THE INFLUENCE OF MINE WASTE CONTAMINATION ON INVERTEBRATES AND FISH IN THE METHOW RIVER VALLEY, OKANOGAN COUNTY, WASHINGTON (U.S.A.)

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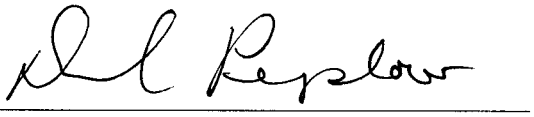
Signature 

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Abstract

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A study of mine-waste contamination effects on Methow River habitat on the eastern slopes of the north Cascade Mountains in Washington state, U.S.A., revealed trace element impacts on invertebrates and fish. Ore deposits in the area were mined for gold, silver, copper and zinc until the early 1950's, but the mines are now inactive. The trace elements arsenic (As), cadmium (Cd), copper (Cu), lead (Pb), and zinc (Zn) were the focus of this study.

The objectives of this study were to determine, (1) the concentrations of Cu, As and other trace elements in sediments and water in the Methow River above and below the Alder Mine, Red Shirt Mill and Alder Mill, (2) whether *in vivo* exposure of caddisfly larvae to Cu and As in microcosm chambers containing contaminated water and sediments induced 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 triploid trout to Cu and As in water or sediments in the Methow River induces the formation of nuclear apoptosis and electron-dense submitochondrial granules in gut epithelial cells and hepatocytes, (4) whether the composition of the electron-dense granules relates to exposure, and (5) the effects of trace element contamination in the Methow River on the growth and developments of caddisfly larvae (*Ecclesomyia spp.*) and trout (*Oncorhynchus mykiss*).

Electron microscopy was used to observe the effect of trace element contamination from mine waste on invertebrates and fish in the Methow River. An above-and-below-mine approach was used to compare exposed and reference populations.

In this study, I 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. The trace elements Cu, As, Cd, Mn, and Pb were significantly higher in Methow River sediments below the mines compared to the reference area above the mines. No effects from mine waste contamination on dissolved metal concentrations in the Methow River were observed. Food chain effects resulting from mitochondrial collapse and the diversion of energy coincides with reduced growth and delayed development in caddisfly larvae and trout in the Methow River. Submitochondrial granules were induced in the mitochondria of live caddisfly larvae and trout exposed *in vivo* to abandoned mine waste contamination in stream water, sediments, and periphyton. The incidence of submitochondrial granules was significantly higher in caddisfly and trout exposed to abandoned mine waste in both controlled microcosms and to contaminants *in situ* in the Methow River below abandoned mines. Elemental analysis of submitochondrial granules by X-ray analysis suggest that bioavailable forms of Cu are present at high concentrations in the environment surrounding the organism, its cells and the mitochondria and in small intestine epithelial cells and hepatocytes 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 suggest both apoptosis and mitochondrial failure are occurring.

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DEDICATION

This is dedicated to the memory of Raymond C. Franklin and to Etta M. Franklin-Peplow, J. Baylie Peplow and Casey S. Peplow.

CHAPTER 1

INTRODUCTION

Mining is one of the oldest activities of human civilization dating back to the Stone Age. Even in our modern society, demands for mining products continue to grow. However, mining often occurs in environmentally sensitive areas where contamination from operating and abandoned mines can cause severe environmental impact. The apparent disregard for the environmental implications has led opponents to characterize mining as a "robber economy." Vernon LaMotte, the mining engineer at Alder Mine from 1939 to 1940 in the Methow Valley in Okanogan County, Washington, described mining as a "liquidation business." In his memoirs, *Stories of the Methow,* LaMotte (1997) noted, "mineral deposits are finite resources that, because they are nonrenewable, come to an end when the deposit is exhausted." Mining also strongly influences local economies and when mines cease operation communities may die.

People were aware of the environmental effects of mining long before the 1960's when Rachel Carson's *Silent Spring* was published (Carson 1962), before strong environmental laws were put in place in the 1970s, and before the 1980's when analytical methods were developed that allowed analysts to determine low concentrations of multiple metals in a single sample. For example, the response of aquatic organisms to toxic water pollution from abandoned mines was recognized over 150 years ago (Davis 1995), and the effect of heavy metals in streams on benthic invertebrates has been noted since the early 1900's (Carpenter 1924). Early research assessing the biological condition of rivers polluted by mine effluent started in 1919 in Wales concurrent with the cessation of lead (Pb) mining in the Aberystwyth district of Cardiganshire. Five years later it was reported that the river Ystwyth was generally barren, except for algae, in comparison to reference streams (Carpenter 1924). Newton (1944) also described the destructive effects of zinc (Zn) pollution from abandoned mines on the same river. Although metalliferous hardrock mines only operate on average from 5 to 15 years until the minerals are

depleted, contamination can occur for hundreds or even thousands of years following the cessation of mining. Contamination of the Rio Tinto estuary in Spain, for example, has occurred due to mining of massive sulfide deposits that began with the Tartessans who developed the first mine over 5000 b.p. (Davis et al. 2000, Palanques et al. 1995).

Mining and ore processing change the topography, hydrogeology, and chemistry of terrestrial and aquatic ecosystems. In metalliferous mining, high volumes of waste are produced because of the low concentrations of metals in the ore. For example, gold (Au), which is commercially viable at less than one-half ounce per ton, creates a huge volume of waste when mined. Mine waste historically has been disposed of at the lowest cost by creating heaps of mine spoils on site. Depending on the parent rock, the mineral being mined, and the extraction methods used, mine spoil heaps can either be inert or contain hazardous constituents.

Pyrite oxidation, a major source of contamination from abandoned mines, is accelerated in mine spoils due to the increased access of air, smaller particle sizes that result from the mining and milling process, the presence of iron-sulfide oxidizing bacteria, and the frequent saturation of mine spoils by infiltrating meteoric water. Acid rock drainage (ARD), or acid mine drainage (AMD), is extremely acidic because pyrite oxidization gives rise to sulfuric acid. ARD accelerates weathering and when surface water infiltrates a mine spoil heap it can leach out soluble salts that contain toxic trace elements at concentrations as high as 0.1 mg L^{-1}(Plumlee et al. 1999).

Many communities in the western United States that are affected by ARD had their origins in the mining boom of the late 1880's. Although mining ceased to be a major economic force after the 1930's, current issues related to the contamination of groundwater, and fish and wildlife habitats is forcing local residents and State and Federal regulatory officials to inventory abandoned mine lands (AML), assess the risk associated with ARD, and remediate sites that pose the greatest risk to environmental health. The precise number of AML sites and the scale of the environmental problem is unknown because a complete inventory, which would take

years to complete, does not currently exist. In the absence of a complete inventory, the USGS has implemented an AML initiative to coordinate activities for the cleanup of Federal Lands affected by ARD. The purpose of the AML initiative is to identify watersheds that would be at greatest risk of environmental degradation from ARD.

In Washington State, most AML occur in environmentally sensitive areas of the Cascade Mountains and Okanogan Highlands of northeastern Washington. Since Hiram Smith, a Washington State legislator, discovered gold near Chopaka Mountain in Okanogan County in 1871, there have been thousands of prospects and mines established in Washington State. Most of the mines were small, unregulated and in operation prior to the 1930's.

Although not included in the AML, the Methow River watershed in the Okanogan County is a watershed at risk of environmental degradation from numerous abandoned mine sites including the Alder Mine, Alder Mill, and Red Shirt Mill, which contain tailings, waste-rock piles, and tunnels that discharge ARD (Peplow 1998). The contamination of a domestic well by waste water and tailings from the abandoned Alder Mill, which ceased operations in 1952, was reported by Spencer (1986) who noted a potential for groundwater contamination. Targets related to groundwater exposure pathways were documented, and a potentially affected human population, estimated to be between 1000 and 1287 residents, was identified within a 6.5-km (4-mile) radius of the Alder Mine and Alder Mill.

As well as the concern about the human population, there is also concern that stream invertebrates and fish could be influenced by ARD from the three sites. I conducted a survey by direct underwater observation (snorkeling) on September 4, 1998 to identify salmonids in the Methow River below the abandoned Alder Mine, Alder Mill and Red Shirt Mill. The species identified during the survey were native steelhead/rainbow (*Salmo gairdneri)* and Chinook salmon (*Oncorhynchyus tshawytscha).* Upper Columbia River summer steelhead, including the Methow river run, were listed under the Endangered Species Act (ESA) as

"endangered" on August 18, 1997. Upper Columbia River spring Chinook salmon, including the Methow River run, were listed under the ESA as "Endangered" on March 16, 1999. Bull trout in the Methow River were listed under the ESA as "threatened" on June 10, 1998. Although not an ESA listed species, summer Chinook also spawn in the Methow River and have experienced a severe decline in numbers of returning adults. Summer Chinook are identified as "depressed" by the Washington Department of Fish and Wildlife. Spawning sites that occur at the junction of Alder Creek and the Methow River have been monitored by the Yakima and Colville tribes and the Chelan County P.U.D. since 1967.

In 1980, the Northwest Power Planning Council (NWPPC) was directed by act of Congress to protect, mitigate and enhance fish and wildlife populations that have been affected by the construction and operation of hydroelectric dams. The NWPPC Fish and Wildlife program recognized the potential impact of abandoned mines on fish habitat and linked the Endangered Species Act and the Clean Water Act by describing the kind of ecological change needed to improve the survival and productivity of endangered fish populations.

