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GENETIC TOXICITY OF PULP MILL EFFLUENT ON JUVENILE CHINOOK SALMON (*ONCHORHYNCHUS TSHAWYTSCHA*) USING FLOW CYTOMETRY

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ABSTRACT

On-site bioassays were conducted at the furthest upstream pulp mill on the Fraser River in British Columbia. Uncontaminated river water was used to dilute treated effluent as discharged from the final diffuser pond. A single cohort of juvenile (8-10gm) chinook salmon (*Oncorhynchus tshawytscha*) was divided into an hypoxic group receiving 65% oxygen saturated water and a normoxic group receiving ambient 88% oxygen saturated water. Both groups were exposed over a period of 30 days to effluent concentrations of 2%, 4%, 8%, and 16%, while the controls received uncontaminated river water. This range of concentrations spanned those encountered by wild juvenile salmon overwintering in the upper Fraser River mainstem.The blood when analyzed by flow cytometry showed significant concentration-dependent clastogenic damage in both the normoxic and hypoxic groups starting at the 4% concentration. A concentration-response curve was determined from the hypoxic data set. Genetic implications of mutagenic damage to natural populations of chinook salmon are discussed along with the utility of the flow cytometer in detecting genotoxic damage. © 1997 IAWQ. Published by Elsevier Science Ltd.

KEYWORDS

Bioassay; chinook salmon; concentration-response; flow cytometry; Fraser River; mutagen; pulp mill effluent.

INTRODUCTION

In British Columbia, stringent provincial and federal government regulations and enforcement have resulted in the significant upgrading of pulp and paper bleached kraft mill effluent (BKME) via treatment and process control by the mills. The acute toxicity of effluents has virtually been eliminated in mills with improved treatment processes. Concurrent with the reduction of lethality has been an increasing interest in the sublethal characteristics of the effluent. The mutagenicity of BKME has been well-documented in bacterial assays (Holmbom *et al.*, 1984; Rao *et al.*, 1995a,b;) and intra-peritoneal injections of XAD-

4/NaOH extracts has been shown to cause micronuclei formation in the liver tissues of trout (*Oncorhynchus mykiss*) (Rao *et al.*, 1995a). Whole paper mill effluent containing pulp, bleaching and dyeing effluent from unspecified processes with no treatment details was shown to cause micronuclei formation in the red blood cells of *Heteropneustes fossilis* (Das and Nanda, 1983). In a field study examining the frequency of micronuclei occurrence in perch populations, Al-Sabti and Hardig (1990) observed an increasing frequency of micronuclei formation in the perch (*Perca fluviatilis*) erythrocytes at a decreasing distance from a Swedish pulp mill on the Baltic Sea over a distance of 7 km. The mill process and treatment were not unspecified.

Flow cytometry is an established (Otto and Oldiges, 1980) precise automated method for quickly examining the relative DNA content of a large population of cells within a sample. The assay is sufficiently sensitive to distinguish between populations of cells whose nuclear DNA content differs by as little as 1 to 2% (Deaven, 1982) and can be performed on a wide array of tissue types. When a clastogen causes chromosomal damage, there is an unequal distribution of nuclear DNA in the resultant daughter cells after division. This increased variation in nuclear DNA content of cells is detected by the flow cytometer as an increase in the coefficient of variation (CV) of the G1 peak. This increase in variation was shown to be dose dependent (Otto *et al.*, 1981).

Flow cytometry has been used to detect environmental mutagenesis in field populations of birds (Custer *et al*, 1994; George *et al.*, 1991), turtles (Lamb *et al.*, 1991; Bickham *et al.*, 1988) and mice (McBee and Bickham, 1988) where contaminated sites were compared with reference sites. Results have been verified by other cytogenetic methods (Bickham *et al.*, 1992; McBee and Bickham, 1988). To our knowledge, the present study is the first occasion where the application of flow cytometry has been used in the detection of DNA damage in fish. The clastogenic effect of BKME on chinook juveniles has not been previously reported.

