ELSEVIER

Aquatic Toxicology 76 (2006) 246–257

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

The effect of chronic chromium exposure on the health of Chinook salmon (*Oncorhynchus tshawytscha*)

Aïda M. Farag [a,*], Thomas May [b], Gary D. Marty [c,1], Michael Easton [d], David D. Harper [a], Edward E. Little [b], Laverne Cleveland [b]

[a] *United States Geological Survey, Columbia Environmental Research Center, Jackson Field Research Station, P.O. Box 1089, Jackson, WY 83001, USA*

[b] *United States Geological Survey, Columbia Environmental Research Center, Columbia, MO 65201, USA*

[c] *Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine, University of California, 1 Shields Ave., Davis, CA 95616-8732, USA*

[d] *International EcoGen Inc., 2015 McLallen Court, North Vancouver, BC, Canada V7P 3H6*

Received 23 September 2004; received in revised form 25 August 2005; accepted 21 September 2005

Abstract

This study was designed to determine fish health impairment of Chinook salmon (*Oncorhynchus tshawytscha*) exposed to chromium. Juvenile Chinook salmon were exposed to aqueous chromium concentrations (0–266 $\mu g\,l^{-1}$) that have been documented in porewater from bottom sediments and in well waters near salmon spawning areas in the Columbia River in the northwestern United States. After Chinook salmon parr were exposed to 24 and 54 $\mu g\,Cr\,l^{-1}$ for 105 days, neither growth nor survival of parr was affected. On day 105, concentrations were increased from 24 to 120 $\mu g\,Cr\,l^{-1}$ and from 54 to 266 $\mu g\,Cr\,l^{-1}$ until the end of the experiment on day 134. Weight of parr was decreased in the 24/120 $\mu g\,Cr\,l^{-1}$ treatment, and survival was decreased in the 54/266 $\mu g\,Cr\,l^{-1}$ treatment. Fish health was significantly impaired in both the 24/120 and 54/266 $\mu g\,Cr\,l^{-1}$ treatments. The kidney is the target organ during chromium exposures through the water column. The kidneys of fish exposed to the greatest concentrations of chromium had gross and microscopic lesions (e.g. necrosis of cells lining kidney tububules) and products of lipid peroxidation were elevated. These changes were associated with elevated concentrations of chromium in the kidney, and reduced growth and survival. Also, variations in DNA in the blood were associated with pathological changes in the kidney and spleen. These changes suggest that chromium accumulates and enters the lipid peroxidation pathway where fatty acid damage and DNA damage (expressed as chromosome changes) occur to cause cell death and tissue damage. While most of the physiological malfunctions occurred following parr exposures to concentrations $\geq$120 $\mu g\,Cr\,l^{-1}$, nuclear DNA damage followed exposures to 24 $\mu g\,Cr\,l^{-1}$, which was the smallest concentration tested. The abnormalities measured during this study are particularly important because they are associated with impaired growth and reduced survival at concentrations $\geq$120 $\mu g\,Cr\,l^{-1}$.

* Corresponding author. Tel.: +1 307 733 2314x11.
E-mail address: aida_farag@usgs.gov (A.M. Farag).

[1] Present address: Animal Health Center, BCMAFF, 1767 Angus Campbell Road, Abbotsford, BC, Canada V3G 2M3.

0166-445X/$ – see front matter
doi:10.1016/j.aquatox.2005.09.011

Therefore, these changes can be used to investigate the health of resident fish in natural waters with high chromium concentrations as well as provide insight into the mechanisms of chromium toxicity.

Keywords: Chinook salmon; Chromium effects; Histology; DNA; Lipid peroxidation; Columbia River

1. Introduction

Chromium (Cr) is abundant in the earth's crust and is used as sodium dichromate in the production of chrome pigments, chrome salts for tanning leather, mordant dying, wood preservatives and anticorrosives. It is biologically significant in the trivalent and hexavalent forms. The hexavalent form is considered the toxic form of Cr because it readily crosses cell membranes. Inside the cell, the hexavelant form is reduced to the trivalent form which complexes with intracellular macromolecules, including genetic material, and is ultimately responsible for the toxic and mutagenic capacities of Cr (Goyer, 1986). Chromium is predominantly excreted in the urine. A major acute response in humans exposed to Cr is acute renal tubular necrosis. Some chronic effects in humans include chronic ulceration and perforation of the nasal septum and allergic skin reactions (Goyer, 1986).

Elevated concentrations of Cr in the Hanford Reach of the Columbia River (northwestern United States) are of particular concern. Columbia River water was used to cool nuclear reactors at the Hanford Nuclear Reservation, and the cooling water was treated with sodium dichromate to prevent corrosion and mineral collection within the pipes (Peterson et al., 1996). Although operations at Hanford were suspended in 1972, the concentrations of Cr in the groundwater upwellings (Hope and Peterson, 1996) exceed the chronic ambient water quality criteria (AWQC, 11 $\mu g\,l^{-1}$) for the protection of aquatic life, established by the United States Environmental Protection Agency (USEPA, 1986). The concentrations of Cr in some pump and treat wells have been increasing since 1999, and recently 275 $\mu g\,Cr\,l^{-1}$ was documented in the water near salmon spawning areas (Don Steffeck, USFWS, oral communication).

While some data exist on the effects of Cr^{6+} on salmon (Olson and Foster, 1956; Buhl and Hamilton, 1991), previous studies did not investigate physiological impairment of parr as a result of Cr exposure. In common carp (*Cyprinus* carpio) Cr^{6+} caused cytogenetic damage as measured by micronuclei induction in erythrocyte cells (Al-Sabti et al., 1994); exposed carp also had depletion of liver and muscle glycogen along with decreased leukocyte counts (Al-Akel and Shamsi, 1996). Further, respiratory inhibition in trout liver mitochondria exposed to dichromate was linked to the reduction of Cr^{6+} (Arillo and Melodia, 1988).

An understanding of individual fish health (pathology) interpreted simultaneously with population level measurements (e.g. survival) can provide a more complete assessment of the effects of potential contaminants (Johnson, 1968). Evaluations based on the residue concentrations and fish health (e.g. microscopic lesions) of fish integrates the actual exposure to pollutants (dose) and effects of those exposures on fish survival and growth (Farag et al., 1994, 1995). Further associations of tissue Cr accumulation, DNA damage, microscopic lesions, oxidative stress and growth reduction would add more weight to a determination of fish health impairment. This weight of evidence approach uses all of the information gathered to determine the health status of a fish population.

Our study of the health status of Chinook salmon exposed to Cr during the parr stage had three objectives: (1) determine the effects of Cr on survival, growth and fish health; (2) determine possible mechanisms of Cr toxicity and (3) develop assessment methods for later use in the health assessments of resident fish in areas where Cr may have been released into the water column.

