehydroabietic acids
[f] not detected (calibration limit)
[g] total of 4 phytosterols: campesterol, stigmastanol, beta-sitosterol, stigmasterol
[h] total of 3 Aromatic Acids: benzoic, p-hydroxybenzoic and 3,5-dichloro-4-hydroxybenzoic acids

Table 2 Leachate chemical characteristics during fathead minnow life-cycle bioassays

Compound or Parameter	Units	Pine 1 Mean	Pine 1 (SD)[a]	Pine 2 Mean	Pine 2 (SD)	Hemlock Mean	Hemlock (SD)	Douglas Fir Mean	Douglas Fir (SD)
TOC	mg/L	62	(7)	83	(10)	90		52	
pH	pH units	8.3	(0.13)	7.9	(0.4)	6.3	(0.5)	7.0	(0.2)
Hardness	mg/L	89	(13)	89	(9.4)	142	(13)	111	(8)
BOD	mg/L	59	(15)	114	(64)	94	(48)	48	(24)
COD	mg/L	154	(16)	206	(36)	300	(106)	190	(42)
Color	mg/L	56	(17)	109	(48)	170	(37)	218	(93)
Conductivity	μmhos/cm	341	(40)	366	(30)	1444	(146)	1124	(54)
Polyphenols	mg/L	6.7	(1.6)	8.1	(3.8)	61	(25)	21	(8)
TSS	mg/L	19	(2.5)	32	(1.1)	NA[b]		6	(3)
Neutral Semi–volatiles[c]	μg/L	NA		785	(639)	13	(18)	21	(53)
Fatty acids[d]	μg/L	31	(36)	88	(49)	ND[f]	[1.0]	5.5	(6.8)
Resin acids[e]	μg/L	1792	(2567)	2319	(1161)	23	(36)	23	(48)
Phytosterols[g]	μg/L	31	(20)	36	(34)	24	(9.9)	10	(6)
Aromatic Acids	μg/L	14	(12)	NA		6.2	(5.8)	---	

[a]standard deviation
[b]Not analyzed
[c] total of 16 neutral semi volatiles
[d] total of 3 fatty acids
[e] total of 7 resin acids
[f] not detected (calibration limit)
[h] total of 4 phytosterols

Short –Term Bioassays

Concentrations of 25% (highest concentration tested) leachate during the first study with pine leachate had no effect on *C. dubia* survival and reproduction, fathead minnow 7-d survival and growth, echinoderm egg fertilization, or bivalve development (Table 3). During the second pine leachate study, three 7-d tests with *C. dubia* and fathead minnows were completed during the fathead minnow life-cycle test. *C. dubia* and fathead minnows were not affected during the first test and survival of both species was not altered during the second test. However, *C. dubia* reproduction and fathead minnow growth were reduced during the second test, and survival and reproduction, and fathead minnow survival and growth were all reduced during a third test (Table 3). Echinoderm fertilization was not

altered at 70% v/v of effluent during three tests with pine leachate (Table 4, Pine 2). Bivalve development was not altered during the first of three tests during the second study with pine leachate. Similar to results with *C. dubia* and fathead minnows, effects increased during the second and third bivalve tests (Table 3, Pine 2).

Hemlock leachate had effects on *C. dubia* survival during the second of two tests, and altered reproduction during both tests (Table 3). Hemlock leachate also reduced survival and growth of fathead minnows in both of two tests at concentrations similar to those affecting *C. dubia.* Hemlock leachate also reduced echinoderm fertilization and bivalve development at less that 20% v/v concentrations (Table 3).

Douglas fir leachate did not alter *C dubia* survival during either of two tests, however, reproduction was altered during the second test. Bivalve development was reduced during two tests, at lower effluent concentrations than altering *C. dubia* reproduction.