METHODS AND MATERIALS

A mobile laboratory was set up at a Fraser River pulp mill at Prince George, B.C. over the winter 1995/96. Juvenile chinook salmon (8 - 10 grams) were exposed to ambient river water dilutions of treated BKME under continuous flow conditions. The chinook salmon were obtained from the Penny Hatchery, a stock whose life history includes an overwintering period in the upper Fraser River. The pulp mill produces 1500 ADT/day of 100% (ClO_2) bleached softwood pulp and utilizes an 8-d aerated stabilization basin to treat approximately 150,000 m^3/day of effluent which is discharged directly into the Fraser River by diffuser. Characteristically, effluent at this mill is acutely not toxic. In the previous year, two non-regulatory static bioassays conducted in full strength effluent over 96h at 4°C resulted in 100% survival. A diving survey (Emmett *et al.*, 1996) confirmed the presence of high densities of juvenile chinook in the shallow nearshore habitat both upstream and downstream of the mill discharge plume.

Effluent was pumped continuously to the mobile laboratory and metered into fish holding tanks to provide dilutions of 16%, 8%, 4% and 2%. The controls received river water. The temperature of effluent discharged in the winter ranged from 20-25°C, which resulted in a small temperature gradient in the experimental tanks ranging from about 1°C in the controls to about 3.5°C in the 16% effluent concentration. We felt that this temperature variation was unlikely to have any significant effect on the expression of the clastogenic response at these low temperatures, since, photoperiod is the main controlling factor of growth during the winter (Clarke, 1992).

Because of possible interactive effects of effluent exposure and moderate under-ice hypoxia, one set of bioassays (Hypoxic) was conducted at 65% air saturation. Ambient river water (0.1 - 0.5°C) pumped from an ice-free area just upstream of the diffuser was in the 85-88% air saturation range and represented the (Normoxic) control. Hypoxic water was produced by passing river water through a vacuum degasser. The hypoxic bioassay was conducted in 200L oval tubs receiving 10L/min. The normoxic bioassay was carried out in 70L annular tanks with a flow rate of 2L/min. Fish were fed at 1% body weight per day over the 30 day exposure period. At the end of this period, 20 fish per treatment were sacrificed. After MS-222 (20

mg/L) anesthesia, blood was collected from the severed caudal peduncle into heparinized microhematocrit tubes and kept on ice until delivery to the West Vancouver Laboratory (usually within 24 hours). No blood samples were received from the 2% hypoxic group and half the normoxic group.

In preparation for flow cytometry, 1 microlitre of chinook red blood cells was treated with 50 ml DNA-Prep LPR (Coulter Inc.) followed by 800 ml of propidium iodide (Coulter DNA-Prep Stain). Samples were left for 15 minutes prior to quantification of nuclear fluorescence by the Coulter Epics XL Flow Cytometer equipped with a 488 nm Argon laser. In order to minimize variation from sample preparation, cells were simultaneously analyzed for nuclear fluorescence and side scatter. The flow cytometer is coupled with a computer program which determines (for the gated G1 cell population) both the mean and standard deviation of each fish for the 10,000 nuclei sampled as well as the full-peak percent Coefficient of Variation (CV), defined as the mean divided by the standard deviation x 100. Chicken red blood cells were used as the standard to control for instrument drift. The chicken red blood cells were usually run after each of the treatments and the control. The chinook red blood cell CVs were corrected for instrument drift by subtraction of the chicken CVs the resulting difference value (CVadj) being the parameter analyzed.

In the notched box plots (SYSTAT software by SPSS Inc.), the box represents the spread of the central 50% of the data and the tails each represent 25% of the data within the outlier cutoffs. The asterisks and circles represent points that are beyond the outlier cutoffs. The line within the box represents the median value. The notch represents the 95% confidence interval of the median (Hoaglin *et al.*, 1983).

The CVadj's were then analyzed using a one way ANOVA using SYSTAT software. Probability plots of the CVadj were utilized to examine normality. Where the F test showed a significant difference between factor level means, a Bonferroni means test (Neter *et al.*, 1985) was used to examine the differences between treatments and the control and between the treatments themselves.

RESULTS AND DISCUSSION

The notched boxed plot graphs (Figure 1 and Figure 2) show the distributional form of the data sets for both the hypoxic and normoxic bioassays. In the construction of these plots the BKME concentration values are treated merely as categories and no relationship is assumed between them.