2. Methods

2.1. Exposure conditions

Experimental water was prepared by blending laboratory well water with deionized water produced by reverse osmosis, eliminating the use of surface water and the potential for fish pathogens to be introduced

to the experiment and influence test results. Experimental water was produced in 5600 l batches where the range of alkalinity was 76–89 mg l^{-1} $CaCO_3$, conductivity was 166–180 μS cm^{-1}, hardness was 76–86 mg l^{-1} as $CaCO_3$ and pH was 7.6–8.0. Unless otherwise indicated, all water used in the experiments was prepared in this way. Chromium concentrations tested in the experimental water ranged from 0 to 266 μg l^{-1}. The water temperature was manipulated to match seasonal conditions: December–March, 5 °C and March–July, 10 °C (Wiggins et al., 1997). The water temperature during the experiment ranged between 9.9 and 11.8 °C except for 1 day it reached 12.8 °C.

Gametes and eyed embryos of fall-run Chinook salmon were obtained from the McNenny State Fish Hatchery, Spearfish, South Dakota. Adult brood fish from the McNenny Fish Hatchery are examined and tested for disease and parasite infection during spawning, and the eggs were collected from adults that were certified disease-free prior to shipment to Jackson, Wyoming. The Chinook salmon from the McNenny Fish Hatchery were originally introduced to Lake Oahe in 1982 and should have no history of pre-exposure to contaminants in the Hanford Reach of the Columbia River.

2.2. Experimental design

Eyed eggs were maintained in a Heath^{R} incubator at a temperature of 8 ± 1 °C and hardness of approximately 150 mg l^{-1} as $CaCO_3$. Mortalities were documented and removed daily. At hatch, the fish were moved to flow-through culture tanks that had a flow of 4 l min^{-1}. The fish were fed at least a 5% wet weight ration of Biodiet (Bio-Oregon, USA—protein 47.0%, carbohydrate 15.5% and fat 7.0%) daily. The daily food ration was split between two feedings.

The experimental phase began during the parr stage of development by randomly distributing 35 fish each to 12 test chambers receiving experimental water with a flow-through proportional diluter system. The circular chambers had a 20 l capacity with dimensions of 43.2 cm × 35.6 cm. The fish were allowed to acclimate in the experimental chambers and water, without chromium added, for 12 days before the start of the experiment. The experiment was conducted for 134 days beginning with juvenile fish (approximately 60 days post swim-up).

Chromium in stock solutions was delivered to eight test chambers via automatic pipettes (Micromedic Systems AP, Model #25000FW). Experimental concentrations of 24 and 54 μg Cr l^{-1} (referred to from this point as 24 and 54) were maintained in each of four replicate chambers. Four chambers without Cr added were used for reference. Thus, a total of 12 experimental units (four reference, four with 24 μg Cr l^{-1} and four with 54 μg Cr l^{-1}) were maintained until day 105. Neither growth nor survival of parr was affected as a result of exposure to these concentrations for 105 days. On day 105 concentrations were increased from 24 to 120 μg Cr l^{-1} and from 54 to 266 μg Cr l^{-1} until the end of the experiment on day 134. Exposure concentrations were increased after the initial 105 days in an attempt to produce an observable response (e.g. behavioral abnormality, reduced growth and mortality) that could be related to fish health assessment parameters. Each chamber received 8 l h^{-1} for 10 volume additions per day. Experimental units were checked daily for mortality and behavior.

At day 105 and at the termination of the experiment (day 134), samples were collected for fish health measurements. Fish were not fed for 24 h prior to sampling. Necropsy assessments were performed on 13 fish sacrificed with iced water, the fish were blotted dry and lengths and weights were recorded. Of the 13 fish sampled, one whole fish was collected from each replicate chamber for measurements of whole fish metal accumulation. Organs for histopathology were collected from two fish from each replicated chamber and fixed in 10% neutral buffered formalin: kidney, skin, skeletal muscle, gill, liver, pyloric caeca, exocrine pancreas and spleen. For measurements of lipid peroxidation and tissue metals, gill filaments, liver (free of the gall bladder), the entire kidney and pyloric caeca were removed immediately from the 10 individual fish per chamber, frozen with liquid nitrogen, and stored at −90 °C. The sets of 10 samples were ground with liquid nitrogen and composited by organ to result in one sample from each replicate chamber. Aliquants of these composites were measured for lipid peroxidation and tissue Cr.

Blood samples were collected from 10 fish (note: blood was collected from the fish used for individual organ collection described above) from each replicate

and frozen at −90 °C for measurements of the variation in nuclear DNA content. This type of analysis measures post-repair DNA damage. The mutagenic event has occurred in a prior cell generation (i.e. the stem cells or transit cells) and the damage (in the form of translocations, deletions or lost chromosome bits) has been transmitted to the daughter cells and effectively fixed in that particular cell line. One of the 10 sample lots was randomly selected from each of four sets of replicates and analyzed for DNA damage. All cells were stained using a modified whole cell method (Clevenger et al., 1985). The frozen red blood cells were thawed rapidly at 37 °C and washed twice in phosphate buffer saline. The cells were counted with a Coulter Counter and then fixed in 1.0 ml 0.5% paraformaldehyde for 10 min at 4 °C. The presence of RNA in a sample can interfere with DNA readings. To remove RNA, 0.1 ml of 1.0 mg ml^{-1} Rnase was added and the samples were incubated for 20 min at 27 °C. The cells were then suspended in propidium iodide, a fluorescent dye that binds stoichiometrically to DNA. An Epic Elite Flow Cytometer (Coulter Corp.) with an argon laser (488 nm) was used to quantify the nuclear DNA content. The samples were run at a rate of 150 s^{-1}. DNA Check Beads (Coulter Corp.), human lymphocytes and chicken erythrocyte nuclei were used as external biological controls for quality assurance/quality control (QA/QC). The data were analyzed with Elite Software (Coulter Corp.) to produce the mean channel of full peak coefficient of variation (CV) values. The CV value represents the variation in nuclear DNA content for approximately 5000 individually measured cells that were analyzed from the blood sample of each fish. The difference between the sample CV and the CV of the external or internal control is defined as the CV difference (CV DIF) and was the discriminating value used in the subsequent least squares analysis (Misra and Easton, 1999). Using these controls make the measurements more precise than previous methods. This more precise measurement used along with modern statistical evaluations of the DNA damage data provides a novel and valuable approach to interpretations of DNA damage measurements (Easton et al., 2002). We also used a pre-repair DNA damage methodology examining strand breakage by means of gel electrophoresis. No significant differences were noted in gill and liver using this method (data not presented).

