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Technical Article

Cell Pathology and Developmental Effects of Mine Waste Contamination on Invertebrates and Fish in the Methow River, Okanogan County, Washington (USA)

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Abstract. A study of the effects of mine waste on Methow River habitat in the north Cascade Mountains in the state of Washington, U.S.A. revealed impacts of sediment metal contamination on invertebrates and fish. The objectives were to determine: (1) effects of metal contamination on the growth and development of caddisfly larvae (*Ecclesomyia spp*.) and trout (*Oncorhynchus mykiss*), (2) whether *in vivo* exposure of caddisfly larvae in microcosms to metal contaminants induces nuclear apoptosis and the formation of electron-dense granules in gut epithelial cell mitochondria, (3) whether *in situ* exposure of trout and caddisfly larvae to sediment metal contamination induces nuclear apoptosis and the formation of electron-dense granules in mitochondria of gut epithelial cells and hepatocytes, and (4) whether the composition of the mitochondrial granules reflects sediment metal contamination. Electron microscopy was used to detect the cytotoxic effects of metal contamination. An above-and-below-mine approach was used to compare exposed and reference populations. Copper contamination in sediments was associated with effects on trout and caddisfly larvae at the cellular level and with secondary effects related to reduced body weights and delayed development occurring at higher levels of biological organization. Electron dense granules in the mitochondria of exposed caddisfly and trout mitochondria were detected. Elemental analysis of mitochondrial granules by X-ray analysis suggested Cu was being sequestered by mechanisms that normally regulate transient cell Ca concentrations. Chromatin compaction, margination, and the observation that large vesicles with bi-layer membranes were being expelled from the nuclei of affected cells suggest that apoptosis was also occurring.

Key words: Apoptosis; caddisfly (*Ecclesomyia spp.*); cell-level effects; mitochondrial granules; reduced growth and development; river sediments; trace element contamination; trout (*Oncorhynchus mykiss)*

Introduction

Contamination from abandoned mines has been shown to cause adverse biological effects at all levels of biological organization in the Methow River Watershed, Okanogan County, Washington, U.S.A. (Peplow and Edmonds 2005). Acid mine drainage (AMD) originates at the Alder Mine, Alder Mill, and Red Shirt Mill; these contain tailings, waste rock piles, and mine openings (Peplow 2004, 2005). The ore deposits were mined for Au, Ag, Cu, and Zn until the early 1950's.

Usually, resource managers and ecologists evaluate: 1) effects of contaminants on nutrient cycling or energy flow at the ecosystem level, 2) reduced diversity or abundance at the community level, and 3) reduced growth or increased mortality among individual members of endangered species at the population level. However, the degree to which cause and effect are related (*i.e.* specificity) and our knowledge of the mechanisms of toxicity is lowest at these higher levels of biological organization (Hodson 1990; Clements 2000). At lower levels of biological organization, effects may be more easily linked to cause, occur more rapidly, and may provide early warnings of toxicological effects on populations.

Indicators of toxicity, such as morphological changes at the tissue level, ultrastructural changes at the cellular level, and biochemical changes at the molecular level better reveal cause and effect relationships. At the cellular level, electron microscopy is useful for the diagnosis of toxicological and metabolic disorders, even when evidence at higher levels is not evident (Phillips et al. 1987). Electron microscopy has been particularly useful for studying the development and incidence of apoptosis (programmed cell death). Apoptosis is a form of cell death that is well controlled by a genetic program, and which may be activated by inner or exterior signals. Although apoptosis accounts for the occasional deletion of cells in normal tissues, it also occurs at increased levels as the result of pathological conditions. In normal tissues, apoptosis is cryptic, can be seen only in scattered single cells or small groups of cells, is extremely rapid, and remains visible for only a matter of hours after it occurs. This, taken in conjunction with the small size of apoptotic cells, means that evidence of apoptosis in normal tissues is rare, but could be used to indicate toxicity when it causes the incidence of apoptosis to increase.

Electron microscopy has also been used to observe the effect of divalent cations on *in vitro* cell cultures

bathed in media containing Ca, St, Pb, Mn, Ba, and Mg. Peachy (1964) and Walton (1973) showed that divalent cations accumulate as spherical electron-dense granules in the matrix of mitochondria. Since experimental studies suggest that these granules are concerned with the regulation of the internal ionic environment of the mitochondria (Peachy 1964), the electron-dense particles observed in mitochondria should correspond to the bioavailable metals in the environment surrounding the mitochondria, cell, and organism. The presence of mitochondrial granules that accumulate metals was also found to coincide with the toxicity data for aquatic organisms (Argese 1996). The EC50 (50% of the effective concentration) data for mitochondrial granules in *in vitro* cultures, compared to *in vitro* toxicity data from a variety of other bioassays, suggests the matrix granules induced by divalent cations in solution could be used to indicate that metals are present at concentrations that are toxic for fish and aquatic invertebrate species.

It is not known, however, if the presence of apoptosis and mitochondrial granules at the subcellular level occurs following *in vivo* exposure to trace element contamination under field conditions, or if their presence can be related to toxicity at higher levels of biological organization. If this process occurs under natural conditions, the identification of metals that are contained in electron-dense mitochondrial granules could serve as both a chemical and biological indicator of metal contamination. This combines the specificity that comes with observing the initial interaction of the toxin with the organism at the molecular level and the disease process responsible for effects at higher levels of biological organization.

The objectives of this study were to determine: 1) the effects of copper contamination on the growth and developments of caddis fly larvae (*Ecclesomyia spp.*) and trout (*Oncorhynchus mykiss*), 2) whether *in vivo* exposure of caddisfly larvae in microcosms to sediment copper contamination induces nuclear apoptosis and the formation of electron-dense granules in the matrix of gut epithelial cell mitochondria, 3) whether *in situ* exposure of caddisfly larvae and trout to trace element contamination induces the formation of nuclear apoptosis and electron-dense mitochondrial granules in gut epithelial cells and hepatocytes, and (4) whether the composition of the mitochondrial granules is related to exposure.

Methods

Location

Study sites were located in the Methow River basin near the town of Twisp in Okanogan County, Washington (Figure 1). The Methow River basin is located in north central Washington, east of the Cascade Mountains, and is bordered by Canada on the north. Draining nearly 4,662 km^1, the Methow River flows southward through western Okanogan County and empties into the Columbia River at km 843 near the town of Pateros (Andonaegui 2000). Three abandoned mine and mill sites are located south of Twisp near the Methow River: the Alder Mill, Red Shirt Mill, and Alder Mine (Figure 1). The mined deposits were sulfide ores composed largely of chemically precipitated silica.

The Alder Mill (48°.21'.13.5"N, 120°.07'.31.6"W, elev. 575 m) is located approximately 1.6 km south of Twisp (Figure 1) and approximately 500 m west of the Methow River at river mile (RM) 39.5 (63.4 km from the confluence of the Methow and Columbia Rivers). The Mill consists of several buildings and two tailings impoundments. The impoundments contain approximately 56,000 m^3 of material. Inputs and springs supplied by Alder Creek feed the upper impoundment creating a contaminated wetlands environment. The pH of the ground water in the tailings impoundment was $< 3.0 \pm 0.4$.