Table 3 Summary of results from bioassays with wood chip leachates

Test and Endpoint		IC25 or no observed effect concentration as v/v% leachate			
		Pine 1	Pine 2	Hemlock	Douglas Fir
C. dubia 7-d – IC25s					
Survival –	Test 1	>25%	>100%	>100%	>100%
Reproduction –	Test 1	>25%	>100%	37%	>100%
Survival –	Test 2	>25%	>100%	75%	>100%
Reproduction –	Test 2	>25%	70%	75%	64%
Survival –	Test 3		40%		
Reproduction –	Test 3		15%		
Fathead minnow 7-d – IC25s					
Survival –	Test 1	>25%	>100%	58%	
Growth –	Test 1	>25%	>100%	57%	
Survival –	Test 2	>25%	>100%	82%	
Growth –	Test 2	>25%	60%	94%	
Survival –	Test 3		98%		
Growth –	Test 3		41%		
Echinoderm fertilization –	Test 1	>20%	>70%	15%	
IC25s	Test 2		>70%	19%	
	Test 3		>70%		
Bivalve development –	Test 1	>20%	>70%	14%	31%
IC25s	Test 2	36%	42%	18%	13%
	Test 3		13%		
Fathead minnow 28-d growth - IC25s					
Egg hatchability		>25%	>100%	>100%	>50%
Juvenile survival		>25%	>100%	>100%	>100%
Juvenile growth		>25%	>100%	>100%	>100%
Fathead minnow life-cycle bioassay –IC25s					
Total eggs		>25%	>100%	31%	4%
Eggs/female/day		>25%	>100%	30%	10%
Egg hatchability		NDR[a]	>100%	>100%	52%
Adult survival male and female		>25%	>100%	>100%	>100%
Bioindicators – No observed effect concentrations					
Egg size		>25%	>100%		
Gonad/somatic index-males		>25%	>100%	>100%	>100%
Gonad/somatic index-females		>25%	>100%	>100%	>100%
Liver somatic index-males		>25%	>100%	>100%	>100%
Liver somatic index-females		>25%	>100%	>100%	
Condition factors-males		>25%	>100%	>100%	>50%
Condition factors-females		>25%	50%[b]	>100%	>100%

[a]NDR – IC25 at 4% but data did not have a dose-response.
[b] Significant increase at 100% effluent

Fathead minnow life-cycle tests

Fathead minnow survival and growth at 28 days was not altered by any of the leachates in these studies (Table 3). During the first study with 25% pine leachate, none of the end-points measured, including egg production, adult survival, egg size, GSIs, LSIs, or Ks were reduced. Similar results were found during the second life-cycle study with pine leachate when fish were exposed to 100% of the leachate. The only significant change was an increase in K of females in 100% effluent.

Hemlock leachate reduced the production of eggs by fathead minnows. The IC25 for the total eggs produced and the eggs/female/day were approximately 30% v/v of hemlock leachate (Table 3). Egg hatchability, adult survival, GSIs, LSIs and Ks were not altered by 100% leachate.

Douglas fir leachate had the greatest effect on fathead minnow reproduction during the life-cycle tests. The IC25 for total egg production was 4 v/v% of leachate and the IC25 for eggs/f/day was 10% leachate. Egg hatchability was also reduced by 25% at 52% v/v of leachate.

Discussion

Pine, hemlock and Douglas fir leachates had effects on *C dubia* reproduction during at least one bioassay when 100% leachate was tested. Pine and hemlock leachates also reduced *C. dubia* survival during at least one test, and reduced fathead minnow survival and growth during at least one bioassay. Overall these leachates were not as toxic as those of aspen where 1 to 2% v/v of leachate was acutely toxic to trout and *Daphnia* (10). The concentration of chips used for the aspen leachates was about 10% (fir) to 80% (pine and fir) greater than in these tests and may have contributed to the difference, but a more significant difference may be the presence of bark in the aspen chips. Bark contains high concentrations of chemical components such as tannins that may be toxic to plants and animals (6,7,8). Differences in species of trees may also contribute to the greater toxicity of the aspen leachate.

