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Barry R. Taylor

J. Stephen Goudey

N. Bruce Carmichael

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TOXICITY OF ASPEN WOOD LEACHATE TO AQUATIC LIFE: LABORATORY STUDIES

BARRY R. TAYLOR,*† J. STEPHEN GOUDEY‡ and N. BRUCE CARMICHAEL§
†Golder Associates Ltd., 1011-6 Avenue S.W., Calgary, Alberta T2P 0W1, Canada
‡HydroQual Laboratories Ltd., #3, 6125-12 Street S.E., Calgary, Alberta T2H 2K1, Canada
§Environmental Protection, British Columbia Ministry of Environment,
1011-4 Avenue, Prince George, British Columbia V2L 3H9, Canada

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Abstract—Trembling aspen (*Populus tremuloides* Michx.), a common hardwood tree throughout Canada, is being harvested at increasing rates for use in paper and building materials. Piles of aspen logs have been observed to produce a dark, watery, acutely toxic leachate. A laboratory study was undertaken to elucidate the nature, strength, and persistence of aspen leachate toxicity and the chemical composition of the leachate. Leaching from aspen chips in the laboratory was rapid, with 1% mass loss in the first 24 h. Another 2 weeks of immersion was necessary to remove all remaining leachable material (3% total). Fresh aspen leachate derived from a 1:9 wood-water mixture (35 d immersion) was characterized by amber color, low pH (4.0), extremely high BOD (>2,600 mg/L), and high conductivity (1140 μS/cm). The leachate was rich in phenols (30 mg/L), organic carbon (2,480 mg/L), and organic nitrogen (13 mg/L). Median acutely toxic concentrations of leachate were consistently 1 to 2% of full strength for trout and *Daphnia*. Inhibition of bacterial metabolism began at concentrations below 0.3%. Leachate was less toxic to plant life but inhibited algal growth at concentrations of 12 to 16%. Toxicity of aspen leachate persisted at the same level as in fresh leachate for more than 2 months unless artificial aeration was provided. Persistence was even greater at low temperature (5°C). Aged leachate underwent a transition marked by a rise in pH and dissolved oxygen concentration, a small decline in conductivity, and a color change, from amber to black. Toxicity declined abruptly when the supply of labile toxicants was exhausted, but it sometimes increased again from the products of microbial metabolism. Oxygen depletion, low pH, and phenolic compounds contribute to the toxicity of aspen leachate, but much of the toxic effect must be attributed to other, unidentified constituents.

Keywords—Aspen Toxicity Forestry Phenols Leachate

INTRODUCTION

Use of aspen (*Populus tremuloides* Michx.) wood as a source of fiber has increased sharply in the last decade, both for production of pulp and for building materials such as veneer, plywood, and oriented-strand board. For example, in the Canadian province of Alberta, where several pulp mills designed to use aspen have recently been built or expanded, *Populus* species accounted for 33% of the growing stock in 1990 and 1991, and 25% of the harvested volume [1]. Given the very large number of aspen trees in the boreal forest of northern Canada, use of aspen is projected to increase further in the future as novel processes for treating hardwood fibers are perfected. There is an estimated 1 to 2 × 10^9 m^3 of merchantable aspen wood in mature, nonreserved forest stands throughout Canada [2].

Because of the susceptibility of forest soils to damage from heavy equipment during spring thaw, normal practice is to harvest trees in winter and stockpile logs in a woodyard until needed in the spring. To reduce problems from oils in fresh wood, kraft pulp mills using aspen have traditionally piled logs in the woods for up to 1 year after cutting [3]. In northern British Columbia (BC), piles of aspen logs cut in winter have been observed to produce a dark, watery leachate, locally termed blackwater, during or after spring melt (B. Kielo, personal communication); the leachate presumably consists of sap and soluble materials leached from the wood cells by the passage of water, perhaps augmented by prolonged contact with slowly melting snow or by structural damage from freezing and thawing.

Live aspen wood is known to contain a substantial mass of water-soluble material that is susceptible to removal by leaching. Besides sugars and other contents of sap and cell cytoplasm, the leachable component contains a suite of phenolic compounds [4,5]. The phenolics form a defense mechanism, serving to prevent microbial infection of wounds [5] and to discourage grazing by wood-boring insects [6] or browsing by mammals or birds [7,8].

Phenols are present in all aspen wood tissue, but they are most abundant in the bark and cambial tissue. Up to 10% of the mass of bark can be phenolics [8]. To avoid toxicity to the tree itself, the phenolics in bark are bound to a simple sugar, creating a phenolic glycoside. Salicin is the most abundant glycoside in aspen, but several other similar compounds may be present. Some free phenol is also present in the heartwood, where the tree cells are nonvital, and autotoxicity would not be a problem [9,10].

Blackwater from aspen woodyards is rich in phenols, has a very high biochemical oxygen demand and may be highly toxic to aquatic life (BC Ministry of Environment, unpublished data). Streams or lakes near aspen woodyards may thus be threatened by uncontrolled runoff containing aspen leachate. As the rate of aspen cutting increases, blackwater could become a serious problem for wood cutters, pulp mills, and board manufacturers.

As aspen leachate is a novel problem, little is known about its production, persistence, and chemical composition, nor have all the components of toxicity been firmly identified. Such basic information is crucial both to establishing the severity of the problem and to designing procedures to deal with it. To rectify

* To whom correspondence may be addressed.

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this deficiency, a laboratory study was undertaken to elucidate the nature, strength, and persistence of aspen leachate toxicity and the chemical composition of the leachate.

Specific objectives of the study were (1) to determine the mass of water-soluble material in aspen wood and the rate and quantity of leaching loss; (2) to determine the toxicity of aspen leachate to aquatic life and the rate at which toxicity decays; (3) to establish the general chemical nature of the leachate and to find simple parameters (such as conductivity) that may be used to predict toxicity or trace dilution of the leachate in a water body; and (4) to isolate the main classes of compounds responsible for acute toxicity. The information gained in this study was used to refine the experimental design of a related field study designed to produce blackwater typical of that produced by wood-harvesting operations (B. Taylor and B. Carmichael, manuscript in preparation).

METHODS

Aspen trees were harvested during leaf fall in October 1991 from a 10-ha stand in Turner Valley, Alberta, in the Rocky Mountain foothills. Five young healthy trees, ranging 28 to 64 years in age (mean 44.8 years) and 9.7 to 15.7 cm butt diameter (mean 13.6 cm), were cut at ground level with a chain saw. Trees were topped, limbed, and cut into 2.5-m (8-ft) lengths in the field and then reduced to chips (0.5 to 10 cm in length) with a commercial chipper. Short logs from each tree were retained to determine age by counting annual rings. Aspen chips were stored without drying in black plastic bags, in darkness, at 5°C.

To address the objectives of the study, four related experiments were undertaken: (1) leaching mass loss from dried wood; (2) production of concentrated leachate (35 d); (3) decay of leachate with and without aeration (65 d); and (4) confirmation of rapid decay with aeration (20 d). In experiment 1, the total mass of soluble material in aspen wood was determined. A large volume of concentrated leachate was then produced (experiment 2) by leaching fresh aspen chips for 35 d, while selected chemical and physical attributes of the leachate were monitored. In experiment 3, the leachate produced in experiment 2 was divided into two aliquots, and changes in toxicity and physical-chemical characteristics with and without aeration were monitored for 65 d. Finally, the nonaerated leachate from experiment 3, which had shown very little change in toxicity and chemical indicators over the 65-d period, was aerated for 20 d (experiment 4) to confirm the profound changes in toxicity observed in the aerated sample in experiment 3. Note that because the last three experiments are connected, the last day of experiment 2 was the first day of experiment 3, and the last day of experiment 3 was the first day of experiment 4. The first day of each experiment is day 0.