The Alder Mine (48°.19'.24.1"N, 120°.09'.38.4"W, elev. 1043 m) is an inactive mine located approximately 4.8 km southwest of Twisp (Figure 1). The site consists of an open adit on the north, an adit retention pond, an open pit, and waste rock dumps. The site is on the north slope of a north-trending ridge. Slopes at the site range from 50-80%. Estimates

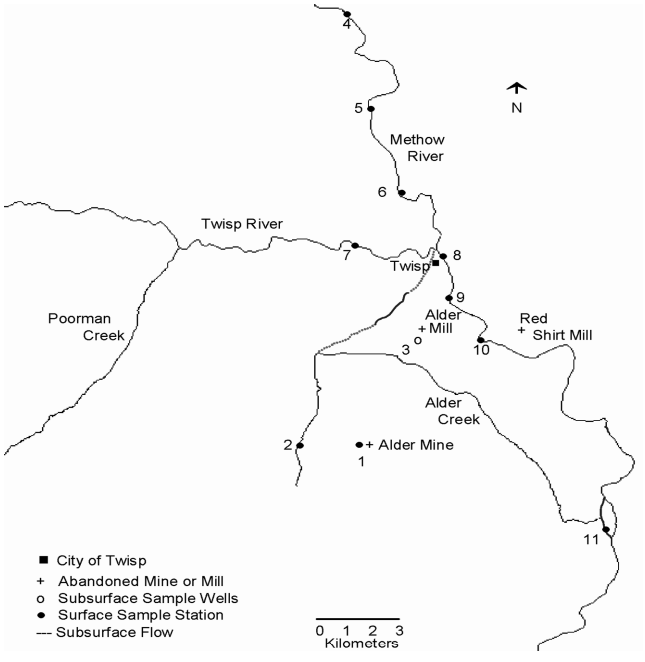

Figure 1. Map showing study area, abandoned mine and mill sites, and sample stations

from aerial photographs indicate that waste rock covers approximately 3.2 ha (US EPA 2000). The flow rate of drainage from the north adit ranges from 5-15 L min^{-1}. The pH of AMD discharged at the mine adit was 2.9 $\pm$ 0.2.

The Red Shirt Mill (48°.21'.05.0"N, 120°.06'.08.1"W, elevation 487 m) is located approximately 1.6 km southeast of Twisp (Figure 1) and east of the Methow River at RM 39.5 (63.4 km from the confluence of the Methow and Columbia rivers). The mill consists of a single building and a tailings pile. The tailings pile is estimated to cover approximately 4,650 m^2 of surface area and contains less than 30,600 m^3 of material. Approximately 4 m^3 of tailings are recruited annually by the Methow River. The site is located adjacent to the Twisp city limits, and residences with private ground water wells are located on and adjacent to the site.

Sample Stations

Sample stations were located near the town of Twisp in Okanogan County, Washington, and the Methow River at river mile (RM) 39.5 (Figure 1). Sample station 1 and 3 are sources of AMD. Sample station 2 is a mixing zone where AMD from Alder Mine enters Alder Creek. Stations 4-7 are reference sites upstream from the abandoned mine sites and stations 8-11 are treatment sites located downstream from areas in the Methow River affected by subsurface flows of ground water from the abandoned mine sites.

There is extensive faulting and calcite-filled fractures in the area where the abandoned mine sites in this study are located. The Methow River has an alkalinity of 103 $\pm$ 14 mg L^{-1} (as $CaCO_3$) and a pH of 7.2 $\pm$ 0.5, which is typical of a system dominated by bicarbonate (Stumm and Morgan 1996).

A tailings sample taken 200 cm below the surface was analyzed using wave dispersive analysis by X-rays (microprobe). Plagioclase feldspars were the most common at 26%; minor components were quartz at 14%, chlorite at 12%, and chlinopyroxine, hornblende, magnetite/hematite, muscovite, and zircone at 5-6%. Numerous minerals, including ilmenite, occurred at levels < 1.

While dissolved metal concentrations are less than the limits of detection by ICP-AES in the Methow River, metal contamination in the sediment was a potential concern. Sand and silt fractions of the river sediments were similar to the tailings: plagioclase feldspars at 26%, quartz at 14%, chlorite at 12%, and hornblende, chlinopyroxine, magnetite/hematite, muscovite, and zircone at 5 - 6%. Trace minerals include apatite, epidote, and ilmenite. SEM-EDS analyses indicated that the coatings on the sediment mineral particles are iron, e.g. siderite ($FeCO_3$) or goethite (FeOOH).

Five trace elements (Al, As, Cu, Mn, and Zn) exceeded background concentrations in both the AMD and Methow River sediments. Three (As, Cu, and Mn) were considered compounds of potential ecological concern (COPECs) because they were detected at concentrations that exceeded both estimated background concentrations and benchmark values for toxicity. The CF was greatest for As and Cu at 9 and 7 respectively. Aluminum, Mn, and Zn were concentrated over background but did not exceed benchmarks for aquatic organisms.

Water samples were collected from Methow and Twisp River stations 4 and 5 above the mines and from Methow River stations 9 and 10 below the mines once monthly for four months prior to the *in situ* microcosm test of trout toxicity (January through April 2001) and once-weekly in May and June during the five-week exposure period in the *in situ* trout exposure study. Water samples were collected in pre-cleaned Teflon® bottles. Subsamples were filtered (Gelman 0.45µm, disposable 25 mm sterile disposable Acrodisc® filter) for determination of dissolved trace element concentrations. All water samples for metals analysis were preserved to pH < 2 with 0.15% nitric acid and stored at 4°C. All analyses were performed within 30 days of sample collection.

Sediment samples were collected using plastic scoops at a shallow depth (<5 cm) and immediately wet sieved in ambient water through a 63 µm sieve. Periphyton was removed from tile using a soft nylon brush (1.5 x 0.5 cm). Sediment samples (oven-dried at 105°C for 24 hours) and periphyton samples (oven-dried 24 h at 60°C) were ground and analyzed for metals. All water and sediment sampling equipment was cleaned by washing with Liquinox® detergent and sequential rinses with distilled water, dilute nitric acid, and deionized water.

Samples of water, sediment, and periphyton were analyzed for trace elements at the University of Washington, College of Forest Resources Analytical Laboratory in Seattle, Washington. Dissolved metal concentrations (0.45µm filtered) were determined after digestion with hot nitric acid using ICP atomic emission spectrophotometry (ICP-AES; Thermo Jarrell Ash ICAP 61E, EPA Method 3050).

General Water Chemistry

Water quality parameters were analyzed during the caddisfly and trout exposure studies described below.

A YSI model 85 meter was used to measure dissolved oxygen (DO) and temperature. Where flow was inadequate for the YSI probe, DO was also determined in the field using the Winkler Titration method (LaMotte Test Kit Model 221788). Alkalinity was measured in the field using the LaMotte Direct Read Titration Kit (Model 221780). A Piccolo Model HI 1295 temperature-compensated digital pH meter was used to measure pH. Dissolved oxygen and pH meters were standardized daily before and after use.

Effects of Copper in the Methow River on Trout Body Weight

Hatchery-raised triploid trout (*Oncorhynchus mykiss)* were used as experimental surrogates to the protected fish populations in the Methow River to look for evidence of toxicity and determine whether exposure to contaminants in the Methow River reduced growth. Eighty two 15-week-old hatchery-raised triploid trout (*Oncorhynchus mykiss)* weighing 35 g were transferred from a nearby hatchery (Trout Lodge, Quincy, WA) in May 2001. The 82 individuals were equally divided into two pens and maintained for 35 days.

One pen was located in a Methow River side channel downstream from the abandoned mine site (station 11) and the other pen was located upstream from the abandoned mine sites (station 7). Two fish pens were constructed from aquaculture netting on a PVC pipe frame that measured 1.1 m on each side. Both pens had 1.6 cm rebar extending 0.5 m through two parallel bottom sections, which were weighed down with four large stones from the river to secure the pen in place. Fish, maintained in the pens for 5 weeks from 7 May 2001 to 13 June 2001, were fed (Rangen 3/32 EXTR 400 Slow Sink food #4974) once daily in the morning (0700-0800 PDT) at 4% of their body weight each day. Visual examination during feeding revealed that the fish readily ingested the food provided and were satiated daily. Each pen was monitored daily for morbidity and mortality throughout the exposure period. At the end of the 5 week exposure period, exposed trout in the pen at station 11 were weighed. Trout in the pen at station 7 were also weighed as a reference.