Although effects on *C. dubia* and fathead minnow were observed in short-term tests with each type of leachate during these studies, the sources of these effects are probably not the same for each leachate. The pine leachate contained resin acid concentrations more than 1000 times greater than the hemlock leachate and the effects on *C. dubia* and fathead minnows increased as the concentration of resin acids in pine leachate increased (Table 1, Table 3). The concentrations of resin acids in the pine leachate are high enough to cause acute toxicity to daphnids such *Daphnia magna* (35) and fathead minnows (36). Concentrations of resin acids altering growth of fathead minnows and reproduction of *C. dubia* have not been reported, but during the tests reported in this paper, the concentration of leachate causing sublethal effects were similar to acutely lethal effect concentrations with acute to chronic ratios between 1:1 and 1:4 when acute toxicity was observed. Acute to chronic ratios of 1:10 are usually considered normal for effluents (33) and NCASI work with pulp mill effluents found that effluents from modernized mills with pine furnish rarely caused mortality of *C. dubia* even if the effects on reproduction occurred at less than 20% effluent. Also, survival and growth of fathead minnows are rarely reduced by pulp mill effluents during 7-d tests (5, 12-18). The concentrations of resin acids in mill effluents are seldom near the concentrations found in these pine leachates, but the mill effluents often reduce *C. dubia* reproduction. The observations of a) high resin acid concentrations, b) low acute to chronic ratios and c) survival effects on both *C. dubia* and fathead minnows at similar leachate concentrations indicate that the cause of effects by pine leachate during short term tests are probably not the same as those causing effects by mill effluents. Mill operations such as tall oil recovery remove much of the resin acids, fatty acids and phytosterols, and treatment systems further reduce the amounts found in final effluents. Thus, differences between wood leachate components and final mill effluents are expected.

Resin acid concentrations of 3200μg/L and other components in the pine leachate did not alter echinoderm fertilization (Table 1 and Table 3). High molecular mass effluent components (HMM) such as tannin and lignins (e.g. polyphenols) in mill effluents are known to have effects on the echinoderm fertilization test (6, 29, 30,31). During the second study with pine leachate, methods were altered to 1) increase this type of HMM material in the pine leachate by increasing the temperature during leaching and 2) decrease the effects of resin acids in the leachates by employing foam fractionation to reduce the amount of resin acids in the leachate. Some increases in polyphenols, a measure of tannins and lignins and thus HMM material, were measured during the second study with pine leachate. Even so, the amount of HMM material did not reach the amount found in pulp mill effluents (5, 12-18) Echinoderm fertilization was not affected by resin acid concentrations greater than 3000 ug/L in pine leachate, and the amount of HMM in the leachate was also not great enough to cause any

changes in the test. The bivalve development test is also known to be affected by HMM. However, three bioassays during the second evaluation of pine leachate indicate that bivalve test results followed the pattern of increasing resin acids, similar to the *C. dubia* and fathead minnow bioassays. Bivalve development is probably affected by the concentration of resin acids. NSVs could also contribute to the greater effects during the third set of bioassays with the pine leachate, however, the low level during the second set of tests indicates this cannot be a major source of toxicity. Phytosterols can also be ruled out as a source of effects noted, since they were highest during the first set of tests when no effects were found with any of the four types of short-term bioassays (Table 1, Table3).

Hemlock leachate affected survival and/or sublethal endpoints in all four types of short-term bioassays. The hemlock leachate was substantially higher in polyphenols than other leachates and the concentrations present were within the range of pulp mill effluents known to have effects on *C. dubia* reproduction, echinoderms and bivalves. The severity of effects also followed the concentration of polyphenols, being less during the second set of bioassays. Levels of polyphenols have been shown to significantly correlate to fathead minnow reproduction results (5, 19). Thus polyphenols or HMM may have an effect during these tests. However, pulp mill effluents, even those with higher polyphenols levels, rarely affect *C. dubia* survival or fathead minnow survival and growth. For this reason, additional unknown toxicants not found at toxic levels in effluents are probably present in this leachate.

Douglas fir leachate had levels of chemical components that were similar to or less than the hemlock leachate, including polyphenols. The second set of bioassays had effects on *C. dubia* reproduction and both tests reduced bivalve development. The effects during the tests also followed the concentration of polyphenols in the leachate. Although resin acids and COD also increased during the second set of tests, none of these approached levels found in the pine leachate when no effects were found in the same types of tests.