Leaching losses from aspen chips were measured based on the method of Taylor and Parkinson [11]. Aspen chips were first dried at 60°C to constant weight and sieved (4.0-mm mesh) to remove sawdust and small chips; 20-g aliquots were then immersed in 0.9 L of University of BC tap water in glass bottles and stored without agitation at 5°C. At intervals from 1 to 28 d, six replicates were removed, redried (60°C), and reweighed, to determine mass loss. Moisture content of fresh wood was also determined by weighing six 50-g samples before and after drying at 60°C. Fresh aspen had a moisture content of 45.3 ± 1.73%.

In experiment 2, large volumes of more concentrated leachate were produced for toxicity tests by immersing 50 kg fresh weight (about 28 kg dry weight) of aspen chips in 250 L of dechlorinated tap water in a 280-L vat lined with polyethylene. The ratio of aspen to water was thus 8.9:1 by dry mass. Leaching proceeded at room temperature. The vat was mixed once each day, and conductance, pH, DO, and absorbance (465 nm) were measured. Water losses from sampling or evaporation were replaced. Changes in toxicity of the leachate were monitored daily with the Microtox brand bacterial luminescence assay [12]. The bacterial luminescence test is ideal for this application because it is fast and requires a very small sample volume.

Leaching continued for 35 d, until chemical and physical characteristics ceased to show rapid changes. The chips were removed by passing the solution through a fiberglass screen (2-mm mesh) into 20-L polyethylene carboys. All leachate not used immediately was stored at 5°C, air excluded, in darkness.

In experiment 3, the decomposition of toxic compounds and the decline in toxicity of leachate with time were examined by placing 50 L of fresh leachate (1 day after removing the chips) in each of two 80-L lined containers; one container was gently aerated with oil-free compressed air (approximately 300 mL/min), and both were maintained at 16 to 18°C. A third, smaller sample (500 ml) was maintained without aeration at 5°C to check the effect of low temperature. Toxicity was monitored with the bacterial luminescence assay each week, or more frequently during periods of rapid change. The rainbow trout acute 96-h bioassay (static) and the *Daphnia magna* acute 48-h bioassay (static) were run on the fresh leachate (day 0, 1 day after removing the wood) and on aerated and nonaerated samples on day 65, using standard protocols [13,14]. (Dilution series varied according to toxicity in preliminary tests, but followed the series: 100%, 33%, 10%, 3%, 1%, etc.) On both dates, trout, *Daphnia,* and bacterial luminescence tests were repeated on split samples by the Aquatic Toxicity Laboratory of BC Ministry of Environment, Vancouver. Several other tests, namely the 7-d *Ceriodaphnia* survival and reproduction assay [15], the 96-h algal growth inhibition assay [16], and the 4-d lettuce seed germination test [17], were run on day-0 leachate to determine the effect of the leachate on a broad range of organisms. Erratic and unpredictable mortality of adults in the *Ceriodaphnia* test (four attempts) precluded calculation of fecundity. Additional chemical and physical characteristics of the leachate (5-day BOD, COD, color, resin and fatty acids, nitrogen, total phenols, and total organic carbon) were determined by Zenon Laboratories, Burnaby, BC, Canada [18].

Intense monitoring of the leachate samples in experiment 3 ended after 55 d. On day 65, when it was evident that no further rapid changes in physical and chemical indicators were taking place, samples were taken from both vats for toxicity testing (bacterial luminescence, trout, *Daphnia*) and for reanalysis of chemical composition (Zenon Laboratories). The previously nonaerated vat was then aerated at 300 mL/min (experiment 4) to confirm the abrupt change in toxicity and chemical characteristics observed in the aerated sample in experiment 3 (see Results). Twenty liters of leachate were available when aeration began, and 250 ml were removed daily for chemical monitoring (absorbance, pH, conductivity, DO) or possible toxicity testing. Aeration of the sample continued for 20 d. Samples taken at approximately 2-d intervals were analyzed for total phenols content and BOD. Toxicity was tested with the bacterial luminescence assay every second day, and with lettuce seeds, trout, and *Daphnia* on day 0 (=day 65 of experiment 3) and days 8 and 20. The trout and *Daphnia* tests were repeated by the BC Ministry of Environment. All samples not tested at once

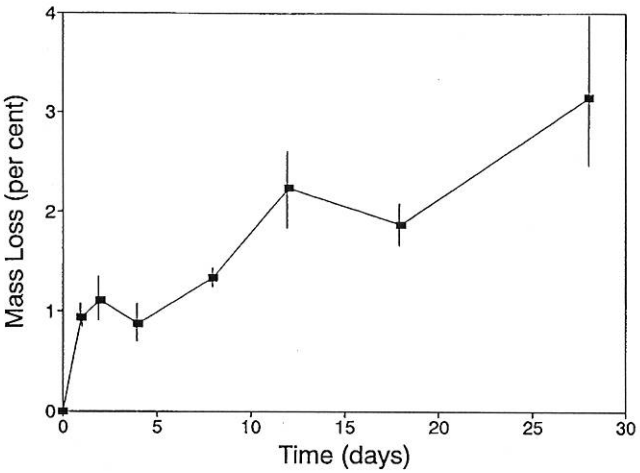

Fig. 1. Temporal pattern of leaching loss (percent of original dry mass) from aspen wood chips immersed in water. Error bars are standard errors.

were stored in darkness at 5°C, air excluded, for no more than 3 days.

A preliminary separation and chemical analysis of the leachate was undertaken to attempt to isolate the chemical fractions responsible for the toxicity. For this investigation, 65-d-old nonaerated leachate and leachate aerated for 20 d in experiment 4 were fractionated using a procedure based on the U.S. Environmental Protection Agency toxicity identification evaluation approach [19]. First, a 200-ml aliquot of the leachate was passed through a C18 column (Supelco Canada, Oakville, Ontario, Canada), which binds organic compounds more or less strongly according to their hydrophobicity. The columns were then extracted sequentially with 4-ml aliquots of methanol in water. Methanol concentrations of 25%, 50%, 75%, and 100% were used, and the leachate that passed through the column constituted a fifth fraction. The fractions containing methanol were concentrated 50 times relative to the water fraction (200 ml into 4 ml). The methanol fractions were therefore diluted with water to bring the final volume up to 200 ml. This step also removed potential toxicity from methanol itself.

Relative toxicity of the five fractions was determined with the bacterial bioluminescence assay. The most toxic fractions were then analyzed by Zenon Laboratories with gas chromatography–mass spectrometry to characterize the major classes of compounds and individual compounds potentially associated with the toxic fraction [20]. Phenanthrene D10 was added to each sample as an internal standard so that the approximate concentration of each compound could be calculated.

Where appropriate, ordinary least-squares regression was used to objectively distinguish trends in chemical or physical characteristics as the leachate aged. This application proved useful where subtle changes through time could be obscured by day-to-day variation in the data. Regressions were also used to describe the slope of linear trends.

RESULTS

Leachable mass and leaching rate (experiment 1)

The first leaching experiment produced reasonably precise estimates of mass loss (Fig. 1). Leaching was evidently rapid and appeared to have two phases, represented by a loss of about 1% mass in the first 24 h, followed by a more gradual loss from 4 to 28 d. Leaching loss during the latter period could be described with a weak ($r^2 = 0.37$) but significant ($p < 0.05$) linear regression with a slope of 0.086% per day. The total mass of leachable material was about 2 to 4% of dry mass of wood (Fig. 1).