Wild Caddisfly Larvae Body Weight and Instar Development

One-hundred caddisfly larvae (*Ecclesomyia spp.*) were collected in June 2001 from each of the four sample stations (stations 4 - 7) located upstream from the abandoned mines in the Methow and Twisp Rivers and compared to 100 larvae from each of the four sample sites located in the Methow River downstream from the abandoned mine sites (stations 8 - 11). Within one hour following collection, larvae were removed from their cases, blotted dry using Whatman #40 filter paper to remove surface water, and weighed. Body weight was recorded as the mean weight in g per 100 larvae. After weighing, the larvae were preserved in 70% ETOH. Head capsule widths were measured at a later date using a slide micrometer and a dissecting microscope to compare relative age distribution. Instar groups and corresponding size ranges were identified based on a frequency distribution histogram of head capsule width data, which were ranked in ascending order and placed into 7 instar groups. Head capsule widths that comprised the horizontal portions of graph were assumed to be from the same instar groups and vertical portions of the graph were assumed to be transitions between instar groups. The midpoint of each transition range defined the size range for each instar group.

Caddisfly Cytotoxicity in a Static Microcosm

To determine whether mitochondrial granules and apoptosis could be induced *in vivo* by exposure to copper contamination from abandoned mines, caddisfly larvae were exposed to copper-contaminated periphyton from station 2. Ten mostly 4th and 5th instar caddisfly larvae (*Ecclesomyia* spp.) were collected in June 2001 from sample station 5 and added to a tray, 10 cm wide x 15cm long x 6 cm deep. The tray contained a 25 x 25 cm terracotta tile incubated for one year at station 2, where it was colonized with trace-element contaminated periphyton, and 500 ml of metal-free water from Methow River station 4. The tray was maintained in an insulated chest 46 cm wide x 76 cm long x 46 cm deep that contained ice approximately 20 cm deep in the bottom. The chest and tray was maintained in a shaded area in the vicinity of station 7. The tray was supported on the ice by a 2.5 cm thick piece of styrofoam. The ice chest was covered by Plexiglas approximately 0.5 cm thick. Medical-grade oxygen was fed continuously to the headspace over the tray at a rate of 0.25 - 0.5 L min^{-1} to promote oxygenation of the water in the tray.

One water sample was collected from the microcosm chamber at the beginning of the test and from the periphyton test chamber at the end of the 24 h exposure period. Periphyton was removed from the tile at the end of the exposure period using a soft nylon brush (1.5 x 0.5 cm). The periphyton was dried for 24 h at 60°C, ground, and analyzed for Cu. Temperature, pH, dissolved oxygen, and alkalinity were measured three times during the study.

Larvae were exposed for 24 hours. At the end of the exposure, two larvae were dissected and samples of

columnar epithelial cells from the small intestine were collected and prepared for examination by transmission electron microscopy (TEM). One larva from station 5 was dissected and preserved at the beginning of the study for use as a control. Tissue samples were analyzed to determine whether mitochondrial granules and apoptosis (i.e. condensation and margination of nuclear heterochromatin, and presence of nuclear vesicles) were detectable in larvae exposed to periphyton contaminated by abandoned mine waste.

Methow River Microcosm Caddisfly Cytotoxicity

Caddisfly larvae from a side channel of the Methow River, upstream of the abandoned mines, were placed in microcosm cages and exposed to conditions at station 10, downstream of the abandoned mines, to determine if mitochondrial granules and apoptosis could be induced in the larvae after exposure to ambient conditions. In June 2001, 25 caddisfly larvae (*Ecclesomyia spp.*), collected from sample station 5 (Figure 1) was added to a 30 cm square basket made of 0.5 cm wire mesh. The basket was secured to rebar driven into a side channel in the Methow River at station 10, where the water depth was ≈ 10 cm. The basket contained a flat stone covered with periphyton ≈ 30 cm in diameter (4 cm thick) from the side channel at station 10. Larvae were maintained for 14 days. Temperature, pH, dissolved oxygen, and alkalinity were measured *in situ* at each sample site.

Periphyton was removed from the stone in the microcosm at station 10 at the end of the exposure period using a soft nylon brush (1.5 x 0.5 cm). The periphyton was dried for 24 h at 60°C, ground, and analyzed for metals (ICP-AES). Water samples were collected once monthly for four months from January through April at station 10 and five times in June during the *in situ* caddisfly cytotoxicity test.

Larvae were monitored daily, and at the end of the 14 day exposure period, five larvae were removed, dissected, and samples of small intestine were collected for cytotoxicity studies. One wild larva from station 5 was dissected and sampled for use as a control.

Wild Caddisfly Larvae Cytotoxicity

Five wild larvae from the side channel where the *in situ* microcosm caddisfly cytotoxicity test was performed (station 10) were also collected at the end of the exposure period (June 2001). One wild larva from station 5 was dissected and sampled for use as a control. The incidence of mitocondrial granules was determined by observing 25 mitochondria per larvae and counting the number of granules per mitochondrion.

Methow River Trout Cytotoxicity

At the end of the 5 week exposure period in June 2001, five juvenile trout from the pen at station 11 were euthanized (0.1% MS-222, pH 7) and dissected, and liver samples were collected for cytotoxicity analysis. Samples from five juvenile trout from the pen at station 7 were also collected for use as references. The incidence of mitocondrial granules was measured by observing 25 mitochondria per larvae and counting the number of granules per mitochondrion.

General Cytology Techniques

Juvenile trout and caddisfly larvae from each cytotoxicity study were dissected. Sections of trout liver and caddisfly larvae small intestine < 2 mm in diameter were collected and preserved in the field in 2.5% glutaraldehyde and 0.1M sodium-cacodylate buffer. The tissue samples were then fixed for 1 h in a final concentration of 1% OsO_4. After dehydration in a graded series of ethanol concentrations and embedding in Embed 812, the sections were cut into silver-gray or white sections (approximately 85 nm thickness) using a Reichert/Jung Ultra-cut E. After the sections were collected onto Au grids and stained with 4% aqueous uranyl acetate for 45 min., the sections were examined and photos taken using a Jeol JEM 1010 transmission electron microscope, operated at 80 keV, located at the University of Washington Zoology Department. Tissues were examined at 1100X for evidence of nuclear apoptosis (Zhao 2001). Magnification was then increased to 34 - 64,000X and the tissues were scanned to observe the mitochondria. Electron-dense spheres > 300A (Peachy 1964, Rouiller 1960) were counted and the average number of granules per mitochondrion were calculated.

X-ray Analysis of Metals in Mitochondrial Granules in Caddisfly and Trout

Tissues for the analysis of metals in the mitochondrial granules were collected on Au grids. Energy Dispersive Spectroscopy (EDS) analyses were carried out at 100 keV using a Joel 1200EX STEM scanning transmission electron microscope with a spot size of approximately 9 nm in STEM mode. The sample was scanned at low magnification until a group of granules was located. The magnification was then increased to about 50,000 x and a 0.4 x 0.4 μm scan window was placed over an individual granule. EDS analysis of the granule was performed for 100 s. The X-ray analysis system used was a ThermoNoran Voyager 4 with a light element X-ray detector mounted horizontally on the TEM column. Background composition was determined by scanning an area not containing a granule.

Statistical Analysis

Unreplicated experiments involving matched paired sites were conducted mimicking a classical treatment-control design (Hurlbert 1984; Wiens and Parker 1995). Thus, it was assumed that other environmental factors besides trace elements that could influence the response were equal among experimental units and samples, and that treatment and controls were from single statistical populations. Multiple samples were collected from each experimental unit (*i.e.* treatment and control). Inferential statistics were used to determine whether there was a significant difference between trace element concentrations, body weight, life stage, and the incidence of mitochondrial granules at the treatment and control locations and to provide an objective estimate of the probability of observing a difference between a treatment and the control under the null hypothesis of no treatment effect.

Chemistry data were reported as the mean concentration (*i.e.* m_t = downstream treatment mean for stations 9 and 10 and m_c = upstream control mean for stations 4 - 5) ± the standard error of the mean (SEM). The one-tailed *t*-test for the hypothesis H_o: $m_t \leq m_c$ and H_a: $m_t > m_c$ was used to compare the treatment mean to the reference mean. Contaminants of potential ecological concern were identified as those trace elements that were at higher concentrations in the downstream samples (stations 9 and 10) compared to upstream samples (stations 4 and 5).