The most common contaminants found in the vicinity of mining areas are As, Cd, Cr, Cu, Pb, Mn, Hg, and Zn (Roberts 2001). This study focused on the trace elements As, Cd, Cu, Pb, and Zn from the abandoned Alder Mine, Red Shirt Mill and Alder Mine and their effects on fish and invertebrates in the Methow River ecosystem south of Twisp. The objectives of this study were to determine, (1) the concentrations of Cu, As and other trace elements in sediments and water in the Methow River above and below the Alder Mine, Red Shirt Mill and Alder Mill, (2) whether *in vivo* exposure of caddisfly larvae to Cu and As in microcosm chambers containing contaminated water and sediments induce 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 triploid trout to Cu and As in water or sediments in the Methow River induces the formation of nuclear apoptosis and electron-dense submitochondrial

granules in gut epithelial cells and hepatocytes, (4) whether the composition of the electron-dense granules relates to exposure, and (5) the effects of trace element contamination in the Methow River on the growth and developments of caddisfly larvae (*Ecclesomyia spp.*) and trout (*Oncorhynchus mykiss*), .

This dissertation contains 4 chapters. Chapter 2 contains descriptions of the three sites including locations, soils, vegetation, climate and sampling station locations. A discussion and review of literature related to the biogeochemistry and toxicity of Cu and As is presented in Chapter 3. In Chapter 4, I present water and sediment chemistry data from above and below the mines, and data on the effects of trace element contamination on growth and development of caddisflies and trout. I also present cytological evidence that exposure to trace elements in water and sediments induces the formation of electron-dense granules in mitochondria and nuclear apoptosis in caddisfly larvae and trout exposed *in vivo* to mine waste contamination. Quality assurance and quality control (QA/QC) data are presented in Appendix 1.

CHAPTER 2

STUDY SITES

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^2, 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: Alder Mill, Alder Mine, and Red Shirt Mine (Figures 1 and 2).

Alder Mill

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 (Figures 1 and 2E) 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 groundwater in the tailings impoundment was 3.0 ± 0.4.

Alder Mine

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 (Figure 2A, B, C, D). The site is on the north slope of a north-trending ridge. Slopes at the site range from 50-80%.

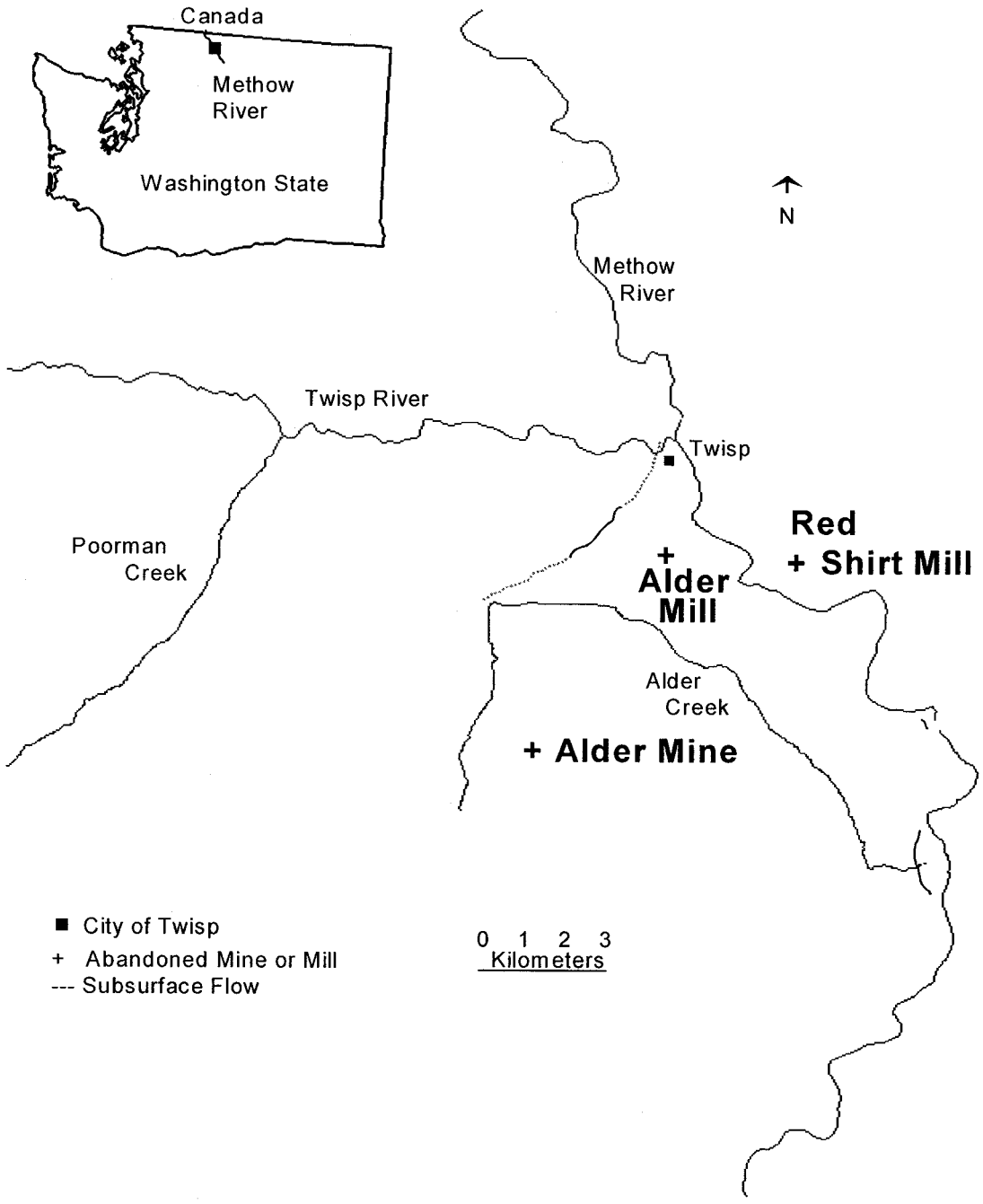

Figure 1. Map showing study area and the abandoned mine and mill sites.

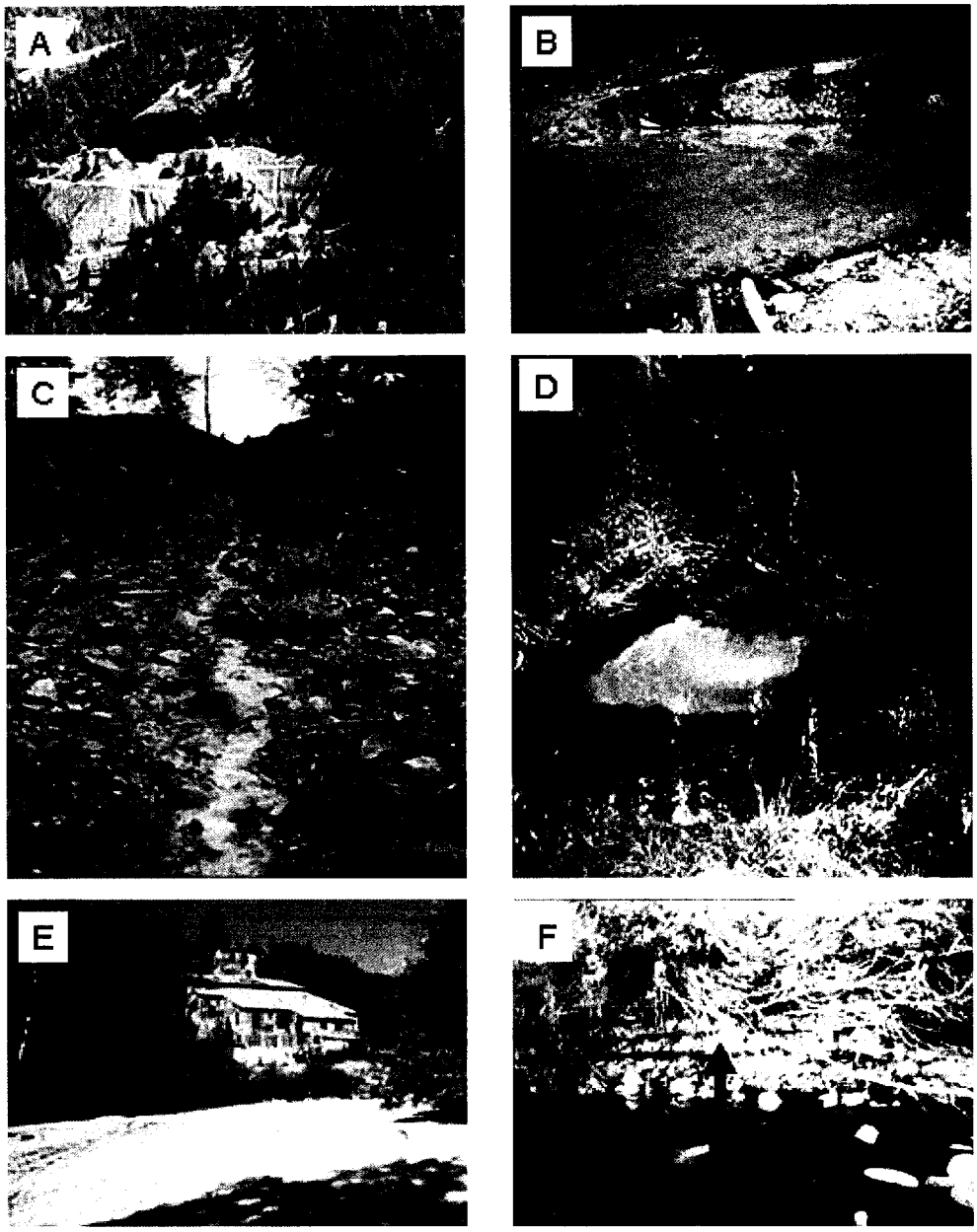

Figure 2. Disturbance at study sites and sources of contamination affecting environmental health. (A) Alder Mine from southeast showing glory hole at top and waste rock covering approximately 16 ha, (B) Acid mine drainage from adit at Alder Mine, (C) malachite precipitation in channel below waste rock pile at Alder Mine, (D) Acid rock drainage entering Alder Creek at upwelling resulting in precipitation of Cu as malachite, (E) Alder Mill with tailings pile in foreground, and (F) Lens of tailings in bank of Methow River at the Red Shirt Mill.