Production of leachate for toxicity testing (experiment 2)

A much more concentrated leachate was produced in the main experiment, where the dilution ratio was 9:1 on an aspen dry-mass basis. Leaching began immediately when chips were immersed in water. Conductivity, a simple surrogate of the concentration of ions in solution, showed a detectable increase within 3 h and continued to increase throughout the 35-d extraction, reaching over 1000 μS/cm (from an initial 388 μS/cm). Conductivity in control buckets containing 20 L of tap water only was consistently 340 to 350 μS/cm. Light absorbance in the vat containing aspen chips increased sharply on the first day as the water turned a bright amber color, but there was no further change for the remaining 34 d. Leachate extracted at the end of the experiment was still a transparent amber color, quite dissimilar to the inky blackwater observed in the field.

The pH of the leachate declined slowly, from 7.6 to 6.6 on day 1, then to 4.0 by day 15; pH remained near 4.0 for the next 20 d. Dissolved oxygen concentration dropped precipitously on the first day, from more than 9 to less than 3 mg/L, but never quite reached zero. Rather, DO hovered around 1.0 to 2.5 mg/L for the remaining month of the extraction, despite the evident organic richness of the leachate. Dissolved oxygen content of pails containing water only never varied far from 8.0 mg/L.

Toxicity became apparent swiftly. Inhibition of bacterial luminescence was detectable after just 4 h, when undiluted leachate reduced light emissions to 9% of that in controls. Bacterial luminescence in full strength leachate was zero after 24 h, and after 5 d, light production was detectable only in solutions containing less than 2% aspen leachate.

Leachate aging and the effect of aeration (experiment 3)

The aerated and nonaerated samples, being derived from the same leachate, were initially identical in measured characteristics: DO was 0.2 mg/L, having declined further from 2.2 mg/L once the leachate was removed from the vat. Both samples were acidic (pH 4.0), amber-colored (absorbance 530 to 560), and rich in dissolved ions (conductivity >1,000 μS/cm). Their characters began to diverge quickly once aeration began.

Dissolved oxygen concentrations in the vat without aeration remained near zero, and never exceeded 1 mg/L, indicating a strong and persistent oxygen demand (Fig. 2A). Dissolved oxygen concentration in the aerated sampled began to rise after 6 d, and after 21 d, it had risen to concentrations in excess of those necessary to support most species of lentic-water aquatic life (Fig. 2A). By the end of the 55-d incubation, DO levels in the aerated vat exceeded 7 mg/L.

Conductivity, pH, and absorbance also showed very little change in the absence of aeration (Fig. 2), and after 60 d, the leachate retained its original amber color. There was a significant increase in pH ($r^2 = 0.50$, $p < 0.05$) in the nonaerated sample, but the rate of change was negligibly small (0.0066 units·d^{-1}). Regression against time showed no significant change in conductivity ($p > 0.10$), and the equation for absorbance had a slight negative slope (-1.78 d^{-1}, $r^2 = 0.60$, $p < 0.05$).

In the aerated vat, in contrast, the pH began to rise at about the same time as DO and had shifted to alkaline by 16 d (Fig. 2B). Conductivity declined, but the change was small, only about 10% over the 2-month period. Most of the decline oc-

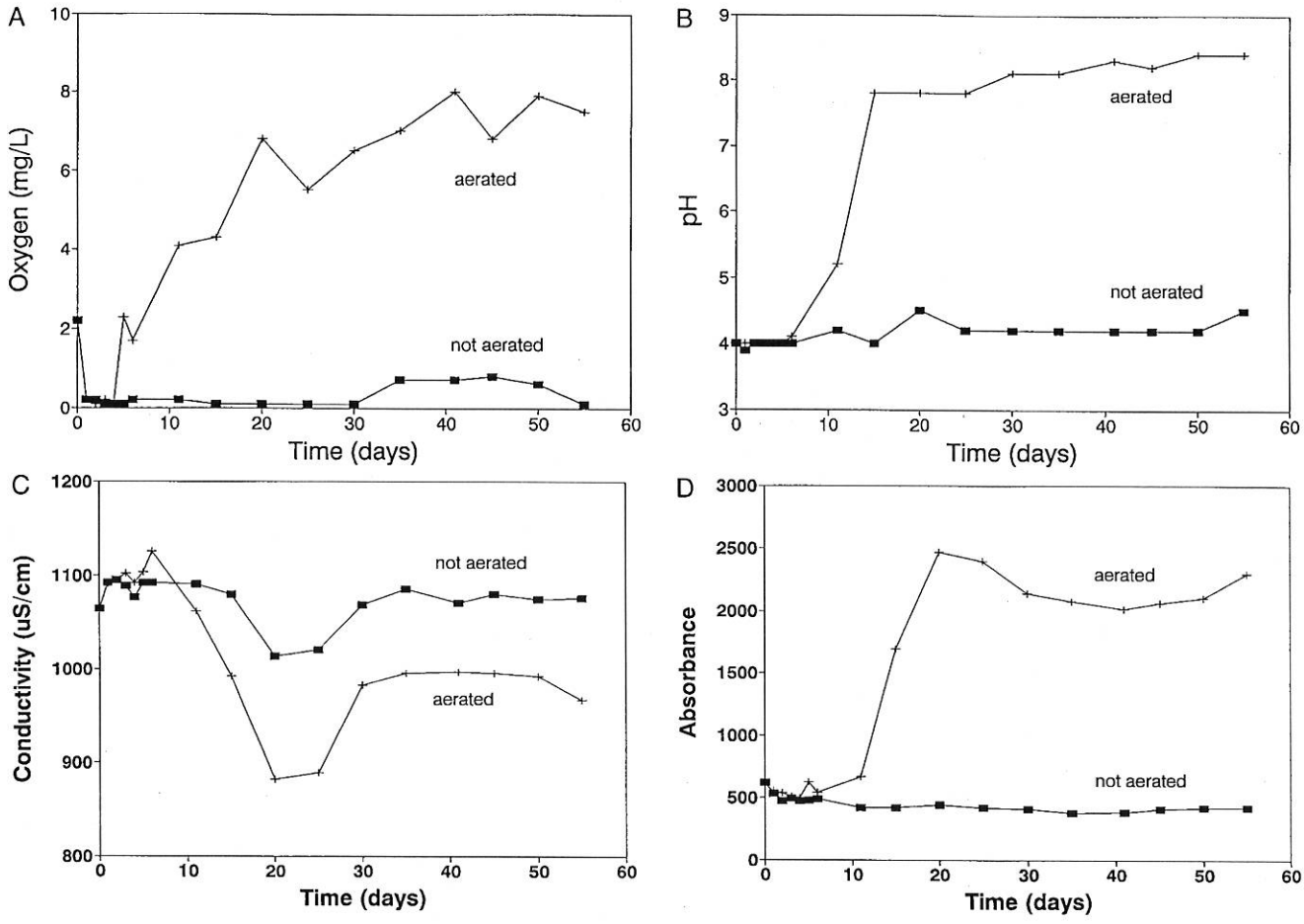

Fig. 2. Change in (**A**) dissolved oxygen concentrations, (**B**) pH, (**C**) electrical conductance, and (**D**) light absorbance at 465 nm in aspen wood leachate with and without aeration.

curred during the 2-week period from day 6 to day 20, and conductivity increased again from day 26 to day 30 (Fig. 2C). The most conspicuous change in the aerated sample was an abrupt darkening, from transparent amber to opaque jet black. The color change was marked by a sudden rise in absorbance, which increased by 2.5 times over a period of less than 4 d (Fig. 2D). In fact, the visible color change occurred overnight. The advent of the color change was signalled by increases in DO, a decline in conductivity, and a large step increase in pH (Fig. 2). The pH continued to increase linearly ($r^2 = 0.91, p < 0.05$) but at a much slower rate (0.017 d^{-1}) after the color change. After day 17, the aerated leachate was indistinguishable from blackwater observed in the field with respect to all of the measured characteristics (BC Ministry of Environment, unpublished data).