Data were reported as the mean body weight per fish (*i.e.* m_t = treatment mean and m_c = control mean). The one-tailed *t*-test for the hypothesis H_o: $m_t \leq m_c$ and H_a: $m_t > m_c$ was used to compare the treatment mean to the control mean. The Kolgomorov-Smirnov test was used to compare the cumulative frequencies of caddisfly larvae from the Methow River above and below the abandoned mines (Zar 1996). The mean number of granules per mitochondrion (i.e. m_t = treatment mean and m_c = control mean) were evaluated using the one-tailed *t*-test for the hypothesis H_o: $m_t \leq m_c$ and H_a: $m_t > m_c$. All statistical analyses were performed using Minitab statistical software (release 9). A significant difference was determined to exist at a $p < 0.05$ level. Data on the number of granules per mitochondrion were also evaluated at the 10% level of significance ($p < 0.1$).

Results

Copper in Methow River Water and Sediments

While dissolved metal concentrations were less than the limits of detection by ICP-AES in Methow River water, sediment Cu concentrations were significantly higher at stations 8 - 11 below the mines compared to controls at stations 4 - 7 (Table 1, $p < 0.05$) above the mines, and exceeded toxicity benchmarks for aquatic biota (Jones and Suter 1997). At the sample stations above and below the mines, the other parameters (*i.e.* pH 6.6 - 8.2, temperature $\leq 13^{o}$C, dissolved oxygen $\geq$ 9 mg L^{-1} and alkalinity > 83 mg L^{-1} $CaCO_3$) were within the ranges normally tolerated by aquatic insects (Jobling 1995; Pickering 1981; Roberts 2001).

Wild Caddisfly Larvae Body Weight and Instar Development

The mean live body weight of caddisfly larvae (*Ecclesioymia spp.*) was lower in the Methow River below the mine sites (stations 8 - 11) than upstream at stations 4 - 7 [2.3 ± 0.5 g (SD) vs. 1.2 ± 0.2 g 100-larvae^{-1}, $p < 0.02$]. Growth patterns were also different between exposed larvae (stations 8 - 11), for which five larval stages were identified, and reference larvae (stations 4 - 7) with seven larval stages. Development of the exposed larvae lagged behind the reference larvae; Figure 2 shows that 84% were mostly 4th instar larvae and only 8% were 5th instar. The reference site had fewer 4th instar (63%) and more 5th instar (35%) larvae. The frequency distributions of caddisfly larval instars were significantly different based on the Kolmogorov-Smirnov goodness of fit test for discrete data ($p < 0.05$).

Effects of Copper in the Methow River Sediments on Trout Body Weight

The mean body weight of trout in the exposed group downstream from the abandoned mines (station 11) was significantly less than the body weights of the upstream control group (station 7) [65g ± 10 (SD) vs. 71 ± 9, $p < 0.05$]. Mortality among the trout in the test group downstream from the abandoned mines also exceeded the upstream control group. Three fish died within 96 hours following the beginning of exposure

Table 1. Concentrations of Cu in Methow River sediment (µg g^{-1}) at sample stations above and below mines from January to June, 2001; TEC is the threshold effects concentration.

	Stations Above Mines				**Stations Below Mines**			
	4	5	6	7	8	9	10	11
Replication								
1	21	19	17	15	25	120	94	65
2	17	13	18	21	23	280	98	62
3	18	15	15	13	24	154	78	77
4	14	15	11	17	27	131	45	74
Mean	18	16	15	16	24	171	79	70
Mean ±SEM: 16 ± 1					Mean ± SEM: 86 ± 16			
					t-Test: *p=0.00* TEC 28			

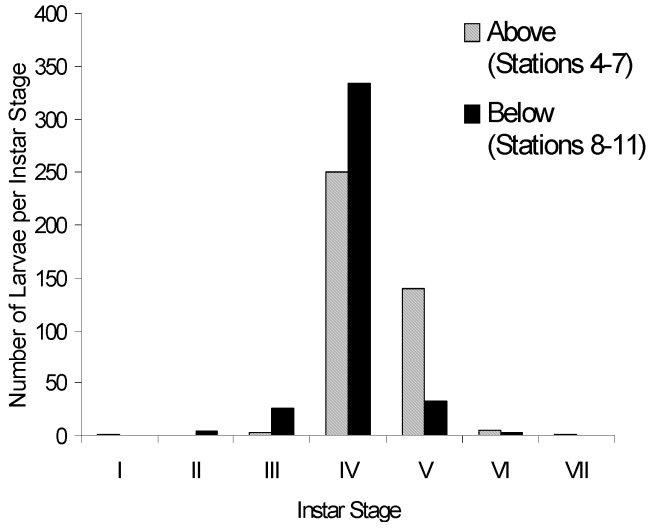

Figure 2. Frequency distribution of caddisfly larvae (*Ecclesomyia spp.*) instars in the Methow River above and below the abandoned mine sites.

compared to no deaths in the control group. Two dead indigenous Coho parr were also encountered at station 11 during the study period.

Caddisfly Cytotoxicity in Static Microcosms

Treatment Condition

The station 1 periphyton treatment contained 292 mg Cu kg^{-1}. Copper was not detectable in the water sampled above the periphyton when it was added to the tray but at the end of the exposure period the dissolved Cu concentration was 26 µg L^{-1}. The other parameters measured (*i.e.* pH 8.6, temperature $\leq$ 9º C, dissolved oxygen $\geq$ 17 mg L^{-1} and alkalinity 219 mg L^{-1} $CaCO_3$) were within the ranges tolerated by aquatic insects (Merritt and Cummins 1996; Ward 1992).

Cytotoxicity

The nuclei and mitochondria of small intestine epithelial cells from the control larva (Figures 3 A and B) and from control trout (Figures 3 C and D) generally appeared to be normal in appearance. Little variation was found in their shape and size. The nuclei were round to oval and measured 5-8 µm in diameter. The nuclear envelope consisted of two visible layers of membrane. The heterochromatin appeared granular or slightly aggregated and sparsely dispersed throughout the nucleus.

The mitochondria of small intestine epithelial cells from caddisfly larvae exposed to Cu contaminated periphyton also appeared to be normal in appearance (Figure 4A). However, morphological changes that are characteristic of nuclear apoptosis were observed in the nuclei. Extensive condensation of nuclear heterochromatin into sharply distinct masses that were often found along the margins of the nuclear envelope was observed in cells from the exposed caddisfly (Figure 4B). In nuclei showing chromatin compaction, chromatin-free nuclear vesicles, which evolved from the nuclear envelope and had no chromatin, were expelled from apoptotic nuclei (Figure 4C). An enlargement of Figure 3C shows the membranes of the apoptotic bodies were also a bilayer derived from the nuclear membrane (Figure 4D). Numerous objects that appeared to be apoptotic bodies containing compacted chromatin and chromatin-free nuclear vesicles were also observed (Figure 4E). In treatments where caddisfly larvae were exposed to periphyton and streamwater contaminated by Cu, electron-dense granules were scattered randomly among the mitochondria and within the matrix between the cristae (Figure 4F).

Methow River Microcosm Caddisfly Cytotoxicity

The concentrations of Cu (294 mg kg^{-1}) in periphyton from Methow River below the Red Shirt Mill at station 10 were similar to concentrations in station 2 periphyton from Alder Creek below Alder Mine in the static toxicity test. The other parameters measured (i.e. pH 7.4, temperature $\leq$ 17º C, dissolved oxygen $\geq$ 9 mg L^{-1} and alkalinity 105 mg L^{-1} $CaCO_3$) were within the ranges normally tolerated by aquatic insects (Merritt and Cummins 1996; Ward 1992).

Submitochondrial granules were induced in all five caddisfly larvae that were translocated from reference station 5 and maintained in the microcosm at station 10 below the Red Shirt Mill for 14 days (Figure 5 A and B). The mean number of electron-dense granules was significantly greater than in the control at both the 10 and 5% levels of significance (Table 2).