Estimates from aerial photographs indicate that waste rock covers approximately 3.2 ha (USEPA 2000). The flow rate of drainage from the north adit ranges from 5-15 L min^{-1}. The pH of AMD discharged at station 1 was 2.9 $\pm$ 0.2 and subsurface samples from stations 2-4 were 4.2 $\pm$0.1.

Red Shirt Mill

The Red Shirt Mill (48°.21'.05.0"N, 120°.06'.08.1"W, elev. 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 (Figure 2F). The tailings pile is estimated to cover approximately 4,650 m^2 of surface area that extends to the bank of the Methow River and contains less than 30,600 m^2 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 groundwater wells are located on and adjacent to the site.

Topography, Soils, Climate, Hydrology and Vegetation

Topography within the Methow River basin ranges from mountainous terrain along the Cascade Crest to a gently sloping, wide valley found along the middle reaches. Elevation ranges from 2600 m in the headwaters of the basin to approximately 240 m at the confluence of the Methow and Columbia Rivers (Andonaegui 2000). Soils in the valley consist of sandy loams that are underlain by alluvium and glacial outwash with very rapid permeability (Waitt 1972). The major groundwater aquifers of the Methow Valley exist in layers of unconsolidated sediments underlain by bedrock. Groundwater occurrence, movement and availability are primarily related to recharge sources and the configuration of depositional sediments.

The climate in the Methow Valley is influenced by the Cascade Mountain rain shadow. Mean annual precipitation ranges from 25 to 38 cm and the mean annual temperature is below

10°C (USFS 1999). Precipitation is seasonal with roughly two-thirds occurring between October and March. Summers are generally hot and are characterized by extended dry periods. Precipitation increases in the fall and generally peaks in the winter with most precipitation in the basin occurring as snow between December and February. Since most of the precipitation occurs as snow, the seasonal distribution of runoff is strongly affected by snow storage.

Flows in the Methow River exhibit a strong peak during spring and early summer with roughly 60 percent of the mean annual discharge occurring during May and June (Milhous 1976). Streamflow remains relatively high during July, but decreases substantially from August through October in response to a reduced snowpack, low precipitation, and decreased soil moisture. Streamflow in the Methow River at Pateros generally reaches an annual low during late September and early October, with some sections going subsurface during dry years. Winter flows typically remain low in response to low autumn precipitation and freezing winter temperatures. Runoff between years is also highly variable as reflected in USGS streamflow data. Maximum and minimum flows for the Methow River at Twisp was 1359 m^3 (May 1948) and 4 m^3 (September 1926), respectively. Alkalinity was 103 ± 14 mg $CaCO_3$ L^{-1} and pH was 7.2 ± 0.5.

Vegetation at the site is characterized by Douglas-fir (*Pseudotsuga menziesii*) and ponderosa pine (*Pinus ponderosa)*, which dominate the overstory. Pinegrass (*Calamagrostis rubescens*) dominates the understory to the extent that other species are inconspicuous. Shrubs are normally a minor component of the stand. Soil texture is sandy loam to sand and the parent material is granitic rock. Soils are spodosols.

Aquatic and Terrestrial Vertebrates

The stream and rivers in the area are utilized by resident trout and salmon for habitat and spawning. Beaver ponds and cattail marshes provide nesting sites for waterfowl, game and songbirds. Endangered and threatened species of juvenile salmonids, including bull trout,

steelhead, and Chinook salmon use the Methow River and Alder Creek as rearing habitat. Two amphibians, the Pacific treefrog (*Hyla regilla*) and the spotted frog (*Rana pretiosa*) were observed in the study area. Blackbear (*Ursus americanus*), muledeer (*Odocoileus hemionus*), snowshoe rabbits (*Lepus americanus)* and the bobcat (*Felis americanus*) were also observed in the study area. Unidentified species of bats have also been observed leaving the mine adits.

Water quality is considered to be good at the confluence of Alder Creek and the Methow River near the town of Twisp. Spring Chinook, Summer Chinook, Steelhead/Rainbow and Bull trout have been found to occur in the Methow River and a survey in a side channel of the Methow River below its confluence with Alder Creek, conducted by direct underwater observation (snorkeling), identified native steelhead/rainbow trout and Chinook salmon. Two redds in the Methow River at the Red Shirt Mill were identified on 10/10/00 and 10/23/00. Two parr (presumably coho) were observed on 1/27/01 in the last pond on Alder Creek after ice melt and before water levels were sufficiently high to provide outlet.

CHAPTER 3

REVIEW OF LITERATURE ON BIOGEOCHEMISTRY OF COPPER AND ARSENIC

Introduction

The weathering of rocks associated with mines that contain sulfide minerals generates acid rock drainage (ARD) containing dissolved metals and sulfate (Paulson and Balistrieri 1999). The geology of the mineral deposit, climate, hydrology and biogeochemical processes determine the composition of natural waters draining sulfide mineral deposits (Plumlee 1999). Acid rock drainage can be entirely natural or enhanced by mining activities. Mining and mineral processing accelerate the weathering process by increasing the exposure of sulfide minerals to air, which accelerates the oxidation and the production of ARD. It should be noted that not all drainage from sulfide mineral deposits is acidic nor must be acidic to transport dissolved metals. This review will focus on the biogeochemistry of copper (Cu) and arsenic (As) found in ARD from hydrothermal sulfide-mineral deposits and waste piles. Other ions, pH and redox conditions will also be discussed when they are relevant to the description of Cu and As biogeochemistry.

Massive Sulfide Deposits

Most sulfide minerals are associated with igneous intrusions that release high-temperature gasses and fluids. These hydrothermal fluids travel long distances through host rocks, react with minerals they encounter, and cause metamorphism. At lower temperatures and pressures, sulfide minerals are formed from hydrothermal solutions. Upon cooling, ore deposits are formed that contain minerals that are thermodynamically unstable in oxidizing environments, are in excess of their normal abundance, and occur at concentrations suitable for extraction by commercial mining operations. Elements that have an affinity for sulfur and are contained in hydrothermal deposits include arsenic (As), cadmium (Cd), gold (Au), lead (Pb), mercury (Hg), molybdenum (Mo), silver (Ag), tin (Sn), and zinc (Zn) (Foster 1969). Trace elements that exist in sulfide phases typically alter to a variety of secondary minerals (Nordstrom 1982).

Oxidation of Metal Sulfide Rocks

Mining exposes metal sulfide minerals in waste rock dumps and tailing heaps to conditions in which they are unstable. Chemical weathering occurs when minerals are exposed to water and oxygen at the earth-atmosphere interface. Pyrite is oxidized to sulfuric acid and soluble iron sulfates, all of which promote the dissolution of other sulfide minerals. The presence and concentrations of individual elements in drainage waters reflect the abundance of that element's metal sulfide mineral in the deposit drained by the water (Plumlee 1999).

Although the solubility and simple dissolution of sulfide minerals in water is generally low, they are unstable in the presence of oxygen and ferric iron (Moses et al. 1987). After the reaction sequence is initiated (Table 1, Reaction 1), a cycle is established in which Fe^{2+} is oxidized by oxygen to Fe^{3+}, which is subsequently reduced by pyrite and results in the generation of more Fe^{2+} (Table 1, Reactions 2 and 3). The oxidation of iron pyrite (FeS_2) and the release of acidity into water draining mine waste is illustrated in Table 1, Reactions 4 and 5. Only pyrite produces acidity during oxidation and not the other metal sulfides (Table 1, Reaction 10).

Singer and Stumm (1970) studied the relative rates of pyrite oxidation by oxygen and ferric iron. The reduction of Fe^{3+} by pyrite, both in the presence and absence of oxygen, was rapid (e.g., 50 minutes to reduce the initial ferric iron concentration 50%) whereas in the absence of ferric iron, no oxidation of pyrite was observed after 1 week. Their conclusion was that the major oxidant of iron pyrite is ferric iron as indicated in the propagation cycle, which are depicted in reactions 2 and 3 (Table 1). Laboratory studies by Singer and Stumm (1970) also showed that the rate of oxidation of Fe^{2+} by oxygen in abiotic systems was slow compared to the rapid oxidation of pyrite by ferric iron (e.g., 1000 days to reduce the ferrous iron concentration 50%). Since reaction 2 (Table 1) is so much slower than reaction 3 it is considered to be the rate limiting step in the pyrite and ferrous iron oxidation cycle.

Table 1. Some important reactions that control oxidation and reduction of pyrite, precipitation of secondary phases, neutralization, and adsorption.