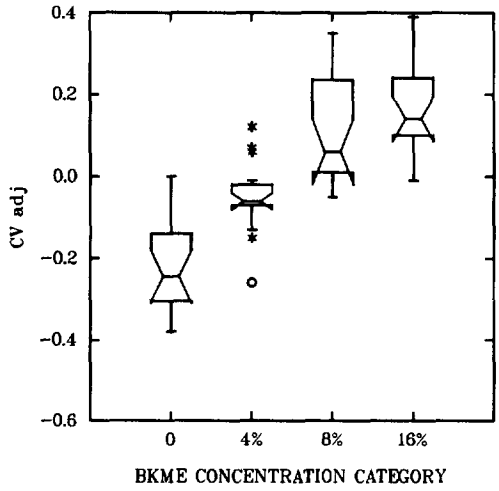

Figure 1. Clastogenic response induced in juvenile chinook salmon after a 30 day exposure to BKME under hypoxic (65 %A.S.) conditions.

The one-way ANOVA (Table 1) indicated that significant effects occurred between the various concentration treatments for both experiments.

In comparing least squared means (Table 2), there was no difference between the hypoxic and normoxic groups within a dose. Even at the 16% level where there was the greatest difference, this difference was not significant. Thus, with respect to the clastogenic response as measured by flow cytometry, the difference in dissolved oxygen concentration between the hypoxic and normoxic groups had no effect. A comparison of means within the hypoxic and normoxic data sets (Table 3 and Table 4) showed that significant clastogenic damage had occurred in the juvenile chinook salmon over the 30 day exposure period and that the response was concentration dependent.

Table 1. One-way anova of phase 1 data sets

Data Set	Source	SS	df	MS	F	P
Hypoxic	Treatment	1.588	3	0.529	41.964	<0.001
	Error	0.832	66	0.013		
Normoxic	Treatment	0.982	4	0.246	22.952	<0.001
	Error	0.449	42	0.011		

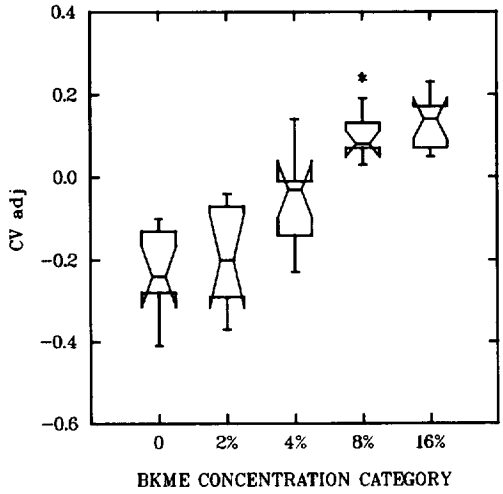

Figure 2. Clastogenic response induced in juvenile chinook salmon after a 30 day exposure to BKME under normoxic (88 %A.S.) conditions.

Table 2. Least squares means and standaed errors by exposure concentration for phase 1 data

Concentration	Hypoxic			Normoxic		
	LS Mean	SE	(N)	LS Mean	SE	(N)
Control	-0.226	0.028	16	-0.229	0.033	10
2%				-0.193	0.034	9
4%	-0.051	0.027	17	-0.057	0.034	9
8%	0.111	0.025	20	0.106	0.034	9
16%	0.169	0.027	17	0.111	0.033	10

A similar concentration-response effect for genetic damage from paper mill effluent was reported by Das and Nanda (1983) in *H. fossilis*. Their peak effect after 30 days exposure occurred at 20% concentration. In the present study, the response appeared to reach an asymptote at 16%. The concentration gradients utilized by Das and Nanda (1983) of 10%, 20%, 40% and 60% did not allow for defining the shape of the concentration-response curve sufficiently to derive a relationship. Rao *et al.* (1995a) prepared XAD-4/NaOH extracts from whole filtered BKME which was injected into trout 3 times at doses of 1ml (8.3 ml/g), 4ml

Table 3. Pairwise comparison probabilities of hypoxic means after bonferroni adjustment

Conc.	0%	2%	4%	8%	16%
0%	1				
2%					
4%	0.000		1		
8%	0.000		0.000	1	
16%	0.000		0.000	0.72	1

Table 4. Pairwise comparison probabilities of normoxic means after bonferroni adjustment

Conc.	C	2%	4%	8%	16%
C	1				
2%	1	1			
4%	0.008	0.076	1		
8%	0.000	0.000	0.018	1	
16%	0.000	0.000	0.010	1	1