Wild Caddisfly Larvae Cytotoxicity

Mitochondrial granules were induced in the seven wild larvae from Station 10 that were analyzed to determine whether matrix granules were being formed under natural conditions (Figure 5C). The mean number of electron-dense granules was significantly greater than in the control at both the 10 and 5% levels of significance (Table 2). Condensation of nuclear heterochromatic into sharply distinct masses along the margins of the nuclear envelope was observed (Figure 5E).

Methow River *In Situ* Microcosm Trout Cytotoxicity

The nuclei and mitochondria of control fish hepatocytes (station 7) generally appeared to be normal

in appearance (Figure 3C, D). Little variation was found in their shape and size. The nuclei in the reference samples were generally round to oval and the nuclear envelope consisted of two visible layers of membrane. The heterochromatin was only slightly granular or aggregated and sparsely dispersed throughout the nucleus. In all five fish sampled after exposure to conditions downriver from the mines at station 11, the mean number of electron-dense granules (Figure 5D) was significantly greater than in the control at the 5% level of significance (Table 2). The condensation of nuclear heterochromatin into sharply distinct masses along the margins of the nuclear envelope, and chromatin-free nuclear vesicles, which evolved from the nuclear envelope, were seen being expelled from apoptotic nuclei (Figure 5F).

X-ray Analysis of Metals in Mitochondrial Granules

The EDS-spectra for mitochondrial granules are shown in Figure 6. After the elements that are found in the system background spectra (Figure 6A) and the grid material (*i.e.* Au) were taken into consideration, it was determined that the wild caddisfly larva tissue contained Cu (Figure 6B). Figure 6A is a spectrum of the mitochondrial matrix in trout hepatocyte tissue from station 11, where no granules were observed. Peaks for C, Si, and O occurred, which are characteristic for background analyses not including the grid.

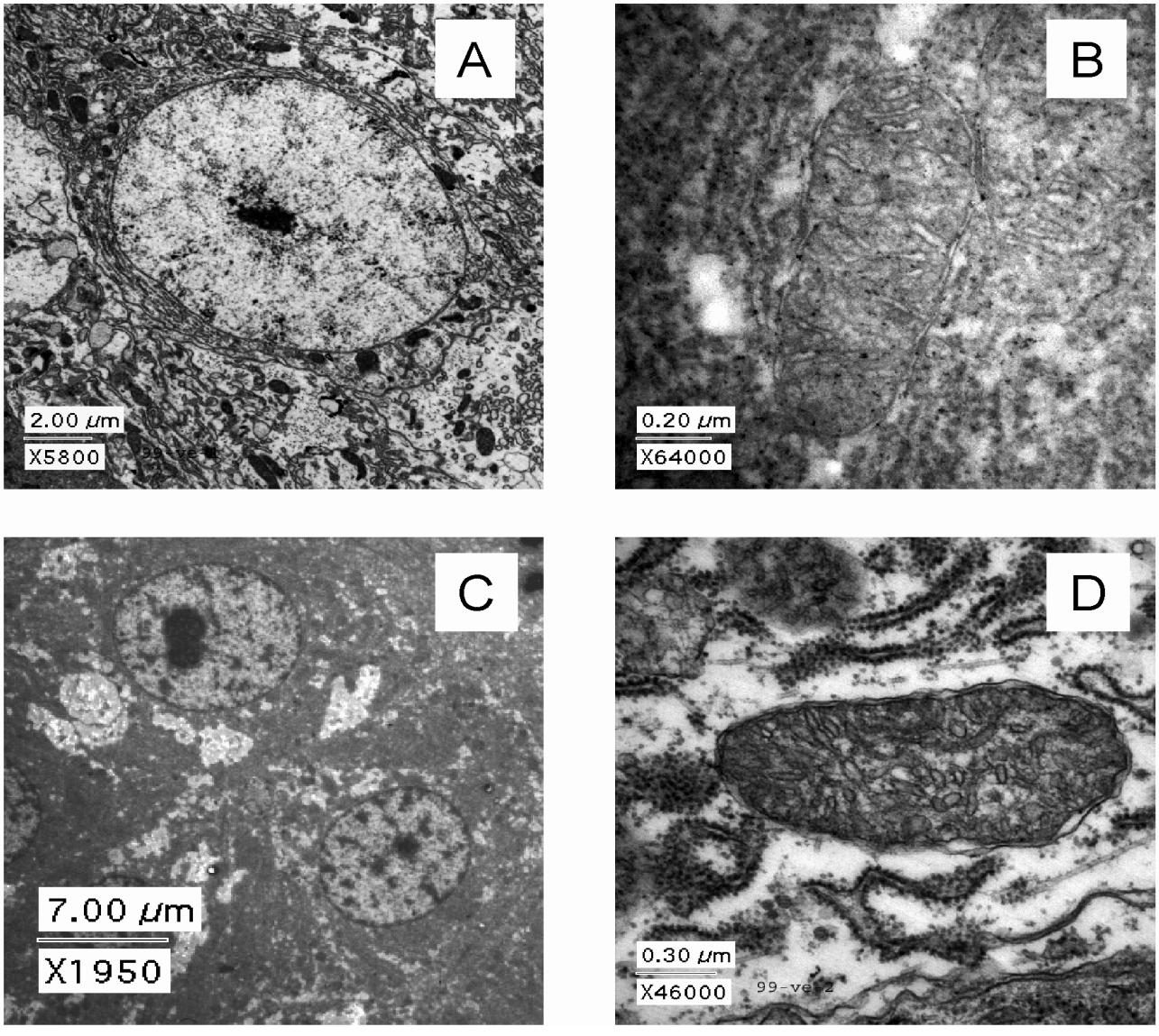

Figure 3. Normal nuclei and mitochondria in caddisfly small intestine epithelial cells and trout hepatocytes: *(A)* caddisfly larva normal nucleus from station 5, X5800, *(B)* caddisfly larva normal mitochondrion from station 5, X64000, *(C)* normal nucleus from juvenile triploid trout hepatocyte from station 7 (Figure 1), X1950, and *(D)* normal mitochondrion from juvenile triploid trout hepatocyte from station 7, X46000.