2.3. Analyses of water and tissue

Treatment water was monitored once per week for dissolved oxygen, pH, alkalinity, hardness, conductivity and total Cr concentrations. Samples of 100 ml of water from each treatment were filtered using a Nalgene® 300 filter holder. Each filtered sample was transferred to a pre-cleaned, 125 ml I-Chem® polyethylene bottle, acidified to 1% HNO_3 and analyzed with ICP-MS. At each time of total Cr sampling, one additional sample was extracted from the low, middle and high Cr treatments and speciated for Cr^{6+}. The water was buffered and eluted through a AG-50W-X8 cation exchange column. Collected eluant containing Cr^{6+} was oxidized with HNO_3 and analyzed by ICP-MS. The instrument detection limit was 0.041 ng ml^{-1}, the greatest method detection limit was 0.74 ng ml^{-1}, and the greatest limit of quantitation was 3.6 ng ml^{-1}. For analysis of Cr in tissue, samples were lyophilized, acid digested with microwave heating and analyzed by ICP-MS (PE.SCIEX Elan 6000). For measurements of tissue Cr, the instrument detection limit was 0.041 ng ml^{-1}, the greatest method detection limit was 1.62 ng ml^{-1} and the greatest limit of quantitation was 5.35 ng ml^{-1}. The recoveries of Cr from all method spikes, including blanks, samples and reference tissues, ranged from 97 to 117% and averaged 103%. Method duplicates prepared by splitting tissue samples had relative percent deviations (%R.S.D.) of <11%. Also, a reference material was replicated through the cryo-grinding procedure. The %R.S.D. for this sample was 6.6% and the measured concentration was not significantly different from reference material that was not cryo-ground. Therefore, the grinding procedure had no effect on the concentrations of Cr in the samples.

For histopathological examination, each fish was assigned a random number so that tissue processing and the subsequent examinations were performed "blind". Tissues were processed into paraffin, sectioned at 4 μm, stained with hemotoxylin and eosin, and the sections were viewed with light microscopy. Lesions were scored as none (0), mild (1), moderate (2) or severe (3). For example, scores for peritubular fibrosis in the kidney were assigned as follows: score = 0, no peritubular fibrosis in sections; score = 1, fewer than four foci of peritubular fibrosis in the section; score = 2, four or more foci of peritubular fibrosis in the section and score = 3, peritubular fibrosis displaces most of the

interstitial cells and involves >75% of the kidney volume.

Markers of energy storage and measures of metabolism included hepatocellular glycogen depletion (scored as in Marty et al., 1997), pancreatic zymogen granule depletion/atrophy and lipid depletion of mesenteric adipose tissue. Pancreatic zymogen granule depletion/atrophy was scored on a relative scale as either none (score = 0) for cells with abundant brightly eosinophilic granules, or as mildly depleted (score = 1) for cells with fewer brightly eosinophilic granules. No fish had more than mild depletion of zymogen granules.

A fluorometric assay (Dillard and Tappel, 1984; Fletcher et al., 1973) was used to measure products of lipid peroxidation. This assay measures the relative intensity of fluorophores formed during lipid peroxidation, and it has been used on fish tissues collected from sites contaminated with metals (Farag et al., 1995). A chloroform–methanol extraction of tissue preceded the fluorometric measurement. Two hundred milligrams of ground tissue were combined with a 2:1 mixture of HPLC-grade chloroform:methanol (7 ml for a 200 mg sample) in a glass homogenizer. The tissue was processed five times in the homogenizer with a glass pestle, diluted with an equal volume of water, and homogenized two additional times. The mixture was then vortexed for 1.5 min and transferred to a Corex tube. The mixture was centrifuged at $1200 \times g$ for 1.5 min and the chloroform layer was removed. Flourescence was measured (Hitachi f-2000) at a wavelength of 435 nm emission during excitation at 340 and 360 nm.

All measurements performed for the 24 and 24/120 $\mu g\,l^{-1}$ treatments contain three rather than four replicates because one replicate failed before day 105, the first sampling day of the parr experiment. These fish died rapidly between days 101 and 105 and the cause could not be determined by microscopic examination of the tissues. Additionally, the water chemistry did not indicate any dysfunction of the diluter. Therefore, these mortalities were not included in the survival calculations, and there were no fish tissues available from the fourth replicate for the physiological measurements.

2.4. Statistical analyses

Statistical analyses were performed using SAS system software, Version 6.11 (SAS Institute Inc., Cary, NC), SYSSTAT (SPSS, Chicago, IL) or Toxstat 3.4 (West Inc. and University of Wyoming, 1994). Multivariate analysis of variance (MANOVA) followed by Tukey means comparisons was performed on all data that met the assumptions of homogeneity and normality. The dependent variables included survival, growth, concentrations of products of lipid peroxidation and concentrations of metals in tissues. The number of replicates for each experiment was four (unless otherwise indicated). If the data did not meet these assumptions and could not be transformed to do so, non-parametric statistical analyses were performed. Statistical significance was assigned at $P \leq 0.05$. The observations noted during the microscopic examinations were not subjected to statistical analyses. The data for DNA damage determination consisted of coefficient of variation values measured by flow cytometry and were analyzed with a weighted least squares procedure (Misra and Easton, 1999).

3. Results

The concentrations of Cr measured in the chambers throughout the experiment were ±10% of the nominal concentrations (Table 1). Therefore, nominal concentrations will be used from this point. The general agreement of Cr^{6+} with the nominal concentrations confirmed that virtually all of the Cr remained in the Cr^{6+} throughout the experiment.

Dose-dependent changes in Cr exposed kidneys included gross and microscopic abnormalities, elevated products of lipid peroxidation, elevated concentrations of Cr in the kidney and reduced growth and survival. Also DNA damage was noted in the red blood cells.

Table 1
Concentrations of total Cr and Cr^{6+} in the water used to expose early life-stage of Chinook salmon to Cr (NS indicates that Cr^{6+} concentrations were not sampled for that treatment)

Nominal chromium ($\mu g\,l^{-1}$)	Measured total chromium ($\mu g\,l^{-1}$)	Measured Cr^{6+} ($\mu g\,l^{-1}$)
0	<1.5	NS
5	5.1	7.2
11	11.6	NS
24	25.2	29.8
54	56.5	NS
120	123.2	133.4

Table 2
Mean percent survival, weight and length of Chinook salmon during an experiment where parr were exposed to 24 or 54 μg Cr l^{-1} to day 105 (on day 105, the concentrations of chromium were increased from 24 to 120 μg Cr l^{-1} and from 54 to 266 μg Cr l^{-1} until the experiment ended on day 134. Percent survival was determined to day 105 and, to day 134. Different superscript letters (a and b) designations indicate a significant difference at $P \leq 0.05$ within a sample day. S.E.M. in parentheses)

Nominal chromium (μg l^{-1})	Sample day	*N*	Percent survival	Weight (g)	Length (mm)
0	105	4	87.8[a] (4.0)	6.0[a] (0.5)	86.3[a] (2.1)
24	105	3	92.3[a] (3.7)	6.0[a] (0.2)	87.3[a] (0.3)
54	105	4	91.3[a] (0.5)	4.7[a] (0.6)	84.3[a] (1.9)
0	105–134	4	96.8[a] (1.9)	9.6[a] (0.24)	100.8[a] (2.3)
24/120	105–134	3	84.3[a] (5.8)	6.8[b] (0.6)	93.0[a] (3.2)
54/266	105–134	4	69.8[b] (3.7)	8.5[a] (3.7)	98.0[a] (2.7)

While most of the physiological malfunctions occurred after exposures to ≥120 μg Cr l^{-1}, significant DNA damage occurred after exposures to 24 μg Cr l^{-1}, the lowest dose level. A detailed description of the results follows.