Aspen leachate was very toxic to aquatic life (Table 1). The median lethal concentration (LC50) to trout was only 1 to 1.8% of undiluted leachate (5% in a preliminary test), and to *Daphnia*, 1.7 to 3.4%. Toxicity as measured by the bacterial luminescence test was even greater, with most EC50s in the range 0.2% to 0.3%, and one result less than 0.1%. Agreement between toxicity tests performed on split samples by two different laboratories was excellent (Table 1). The leachate was also strongly inhibitory to algal growth, although the effective concentrations were about an order of magnitude greater than for animals. The seed germination test showed no effect of the leachate, and root elongation was significantly reduced only at relatively high concentrations (Table 1). Evidently, the toxicity of aspen leachate to plant life is comparatively weak.

The toxicity of the leachate, as indexed by the bacterial luminescence assay, was virtually constant for both the vat without aeration and the sample stored at 5°C, with EC50s of 0.3 to 0.4% and 0.2 to 0.3%, respectively, over the 55 d of monitoring. Toxicity of the aerated sample, on the other hand, began to decline (bacterial luminescence EC50 increased) at about day 6, and the EC50 reached a peak of 10% by day 25 (Fig. 3). Surprisingly, toxicity increased sharply again, and by day 35, the EC50 had declined to approximately 2%. A second increase 10 d later again raised the EC50 to 10% by the end of the 55-d monitoring period. The period of the first increase in EC50 corresponds to the increase in pH and DO content of the leachate, and especially with the sudden drop in conductivity seen in Fig. 2C. The subsequent decline in EC50 also corresponds with the increase in conductivity from day 25 to 30 but has no counterparts in the other physical or chemical data.

An acute *Daphnia* toxicity test was run on day 20 to confirm the bacterial luminescence results. The LC50 for aerated leachate, with the 95% confidence interval in parentheses, was 31% (25 to 50%), confirming that the decline in toxicity was real. The LC50 for the vat without aeration was 2.2% (1.2 to 3.1%), the same as for fresh leachate (Table 1).

Bacterial luminescence assays on the leachate after day 55 indicated that the second decline in toxicity of aerated leachate continued (EC50, day 64: aerated, 29%; not aerated, 0.34%;

Table 1. Toxicity of fresh aspen wood leachate (28 kg aspen [dry mass] in 250 L of water, 35 d)

Test	Laboratory[a]	LC50/EC50 (%)	95% C.I.
Trout, acute (96 h)	HQ[1]	5.0	2.5–10
	MOE[1]	1.3	1.0–1.8
	HQ[2]	1.2	Not calculated
	MOE[2]	1.6	1.0–1.6
Daphnia, acute (48 h)	HQ[3]	≈3	Not calculated[b]
	MOE[3]	1.7	1.0–3.0
	HQ	1.5	0.6–2.5
	HQ[4]	1.0	0.6–1.2
	MOE[4]	3.0	1.8–3.2
		3.4	3.2–5.6
Bacterial luminescence (15 min)	HQ	0.23	0.19–3.0
	HQ[5]	0.20	0.17–0.23
	MOE[5]	0.29	0.27–0.31
	MOE	0.085	0.047–0.141
Algal growth (72 h)	HQ	16	Not calculated
	HQ	11.9	Not calculated
Lettuce seed germination	HQ	>100	Not calculated
Lettuce root elongation	HQ	25	Not calculated

Superscript numbers indicate results from split samples.
[a] HQ = HydroQual Laboratories: MOE = BC Ministry of Environment Aquatic Toxicity Laboratory.
[b] 3% dilution caused 50% mortality plus 40% immobility.

5°C, 0.27%). Consequently, the toxicity of aerated leachate to trout was tested on day 65 to confirm the decreasing toxicity. Leachate from the aerated vat was not toxic to trout, even at full strength, and EC50s in the replicate bacterial luminescence tests had increased by two orders of magnitude (Table 2). Hence, the aerated leachate could be considered only marginally acutely toxic. By comparison, all three tests indicated that toxicity of nonaerated leachate was barely different on day 65 from day 0 (Table 2). The leachate was still very toxic to rainbow trout and *Daphnia*, with LC50s of only 2 to 3%.

Preliminary chemical and physical data on aspen leachate at the beginning (day 0) and at the end (day 65) of the first aging experiment are displayed in Table 3. The low DO content of the fresh leachate is a result of its high biochemical oxygen demand, more than 2,600 mg/L. The leachate was also rich in phenols, as expected, but the concentrations of resin and fatty acids were too low to be of toxicological significance [21,22]. While the wood did leach significant quantities of total nitrogen, neither ammonia nor nitrite would pose a toxicity problem in most natural waters (Table 3). The high total carbon content presumably reflects the sugar content of aspen sap. Conductivity measured by Zenon Laboratories (Table 3) was always slightly greater than that measured in our laboratory (Fig. 2C), reflecting either a bias in one measurement or changes occurring during shipping.

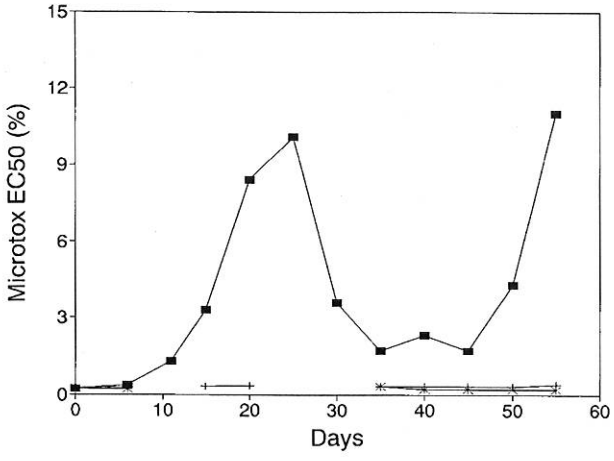

Fig. 3. Change in toxicity (EC50, as a percentage of undiluted sample), of aspen leachate with (■) and without (+) aeration, and at 5°C (*), according to the 15-min bacterial luminescence assay.

After 65 d, the nonaerated sample still retained many of the chemical characteristics of the original material. Significantly, rather than declining, the concentration of total phenols in the nonaerated vat apparently increased to over 80 mg/L (Table 3). Phenols were nearly eliminated in the aerated sample, and total organic carbon showed a further decline compared with the sample without aeration. While still high, BOD was almost two orders of magnitude less than without aeration, and pH had risen to neutrality (Table 3). The high light absorbance at 465 nm is consistent with the dark color of aerated samples, although some color increase was also evident without aeration.

The full set of toxicity tests on the less toxic, aerated leachate required all of the remaining material. Monitoring of that sample was therefore terminated. Aeration of the remainder of the previously nonaerated sample then commenced to ascertain whether the transformation from amber to black could be repeated.

Confirmation of aeration effects (experiment 4)

Aeration of the previously nonaerated sample produced an identical response to the first (65-d) aeration, except that physical-chemical changes were slightly more rapid and more variable. Light absorbance and pH followed nearly identical temporal patterns (Fig. 4A) and consequently were highly correlated ($r^2 = 0.81, p < 0.001$). Absorbance was low and nearly constant for the first week, but it underwent a step increase on days 8 through 10, as the leachate turned black. The pH displayed the same sigmoid pattern, except that pH began to rise slowly from the outset (Fig. 4A). Concentrations of DO varied erratically, especially in the first week, making temporal trends difficult to discern (Fig. 4B). There was a significant increase in DO from the onset of aeration to day 9 ($r^2 = 0.60, n = 9, p < 0.05$); beyond the ninth day, DO appeared to stabilize near 6 to 7 mg/L. Electrical conductance (Fig. 4B) declined erratically for the first 2 weeks ($r^2 = 0.32, n = 14, p < 0.05$), from over 1100 to about 950 μS/cm, then increased again for the remaining

Table 2. Toxicity of aspen wood leachate after 65 d, with and without aeration; values are LC50s or EC50s, as a percent of undiluted sample (95% C.I.)