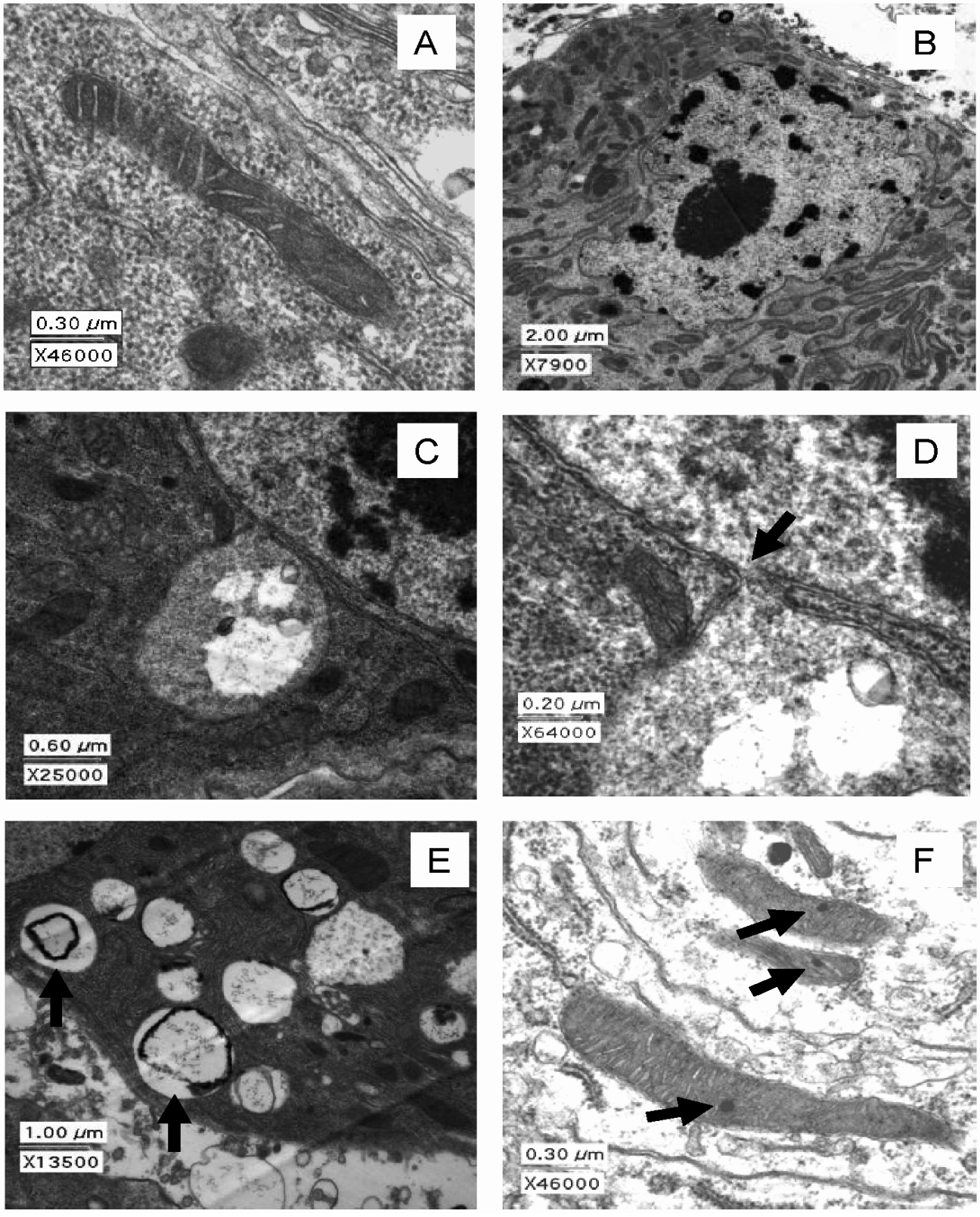

Figure 4. Mitochondria and nuclei in caddisfly larvae small intestine columnar epithelial cells from the static microcosm caddisfly cytotoxicity test after exposure to periphyton containing Cu from Station 2: *(A)* mitochondria with no apparent effects, *(B)* chromatin in the nucleus showing condensation and margination, *(C)* apoptotic body containing chromatin being expelled from the nucleus, *(D)* enlargement of Figure C showing bilayer membranes of vesicle, *(E)* apoptotic bodies phagocytized by interhepatocytic cell, *(F)* electron-dense granules (arrows) within the mitochondria of cells from larvae exposed to contaminated periphyton.

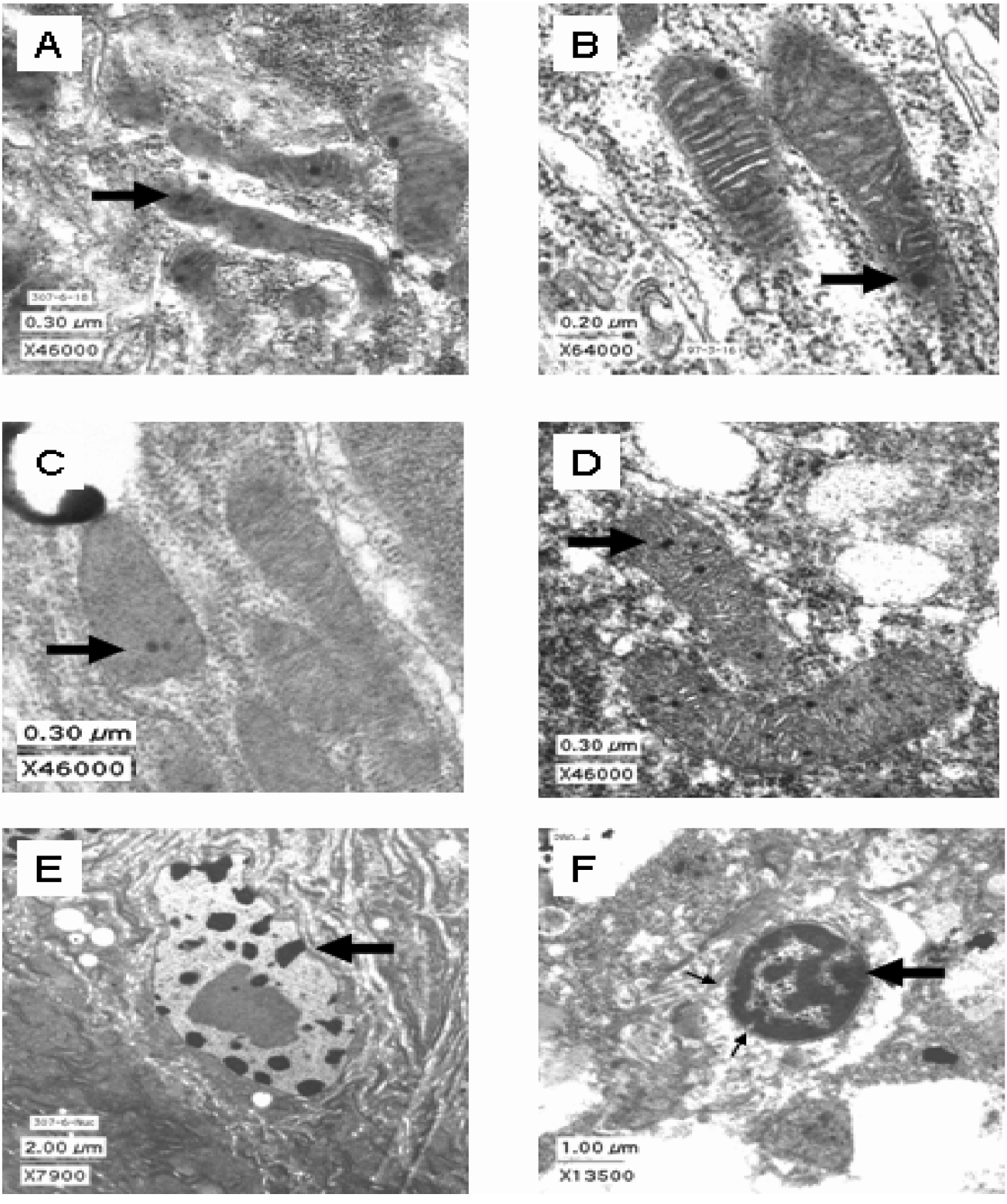

Figure 5. Mitochondria and nuclei from caddisfly larvae and trout exposed *in situ* to Cu in Methow River sediments: *(A)* mitochondrial granules (arrow) in caddisfly from *in situ* microcosm caddisfly cytotoxicity test; *(B)* mitochondrial granules (arrow) from caddisfly caged at station 10 below the mines; *(C)* mitochondrial granules (arrow) in wild larvae from station 10 below the mines; *(D)* mitochondrial granules in hepatocyte mitochondria (arrow) from test trout at station 11 below the mine; *(E)* chromatin condensation and margination of apoptotic nucleus (arrow) in wild caddisfly from station 10 below the mines; and *(F)* chromatin condensation and margination of apoptotic nucleus (large arrow) in hepatocytes from trout at station 11 below the mines. Chromatin-free nuclear vessicles (small arrows) are seen being expelled from the nucleus.

Table 2. Incidence of spherical electron-dense granules in mitochondria of caddisfly and trout exposed to mine waste contamination. The Dunnet's test was used to compare control means to each other group mean. The data are numbers of spherical electron-dense granules observed in the mitochondria of caddisfly larvae (*Ecclesomyia spp*) small intestine epithelial cells (*A*-*C*) and the mitochondria of juvenile triploid trout hepatocytes (*D*). H_o was that $m_{test} \leq m_{control}$. H_a was that $m_{test} > m_{control}$.