Effects on growth and survival were related to exposure concentrations and duration. Chromium exposure had no significant effect on growth and survival after 105 days (Table 2), but survival decreased to 69.8% in fish exposed to 54/266 from days 105 to 134. There was also a trend of decreased survival in the 24/120 treatment at 134 days (84.3%) compared to the reference (96.8%). By day 134, the mean weight of fish in the 24/120 treatment was 6.8 g, significantly less than the reference (9.6 g). However, fish in the 54/266 treatment (mean weight 8.5 g) were not significantly different in size than the reference fish (Table 2).

The concentrations (μg g^{-1} dry weight) of Cr in the organs of fish increased when fish were exposed to Cr in the water (Table 3). At day 105, gill, kidney and whole body tissues from the 54 μg Cr l^{-1} treatment had elevated concentrations of Cr (25.7, 25.6 and 8.8, respectively) compared to fish in water without Cr (7.4, 5.5 and 1.5, respectively). At day 134, gill, kidney, liver and pyloric ceca from fish in both Cr treatments were significantly greater than the reference fish (Table 3). And fish from the 54/266 treatment had greater concentrations of Cr in gill, liver and pyloric caeca than those from the 24/120 treatment. However, while a trend existed, there was no longer a significant increase in whole body concentrations of Cr at day 134, indicating the limited usefulness of whole body Cr measurements with age.

The most significant gross finding was multifocal discoloration of the margin of the kidneys. The entire margin of affected kidneys was uniformly white, and the white edge was less than 1 mm wide. This abnormality affected 2, 9 and 19% of fish from the 54, 24/120 and 54/266 μg Cr l^{-1} treatments, but none of the fish from the reference or the 24 μg Cr l^{-1} treatment.

Table 3
Mean concentrations of Cr (μg g^{-1} dry weight) in various tissues collected during an experiment where Chinook salmon parr were exposed to 24 or 54 μg Cr l^{-1} to day 105 (the concentrations of chromium were increased from 24 to 120 μg Cr l^{-1} and from 54 to 266 μg Cr l^{-1} for the remainder of the experiment that ended on day 134. Different superscript letters (a–c) designations indicate a significant difference at $P \leq 0.05$ within a tissue and within a sample day. S.E.M. in parentheses)

Nominal chromium (μg l^{-1})	Day	*N*	Gill (μg g^{-1})	Kidney (μg g^{-1})	Liver (μg g^{-1})	Pyloric caeca (μg g^{-1})	Whole body (μg g^{-1})
0	105	4	7.4[a] (3.8)	5.5[a] (2.6), $N=3$	0.9[a] (0.4)	0.6[a] (0.2), $N=3$	1.5[a] (0.4)
24	105	3	14.8[a] (6.7)	9.6[a] (4.8)	4.0[a] (2.1)	0.7[a] (0.2)	6.6[a] (2.4)
54	105	4	25.7[b] (1.5)	25.6[b] (5.6)	3.0[a] (0.4)	1.2[a] (0.2)	8.8[b] (2.1)
0	134	4	1.3[a] (0.3)	2.8[a] (1.0)	0.4[a] (0.1)	0.3[a] (0.1)	0.2[a] (0.1)
24/120	134	3	29.4[b] (2.0)	30.2[b] (7.9)	4.6[b] (0.6)	2.1[b] (0.4)	6.4[a] (2.7)
54/266	134	4	35.4[c] (1.1)	29.6[b] (2.3)	7.2[c] (0.5)	3.7[c] (0.4)	6.5[a] (3.6)