Test	Laboratory[a]	LC50 or EC50	
		Not aerated	Aerated
Trout, acute (96 h)	MOE	2.2 (1.0–3.2)	>100 (no mortalities)
Daphnia, acute (48 h)	MOE	2.83 (2.23–3.58)	
Bacterial luminescence (15 min)	HQ	0.34 (0.27–0.41)	29 (15–55)
	MOE	0.48 (0.45–0.52)	36.3 (26.1–50.4)

[a] HQ = HydroQual Laboratories; MOE = BC Ministry of Environment Aquatic Toxicity Laboratory.

week of the experiment ($r^2 = 0.87$, $n = 8$, $p < 0.01$). All of these patterns are similar to those observed in the first aeration test.

Toxicity of the second aerated leachate was tested at the outset, on day 8, when black color first appeared, and on day 20, at the end of the experiment (Table 4). Before aeration began (day 0), the leachate was highly toxic in all three acute tests. By day 8, there was only a marginal decrease in toxicity in the bacterial luminescence and *Daphnia* tests, and the leachate still had an LC50 in the trout test near 2% (Table 4). Even after 20 d, when the leachate had turned black and physical-chemical attributes appeared to have stabilized, it was still acutely toxic, although EC50s had increased by roughly an order of magnitude (Table 4).

Figure 5 traces changes in BOD and total phenols content of the leachate during aeration. Notwithstanding an apparent increase on day 2, BOD declined rapidly (83 ± 15 mg L^{-1} d^{-1}) and linearly ($r^2 = 0.786$, $p < 0.01$) throughout the aeration period, although it still exceeded 1,000 mg/L on day 20 (Fig. 5). Phenolics content varied erratically from one day to the next, but there was no clear trend in the data, and the concentration on day 20 (76 mg/L) was virtually the same as on day 0 (79 mg/L). While the high phenols content is consistent with the high toxicity of the leachate, there is poor correspondence between day-to-day variation in phenols content (Fig. 5) and measured fluctuations in toxicity (Table 4).

Table 3. Chemical composition of fresh and aged leachate from aspen wood chips (units are mg/L unless specified otherwise)

Parameter	Day 0	Day 65	
		Not aerated	Aerated
True color (units)	500	600	500
Color 465 nm (units)	222	1,750	3,450
pH (units)	4.0	4.7	7.5
Conductivity (μS/cm)	1,140	1,130	1,170
BOD_5	>2,600	1,660	550
COD	5,170	4,960	1,780
Total organic carbon	2,480	1,780	710
Phenols	29.9	83.1	1.0
Resin acids	0.035	0.042	0.011
Fatty acids	2.15	0.245	0.097
Total nitrogen	13.3	10.3	13.3
Organic nitrogen	12.8	9.8	13.0
Total ammonia	0.42	0.50	0.28
Nitrate + nitrite	0.040	<0.02	<0.03

Identification of toxic compounds

For comparison, toxicity in the bacterial luminescence test of the various fractions from the C18 column is expressed as toxic units, defined as 100/EC50 (Table 5). In the aerated leachate, none of the five chemical fractions produced significant toxicity in the bacterial luminescence test. Reductions in light output from full-strength fractions ranged from 37 to 72%, equivalent to EC50s near or beyond 100% (1 toxic unit or less).

In contrast, substantial toxicity was associated with every fraction of the nonaerated leachate except the pure methanol fraction (Table 5). Given the high concentration of organic matter in the aspen leachate, the possibility cannot be excluded that the C18 column was overloaded and that some nonpolar material remained in the water fraction that passed through the column. In the remaining fractions, the increasing toxicity of the leachates with increasing proportion of methanol in the elutant, to a maximum in the 75% methanol fraction, suggests that the toxic fraction comprises largely nonpolar compounds. Water fraction may reflect a second fraction containing polar compounds, or it may be an artifact of overloading the column.

Gas chromatography–mass spectrometry analysis was performed on the water fraction and the 75% methanol fraction from the nonaerated leachate, the two fractions that were most toxic in the bacterial luminescence assay. Some 17 compounds, representing more than 90% of the total peak area, were identifiable in the water fraction (Table 6). With a few exceptions such as cyclohexanediol and octanoic acid, the components were all phenolic compounds or various substituted benzenes; benzoic acid and phenol were by far the most abundant compounds. The 75% methanol fraction produced numerous peaks, of which only four, representing more than 75% of total peak area, were identifiable (Table 6). Nonanoic acid is the only identified compound not also found in the water fraction. As this was a preliminary investigation, no confirmatory testing was undertaken; hence, the contribution of specific compounds to toxicity of the leachate is unknown.

DISCUSSION

It is evident from these preliminary experiments that fresh aspen wood leaches readily and that the leachate so produced is very toxic to aquatic life. A 1% solution of the stronger aspen leachate was acutely toxic to trout, and similarly dilute solutions were toxic to *Daphnia* or bacteria. Further, the leachate has a very high oxygen demand for decomposition (BOD), and in large quantities of leachate, passive atmospheric aeration was completely insufficient to maintain dissolved oxygen concen-

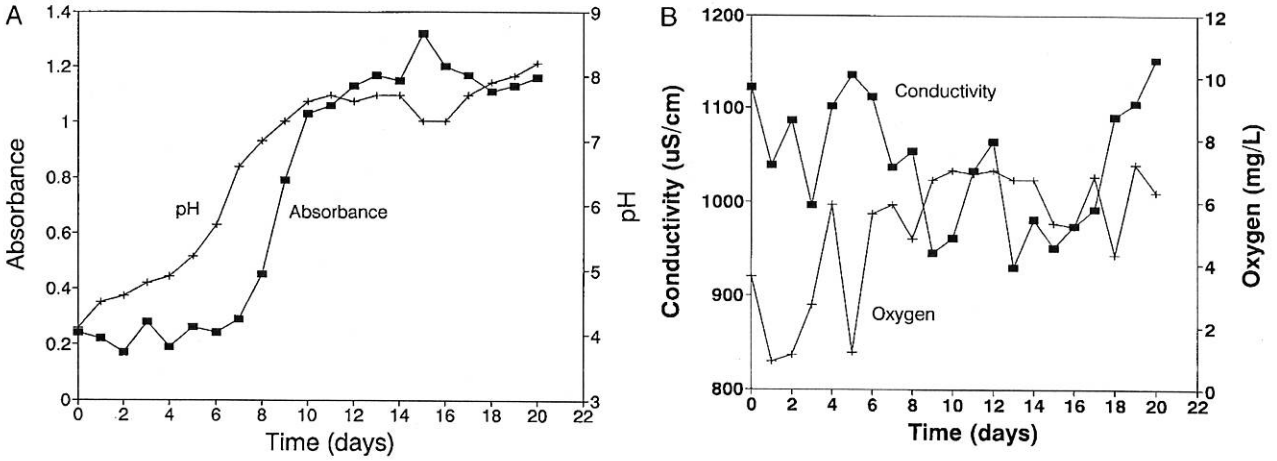

Fig. 4. Change in (**A**) light absorbance (465 nm) and pH, (**B**) dissolved oxygen concentration and electrical conductance, in the second aeration trial. Aspen leachate was aged 65 d without aeration before aeration (300 ml/min) began.

trations significantly above zero. Thus, the leachate could pose a threat to aquatic ecosystems through direct toxicity and through deoxygenation of the water column.