Caddisfly i*n situ* microcosm exposure test: larvae from station 5 in cage at station 10			Wild caddisfly larvae from station 10			Trout exposed group from station 11 compared to control group from station 7		
Mean # of Granules/Mitochondria			Mean # of Granules/Mitochondria			Mean # of Granules/Mitochondria		
Replicate	Test	Control	Replicate	Test	Control	Replicate	Test	Control
1	0.72	0.00	1	0.12	0.00	1	2.00	0.00
2	1.04	0.39	2	0.92	0.39	2	1.32	0.00
3	0.64	0.06	3	0.28	0.06	3	1.24	0.00
4	0.84	0.29	4	0.72	0.29	4	2.20	0.00
5	0.08		5	0.84		5	1.72	
			6	0.16				
			7	1.04				
Mean	0.66	0.19	Mean	0.58	0.19	Mean	1.70	0.00
	$p = 0.04$			$p = 0.00$			$p = 0.00$	
Conclusion: H_0 (5%)		Reject	Conclusion: H_0 (5%)		Reject	Conclusion: H_0 (5%)		Reject
H_0 (10%)		Reject	H_0 (10%)		Reject	H_0 (10%)		Reject

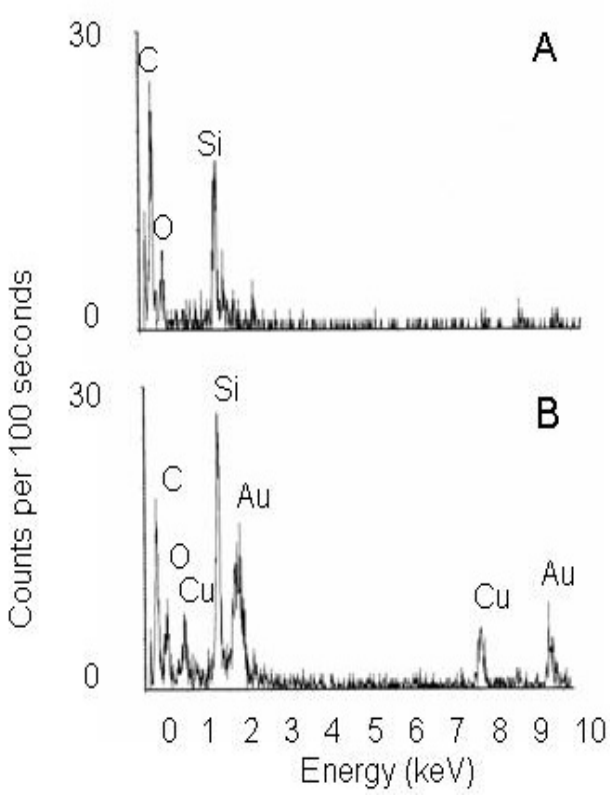

Figure 6. Energy dispersive x-ray analysis (EDX) spectra showing composition of electron-dense spheres in matrix of caddisfly and trout mitochondria: *(A)* System background is typical spectra from the analysis of the mitochondrial matrix not including the grid material or matrix granule; and *(B)* Cu was sequestered by matrix granules in hepatocyte mitochondria of caddisfly and trout in the Methow River. Background included Au from the grid.

Discussion

Mitochondrial Granule Formation and Apoptosis in Microcosms

Results from the static microcosm caddisfly cytotoxicity test showed that caddisfly larvae (*Ecclesiomyia spp.*) exposed *in vivo* to Cu (292 mg kg^{-1}) in periphyton develop signs of nuclear apoptosis and form electron-dense granules in the matrix of gut epithelial cell mitochondria. It is not clear, however, whether the concentration of Cu in the dissolved fraction (i.e. 26 µg L^{-1}), apparently leached from the periphyton, contributed to the formation of mitochondrial granules and apoptotic bodies.

Much of the knowledge regarding acute toxicity and the effects of dissolved Cu in the aquatic environment is based primarily on laboratory studies. Acute toxicity studies for Cu have shown that while it is an essential element for aquatic organisms, dissolved Cu is toxic to some invertebrate larvae at concentrations exceeding 16 µg L^{-1} (Anderson et al. 1980). While caddisfly larvae appear capable of tolerating dissolved Cu at concentrations as high as 6200 µg L^{-1} (Remwoldt et al. 1974), there is evidence that benthic organisms are sensitive to sediment Cu concentrations as low as 28 µg g^{-1} (Jones and Suter 1997).

Trace Elements in Methow River Sediments

The mean sediment Cu concentration at stations 8 - 11 below Twisp (i.e. 86 ± 16 mg kg^{-1}) was higher ($p < 0.05$) than at the upstream stations 4 - 7 (i.e. 16 ± 1 mg kg^{-1}). The mean sediment Cu concentration at stations

below Twisp also exceeded 42 mg kg^{-1}, the estimated natural background value for the Methow River (Boleneus and Chase 1999, San Juan 1994).

In studies in the upper Stillwater River basin, Montana, Amacher et al. (1995) and Gurrier (1998) showed that adsorption of Cu onto hydrous Fe oxides followed by particle deposition was the dominant removal mechanism in stream waters with a pH > 4.5.

Formation of Mitochondrial Granules and Apoptosis

Caddisfly larvae and trout exposed in situ to ambient concentrations of Cu in Methow River sediments and periphyton also resulted in electron-dense granules and apoptosis. The lack of mitochondrial granules in samples from the trout reference population compared to an observed background level in the caddisfly reference group could be due to differences in feeding habits and the pelagic behavior of trout compared to the benthic mode of caddisfly larvae (Jobling 1995; Ward 1992).

X-ray Analysis of Mitochondrial Granules

Since experimental studies suggest that mitochondrial granules are involved with the regulation of the internal ionic environment of the mitochondria (Peachy 1964), the electron-dense particles observed in mitochondria should correspond to the metals in the environment that are bioavailable. The accumulation of Cu as spherical granules in caddisfly larvae small intestine epithelial cell mitochondria and in the hepatocytes of trout suggest that bioavailable forms of these elements are present at high concentrations in the environment surrounding the organism, its cells and the mitochondria.

Effects of Trace Elements on Caddisfly and Trout Body Weight and Development

The observation that larvae in the Methow River downstream from the mines were predominantly stage-four instars while upriver stage-five instars were more prevelant suggests that metal toxicity at the mitochondrial level is associated with delayed caddisfly development equal to approximately one-month based on differences in available life-history histograms for *Ecclesiomyia spp.* (Irons 1987; Merrit and Cummins 1996). Furthermore, the lower body weights of microcosm trout and wild caddisfly larvae (*Ecclesiomyia spp.*) downstream from the mine sites in the Methow River suggests there may be a diversion of energy from growth to tissue repair (Ishak and Sharp 1987).

There is evidence that, aside from ATP problems, metal toxicity leads to mitochondrial collapse followed by the release of cytochrome C that activates caspases and mediates apoptotic cell death (Dragan 2001; Liu et al 1996; Kluck 1997; Yang 1997; Zhao et al. 2001). Copper, for example, is an essential trace element utilized as a cofactor to cytochrome C, which is involved in oxygen metabolism (Halliwell et al. 1999). At toxic concentrations, Cu^{2+}, or its low molecular weight complexes, causes the formation of reactive oxygen species, such as hydrogen peroxide, and induces apoptotic cell death. This is preceded by the up-regulation of Bax (Zhai 2000), the loss of mitochondrial membrane potential or permeability transition (Pourahmad and O'Brian 2000), and the release of cytochrome-C into the cytosol. Cytochrome-C is responsible for the initiation of apoptosis (Zhao 2001).

The precipitation of metals in the matrix of mitochondria is a potential indicator of mitochondrial failure (Halliwell and Gutteridge 2002). Because of a large negative membrane potential, mitochondria are effective barriers of cycstolic metal transients. Metals enter the mitochondrial matrix via a transporter and at low levels stimulate the Kreb's cycle and oxidative phosphorylation. At high levels of metals or when the membrane becomes permeable, loading can result in a catastrophic, irreversible collapse of mitochondrial membrane potential (*i.e.* permeability transition) that not only prevents ATP production, but also increases free radical production (Kamp 2002). The lowered ATP availability reduces the ability of the cell to bail metals out of the cytoplasm into the extracellular volume, which increases cystolic metal concentrations and free radical production further accentuating mitochondrial instability.