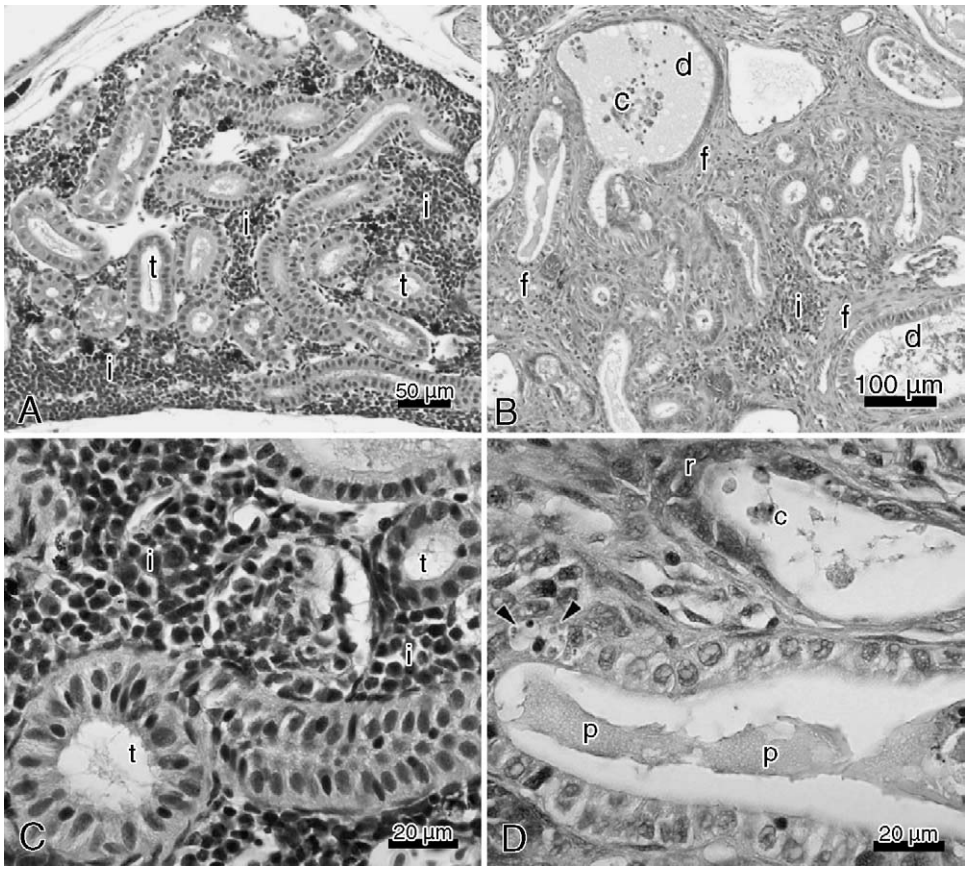

Fig. 1. Microscopic features of kidneys from juvenile Chinook salmon sampled on Day 134; hematoxylin and eosin stain. (A and C) Normal kidney from a fish exposed to 0 μg Cr l^{-1} for 134 days and (B and D) abnormal kidney from a fish exposed to 24 μg Cr l^{-1} for 105 days followed by 120 μg Cr l^{-1} from days 105 to 134. In the normal kidney, sectional area of tubules (t) and basophilic interstitial hematopoietic cells (i) is about equal. In the kidney exposed to chromium, tubules are dilated (d), their lumina contain scattered cellular debris (c) and eosinophilic protein (p) and the tubular epithelial cells vary from necrotic (arrowheads) to attenuated. Some attenuated tubular epithelial cells stain basophilic: evidence of regeneration (r). Tubules are separated by abundant eosinophilic fibrosis (f) that seems to compress remnants of interstitial cells (i).

The most severe microscopic lesions were also in the kidney. Necrosis (death), fibrosis (scarring) and dilation of tubular lumina affected kidneys from 24/120 to 54/266, but not reference kidneys (Fig. 1, Table 4). Kidneys from the greatest doses also had decreased scores for interstitial blood forming cells (also called hematopoietic cells). Interstitial blood forming cells in the kidney normally develop into erythrocytes or leukocytes. Decreased scores for interstitial blood forming cells in all groups of exposed fish provide evidence that exposed fish might have been anemic or had decreased leukocyte numbers (Table 4), but further blood analyses were not performed.

The gill and spleen had subtle microscopic changes. The gill lamellar epithelial cells tended to be thicker in the 24/120 (0.44) and 54/266 (0.25) compared to the reference (0.08) (Table 5). Apoptosis, or programmed cell death, was more prominent than the reference (0.17) in chloride cells of gills from the 24 (0.67), 24/120 (0.67) and 54/266 (0.83) treatment groups (Table 5). Apoptotic chloride cells had condensed hypereosinophilic cytoplasm, and nuclear pyknosis and karyorrhexis (break up of nucleus into small pieces) were common. Apoptotic cells were most common in the notch between adjacent lamellae, but they also occurred along the sides and tips of lamella. Scores

Table 4
Mean scores (S.E.M. in parentheses) for microscopic changes in kidney of Chinook salmon parr exposed to 24 or 54 μg Cr l^{-1} for 105 days (chromium concentrations were then increased from 24 to 120 μg Cr l^{-1} and from 54 to 266 μg Cr l^{-1} until the experiment ended on day 134. Microscopic changes were scored as none (0), mild (1), moderate (2) or severe/marked (3))

Nominal chromium (μg l^{-1})	Day	*N*	Kidney			
			Interstitial blood forming cells	Necrosis (death) of cells lining tubules	Fibrosis (scarring) around tubules	Dilation of tubular lumen
0	105	12	1.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
24	105	9	0.78 (0.15)	0.00 (0.00)	0.00 (0.00)	0.11 (0.11)
54	105	12	0.92 (0.08)	0.00 (0.00)	0.00 (0.00)	0.08 (0.08)
0	134	12	0.92 (0.08)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
24/120	134	9	0.44 (0.18)	0.56 (0.29)	0.44 (0.34)	0.33 (0.24)
54/266	134	12	0.67 (0.14)	0.50 (0.19)	0.42 (0.23)	0.50 (0.19)

for amphophilic spherules in the spleen were greater in the 24/120 and 54/266 compared to the reference on day 134. Amphophilic spherules were usually homogeneous, about 7 μm in diameter, adjacent to a nucleus, and surrounded by a delicate cell membrane (Fig. 2). Three fish had amphophilic spherules scored as moderate (none were severe), and all three fish were sampled on day 105 after exposure to 24 μg Cr l^{-1}. The nature of these spherules was not determined, but their staining was distinct from erythrocyte cytoplasm.

Microscopic changes in the liver and pancreas provided evidence of differences in energy storage and metabolism (Table 6). At sample day 105, control fish had relatively abundant glycogen stores in hepatocytes and mild zymogen granule depletion in exocrine pancreas cells: consistent with depletion of pancreatic enzymes during active feeding to enhance glycogen stores. By day 134, the pattern had reversed; hepatocyte glycogen depletion was relatively greater in control fish while pancreatic zymogen granule depletion was less than in controls (Table 6). At day 105, fish exposed to Cr sometimes had lipid droplets in hepatocytes, whereas control livers had no hepatocellular lipid (Table 7). Scores for hepatocellular lipid were low for all groups sampled at day 134.

The CV DIF in DNA measurements decreased with increasing dose at day 105 (Table 7). The significantly smaller numbers indicate greater amounts of DNA damage in fish exposed to 24 and 54 μg Cr l^{-1} when compared to the reference (1.218 and 1.130 versus 1.730). There was a negative relationship among treatment groups for variations in DNA at day 134. The CV DIF values actually decreased significantly when compared to the reference at day 105.

Again, lipid peroxidation measurements point to the kidney as the target organ of toxicity due to Cr exposure. There were no significant differences in the relative intensity of products of lipid peroxidation among

Table 5
Mean scores (S.E.M. in parentheses) for microscopic changes in gill and spleen of Chinook salmon parr exposed to 24 or 54 μg Cr l^{-1} for 105 days (chromium concentrations were then increased from 24 to 120 μg Cr l^{-1} and from 54 to 266 μg Cr l^{-1} until the experiment ended on day 134. Microscopic changes were scored as none (0), mild (1), moderate (2) or severe/marked (3))

Nominal chromium (μg l^{-1})	*N*	Day	Gill		Spleen
			Lamellar epithelial thickness	Apoptosis (death) of chloride cells	Eosinophilic spherules
0	12	105	0.17 (0.11)	0.33 (0.14)	0.08 (0.83)
24	9	105	0.56 (0.17)	0.67 (0.17)	0.00 (0.00)
54	12	105	0.00 (0.00)	0.27 (0.14)	0.18 (0.12)
0	12	134	0.08 (0.08)	0.17 (0.11)	0.17 (0.11)
24/120	9	134	0.44 (0.18)	0.67 (0.17)	0.33 (0.17)
54/266	12	134	0.25 (0.13)	0.83 (0.11)	0.42 (0.15)

Table 6
Mean scores (S.E.M. in parentheses) for microscopic changes in the liver and pancreas of Chinook salmon parr exposed to 24 or 54 μg Cr l^{-1} for 105 days (chromium concentrations were then increased from 24 to 120 μg Cr l^{-1} and from 54 to 266 μg Cr l^{-1} until the experiment ended on day 134. Microscopic changes were scored as none (0), mild (1), moderate (2) or severe/marked (3))