(1) $FeS_2(s) \xrightarrow{(+O_2)} Fe^{2+} + \text{S-compound}$

(2) $Fe^{2+} + O_2(aq) \longrightarrow Fe^{3+}$

(3) $Fe^{3+} + FeS_2(s) \longrightarrow Fe^{2+} + SO_4^{-2}$

(4) $FeS_2(s) + {}^7/_2O_2(aq) + H_2O \longrightarrow Fe^{2+} + SO_4^{2-} + 2H^+$

(5) $FeS_2(s) + 14Fe^{3+} + 8H_2O \longrightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$

(6) $Al(OH)_3(s) + 3H^+(aq) = Al^{3+}(aq) + 3H_2O(l)$

(7) ${}^1/_2Al_2Si_2O_5(OH)_4(s) + 3H^+(aq) = Al^{3+}(aq) + Si(OH)_4^0(aq) + {}^1/_2H_2O(l)$

(8) $CaCO_3\,(calcite) + 2H^+(aq) \longrightarrow Ca^{2+} + H_2O + CO_2$

(9) $CaCO_3\,(calcite) + CO_2 + H_2O \longrightarrow Ca^{2+} + 2HCO_3^-$

(10) $ZnS(s) + 2O_2 \longrightarrow Zn^{2+} + SO_4^{2-}$

(11) $FeS_2(s) + {}^{15}/_4O_2(g) + {}^7/_2H_2O(l) \longrightarrow Fe(OH)_3(s) + 2SO_4(aq) + 4H^+(aq)$

(12) $Fe^{3+} + 3H_2O \longleftrightarrow Fe(OH)_3(s) + 3H^+$

Bacterial Catalysis of Oxidation and Reduction Reactions

Field investigations of the oxidation of Fe^{2+} in mine waters indicate the rate of this reaction is more rapid than laboratory studies at the same pH (Singer and Stumm 1970). Although several agents have been shown to catalyze the oxidation of Fe^{2+}, microorganisms appear to exhibit the greatest effect in accelerating the oxidation of ferrous iron. Comparisons between the rates of oxidation of Fe^{2+} after inoculation with sterilized and non-sterilized mine water showed that microbial activity accelerated the reaction by a factor larger than 10^6.

According to the biological continuum concept, for any given parameter or group of parameters, a microbial species or group will grow within a range defined by minimum and maximum levels. The optimal ranges over which different microbes grow vary, overlap and form a continuum. The distribution of microbes on this continuum fits a normal distribution curve with most microbial growth occurring in a restricted range and with limited growth occurring in the tails that represent lower and upper extremities. Most organisms important to biogeochemical processes exist in these extremities. Tailings are often heterogenous mixtures of rocks, gravel, coarse- to fine-grained sand, silt and clay and the complex geochemical nature of the environment is paralleled by an equally complex microbial community.

Bacterial Oxidation of Reduced Sulfur and Ferrous Iron

Thiobacillus, Thiomicrospira, and Thiospera are three genera of colorless sulfur bacteria that are characterized by their ability to gain energy from the oxidation of reduced sulfur compounds. Some members of this group can also use other inorganic sources of energy including hydrogen, ferrous iron and other reduced metal ions (Kuenen et al. 1992). The ability of these organisms to use organic compounds for carbon or energy varies. Most strains of colorless sulfur bacteria are able to grow autotrophically. Many are obligate chemolithotrophs and are able to use only carbon dioxide as their carbon source. Some are facultative chemolithotrophs that are physiologically versatile and can grow autotrophically,

heterotrophically, or mixotrophically and can use either inorganic or organic compounds simultaneously for energy and carbon. A small number are chemolithoautotrophs because they derive their energy from the oxidation of reduced inorganic compounds but require an organic source of carbon because they lack the Calvin cycle necessary for carbon dioxide fixation.

All species in the three genera are respiratory and are able to use oxygen as their terminal electron acceptor (Kuenen et al. 1992). Wielinga (1999) described the distribution of bacteria and found large populations in the anoxic zone at and below the groundwater table. Although sulfur- and iron-oxidizing bacteria have been found at or below the anoxic groundwater interface they are widely distributed throughout tailings piles and often overlap with sulfate-reducing bacteria that are generally found in oxic zones containing high concentrations of oxidizable metals (Lovley 1991, Wielinga 1999). Traditionally, it has been thought that Fe^{3+} and SO_4^{2-} reduction occurred only after other, more energetically favorable electron acceptors such as O_2, NO_3^-, and Mn(Iv) were depleted. However, it has been shown that sulfate reduction can occur in oxic environments (Canfield and Marais 1991, Frund and Cohen 1992, Hastings and Emerson 1988, Moser and Nealson 1996). Miller et al. (1987) and Jorgensen (1977) observed both sulfur-oxidizing and sulfate reducing bacteria in the same zone and suggested the existence of microniches to explain the phenomenon.

Of central importance to sulfur and iron oxidation is the simultaneous presence of an electron donor (i.e., reduced sulfur, ferrous iron) and an electron acceptor (i.e. oxygen or nitrogen oxides). Since sulfides are present in at least minor amounts in most rocks, the oxidative dissolution of sulfide minerals is ubiquitous in chemical weathering. When ore bodies containing pyrite (FeS_2), the most abundant sulfide in the earth's crust, are exposed to oxygen and water by mining, the result is often the production of acid rock drainage (ARD) containing sulfuric acid and ferric iron (Langmuir 1997). Ferric iron also interacts with sulfides to form sulfuric acid and ferrous iron and microorganisms that oxidize Fe^{2+} to Fe^{3+} accelerate the

reaction. Because of this, pyrite dissolution is generally controlled by the activity of iron oxidizing bacteria that grow in the narrow zone and gradient where sulfide and oxygen coexist (Schrenk et al. 1998).

Bacterial Reduction of Sulfate and Ferric Iron

There is a progression of redox changes with depth in stratified environments like soils, sediments, and microbial mats. Organic matter is oxidized using different electron acceptors that are consumed in order of decreasing redox potential. This begins with the depletion of oxygen, followed by the reduction of nitrogen oxides, Mn(IV), Fe(III), and ending with the reduction of sulfate to produce sulfide and CO_2 to produce methane (Langmuir 1997). Bacteria that couple the oxidation of organic matter to the reduction of sulfate are important to the biogeochemical cycling of iron and trace elements in the pore water of mine tailings. Ferric oxides strongly absorb and coprecipitate toxic trace elements and there is a lot of evidence to suggest that trace elements are released as a result of Fe(III) reduction (Lovely 1991).

Types of Fe(III) reducing bacteria include those that catabolize fermentable sugars and amino acids under anaerobic conditions, as well as sulfur, hydrogen, organic acid and aromatic compound oxidizing Fe-reducers (Lovely 1991). Natural and contaminating organic compounds are oxidized by Fe(III) reducing bacteria (dissimilatory iron-reducers) that obtain energy for growth by coupling the complete oxidation of organic matter to carbon dioxide with an enzymatic reduction process that utilizes Fe(III) as the sole electron acceptor. Below the zone of metal reduction (e.g., iron) the next major reduced species to appear in pore water is sulfide (Langmuir 1997, Nealson and Stalh 1997). Sulfate is generally stable unless reduced biologically and probably no abiotic sulfate reduction occurs in sediments (Nealson and Stahl 1997). In contrast to ferric iron oxide reduction that releases trace elements, sulfate reducing bacteria, when active, may decrease the concentration of free metal ions that would otherwise be available in the dissolved form (Wielinga et al. 1999).

Neutralization by Carbonates and Other Minerals

Mine-drainage and natural-drainage waters can span a broad range of pH values (Plumlee et al. 1999, Nordstrom et al. 2000). Waters with less extreme pH values can be the result of buffering reactions with $Al(OH)_3$ and aluminosilicate minerals in the deposit host rocks (Table 1, Reactions 6 and 7) (Sposito 1989). Another factor that may affect drainage pH in some deposits is the presence of carbonate minerals (e.g., calcite, Table 1, Reactions 8 and 9). Mine waters that react with carbonate-bearing host rocks can have near neutral pH values concurrent with elevated levels of Cu, Zn, and Cd (Plumlee 1999).

Mineralogical Changes Following Sulfide Mineral Oxidation

Sulfide minerals are strongly oxidized in the upper leached zone of the tailings pile. The sulfide particle shrinks due to dissolution by an oxidation process that has been described as the "shrinking particle" model (Lin 1996). The shrinking particle is comprised of an outer original particle surface, a shrinking unreacted core on the inside and a moving surface in between. Backscattered electron images show unaltered pyrite and pyrrhotite grains in the core and geothite that precipitates as a cement in bands in the reaction rim. Optical microscopy and SEM/EDS analyses reveal pyrrhotite remnants showing wide alteration rims in contrast to pyrites that showed only narrow alteration rims. It was also shown that the intensity of alteration decreases with depth, i.e., the alteration rims are narrower in the accumulation horizon as compared to the leached horizon.

Cu and As Speciation

The mobility, bioavailability and toxicity of trace elements depend on their reactivity and solubility, which are determined by their speciation. Speciation refers to (1) element identity, (2) oxidation state, (3) physical state (i.e., liquid, gas, solid, colloid, inorganic or organic complex), (4) molecular formula, and (5) molecular structure. A major environmental variable that determines the speciation of trace elements in water is pH. For example, Figure 3 shows that as

pH drops below approximately pH 10 or 11, metals such as Cu, Zn, Cd, and Fe tend to increase their solubility in water exponentially (Sylva 1976). A possible explanation for the increase in solubility of Cu, Zn and Cd is related to carbonate speciation and a corresponding increase in the K_{diss} for metal carbonates. Brown et al. (1999) also observed metals movement at high pH (> 7.0) where there was an increase in the movement of Pb, Zn and Cu in limed high-metal biosolids, due possibly to the formation of complexes with organic ligands (e.g., fulvic acids).