Leaching from aspen wood is evidently biphasic, with a rapid loss of about 1% mass on the first day, followed by another 1 to 2% loss over the next 2 weeks or so. (There may be a further increase beyond 2 weeks, but the leaching losses for 18 and 28 days are not significantly different; Student's t test, $p > 0.10$.) Quantitative estimates of leaching loss from wood are few, but our estimates are similar to the 5% loss estimated for twigs of birch [23]. The first-day loss probably represents sap and soluble material in cambial cells that would be immediately exposed to water by the tubular structure of the vascular system, while the slower loss thereafter arose from water penetrating through cell walls of nonvascular tissues.

Of course, our experiments used chipped, whole-wood rounds, from which leachable material would be released much more quickly than from logs. On the other hand, we observed during the second leaching experiment that samples with a high bark content were darker in color and appeared to have lost slightly more mass compared to bark-free samples. The bark is also reportedly the major source of toxic phenolics [4,8], although these are evidently not the only toxins in the leachate. The rapid mass loss during the first day would be mostly from bark and sap and would therefore contain a disproportionate amount of phenols and phenolic glycosides, as well as simple sugars that would produce a high BOD. This conclusion is supported by the first leaching experiment, in which there was a dramatic decline in DO concentrations and a rapid increase in toxicity within the first 24 h. Consequently, while leaching losses from intact logs are likely to be less than losses from chips, they are also likely to contain more of the environmentally significant compounds.

Harvestable aspen trees in virgin stands are usually 80 to 100 years old, and the expected harvest rotation will be 60 to 80 years (D. Parminter, personal communication). The aspen trees used in these experiments were mostly younger than harvestable age (mean age 45 years) and therefore may have been physiologically different from older trees. However, phenolic compounds are produced in large quantities by aspen trees of all ages [8], and the difference in age here is small enough that results should be generally applicable.

The toxicity of leachate from an aspen woodpile is illustrated by considering the volume of water potentially affected by a given mass of wood. Based on the results here, leachate from about a 10:1 (mass of water: dry mass of wood) mixture of

Table 4. Experiment 4: Change in toxicity of previously unaerated leachate (65 d) after aeration (300 ml/min) began; data are LC50s or LC50s as a percent of undiluted sample (95% C.I.)

Test	Laboratory[a]	LC50 or EC50		
		Day 0 (65)	Day 8	Day 20
Trout, acute (96 h)	HQ	0.8 (0.6–1.2)	2.2 (1.6–3.1)	Insufficient sample
	MOE	2.2 (1.0–3.2)	<10	Insufficient sample
Daphnia, acute (48 h)	HQ	1.0 (0.8–1.3)	2.2 (1.6–3.1)	16 (13–25)
	MOE	1.41 (1.15–1.68)	3.6 (3.2–5.6)	13.4 (10–18)
Bacterial luminescence (15 min)	HQ	0.5 (0.3–0.9)	1.6 (1.2–2.1)	10.4 (8.7–12.5)
	MOE	0.7 (0.66–0.85)	1.04 (0.83–1.31)	4.95 (4.39–5.57)
Seed germination	HQ	39 (5–90)	74 (62–87)	37 (23–69)
Root elongation	HQ	23	76	36

[a] HQ = HydroQual Laboratories; MOE = BC Ministry of Environment Aquatic Toxicity Laboratory.

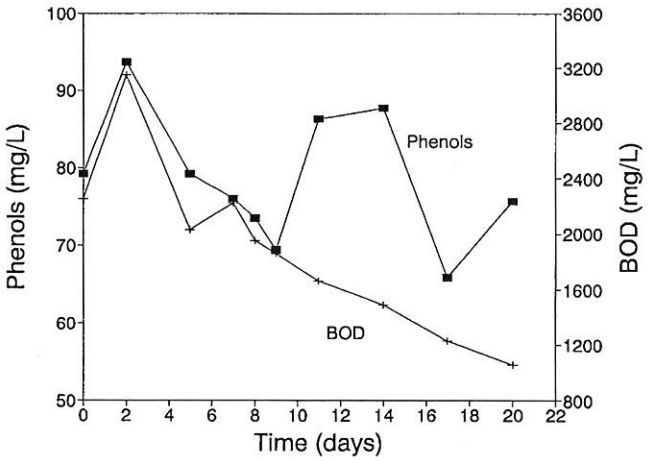

Fig. 5. Change in concentration of total phenols and BOD in the second aeration trial. Aspen leachate was aged 65 d without aeration before aeration (300 mL/min) began.

aspen in water was toxic to trout in the neighborhood of 1% of full strength. Hence, in the worst case, one metric ton of aspen could render 1×10^6 L of water (1,000 m^3) acutely toxic to trout. By comparison, the 60-d inventory of roundwood for a good-size pulp mill using aspen is 60,000 to 80,000 metric tons [3].

The aspen leachate appears to be less toxic to plant life than to animals, given the relatively high concentrations necessary to inhibit algal growth or seed germination. This is perhaps surprising, because phenolic compounds have long been considered plant growth inhibitors [24–27], and the aspen leachate was rich in phenols. Megharaj et al. [28] observed strong inhibition of growth and photosynthesis in two species of green algae at phenol concentrations below 50 μg/L; the nominal concentration of total phenolics in 12 to 16% fresh aspen leachate, the EC50 concentration in the algal growth test, would be 3.6 to 4.8 mg/L. Along with the high doses needed to inhibit seedling growth, this suggests that much of the total phenolics in fresh aspen leachate was not phenol but some less inhibitory compound. However, the lack of effect of leachate on seed germination, compared with that on seedling growth (root elongation), is consistent with other work on phenols [29] that also shows a stronger inhibition of root elongation.

Dissolved oxygen remained at or near zero in pails with aspen wood in the first mass-loss experiment and during the aging of the more concentrated leachate. Significantly, however, DO concentrations were generally 1 to 2.5 mg/L in the large vat in which concentrated leachate was produced. This suggests that the metabolism of microbial decomposers was inhibited in the strong solution to the extent that they could not exhaust all of the available oxygen. Such inhibition would be consistent with the low EC50 concentrations in the bacterial luminescence test.

Table 5. Toxicity in the bacterial luminescence bioassay of different fractions of aged aspen leachate, separated according to relative solubilities in methanol and water; data are toxic units (100/EC50)

Fraction (% Methanol)	Not aerated Day 0	Aerated Day 20
0[a]	5,000	<1
25	322	<1
50	1,430	<1
75	2,000	<1
100	<1	—

[a] Leachate after passage through the C18 column.

Table 6. Major compounds identified in gas chromatography–mass spectrometry scan of the two most toxic fractions of aged aspen leachate; data are approximate concentrations in whole leachate

Compound	Concn. (mg/L) Water fraction (column effluent)	Concn. (mg/L) 75% methanol fraction
Benzoic acid	36	—
Phenol	21	—
Cyclohexanediol	10	—
Nonanoic acid	—	12
Hydroxyphenylpropanoic acid	5	—
Salicylic acid	4	—
Guaiacol	3.6	—
Hydroxymethoxyphenyl acetic acid	2.5	—
Phenylpropionic acid	2.3	—
Octanoic acid	2	0.8
2-Hydroxy cyclohexanone	2	—
C1-phenol[a]	—	4.4
C2-phenol[a]	2	6.6
Hydroxybenzyl alcohol	1.6	—
Catechol	1.5	—
1-(2-hydroxphenyl) ethanone	1	—
3,4,5-trimethoxybenzoic acid	1	—
Phenyl acetic acid	0.8	—
2-Phenoxyethanol	0.6	—

[a] Unidentified phenols substituted with a one-carbon group (C1) or two-carbon groups (C2). C2-phenols may contain a single 2-carbon group or two 1-carbon groups.