Nominal chromium (μg l^{-1})	*N*	Day	Liver		Pancreas
			Glycogen depletion	Hepatocellular lipidosis	Zymogen granule depletion
0	12	105	1.08 (0.26)	0.00 (0.00)	1.00 (0.00)
24	9	105	1.56 (0.18)	0.22 (0.15)	0.67 (0.17)
54	12	105	1.92 (0.19)	0.25 (0.13)	0.58 (0.15)
0	12	134	2.67 (0.14)	0.00 (0.00)	0.58 (0.15)
24/120	9	134	1.67 (0.29)	0.11 (0.11)	0.78 (0.15)
54/266	12	134	2.00 (0.17)	0.00 (0.00)	0.67 (0.14)

the treatment groups for gill, liver, pyloric caeca or whole fish (data not presented). We were able to collect enough kidney tissue to perform one measurement of lipid peroxidation for each treatment. The relative intensities were reference = 76.5, 120 = 134.8 and 266 = 137.7 at a wavelength of 340 excitation and reference = 53.9, 120 = 110.7 and 266 = 112.9 at a wavelength of 360 excitation. These measurements indicate an increase in lipid peroxidation in the kidneys, but statistical analyses could not be used to define the differences.

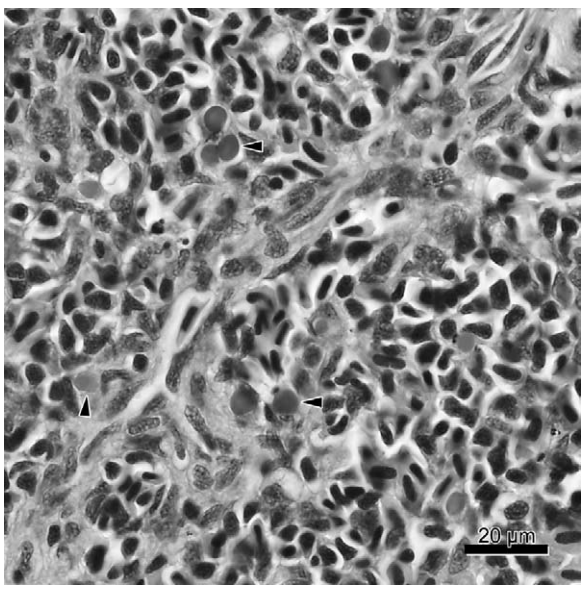

Fig. 2. Microscopic features of the spleen from a Chinook salmon exposed to 24 μg Cr l^{-1} for 105 days; hematoxylin and eosin stain. The splenic parenchyma contains moderate numbers of amphophilic intracytoplasmic spherules (arrowheads).

Table 7
Measurements of DNA alterations in blood of Chinook salmon parr that were exposed to 24 or 54 μg Cr l^{-1} to day 105 (the concentrations of chromium were increased from 24 to 120 μg Cr l^{-1} and from 54 to 266 μg Cr l^{-1} for the remainder of the experiment that ended on day 134. Different superscript letters (a–c) designations indicate a significant difference at $P \leq 0.05$, within a sampling day. The difference between the sample CV and the external or internal control is defined as the CV difference and was the discriminating statistic (Misra and Easton, 1999))

Nominal chromium (μg l^{-1})	Day	*N*	CV DIF
0	105	12	1.730^{a}
24	105	9	1.218^{b}
54	105	11	1.130^{c}
0	134	11	0.411^{a}
24/120	134	9	0.450^{b}
54/266	134	12	0.506^{c}

Note: All probabilities are ≤0.0078.

4. Discussion

The kidney is an important target organ during aqueous Cr exposures. Chinook salmon exposed to dissolved Cr^{6+} accumulate the metal in various organs and develop DNA damage, lipid peroxidation, microscopic lesions, gross abnormalities, reductions in weights and reductions in survival. Changes in the kidney during parr exposure to Cr suggest that post-repair DNA damage, lipid peroxidation and tissue lesions work to cause effects on growth and eventually death of fish. Not only do these changes help explain the mechanism of toxicity of Cr, they can be used to investigate the health of fish residing in contaminated environments.

Data from the multiple types of physiological malfunction that we observed provide the basis for theories about the actions of Cr on individual fish. Necrosis of

tubular epithelial cells in the kidney after chromium exposure was associated with a relative decrease in the number of blood forming cells and scarring (fibrosis) around kidney tubules. Therefore, the microscopic investigation provides evidence that the kidney was the primary site of damage. This finding is supported by the necropsy results where gross abnormalities were noted in the kidneys (9% in 24/120 and 19% in 54/266 $\mu g\,Cr\,l^{-1}$).

Zymogen granules store digestive enzymes that are released after feeding (Ellis et al., 1989). Therefore, slight depletion is expected in normally feeding fish and was observed in fish from the reference (mean score = 1.00 at day 105). However, fish that are not feeding or digesting consistently might accumulate zymogen granules in the pancreatic cells, resulting in lower scores for zymogen depletion. Lesser scores for zymogen depletion were observed in the 24 (0.67) and 54 (0.58) compared to the reference (1.00) at day 105. The zymogen depletion scores decreased in the reference between days 105 and 134, but the presence of these granules remained unchanged for the treatment groups. This phenomenon may define delayed smoltification in Chinook salmon exposed to Cr.

Lipid peroxidation can lead to cell death and tissue damage (Halliwell and Gutteridge, 1985; Wills, 1985). The trend of elevated lipid peroxidation in the kidney is evidence that Cr accumulated in the kidney causes lesions through the peroxidation pathway. Lloyd et al. (1997, 1998) have strongly implicated the Fenton reaction in the mechanism for DNA damage during Cr exposures. The Fenton reaction is a step in lipid peroxidation where transition metals, such as Cr, reduce hydrogen peroxide to hydroxyl radicals. These hydroxyl free radicals generate oxidative DNA lesions and cause the peroxidation of polyunsaturated fatty acids in cells membranes. Other researchers have documented that Cr can cause lipid peroxidation (Susa et al., 1996). And we have now documented DNA damage, lipid peroxidation and necrosis of kidney cells simultaneously as a result of Cr exposure.