Another major environmental variable that determines the chemistry and solubility of elements such as Cu and As in surface and groundwater is oxidation and reduction (redox). All redox reactions involve an oxidizing agent, which is a substance that accepts electrons, and a reducing agent, which is a substance that donates electrons (Sposito 1989). Eh of a solution, measured as the electrical potential, is a measure of the oxidizing or reducing tendency. A large number of electrons would create a reducing environment and in the absence of electrons it would be an oxidizing environment. The pE, the negative common logarithm of the electron concentration, is sometimes used in lieu of Eh (Sposito 1989, Langmuir 1997). pE is related to Eh by the expression:

pE = [Faraday Constant / 2.3 x R (Gas Constant) x T (Absolute Temperature)] x Eh

Brookins (1988) published a reference book of Eh-pH diagrams for 75 elements including Cu and As. Large sets of thermodynamic data were used to create generic Eh-pH diagrams using Gibbs free energy data at standard temperature and pressure from the National Bureau of Standards compilations and the US Geological Survey. Eh-pH diagrams for other temperatures can also be calculated. For many reactions, changes in pressure do not introduce significant errors in the Eh-pH boundries. If reactions are assumed to take place at essentially atmospheric

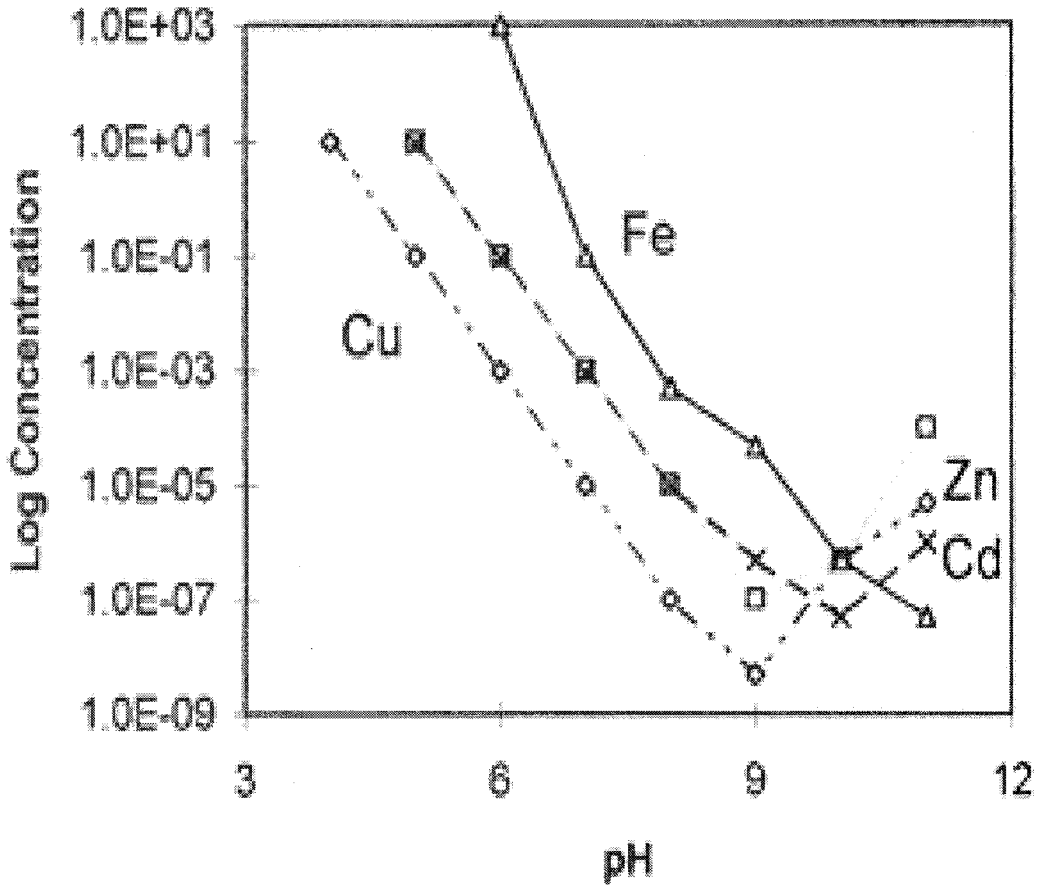

Figure 3. The effect of pH on the solubility of dissolved Cu, Zn, Cd and Fe (Sylva 1975).

conditions (about 25°C and one atmosphere pressure), then Eh-pH stability diagrams can be useful in characterizing chemical systems. The following examples using Cu and As will serve to illustrate this point:

Copper

Chalcocite (Cu_2S) and chalcopyrite ($CuFeS_2$) are the most common copper ores. Copper also occurs in malachite [$Cu_2CO_3 \cdot Cu(OH)_2$], azurite [$2CuCO_3 \cdot Cu(OH)_2$] and cuprite Cu_2O. Copper concentrations in soil vary from 2 to 100 mg kg^{-1} (Aeseth and Norseth 1986). Freshwater Cu concentrations range from <1 to >10^6 μg L^{-1} in natural waters draining unmined mineralized areas (Plumlee 1999). In mine drainage waters the concentration of Cu can range from <1 to approximately 5 mg L^{-1}.

Copper can exist in two oxidation states (+1 and +2). In igneous rocks Cu is usually present as the univalent ion, but like other metallic elements, Cu also exists in minerals in the +2 oxidation state (Aeseth and Norseth 1986). Cuprous copper (Cu^+) is unstable in most freshwater environments and undergoes auto-oxidation to Cu^{2+}. Metallic Cu is not oxidized in pure water, but sulfuric acid dissolves the metal into cupric copper (Cu^{2+}) sulfate. Hydrolysis and precipitation reactions dominate the chemistry of Cu(II) at pH values encountered in most freshwater systems (Sylva 1976).

There are three solid phases for Cu: sulfides , carbonates, and hydroxides. Sulfides and carbonates are important in controlling Cu ion solubility. Precipitation of hydroxides, sulfides, and carbonates occurs when the K_{sp} is exceeded. In the presence of free oxygen (a positive Eh) dissolved Cu^{2+} is stable at pH values of less than approximately 6-7 (Figure 4). With increasing pH, first carbonate and then the hydroxide solids are formed and become the stable phase. In aerated waters at pH 7-8, copper carbonate is the stable phase. For negative values of reduction potential (Eh), the sulfide remains stable over a wide pH range.

The most efficient process by which free Cu(II) is removed from the system is the precipitation of malachite. It appears that pH has a greater effect on malachite precipitation than bicarbonate concentration. The malachite stability range begins at pH values greater than about 6 (Figure 4). Adsorption of free Cu by sand, gravel and suspended clay and colloidal Fe also occurs at pH values greater than about 6.5 (Sylva 1976).

In soils and sediments, however, Cu is readily adsorbed or fixed making it one of the trace elements that moves the least. Total Cu includes six pools: (1) soluble ions, (2) exchangeable Cu, (3) stable organic complexes, (4) Cu adsorbed by hydrous oxides, (5) Cu adsorbed on to a clay-humus colloidal complex, and (6) Cu in the crystal lattice of soil minerals (Alloway 1995). Copper is most commonly associated with organic matter, oxides, and minerals. While it appears difficult to separate Cu adsorbed by these compounds, it appears that exchangeable Cu is lowest in the organic fraction.

Arsenic

Arsenic is characterized by a generally low abundance in most rock types and high concentrations in hydrothermal deposits. For example, the average concentration of As in hydrothermal deposits ranges from 0.05 to 10% whereas in unmineralized rocks the average concentration is from 0.3 to 16 mg kg^{-1} (Sparks 2003). Sulfide minerals of arsenic include realgar $As(II)_4S_4$, and orpiment $As(III)_2S_3$.

Arsenic, which belongs to the Group V elements, has 5 valence electrons that determine its chemical behavior. Arsenic can exist in several oxidation states and can undergo oxidation or reduction when it interacts with mineral surfaces or organic compounds, which act either as oxidants or reductants (Brown et al. 1999). Arsenic is stable in four oxidation states (+5, +3, 0, -3) under Eh conditions occurring in aquatic systems (Ferguson and Gavis 1972). Arsenic metal occurs only rarely, As(III) only at extremely low Eh values (Sparks 2003), and As(V)