The pine leachates had no effect on fathead minnow reproduction or bioindicators of reproductive fitness during two life-cycle tests, even though the concentrations of resin acids were much higher than those from modernized pulp mills, and at times reached levels reported to be lethal in the literature. This indicates that resin acids are unlikely to be causes of any effects on reproduction found during tests with pulp mill effluents. Literature reports indicate that fish can acclimate to resin acids and begin to quickly remove them (37). This may have occurred during this test since short-term tests indicated that some leachate samples would affect the survival and growth of juvenile fathead minnows. NCASI has reported that polyphenols in effluent from kraft mills with pine furnish have the highest correlations with egg production during life-cycle test (5,19). This correlation has continued as additional effluents have been added to the data base (38). Other components such as phytosterols, TOC, and color also have significant but weaker correlations. As found with the leachates, resin acids do not correlate significantly with reproduction end points. The levels of those components that correlate well with reproduction during tests with effluents were lower in the pine leachate than in any mill effluents causing reproduction effects. Thus, while these leachates confirm that resin acids are unlikely to be a cause of effects on reproduction by mill effluents, other components such as polyphenols remain viable candidates for further study.

Hemlock leachate reduced egg production at approximately 30% effluent. Effluents from mills with predominately hemlock furnish have not been evaluated in fathead minnow life-cycle testing. However, most chemical components measured in hemlock leachate are below those found in effluents from mills with pine furnish that reduce egg production. The concentration of polyphenols is the exception, being similar to levels found in effluents from mills with pine furnish. Thus, polyphenols may have contributed to the effects on reproduction observed during this investigation. Given the levels of fatty acids, resin acids, phytosterols, NSVs and aromatic acids measured in pine leachates when no effects were found, these components are unlikely sources of effect during the investigation with hemlock leachate.

The fir leachate also reduced fathead minnow reproduction during life-cycle tests at 4 to 10% v/v, having the greatest effect. However, chemical analyses do not reveal unusually high levels of any of the components measured. Recently effluent from two pulp mills with Douglas fir furnish were investigated in fathead minnow life-cycle bioassays (39, 40). The effluents from those mills had greater effects on fathead minnow reproduction when compared to other effluents from modernized mills with good secondary treatment. Also, as was found in this leachate study, the concentrations of components measured in the mill effluents were among the lower levels measured in all pulp mill effluents tested. This indicates that something other than components currently being measured for all

mill effluents may be present in effluent from mills with fir furnish. Alternatively, measurements of effluent components that are not chemical specific such as polyphenols, TOC, COD, color, etc may evaluate a different group of chemicals that are not similar between pine and fir leachates and effluent. If chemicals more potent to fish reproduction occur within those broad measurements for effluents with fir furnish, then sufficient numbers of effluents from mills with each type of furnish will need to be evaluated to determine their utility as predictors of adverse effects on reproduction.

In summary, in these laboratory studies, pine leachates caused effects on biological endpoints in short-term tests but not in life-cycle tests. Concentrations of resin and fatty acids were higher in the pine leachates than in nearly all pulp mill effluents, and these were the likely cause of effects by the pine leachates. Other components measured in effluents that significantly correlate to reproduction during fathead minnow life-cycle tests were not as high as in the effluents, thus the pine leachates were useful in ruling out resin and fatty acids as causative agents fish reproduction tests with effluents from mills. However short-term and life-cycle bioassays with the pine effluents did not provide additional information on other effluent components that correlate to reproduction over a broad range of tests such as polyphenols, phytosterols, TOC, COD and color. Hemlock and fir leachate both caused effects on fish reproduction during life-cycle tests, indicating that chemicals derived directly from wood furnish can cause effects on fish reproduction. Polyphenol concentrations in hemlock leachate were similar to those measured in mill effluents with pine furnish when effects on fish reproduction were found, and may be causative agents during the life-cycle test. Since effluents from modernized mills rarely cause acute toxicity, the effect of the hemlock leachate on survival of *C. dubia* and fathead minnows probably indicates additional toxicants are present in the leachate that are not found in biologically treated final mill effluents. Douglas fir leachate had the greatest effect during fathead minnow reproduction tests, but had intermediate to low concentrations of most of the measured effluent components. Similar chemical and life-cycle results have been observed with effluents from two mills with fir furnish. Thus, effects derived from furnish are possible, and to understand them, additional studies with effluents from mills with different furnish will be needed.

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