The fibrosis observed in the kidney, the apoptosis observed in the gill, the presence of amphophilic spherules in the spleen and lipid peroxidation noted in the kidney provide evidence that the onset of cell death (possibly mediated through apoptosis) is responsible for the two apparently opposite patterns of DNA damage that we observed in the blood. In the first pattern, DNA damage has a direct relationship with increasing dosage of the water medium with hexavalent Cr over 105 days. In the second pattern, DNA damage decreased inversely with dose below that indicated by the control samples when the juvenile Chinook salmon were exposed to the 24/120 and 54/266 $\mu g\,Cr\,l^{-1}$ from days 105 to 134. The two sets of exposures differ by dose and by duration; both of which can affect the level and pattern of DNA damage (Das and Nanda, 1986). Only after increased exposure from days 105 to 134, was the level of apoptosis greater than in the controls and it appeared to increase with the dose. We believe that the interaction between cell death, possibly mediated through apoptosis, and the DNA damaged cells can explain the reduction in the variance of the nuclear DNA content. At these concentrations, few, if any, cells may be able to survive along the cellular lineage from stem cells to blood cells. A shortage of new blood cells might explain the microscopic observations that revealed smaller scores for interstitial blood forming cells after the 24/120 and 54/266 $\mu g\,Cr\,l^{-1}$ exposures.

Additional investigations to further define the connection between apoptosis and DNA damage during chronic Cr^{6+} exposures are warranted. First, we did not collect the blood samples for DNA damage measurements from the identical fish collected for histology observations. A direct correlation of the prevalence of DNA damage and apoptosis would be more apparent if both measurements were performed on identical fish. Second, in addition to the measurements we performed, immunohistochemical procedures have been developed to directly assess apoptosis (e.g. Sunila and LaBanca, 2003), and similar methods might be adaptable for directly documenting apoptosis in chromium-exposed fish. Using immunohistochemistry along with directly measuring DNA damage in the kidney might provide more information about a conclusion that apoptosis and DNA damage are related during chronic Cr^{6+} exposure.

This observation of reduction in measured DNA damage as a result of increasing exposure to a mutagen has previously been reported by Al-Sabti et al. (1994) (for Cr), Countryman and Heddle (1976) (for ionizing radiation) and by Easton et al. (1997) (for pulp mill effluent). The latter study also demonstrated a dose–response curve that fit a second degree quadratic equation. Easton et al. (1997) suggested an increase in

apoptosis, or an increase in cross linkages that prevent mitosis could explain this phenomenon. Our current study implicates apoptosis as a factor, but we have not excluded the occurrence of cross linkages that might prevent mitosis. Chromium can form intermediates that react with DNA (Outridge and Scheuhammer, 1993; Bridgewater et al., 1994; Xu et al., 1996; Singh et al., 1998). These intermediates can inhibit DNA replication (Patierno et al., 1993) and the DNA repair mechanism (Bridgewater et al., 1994). It is the inhibition of the DNA repair mechanism that may ultimately trigger apoptosis in the hematopoietic tissue.

At day 105, the health of fish was slightly impaired compared to the extensive changes noted at day 134 in fish from the 24/120 and 54/266 $\mu g\,Cr\,l^{-1}$. Elevated variation in nuclear DNA content at day 105 was evidence of DNA damage (Otto and Oldiges, 1980; Otto et al., 1981; Bickham et al., 1988; Easton et al., 1997). Increased lipid in hepatocytes of exposed fish at day 105 is evidence of altered lipid metabolism. Smaller scores for pancreatic zymogen granule depletion among fish exposed at day 105 indicate that fish in these treatments are not digesting food as consistently as the unexposed fish.

Microscopic changes in the liver and pancreas provide evidence that Cr exposure alters smoltification. From days 105 to 134 in reference fish, hepatocellular glycogen stores were depleted and pancreatic zymogen granules were filled. These findings are consistent with the energy demands of smoltification (Mayer et al., 1994; Hong et al., 1996). In fish exposed to Cr, by comparison, scores for hepatocellular glycogen and pancreatic zymogen granules remained unchanged from days 105 to 134.

In summary, the health of fish was significantly impaired in the 24/120 and 54/266 $\mu g\,Cr\,l^{-1}$ treatments. Changes associated with elevated concentrations of Cr in the kidney included DNA damage, gross and microscopic lesions and increased products of lipid peroxidation. It appears that Cr accumulates and enters the lipid peroxidation pathway where fatty acid damage and DNA damage occur and may initiate cytotoxicity and the subsequent tissue damage. These changes translate into gross and microscopic changes. These malfunctions are particularly important because they are associated with changes in growth and survival in this study, which can be related to effects at the population level. Such information can be used to link changes in resident fish with possible alterations in populations of fish.

Acknowledgements

Rick Cordes, Will Sayer, Mike Barnes and Eric Krebs from the McNenny State Fish Hatchery, Spearfish, South Dakota, provided Chinook salmon eggs and expert advice on their rearing. William Brumbaugh measured the Cr in tissues and water. Brad Mueller and Richard Skinker provided technical assistance. Dan Woodward provided input into the experimental design. This study was funded by the USFWS. Don Steffeck, USFWS and Dan Audet, USFWS were project managers. The use of trade names does not imply endorsement by the U.S. Government.

References

Al-Akel, A.S., Shamsi, M.J.K., 1996. Hexavalent chromium: toxicity and impact on carbohydrate metabolism and haematological parameters of carp (*Cyprinus carpio* L.) from Saudi Arabia. Aquat. Sci. 58, 24–30.

Al-Sabti, K., Franco, M., Andrijanic, B., Knez, S., Stegnar, P., 1994. Chromium-induced micronuclei in fish. J. Appl. Toxicol. 14, 333–336.

Arillo, A., Melodia, R., 1988. Effects of hexavalent chromium on trout mitochondria. Toxicol. Lett. 44, 71–76.

Bickham, J.W., Hanks, B.G., Smolen, M.J., Lamb, T., Whitfield Gibbons, J., 1988. Flow cytometric analysis of the effects of low-level radiation exposure on natural populations of slider turtles (*Pseudemys scripta*). Arch. Environ. Contam. Toxicol. 17, 837–841.

Bridgewater, L.C., Manning, F.C.R., Woo, E.S., Patierno, S.R., 1994. DNA polymerase arrest by adducted trivalent chromium. Mol. Carcinog. 9, 122–123.

Buhl, K.J., Hamilton, S.J., 1991. Relative sensitivity of early life stages of arctic grayling, coho salmon, and rainbow trout to nine inorganics. Ecotoxicol. Environ. Saf. 22, 184–197.

Clevenger, C.V., Bauer, K.D., Epstein, A.L., 1985. A method for simultaneous nuclear immunofluorescence and DNA content quantitation using monoclonal antibodies and flow cytometry. Cytometry 6, 208–214.

Countryman, R.I., Heddle, J.A., 1976. The production of micronuclei from chromosomal aberration in irradiated culture of human lymphocytes. Mutat. Res. 41, 321–332.

Das, R.K., Nanda, N.K., 1986. Induction of micronuclei in peripheral erythrocytes of fish heteropneustes-fossilis by mitomycin-c and paper-mill effluent. Mutation Res. 175, 66–71.

Dillard, C.J., Tappel, A.L., 1984. Fluorescent damage products of lipid peroxidation. Methods Enzymol. 105, 337–341.