The toxicity of aspen leachate also appears to be quite persistent under laboratory conditions. The unaerated leachate maintained its original toxicity for 2 months at 16°C, and the slow rates at which pH and DO (which generally indicate the progress of decomposition) were recovering suggest that it would remain toxic for several more months. Leachate toxicity was even more persistent at low temperature: the smaller sample at 5°C showed no discernable change in toxicity over more than 2 months, despite its small volume (500 ml) and free exposure to the air. In that experiment, the aerated leachate turned black after 2 weeks and its toxicity declined; however, in both that and the confirmatory experiment, toxicity remained high, or even increased, for some time after the leachate had turned black. Hence, blackwater encountered in the field might or might not be a serious toxic solution, and its persistence has not been evaluated. The question of how to effectively treat blackwater to enable safe discharge should be investigated.

The change from fresh leachate to blackwater was dramatic in its abruptness and in the uniformity of changes in color, pH, and conductivity. The pattern suggests that the color change comes about when one fraction of the labile organic material is exhausted; organic acids are the most likely class, because their decomposition would cause an increase in pH and a decrease in electrical conductance. The color change probably represents the polymerization of cyclic organic compounds such as benzoic acid to form large-molecular weight humic and fulvic acids. Blackening is routinely observed in the decay of wastewaters containing labile organic matter—for instance, in the treatment of pulp mill effluent. At about the same time as the black color appeared, the sample began to accumulate surface foam, a characteristic of dissolved humic material.

The reiteration of the same pattern of physical-chemical changes when the second vat was aerated (experiment 4) confirms the generality of the response. However, although there was no obvious change in appearance or toxicity during the first 65 d, the nonaerated leachate was nonetheless decomposing during this period. This is apparent from the chemistry data in Table 4, which show a loss of organic carbon, a small increase in pH, and declines in concentrations of minor components such as resin and fatty acids. The dramatic jump in concentration of phenolic compounds, while unexpected, at least indicates microbial activity. The nonaerated vat appears to have been proceeding slowly along the same pathway as the aerated sample; this would account for the somewhat faster transition to blackwater once aeration was begun. The very large BOD of nonaerated leachate, even after 65 d, illustrates that leachate toxicity is likely to persist wherever oxygen supply is limited to passive surface aeration.

While the color change in aging leachate was conspicuous to the naked eye, analytically, it was reflected only in absorbance at 465 nm, and not in true color (full spectrum light absorption), which barely changed from fresh leachate to blackwater (Table 3). We had hoped that the black color, given that it presumably arises from polymerization of phenols and other substituted benzenes, would correlate with the loss of toxicity, and hence could be used as a field marker to distinguish new (i.e., hazardous) from old leachate. Unfortunately, this is not the case, as the toxicity of aerated leachate in experiment 3 increased again after the color change (Fig. 3), and in experiment 4, the leachate turned black without any substantial loss of toxicity (Table 3) or decline in phenolics content (Fig. 5).

It is evident from the bacterial luminescence assay in experiment 3 that the decline in toxicity of aspen leachate is not a linear process, but rather passes through two cycles of decline and increase and decline again (Fig. 3). The relatively small confidence intervals for the bacterial luminescence assay, and the close correspondence of bacterial luminescence results with those of other tests in this and other experiments, strongly suggest that the pattern is real and not an artifact of the assay. Unlike the first decline in toxicity, the second phase was not accompanied by any measured physical or chemical changes, except possibly a marginal fall and rise in light absorbance and conductivity (Fig. 2C,D).

The bacterial luminescence data imply that a toxicant is being manufactured during decomposition of the leachate; the compound or compounds responsible must be labile (because the toxicity did not persist), neutral (because pH was unaffected), and either colorless and nondissociated at neutral pH or present in very small quantities. In the nonaerated leachate, phenolic compounds were evidently manufactured during aging, as evinced by the more than twofold increase in total phenols concentration between day 0 and day 65. If the same process occurred in the aerated sample, the manufactured phenols could have been responsible for the second phase of toxicity. Cleavage of the sugar molecule from phenolic glycosides to release salicylic acid or other phenolic compounds is the most obvious source of the additional phenol.

But while some salicylic acid was found in the gas chromatography–mass spectrometry analysis of 65-d-old leachate, unsubstituted phenol and benzoic acid were relatively far more abundant (Table 6). The concentrations of various compounds estimated from gas chromatography–mass spectrometry analysis are only very approximate, but they do indicate the rank order of abundances. Almost all of the other identifiable compounds were phenolics or substituted benzenes, so it is clear that this group is the largest constituent of the toxic fraction.

There are several possible sources of these phenolic compounds. First, as mentioned earlier, enzymatic hydrolysis of salicin and related phenolic glycosides such as salireposide and salicortin [8] would release salicyclic acid (*o*-hydroxybenzoic acid). Second, aspen wood is unique among commercial hardwoods in that it contains substantial quantities (600 mg/kg) of *p*-hydroxybenzoic acid, which readily produces phenol by decarboxylation [10]. Variously substituted benzoic acids are known to be important intermediates in the bacterial degradation of a wide range of phenolic compounds; further decay leads to cyclohexanones, cyclohexanols, and eventually straight-chain carboxylic acids (see review in Londry and Fedorak [30]). Hence, most of the compounds identified in the toxic fractions of aged aspen leachate were either phenolics derived from the wood or expected products of their incomplete decay.

Yet standard toxicity data suggest that neither phenols nor any other known component of aspen leachate are sufficient to explain the observed toxicity. In very dilute solutions of leachate (<10%), DO could be maintained above 5 mg/L with aeration, but these solutions were still toxic to trout and other organisms. As for phenolics, the LC50 of simple phenol is 5 to 10 mg/L [31,32], and other known, natural phenolic compounds are toxic in the same range [32]. A 1 to 2% solution of fresh aspen leachate (the trout LC50) contains 0.3 to 0.6 mg/L of phenolic compounds, apparently far too little to produce acute toxicity from any of the known compounds.

In all three experiments in which toxicity and chemistry of aspen leachate were measured, there is no correspondence between phenols concentrations and toxicity. Fresh aspen leachate, containing 30 mg/L total phenolics, produced LC50s around 1 to 2% for both fish and invertebrates and an order of magnitude less for bacteria. In the vat without aeration (experiment 3), phenols concentration rose to 83 mg/L after 65 d, yet there was no detectable change in toxicity to any of the test organisms. When the remaining leachate was aerated, there was a gradual decline in toxicity (increase in LC50s of about 10 times) from day 0 through day 8 to day 20, without any corresponding change in phenols concentration (79, 74, and 76 mg/L, respectively). As mentioned earlier, leachate toxicity to plants also does not agree with expectations based on phenols concentration.

There is a conundrum, therefore, because experimental data suggest that phenols are not the chief toxicant in the leachate, but all of the identified compounds in toxic fractions are phenols or their breakdown products (although about 10 to 20% of the constituents have not been identified). Salicin is nontoxic to *Daphnia* at concentrations up to 1,000 mg/L (HydroQual Laboratories Ltd., unpublished data), and salicylic acid is acutely toxic to *Daphnia* at concentrations of 50 to 150 mg/L [33]. Toxicity of benzoic acid is even lower [34]. Thus, we cannot account for the toxicity of aspen leachate with any of the known chemical constituents.