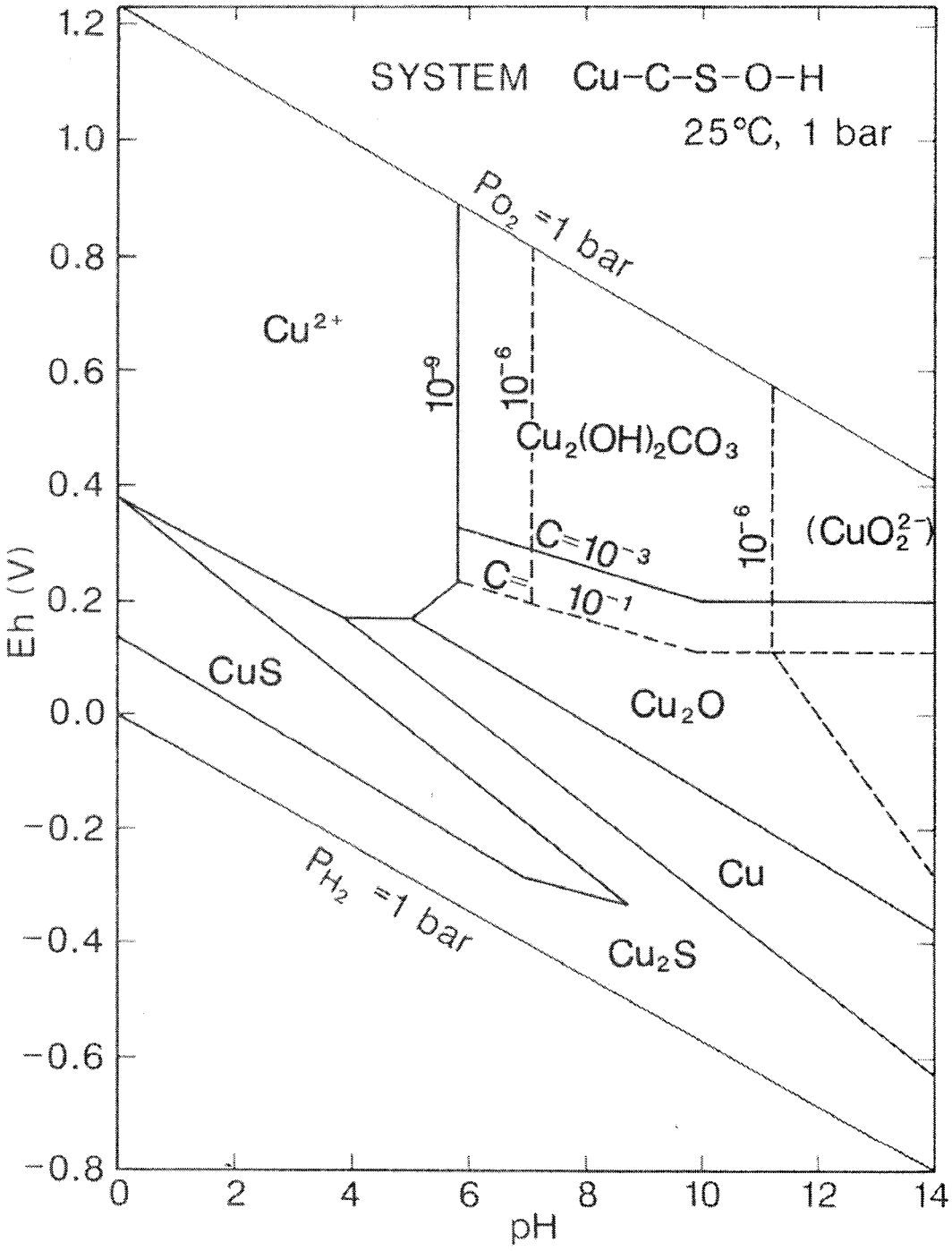

Figure 4. Eh-pH diagram for Copper (Brookins 1988)

occurs in oxidizing environments (Figure 5). Arsenic is characterized by the formation of oxyanionic species (e.g., arsenite [As(III)O_3^{3-}], and arsenate [As(V)O_3^{3-}], which because of their low charge densities as oxyanions form weak bonds with cations that are soluble [Fe(III)As(III)O_3, and Fe(III)As(V)O_3,].

Adsorption

Adsorption is the accumulation of matter at the interface between a solid phase and an aqueous solution phase (Sposito 1989). Surface functional groups that have an electrical charge can adsorb ions. Three types of adsorption to particle surfaces include the formation of (1) inner sphere complexes, (2) outer sphere complexes, and (3) diffuse-ion swarms. The inner-sphere complex mechanism probably involves ionic as well as covalent bonding. The diffuse-ion swarm and the outer-sphere complex mechanisms of adsorption involve, almost exclusively, electrostatic bonding.

Readily exchangeable ions are those that can be replaced easily by leaching with an electrolyte solution with a specific composition, concentration, and pH (Sposito 1989). Ions specifically adsorbed are considered "readily exchangeable" and methods to determine readily exchangeable adsorbed ions must avoid extracting specifically adsorbed ions.

Surface Charge

Solid particles develop electrical charge in two principal ways: either from isomorphic substitutions by ions with differing valence or from the reaction of surface functional groups with ions (Sposito 1989). Different types of surface charge, which can be either positive, neutral or negative, contribute to the net total charge. Permanent structural charge can be created by isomorphic substitution in both primary and secondary minerals. Clay minerals like illite, vermiculite, and smectite can have permanent structural charges up to 100 times greater than from isomorphic substitutions in hydrous oxides.

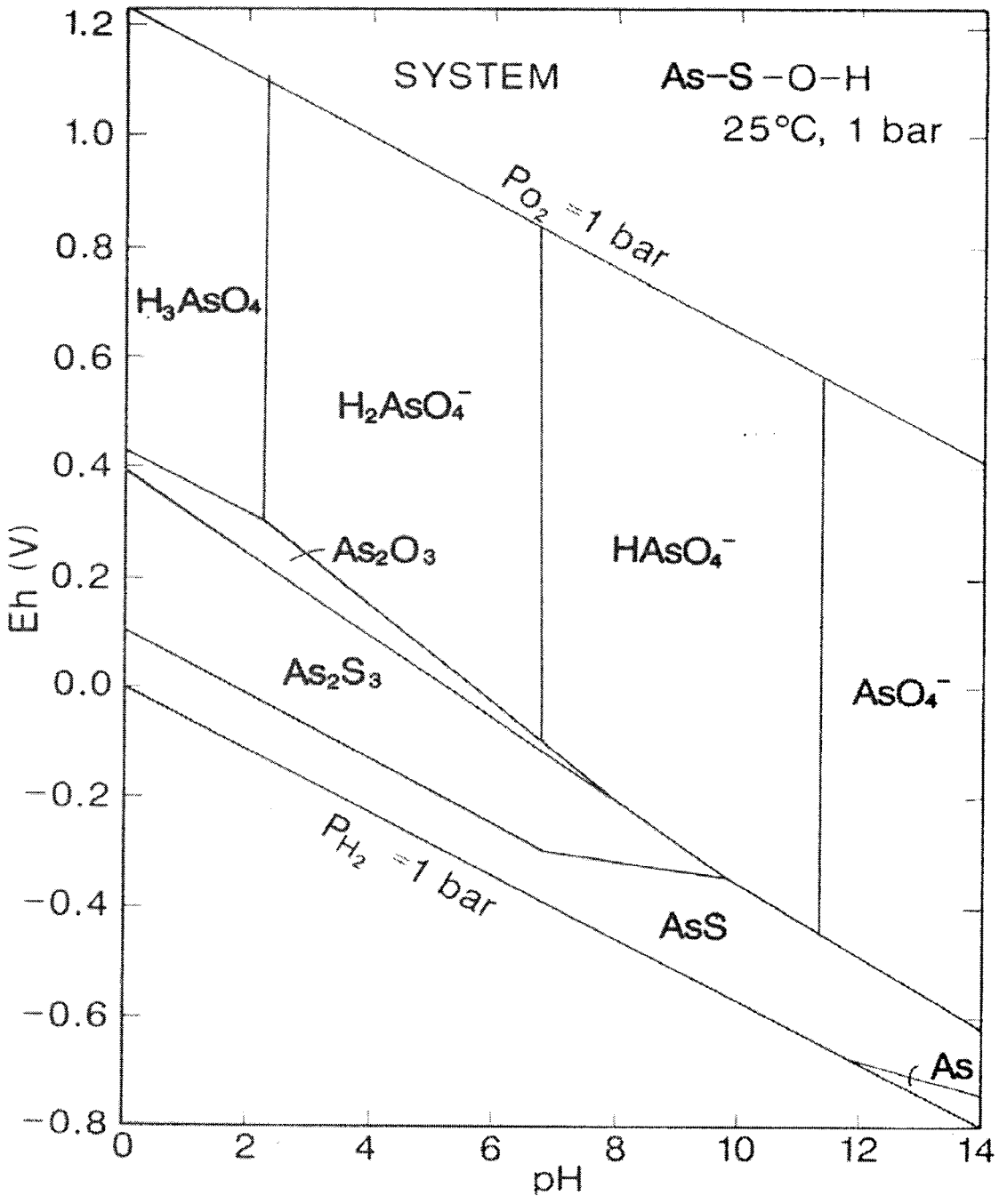

Figure 5. Eh-pH stability diagram for arsenic (Brookins 1988).

Secondary Mineral Coatings

The occurrence of secondary coatings on primary minerals is common, and reactions with the secondary minerals, rather than the primary mineral substrate, probably represents the principal control over the occurrence of trace-elements in pore water. In general, two types of layers characterize the crystalline structure of secondary minerals: Si layers, and layers that are comprised of Al, Fe, or Mg, which are held together by shared oxygen atoms (Sparks 2003). In regards to Fe, it is relatively insoluble and tends to accumulate as amorphous and poorly crystallized oxides known as ferrihydrite (Sposito 1989), which can be quantified by extraction in weak oxalate solution (Tessler 1979). With increasing time, most Fe is found as a crystalline or hydrous oxide, which can be extracted using a reducing solution of citrate-dithionate.

Ion Exchange

In addition to precipitation and coprecipitation, adsorption by amorphous iron oxides occurs when there is an exchange of metal cations and anions with surface ligands. Adsorption of a dissolved ionic species is always part of an exchange reaction that involves a competing ionic species with a lower sorption energy. A sorbent phase with a net negative charge has a cation exchange capacity (CEC), and sorbents with a net positive surface charge have an anion exchange capacity (AEC). Surface charge may be permanent and independent of solution composition, or variable and dependent on solution composition (Langmuir 1997). The sorption energy of an ion depends on (and is proportional to) at least two factors: valency and atomic weight. The sorption energy of hydrogen is higher than divalent cations and competes with other cations even when its concentration is 10 to 100 times less than that of the cations.

Absorptive organic particles and the ions in solution interact to form inner- and outer-sphere complexes. A study that investigated the partitioning of Cu between the particulate and dissolved phases showed that binding to the surfaces of amorphous Fe-oxyhydroxides regulates Cu concentrations in solution (Johnson 1986). Similar results were found in regards to arsenic

mobilization (Cummings et al. 1999). Hydrous ferric oxide strongly adsorbs both As(V) and As(III) and reductive dissolution was found to be the dominant mechanism by which As was released into solution.

The secondary minerals that occur as a result of weathering can have a net negative charge (Sposito 1989). The negative charge has several origins and two will be described here. The first is a result of ionic substitutions within silicate clays (e.g., Mg^{2+} for Al^{3+} in montmorillonite). When this occurs, there is an unsatisfied negative charge inside the crystal lattice. This negative charge, which is internal and is conceptualized as a halo of charge surrounding the outside of the clay particle, is permanent and cannot be neutralized by covalent bonding with cations in solution. The second source of negative charge is on the exposed surface of the clay particle where hydroxide (-OH) radicals are exposed to the ambient solution (Sparks 2003). Depending on pH, the H^+ ion may be bound to the radical and when a number of H^+ are dissociated they leave negative charges ($-O^-$) that attract and bind cations. The binding of cations is reversible, pH dependent, and in equilibrium with the ionic concentrations in the solution. Cation exchange is especially important in regards to iron oxide minerals.