(33.3 ml/g), 7ml (58.3 ml/g), 12 ml (100 ml/g), and 16 ml (133 ml/g). They obtained a dose response for the 1 ml and 4 ml treatments, but the remainder proved lethal to the fish. Although injection of effluent into the fish ensures a known dose being given, the pathway of distribution and uptake are certainly much different than when absorbed over the gills. Dosing the water in a flow-through system is a much more realistic way for fish to be exposed to BKME and the clastogenic properties of the entire effluent examined.

$$Y = B_0 + B_i x + B_2 x^2 + e_i \quad (1)$$

Y = CVadj.
X = Dose
B_0 = mean response of Y when x = 0.
B_1 = linear effect coefficient
B_2 = quadratic effect coefficient
e_i = error term

As an initial attempt to more clearly define the concentration-response curve for chinook salmon juveniles, a second degree quadratic equation of the form was fitted to the 4 different concentration levels for the actual percent values of the normoxic data set using the NONLIN module of SYSTAT (SPSS Inc.). The following function was derived:

$$Y = -0.25828 + 0.06050x - 0.00225x^2 \quad (2)$$

which had a corrected R^2 of 0.703 and represented a highly significant relationship (Table 5). The asymptotic standard errors of the regression parameter estimates (shown in Table 6) are especially large for the B2 regression coefficient which tends to define the curve portion of the quadratic equation. Detectable clastogenic damage is near its maximum level at the asymptote. The apparent falling off of observable clastogenic damage at the 16% level may indicate an increase in mutagen induced cross-linkages between chromatids in the stem and or transit cells that could prevent mitosis or result in apoptosis (Preston, 1981).

Lower 95% Each regression coefficient differs significantly from 0 (Table 6) and so contributes effectively to the concentration response relationship. Figure 3 shows the fit of the quadratic equation to the normoxic data. The fit is essentially determined by the variation around 4 data points, namely the concentration levels of 2%, 4%, 8% and 16%. The data points are presented as jittered in Figures 3 and 4 so that identical values could be seen as point clusters rather than not seen at all. Equation 2 was also fitted to the hypoxic data (Figure 4).

Table 5. Anova results for equation 2

Source	SS	df	MS	F	P
Regression	1.12238	3	0.37413	38.6	<0.001
Residual	0.42581	44	0.00968		
Total	1.54820	47			
Corrected Total	1.43564	46			

Table 6. Asymptotic errors of parameter estimates of equation 2

Parameter	Estimate	Asymptotic s.e.	Lower 95% C.L.	Upper 95% C.L.
B_0	-0.25828	0.02682	-0.31233	-0.20422
B_1	0.06050	0.00964	0.04107	0.07993
B_2	-0.00225	0.00056	-0.00338	-0.00111

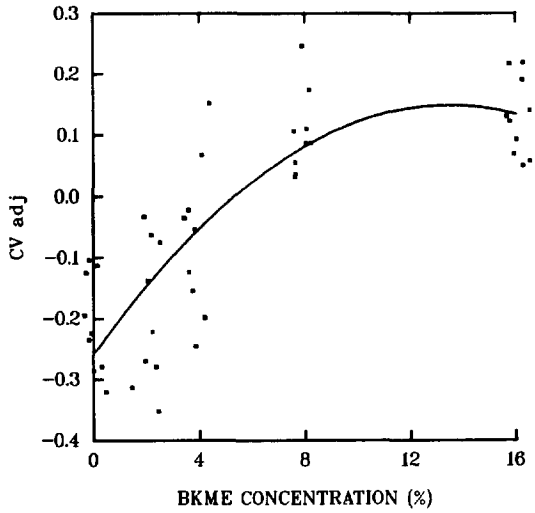

Figure 3. Fit of equation 2 to normoxic data.

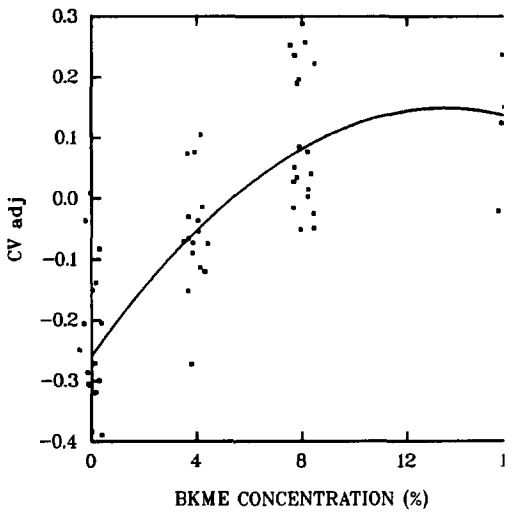

Figure 4. Fit of equation 2 to hypoxic data.