Easton, M.D.L., Kruzynski, G.M., Solar, I.I., Dye, H.M., 1997. Genetic toxicity of pulp mill effluent on juvenile Chinook salmon (*Oncorhynchus tshawytscha*) using flow cytometry. Water Sci. Technol. 35, 347–355.

Easton, M.D.L., Luszniak, D., Von der Geest, E., 2002. Preliminary examination of contaminant loadings in farmed salmon, wild salmon and commercial salmon feed. Chemosphere 46, 1053–1074.

Ellis, A.E., Roberts, R.J., Tyler, P., 1989. The anatomy and physiology of teleosts. In: Roberts, R.J. (Ed.), Fish Pathology. Bailliere Tindall, London, England, pp. 12–54.

Farag, A.M., Boese, C.J., Woodward, D.F., Bergman, H.L., 1994. Physiological changes and tissue metal accumulation in rainbow trout exposed to foodborne and waterborne metals. Environ. Toxicol. Chem. 13, 2021–2029.

Farag, A.M., Stansbury, M.A., Hogstrand, C., MacConnell, E., Bergman, H.L., 1995. The physiological impairment of free ranging brown trout exposed to metals in the Clark Fork River, Montana. Can. J. Fish. Aquat. Sci. 52, 2038–2050.

Fletcher, B.L., Dillard, C.J., Tappel, A.L., 1973. Measurement of fluorescent lipid peroxidation products in biological systems and tissues. Anal. Biochem. 52, 1–9.

Goyer, R.A., 1986. Toxic effects of metals. In: Klaassen, C.D., Amdur, M.O., Doull, J. (Eds.), Toxicology the Basic Science of Poisons, third ed. MacMillan Publishing, New York, NY, pp. 582–635.

Halliwell, B., Gutteridge, J.M.C. (Eds.), 1985. Free Radials in Biology and Medicine. Clarendon Press, Oxford, pp. 139–170.

Hong, J., Bradley, T.M., Tremblay, G.C., 1996. Lactate-dependent gluconeogenesis and atractyloside-sensitive flux through pyruvate carboxylase are reduced during smoltification of Atlantic salmon (*Salmo salar*). J. Exp. Zool. 276, 375–386.

Hope, S.J., Peterson, R.E., 1996. Chromium in River Substrate Pore Water and Adjacent Ground Water: 100-D/DR Area, Hanford Site, Washington. Betchel Hanford, Inc., Richland Washington. BHI-00778 Rev. 0.

Johnson, D.W., 1968. Pesticides and fishes—a review of selected literature. Trans. Am. Fish. Soc. 97, 398–424.

Lloyd, D.R., Carmichael, P.L., Phillips, D.H., 1998. Comparison of the formation of 8-hydroxy-2'-deoxyguanosine and single- and double-strand breaks in DNA mediated by fenton reactions. Chem. Res. Toxicol. 11, 420–427.

Lloyd, D.R., Phillips, D.H., Carmichael, P.L., 1997. Generation of putative intrastrand cross-links and strand breaks in DNA by transition metal ion-mediated oxygen radical attack. Chem. Res. Toxicol. 10, 393–400.

Marty, G.D., Heintz, R.A., Hinton, D.E., 1997. Histology and teratology of pink salmon larvae near the time of emergence from gravel substrate in the laboratory. Can. J. Zool. 75, 978–988.

Mayer, I., Borg, B., Plisetskaya, E.M., 1994. Plasma levels of insulin and liver glycogen contents in one-and two-year old Atlantic salmon (*Salmo salar* L.) during the period of parr-smolt transformation. Fish Physiol. Biochem. 13, 191–197.

Misra, R.K., Easton, M.D.L., 1999. A comment on analyzing flow cytometric data for comparison of mean values of the coefficient of variation of the G1 peak. Cytometry 36, 112–116.

Olson, P.A., Foster, R.F., 1956. Effect of Chronic Exposure to Sodium Dichromate on Young Chinook Salmon and Rainbow Trout. Hanford Biological Research Annual Report, 1955. Atomic Energy Commission, HW-41500, Richland, Washington.

Otto, F.J., Oldiges, H., 1980. Flow cytogenetic studies in chromosomes and whole cells for the detection of clastogenic effects. Cytometry 1, 13–17.

Otto, F.J., Oldiges, H., Gohde, W., Jain, V.K., 1981. Flow cytometric measurement of nuclear DNA content variations as a potential in vivo mutagenicity test. Cytometry 2, 189–191.

Outridge, P.M., Scheuhammer, A.M., 1993. Bioaccumulation and toxicology of chromium: implications for wildlife. Rev. Environ. Contam. Toxicol. 130, 31–77.

Patierno, S.R., Blankenship, L.J., Wise, J.P., Xu, J., Bridgewater, L.C., Manning, F.C.R., 1993. Genotoxin-induced apoptosis: implications for carcinogenesis. In: Hu, V.W. (Ed.), The Cell Cycle: Regulators, Targets and Clinical Applications. Plenum, New York, NY, pp. 331–340.

Peterson, R.E., Raidle, R.F., Denslow, C.W., 1996. Conceptual Site Models for Groundwater Contamination at 100-BC-5, 100-KR-4, 100-HR-3, and 100-FR-3 Operable Units. Bechtel Hanford, Inc., BHI-00917, Richland, Washington.

Singh, J., Bridgewater, L.C., Patierno, S.R., 1998. Differential sensitivity of chromium-mediated DNA interstrand crosslinks and DNA-protein crosslinks to disruption by alkali and EDTA. Toxicol. Sci. 45, 72–76.

Sunila, I., LaBanca, J., 2003. Apoptosis in the pathogenesis of infectious diseases of the eastern oyster Crass Crassostrea virginica. Dis. Aquat. Org. 56, 163–170.

Susa, N., Ueno, S., Furukawa, Y., Sugiyama, M., 1996. Protective effect of Vitamin E on chromium (VI)-induced cytotoxicity and lipid peroxidation in primary cultures of rat hepatocytes. Arch. Toxicol. 71, 20–24.

USEPA (United States Environmental Protection Agency), 1986. Quality Criteria for Water 1986. EPA Report 440/5-86-001, Office of Water Regulations and Standards, Washington, DC.

Wiggins, W.D., Ruppert, G.P., Smith, R.R., Reed, L.L., Hubbard, L.E., Courts, M.L., 1997. Water Resources Data Washington Water Year 1996. U.S. Geological Survey Water Data Report WA-96-1, Tacoma, Washington.

Wills, E.D., 1985. The role of dietary components in oxidative stress in tissue. In: Sies, H. (Ed.), Oxidative Stress in tissues. Academic Press, New York, pp. 197–218.

Xu, J., Manning, F.C.R., Bubley, G.J., Patierno, S.R., 1996. Effects of chromium-damaged DNA templates on DNA replication and transcription. In: Proceedings of the American Cancer Research 36, p. 948.