Iron

The oxidation of pyrite (FeS_2) produces ferric hydroxide solids, sulfate, and hydrogen ions (Table 1, Reaction 11). The ferric iron produced in Reaction 2 (Table 1) can hydrolyze and precipitate as amorphous ferric hydroxide $Fe(OH)_3$ (Table 1, Reaction 12), geothite FeOOH, or jarosite $KFe_3(SO_4)_2(OH)_6$. As the pH rises, the concentration of free iron tends to decrease. Initially, iron forms suspended colloids known as hydrous ferric oxide (HFO, $FeOOH \cdot nH_2O$), which is soluble under acid conditions and nearly insoluble at near neutral pH (Figure 6). After gelatinous HFO precipitates it gradually dehydrates to more stable forms such a goethite. Ferrihydrite coprecipitates other ions which acts as a scavenger adsorbing metal cations and anions (Sparks 2003).

As evident in the Eh-pH diagram in Figure 6, dissolved Fe(II) is released under reducing conditions whereas Fe(III) oxyhydroxides occur in oxygenated environments (Langmuir 1997). In oxidized surface waters and sediments, dissolved iron is mobile below about pH 3 as hydrated Fe^{3+} and Fe(III) inorganic complexes. From pH 5 to 10, Fe(III) is mobile as colloidal ferric oxyhydroxide, which occurs in suspended form in surface- and groundwaters thus influences the chemistry and transport of trace elements by adsorption and coprecipitation reactions (McKnight et al. 1988, Langmuir 1997). Since Fe(III)-oxides strongly adsorb toxic trace metals (Schoer 1985, Tessier 1985, Johnson 1986, Cummings et al. 1999, Benjamin and Leckie 1981), Fe(III) reduction influences their release. The remobilization of Cu and As that were either adsorbed by or coprecipitated with iron-oxides is a consequence of the reduction of the less soluble oxidized species and their dissolution to the more soluble reduced forms. Under reducing conditions below pH 7-8, iron becomes soluble and mobile as uncomplexed Fe^{2+} and absorbed Cu and As are released and enter the dissolved phase.

Transport of Dissolved Cu and As

Water is the prime transporting vehicle and is responsible for the dispersion of trace elements like Cu and As from their source (Zajic 1969). When subjected to erosion, weathering, oxidation, leaching, and groundwater transport, Cu and As can become dispersed over large areas.

Specifically, it appears that that seasonal fluctuations in precipitation and the infiltration of meteoric water can, under certain circumstances, cause the water table to drop in near-surface sulfide mineral deposits, waste rock dumps or tailings piles. After the water table drops water is held in the unsaturated vadose zone, which extends to the phreatic surface. Iron-sulfide minerals in the vadose zone oxidize releasing cations and anions that are subsequently sorbed to Fe-oxides. When the phreatic surface rises following the infiltration of meteoric water, reducing conditions return that release Fe^{2+}, HSO_4^- and the associated ions. Soils saturated by water

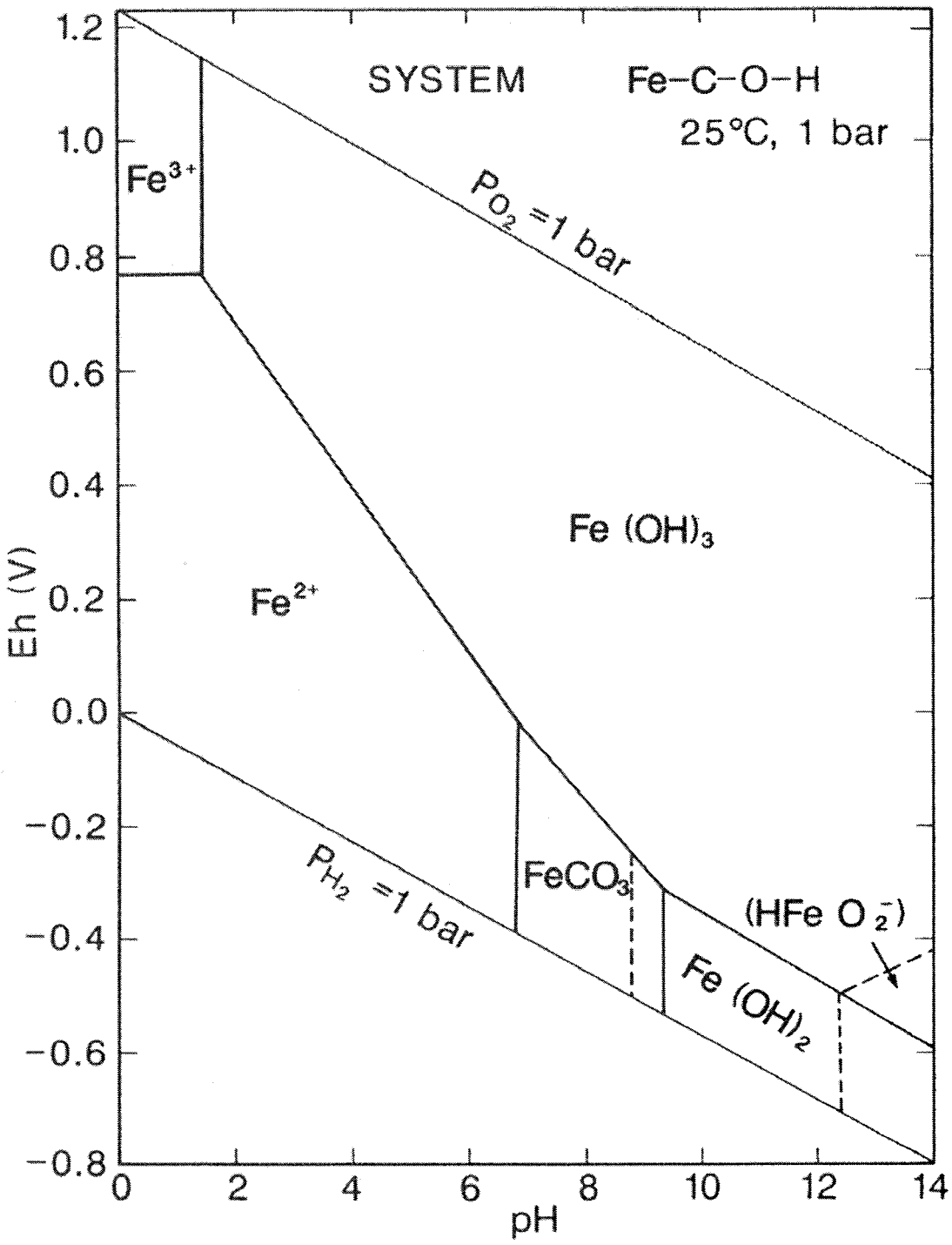

Figure 6. Eh-pH stability diagram for iron (Brookins 1988).

become closed systems with respect to redox reactions, which leads to the depletion of oxygen and the buildup of carbon dioxide from microbial respiration processes (Sposito 1989). Alternating conditions of oxidation and reduction cause iron, trace elements (and sulfate) to cycle between solid and dissolved phases until they are ultimately transported away with surface or groundwater.

Colloid Facilitated Transport

The process of colloid facilitated transport is a potential mechanism for the translocation of adsorbed metal contaminants (Sposito 1989, Kretzschmar et.al. 1999). Solids with a diameter between 0.01 and 10 μm are considered colloids (Sposito 1989, Stumm and Morgan 1996). To be dissolved, therefore, a molecule should have a diameter of < 0.01 μm. Colloids can remain suspended indefinitely and it is estimated that 30-70% of the filterable (0.45 μm) ferric iron occurs as colloidal particles (Langmuir 1997). Coarser particles are considered suspended particles, which can be transported if surface or groundwater flow is adequate. If present, some colloidal solids will pass through a 0.45 μm filter membrane used to distinguish dissolved from suspended metals. According to the colloid facilitated transport model, metals partition between the surfaces of immobile matrix particles, the aqueous phase, and suspended colloidal particles, which are transported with flowing water. While strongly sorbing contaminants can be highly retarded by immobile matrix particles, colloidal carriers can provide another potential rapid transport mechanism.

Cu Adsorption on Fe Oxides

It has been shown that the sorptive process that binds Cu to the surfaces of amorphous Fe-oxyhydroxides and regulates their concentrations in solution are pH dependent and that a positive correlation exists between the extent of binding and pH (Benjamin and Leckie 1981, Johnson and Kendall 1999). Fe-oxyhydroxides are particularly suitable for the adsorption of Cu

because they are relatively abundant and have high specific surface areas. Benjamin and Leckie (1981) measured the adsorption of Cu onto amorphous iron oxyhydroxides and for each metal they found that there was a narrow pH band where adsorption increased from near zero to near 100%. The curves are steep, which gives them the name adsorption edge. Generally speaking, the pH-adsorption edge was constant up to a threshold adsorbate (e.g., Cu) concentration and then increased with adsorbate concentration. There are at least three possible causes for this: (1) the coulombic attraction between the solid and the adsorbate decreases as the metal ions adsorb because the surface charge becomes more positive, (2) there are unfavorable chemical interactions between adjacent adsorbed species, and (3) there are a variety of site types on the solid of varying affinity for the adsorbate (Benjamin and Leckie 1981).