The BKME effluent plume in the Fraser River in the Prince George area shows incomplete mixing over a 14 km stretch of river occupied by pulp mills (Hatfield Consultants, 1993). Although detailed effluent distribution patterns under winter flow conditions remain to be determined, aerial photography taken in September of 1993 by G.A. Borstad and Associates of Sidney, B.C. clearly documented incomplete mixing of effluent from the Prince George mills with Fraser River water. In the winter of 1994, based on conductivity data, we estimated a concentration of 4% effluent in nearshore salmon habitat impacted by the Northwood mill at a distance of 0.5 km from the diffuser discharge (DFO unpublished). Emmett *et al.*, (1996) have shown in their diving survey of the Fraser River at Northwood that juvenile chinook salmon prefer shallow nearshore overwintering habitat. Thus there is ample opportunity for these young salmon to be exposed to potentially genotoxic doses (concentration x time) of effluent for as long or longer than in the present study. Das and Nanda (1983) demonstrated not only a positive relationship between genotoxic effect and concentration of paper mill effluent but also a positive relationship between genotoxic effect and exposure time at the same concentration. The direct comparability of their results is somewhat limited because of a lack of detail on mill process, wood furnish and treatment technology. Even so, the 1% concentration estimated by Hatfield Consultants (1993) to extend from Prince George to Lytton (600 km) during the winter low flow period could possibly present a significant genotoxic hazard given sufficient exposure time.

CONCLUSIONS

BKME representing final treated effluent was shown to cause significant clastogenic activity in erythrocyte precursor cells (stem and transit cells) of juvenile chinook salmon at concentration levels spanning the range present in the upper Fraser River in the Prince George region. The resulting erythrocytes were not sufficiently damaged to undergo apoptosis, but may, owing to chromosome structural damage, have some impairment in their primary function of oxygen transport.

The variability in response of the chinook salmon juveniles at each concentration level of BKME may reflect genetic variation in susceptibility to clastogenic damage as mediated by the variation in the efficiency of the DNA repair mechanism (Evans, 1981) in each fish. Unrepaired double-strand breaks are the basis of chromosome breaks (Painter, 1982). If genetic variability exists for the repair mechanism and fitness traits (i.e. survival and reproductive success) are being ultimately affected then selection could act to shift the gene frequency structure of a local population or stock towards a new adaptive peak which could seriously impact not only responses to future genetic challenges, but also the physiological capability of the stock to survive any new environmental conditions (Wilson, 1988; Diamond *et al.*, 1995).

One of the main consequences of mutagenicity on somatic cells is the greatly increased risk of cancer in the fish of the present generation (Vielkind, 1992). Exposure at the juvenile stage is especially significant, since mutagen induced carcinogenesis has been shown to be much more prevalent in juvenile fish (Schwab *et al.*, 1978; Hawkins *et al.*, 1985) than in adults.

At present, there is no evidence that BKME at the concentrations examined is genotoxic to germ cells. If the germ cells were affected, then the mutations could become fixed in the population and impact future generations. Future research should examine the impact of BKME mutagenic activity on fish germ cells.

The flow cytometer is proving to be a valuable tool in the monitoring and detection of genetic damage in both laboratory and field applications (Gray *et al*, 1979; Bickham *et al*, 1988; McBee and Bickham, 1988; Lamb *et al*, 1991; Bickham *et al.* 1992; Custer *et al*, 1994). The collection and processing of blood in the field can be improved from the present study by treating the heparinized blood with Ham's freezing media and then freezing in liquid nitrogen (see Custer *et al*, 1994) for later storage at -80°C until analysis. When running samples in the cytometer for detection of DNA damage, it is vital to run an internal control such as chicken red blood cells from which the experimental sample is subtracted in order to correct for any machine drift.

Serious consideration should be given to adding the flow cytometry test for genotoxic effects to existing environmental impact assessments of pulp and paper mill discharges. Since live fish are already being sampled as part of existing Environmental Effects Monitoring (EEM) protocols, the small volume of blood required as well as the presumed immunity of the assay to the effects of fish capture and subsequent handling stress, makes the flow cytometry assay for genotoxicity an attractive addition to the present suite of biomarkers.

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