Conclusion

In this study, we showed that it is likely that trace elements from the abandoned mines near the Methow River are affecting benthic invertebrates and fish at the cellular level with secondary effects related to reduced body weights and delayed development occurring at higher levels of biological organization. Results from the analysis of water and sediments showed that Cu was significantly higher in Methow River sediments below the mines compared to the reference area above the mines, and that it caused signs of toxicity in aquatic biota in the Methow River. No effects from mine waste contamination were observed on dissolved metal concentrations in the Methow River.

Sediment metal toxicity likely arises from mitochondrial collapse and the diversion of energy, which is thought to be causing reduced growth and development in caddisfly larvae and trout in the Methow River. *In vivo* microcosm exposure showed that mitochondrial granules are induced in the mitochondria of live caddisfly larvae exposed to periphyton containing Cu-contaminated sediments. Results also showed that the incidence of mitochondrial granules was significantly higher in caddisfly and trout exposed *in situ* to sediments in the Methow River contaminated by Cu from abandoned mines. Elemental analysis of mitochondrial granules by X-ray analysis suggest that bioavailable forms of Cu must be present in the environment surrounding the organism, its cells and the mitochondria of caddisfly and trout from the Methow River. Chromatin compaction, margination, and the observation that large vesicles with bilayer membranes were being expelled from the nuclei of affected cells from caddisfly larvae and fish exposed to Cu suggests that genetically programmed 'apoptotic' cell death is also occurring.

References

Amacher MC, Brown RW, Sidle RC, Amacher JK (1995) Effect of mine waste on element speciation in headwater streams. In: Allen HE, Huang CP, Bailey GW, Bowers AR (eds), Metal Speciation and Contamination of Soil, Lewis, Chelsea, MI, USA, p 275-309

Anderson RL, Walbridge CT, Fiandt JT (1980) Survival and growth of *Tanytarsus dissimilis* (Chironomidae) exposed to copper, cadmium, zinc, and lead. Arch Environ Contam Toxicol 9: 329-335

Andonaegui C (2000) Salmon and Steelhead Habitat Limiting Factors Water Resource Inventory Area 48, Methow Watershed. Washington State Conservation Commission, Olympia, WA, USA. 91 pp

Argese E, Bettiol C, Miana P, Iuzzolino L, Giurin G (1996) Submitochondrial particles as *in vitro* biosensors of heavy metal toxicity. J Aquat Ecosyst Hlth 5: 125-134

Boleneus DE, Chase DW (1999) Digital analytical data from mineral resource assessments of national forest lands in Washington: USGS OFR 99-344B, US Geological Survey, Spokane, WA, USA . Can be accessed online <http://wrgis.wr.usgs.gov/open-file/of99-344> . 65 pp.

Clements WH (2000) Integrating effects of contaminants across levels of biological organization: an overview. J Aquat Ecosyst Stress Recovery 7: 113-116

Dragan YP, Bidlack WR, Cohen SM (2001) Implications of apoptosis for toxicity, carcinogenicity, and risk assessment: Fumonisin B_1 as an example. Toxicol Sci 6: 6-17

Gurrier JT (1998) Distribution of metals in water and sediment and effects on aquatic biota in the upper Stillwater River basin, Montana. J Geochem Explor 64: 83-100

Halliwell B, Gutteridge JMC (1999) Free Radicals in Biology and Medicine. Pergamon, New York, USA. 936 pp.

Hodson PV (1990) Indicators of ecosystem health at the species level and the example of selenium effects on fish. Environ Monit Assess 15: 241-254

Hurlbert SH (1984) Pseudoreplication and the design of ecological field experiments. Ecol Monogr 54(2): 187-211

Irons JG (1987) Life history patterns and trophic ecology of Trichoptera in two Alaskan (U.S.A.) subarctic streams. Can J Zool 66: 1258-1265

Ishak KG, Sharp HL (1987) Metabolic errors and liver disease. In: MacSween RNM, Anthony PP, Scheuer PJ (eds), Pathology of the Liver, Churchill Livingstone, New York, USA, p 118-123

Jobling M (1995) Environmental Biology of Fishes. Fish and Fisheries Series 16, Chapman & Hall, London, UK, 455 pp

Jones DS, Suter GW (1997) Toxicolgical Benchmarks for Screening Contaminants of Potential Ecological Concern. US Dept of Energy, Office of Environmental Management, ES/ER/TM-95/R4, 48 pp

Kamp DW, Panduri V, Weitzman SA, Chandel N (2002) Asbestos-induced alveolar epithelial cell apoptosis: role of mitochondrial dysfunction caused by iron-derived free radicals. Mol Cell Biochem 234/235: 153-160

Kluck RM, Bossy-Wetzel E, Greene DR, Newmeyer DW (1997) The release of cytochrome c from mitochondria: a primary site of Bcl-2 regulation of apoptosis. Science 275(5303): 1132-1136

Liu X, Kim CN , Yang J, Jemmerson R, Wang X (1996) Induction of apoptotic program in cell-free extracts> requirement for dATP and cytochrome c. Cell 86: 147-157

Merritt RW, Cummins KW (eds) (1996) An Introduction to the Aquatic Insects. 3rd Edit, Kendall/Hunt, Dubuque, MN, USA, 862 pp

Peachy LD (1964) Electron microscopic observations on the accumulation of divalent cations in intramitochondrial granules. J Cell Biol 20: 95-109

Peplow D, Edmonds R (2004). Health risks associated with contamination of groundwater by abandoned mines near Twisp in Okanogan County, Washington, USA. Environ Geochem Health 26: 69 - 79

Peplow D, Edmonds R (2005). The effects of mine waste contamination at multiple levels of biological organization. Ecol Eng 24:101-119

Peplow D, Edmonds R (2005) The effects of mine waste contamination at multiple levels of biological organization. Ecol Eng 24: 101-119

Phillips MJ, Poucell S, Patterson J, Valencia P (1987) The Liver: An Atlas and Text of Ultrastructural Pathology. Raven Press, New York, USA, 585 pp

Pickering AD (1981) Stress and Fish. Academic Press, New York, USA, 367 pp

Pourahmad J, O'Brien JT (2000) A comparison of hepatocyte toxic mechanisms for Cu^{2+} and Cd. Toxicology 143(3): 263-273

Remwoldt R, Lasko L, Shaw C, Wirkowski E (1974) The acute toxicity of some heavy metal ions toward benthic organisms. Bull Environ Contam Toxicol 10: 291-294

Roberts RJ (2001) Fish Pathology. 3rd edit, WB Saunders, New York, USA, 472 pp

Rouillier CH (1960) Physiological and pathological changes in mitochondrial morphology. Int Rev Cytol 9: 227

San Juan C (1994) Natural Background Soil Metals Concentrations. Publ 94-115, Toxics Cleanup Program, Washington State Dept of Ecology, Olympia, WA, USA

Stumm, W, JJ Morgan (1996). Aquatic Chemistry. 3rd Edit, John Wiley & Sons, New York, USA, 102 pp

US EPA (2000) Alder Mine Site Phase 1 and Phase 2 Preliminary Assessment/Site Inspection Report. TDD: 99-04-0004. Region 10 Superfund Technical Assessment and Response Team, US Environmental Protection Agency, Seattle, WA, USA, 102 pp

Walton JR (1973) Granules containing lead in isolated mitochondria. Nature 243(5402): 100-101

Ward JV (1992) Aquatic Insect Ecology. John Wiley & Sons. New York, USA, 438 pp

Wiens JA, Parker KR (1995) Analyzing the effects of accidental environmental impacts: approaches and assumptions. Ecol App 5(4): 1069-1083

Yang J, Bhalla K, Kim CN, Ibrado AM, Teng TI, Jones DP (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275: 1129-1132

Zar JH (1996) Biostatistical Analysis. 3rd edit, Prentice Hall, New Jersey, USA, 662 pp

Zhai QH, Ji M, Zheng Z, Yu X, Sun L, Liu X (2000) Copper induces apoptosis in BA/F3 cells: Bax, reactive oxygen species and NF_k B are involved. J Cell Physiol 184: 161-170

Zhao Y, Wu M, Shen Y, Zhai Z (2001) Analysis of nuclear apoptotic process in a cell-free system. CMLS (Cellular and Molecular Life Sciences) 58: 298-306

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