The low pH of aspen leachate could have augmented the toxicity of phenolics and would itself be stressful to aquatic life. In tests with rainbow trout done by the BC Ministry of Environment, the initial pH was measured at each dilution used in the test. The pH at the lowest concentration producing 50% mortality in two tests on fresh leachate were 4.4 (1.8% solution) and 4.5 (1.3%), and after 65 d without aeration, 5.2 (3.2%). Concentrations of leachate toxic to *Daphnia* had pH values ranging from 5.6 to 6.0. It is doubtful, however, that a difference

in pH alone would be sufficient to produce the order-of-magnitude increase in phenol toxicity that would be necessary to account for the observed toxicity. Further research should concentrate on identifying the missing toxic constituent.

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REFERENCES

1. **Ondro, W.J.** 1991. Economics of 15 options for industrial utilization of poplars. Information Report NOR-X-320. Forestry Canada, Edmonton, AB, Canada.
2. **Forestry Canada.** 1990. Selected forestry statistics, Canada, 1990. Information Report E-X-44. Economics and Statistics Directorate, Ottawa, ON, Canada.
3. **Cagampan-Stoute, C.** 1991. Aspen chip seasoning comes of age. *Pulp and Paper J.* **44**:23.
4. **Pearl, I.A.** and **S.F. Darling.** 1971. Hot water phenolic extractives of the bark and leaves of diploid *Populus tremuloides*. *Phytochemistry* **10**:483–484.
5. **Van Sumere, C.F.** 1989. Phenols and phenolic acids. In J.B. Harborne, ed., *Plant Phenolics. Methods in Plant Biochemistry,* Vol. 1. Academic, London, UK.
6. **Lindroth, R.L., J.M. Scriber** and **M.T.S. Hsia.** 1988. Chemical ecology of the tiger swallowtail: Mediation of host use by phenolic glycosides. *Ecology* **69**:814–822.
7. **Basey, J.M., S.H. Jenkins** and **P.E. Busher.** 1988. Optimal central-place foraging by beavers—Tree size selection in relation to defensive chemicals of quaking aspen. *Oecologia* **76**:278–282.
8. **Palo, R.T.** 1984. Distribution of birch (*Betula* spp.), willow (*Salix* spp.) and poplar (*Populus* spp.) secondary metabolites and their potential role as chemical defense against herbivores. *J. Chem. Ecol.* **10**:499–521.
9. **Stranks, D.W.** 1976. Wood preservatives: Their depletion as fungicides and fate in the environment. Forestry Technical Report 10. Canadian Forestry Service, Ottawa, ON, Canada.
10. **Shariff, A.J., R.W. Lowe, D. Berthiaume, J.R.G. Bryce** and **R.A.N. McLean.** 1989. Unexpected source of phenol in the sulphur-free semichemical pulping of hardwood. *TAPPI J.* **72**:177–183.
11. **Taylor, B.R.** and **D. Parkinson.** 1988. Patterns of water absorption and leaching in pine and aspen leaf litter. *Soil Biol. Biochem.* **20**: 647–656.
12. **Environment Canada.** 1992. Biological test method: Toxicity test using luminescent bacteria *Photobacterium phosphoreum*. EPS 1/RM/24. Environmental Protection Series, Ottawa, ON, Canada.
13. **Environment Canada.** 1990. Biological test method: Acute lethality test using rainbow trout. EPS 1/RM/9. Environmental Protection Series, Ottawa, ON, Canada.
14. **Environment Canada.** 1990. Biological test method: Acute lethality test using *Daphnia* sp. EPS 1/RM/11. Environmental Protection Series, Ottawa, ON, Canada.
15. **Environment Canada.** 1992. Biological test method: Test of reproduction and survival using the cladoceran *Ceriodaphnia* sp. EPS 1/RM/21. Environmental Protection Series, Ottawa, ON, Canada.
16. **Environment Canada.** 1992. Biological test method: Growth inhibition test using the freshwater alga *Selenastrum capricornutum*. EPS 1/RM/25. Environmental Protection Series, Ottawa, ON, Canada.
17. **Greene, J.C., C.L. Bartels, W.J. Warren-Hicks, B.R. Parkhurst, G.L. Linder, S.A. Peterson** and **W.E. Miller.** 1989. Protocol for short-term toxicity screening of hazardous waste sites. EPA/3-88-029. U.S. Environmental Protection Agency, Corvallis, OR, USA.
18. **McQuaker, N.R.** 1976. A laboratory manual for the chemical analysis of waters, wastewaters, sediments, and biological materials. Queen's Printer, Victoria, BC, Canada.
19. **Norberg-King, T.J., et al.** 1991. Methods for aquatic toxicity identification evaluations. Phase II: Identification, 2nd ed. EPA/600/3-88/035. U.S. Environmental Protection Agency, Office of Research and Development, Duluth, MN.
20. **U.S. Environmental Protection Agency.** 1986. Test methods for evaluating solid waste: Physical/Chemical methods, (SW-846), 3rd ed. Method 8270. Cincinnati, OH.
21. **Leach, J.M.** and **A.N. Thakore.** 1973. Identification of the constituents of kraft pulping effluent that are toxic to juvenile coho salmon (*Onchorynchus kisutch*). *J. Fish. Res. Board Can.* **30**:479–484.
22. **Leach, J.M.** and **A.N. Thakore.** 1976. Toxic constituents in mechanical pulping effluents. *TAPPI J.* **59**:129–132.
23. **Taylor, B.R.** and **H.G. Jones.** 1990. Litter decomposition under snow cover in a balsam fir forest. *Can. J. Bot.* **68**:112–120.
24. **Wang, T.S.C., T.K. Yang** and **T. Chuang.** 1967. Soil phenolic acids as plant growth inhibitors. *Soil Sci.* **103**:239–246.
25. **Einhellig, F.A.** and **J.A. Rasmussen.** 1978. Synergistic inhibitory effects of vanillic and *p*-hydroxybenzoic acids on radish and grain sorghum. *J. Chem. Ecol.* **4**:425–436.
26. **Einhellig, F.A.** and **J.A. Rasmussin.** 1979. Effects of three phenolic acids on chlorophyll content and growth of soybean and grain sorghum seedlings. *J. Chem. Ecol.* **5**:815–824.
27. **Williams, R.D.** and **R.E. Hoagland.** 1982. The effects of naturally occurring phenolic compounds on seed germination. *Weed Sci.* **30**: 206–212.
28. **Megharaj, M., H.W. Pearson** and **K. Venkateswarlu.** 1992. Effects of phenolic compounds on growth and metabolic activities of *Chlorella vulgaris* and *Scenedesmus bijugatus* isolated from soil. *Plant Soil* **140**:25–34.
29. **Krogmeier, M.J.** and **J.M. Bremner.** 1989. Effects of phenolic compounds on seed germination and seedling growth in soil. *Biol. Fertil. Soils* **8**:116–122.
30. **Londry, K.L.** and **P.M. Fedorak.** 1992. Benzoic acid intermediates in the anaerobic biodegradation of phenols. *Can. J. Microbiol.* **38**: 1–11.
31. **Buikema, A.L. Jr., M.J. McGinnis** and **J. Cairns, Jr.** 1979. Phenolics in aquatic ecosystems: A selected review of recent literature. *Mar. Environ. Res.* **2**:87–181.
32. **Environmental Protection Service.** 1985. Phenol: Environmental and technical information for problem spills. Technical Services Branch, Environmental Protection Programs Directorate, Ottawa, ON, Canada.
33. **Wang, W.H.** and **J.P. Lay.** 1989. Fate and effect of salicylic acid compounds in freshwater systems. *Ecotoxicol. Environ. Saf.* **17**: 308–316.
34. **Verschueren, K.** 1983. *Handbook of Environmental Data on Organic Chemicals,* 2nd ed. Van Nostrand Reinhold, Toronto, ON, Canada.