As Adsorption on Fe Oxides

The most influential parameter affecting arsenic is iron content and mobility appears to be determined by the pH dependence of sorption reactions of arsenic on iron oxide rather than dissolution-precipitation reactions involving arsenic (Cummings 1999). Hydrous ferric oxide strongly adsorbs both As(V) and As(III) (Brown 1999, Cummings et al. 1999). In the presence of excess ferric oxide, reductive dissolution releases ferrous iron and adsorbed As, which was found to be the dominant mechanism by which As was released into solution. Also, when Eh-pH stability diagrams for As and Fe are compared (Figures 5 and 6) As(III) would be mobile in the dissolved phase whereas As(V) would be mobile if adsorbed to colloidal hydrous ferric oxide [$Fe(OH)_3$]. Adsorption by iron oxide comprises three steps including (1) surface adsorption, (2) diffusion into the particle, and (3) adsorption and fixation at positions within the mineral particles (Sposito 1989). Furthermore, adsorption by iron oxide depends on pH and when the pH is less than the Point of Zero Net Proton Charge (PZNPC) the surface is positively charged and adsorbs anions [e.g., $As(III)O_3^{-3}$ and $As(V)O_4^{-3}$].

At near neutral pH values (7-9), slow dissociation of weak arsenic acid (H_3AsO_3) produces arsenite ion ($H_2AsO_3^-$). This partially neutral and partially negative charged arsenite ion is attracted to the positively charged surface below the pH_{PZNPC} resulting in high As(III) removal (Vaishya and Gupta 2002). It may be hypothesized that in the 7-9 pH range, the removal of As may be due to a combination of electrostatic attraction between As anions and iron oxide, and Van der Waals' forces, with the possibility that the former dominates. When the pH is greater than the pH_{PZNPC} the surface becomes negatively charged resulting in a decrease in the removal of As. The decrease in the removal of As at pH higher than pH_{PZNPC} may be the result of a net negative charge on the adsorbent.

When pH is approximately equal to the pH_{PZNPC} the iron oxyhydroxide particles are not repelled and so tend to flocculate and settle out (Langmuir 1999). The exact pH of the PZNPC depends on the species of FeOOH. Iron oxides generally have high PZNPC values (approximately 8) indicating that they have a net positive charge over a wide pH range and would have the ability to adsorb anions. In carbonate buffered streams where the pH is higher than the PZNPCs, iron oxides would absorb cations.

Metal Binding by Acid Volatile Sulfides

It is assumed that a key to the bioavailability of sediment contaminants has been correlated with the interstitial water concentrations free metal ions (Di Toro 1992). In anaerobic sediments, an important binding phase that controls interstitial water concentrations of the metals is an extractable fraction of iron sulfides known as acid-volatile sulfides (AVS). AVS binds, on a mole-to-mole basis, a number of cationic metals of environmental concern (e.g., Cu) forming insoluble sulfide complexes with minimal biological activity (Ankly, 1996). Hansen (1996) recommended that a metal to AVS ratio be used as a more accurate prediction of metal bioavailability but warned that the potential for release of nonavailable metal as a result of oxidation of AVS may be part of the normal seasonal sulfide cycle.

DiToro (1992) demonstrated that when divalent cations are present in sediments they rapidly displace iron in $FeS_{(s)}$ to form metal sulfide precipitates. The replacement reaction occurs for any metal that forms a sulfide more insoluble than FeS. The solubility products (log K_{sp}) decrease from FeS (-22.39) to CuS (-40.94). DiToro (1992) hypothesized that since AVS is such a strong partitioning phase for cationic metals in sediments, metal activity will approximate the metal in excess of the AVS and the net concentration of AVS in excess of the metal concentration could be used to predict the no-acute-metal-toxicity concentration. When [metal]/[AVS] < 1, all of the metal is converted to metal sulfide because of the displacement reaction. When [metal]/[AVS] > 1, all of the iron sulfide has been converted to metal sulfide and the excess metal remains potentially toxic to sensitive organisms. It should be noted that when the AVS concentration is zero, as it is in fully aerobic sediments, other sediment properties such as organic carbon or iron content would control the metal activity.

Bioconcentration and Toxicology of Arsenic and Copper

Many studies have been carried out to measure the bioavailability and potential effects of Cu and As on aquatic insects and fish (Warnick and Bell 1969, Let et al. 1976, Nehring 1976, Chapman 1978, Anderson et al. 1980, Clements and Kiffney 1994, Canivet et al. 2001, Tisler and Koncan 2002). Much of the knowledge regarding the toxicity and effects of trace elements is based primarily on laboratory studies or studies that integrate the laboratory and field approach and evaluate the effects of trace element exposure at different trophic levels, including bacteria, macroinvertebrates and fish. A wide range in reported toxicity values, however, makes it difficult to identify specific threshold values for different trace elements. For example, the 96-hour LC 50 (96-LC50), which has been used to estimate toxicity threshold values, can vary by as much as three orders of magnitude between families and by over one order of magnitude within a species. Nehring (1976) reported a 96-LC50 for *Pteronarcys californica* that was between 10,000 and 13,900 µg-Cu L^{-1}, whereas the 96-LC50 for *Hydropshche belteni* was

8,300 µg-Cu L^{-1} (Warnick and Bell 1969) and for *Tanytarsus dissmilis* it was 16 µg-Cu L^{-1} (Anderson et al. 1980). For *Salmo gairdneri*, the 96-LC50 can range from 17 to 680 µg-Cu L^{-1} (Lett et al. 1976). In addition to differences in toxicity between taxa, toxicity is also related to the chemical characteristics of the water (e.g., including alkalinity, hardness, pH, and the presence of specific organic compounds), interactions with other metals, speciation and complexation (Chakoumakos et al. 1979, Stephenson 1983, Gauss et al. 1985).

Trace element contaminants (e.g., As and Cu) may exist in a variety of forms, both free in solution and adsorbed onto particulate surfaces. When considering their toxicity an important question is whether the contaminant is entering the cells of organisms in the ecosystem. The answer to this apparently simple question is complicated because the contaminant that enters the cell [CH_3 As or $(CH_3)_2$ As] may not be the same as the one that entered the system (e.g., arsenite [As(III)O_3^{3-}] or arsenate [As(V)O_3^{3-}]. Contaminants can also exist in a variety of forms, both free in solution and adsorbed onto particulate surfaces.

It is generally believed that the uptake of adsorbed As is significantly less than the absorption of dissolved As and that to enter the biogical cycle As must be in a dissolved form (Tamaki and Frankenberger 1992). Much of the knowledge regarding the toxicity and effects of dissolved As in the aquatic environment is based primarily on laboratory studies that indicate As toxicity, although highly species specific, generally occurs at concentrations exceeding 17 mg kg^{-1} in sediments and at least 200 µg L^{-1} in the dissolved fraction (Canivet 2001, Tisler 2002). Similar toxicity studies for Cu have shown that while it is essential element for aquatic organisms dissolved Cu can also be toxic at concentrations exceeding 16 µg L^{-1} (Anderson et al. 1980), and there is clear evidence that benthic organisms are sensitive to sediment concentrations > 28 µg g^{-1} (Jones and Suter 1997).

The first step in the uptake of contaminants by organisms is entry through an apical epithelial cell membrane, which is the organism's interface with the external environment. It

appears that contaminants can enter apical cell membranes by at least three routes (Simkiss 1996): lipids, aqueous, and endocytic. The lipid route is a function of the cell membrane, which is a bilayer of lipid molecules that are arranged so that the hydrophilic groups are facing outward and the hydrophobic groups are facing inward. It is generally considered that the flux of materials across the cell membrane is largely dependent upon their lipid solubility and this is modeled by the octanol/water partition coefficient. The more lipid soluble a molecule is, the greater the octanol/water coefficient and the greater its flux into an organism for any given concentration gradient.

In the aqueous route the lipid membrane is also selectively permeable to cations, which is explained by the presence of aqueous pores that are formed by proteins that loop in and out of the membrane (Luecke et al. 1999). It appears that selectivity is based on the interaction between the hydration and dehydration of the ion and on the binding and release of the cation from the ligands in the channel wall. Some channels are selectively permeable to cations (Simkiss 1996). Membrane-bound enzymes that split adenosine triphosphate (ATP) can use the chemical energy released to drive ions against their electrochemical gradients.

In the endocytic route, trace element contaminants associated with bacteria in the particulate form may be preferentially processed into the digestive system as a nutrient source and sorted by well defined mechanisms so that they are phagocytosed into specific digestive cells (Simkiss 1996). This phenomenon includes processes that range from specific receptor-mediated events to the simple assimilation of food particles. These are fundamental cellular processes that link the endocytosis of food particles to intracellular lysosomal digestion. The uptake of iron is one example of a very specific type of endocytosis.

Arsenic

Much of the knowledge regarding the toxicity and effects of As in the aquatic environment is based primarily on laboratory data, which indicates that toxicity is highly species specific and occurs when As concentrations exceed at least 200 $\mu g\ L^{-1}$ (Canivet 2001, Tisler 2002).

Bioconcentration of As occurs in aquatic organisms, especially algae and lower invertebrates at the base of the food chain, that are exposed to arsenic that has accumulated in sediments (Mason et al. 2000). However, to enter the biogical cycle, As must be in a dissolved form (Tamaki and Frankenberger 1992). Studies of the bioavailability of As suggest that the absorption of As in ingested dust, soil, or sediments is significantly less than the absorption of dissolved As. When soils, mining, and smelter wastes containing As were incubated *in vitro* in simulated gastrointestinal fluids, only a fraction of the As (i.e., < 11%) became bioavailable (DHHS 2000). While predators may accumulate As from the surrounding water or from feeding on other fish, there was no evidence of biomagnification and As concentrations in freshwater organisms tend to decrease with increasing trophic level (Mason et al. 2000).

Both arsenate and arsenite are well absorbed by the oral route when ingested with food (Davis et al. 1992). Once absorbed, arsenites are partially oxidized to arsenates and arsenates are partially reduced to arsenites, resulting in a mixture of As(III) and As(V) in the hemolymph or blood (Vahter and Norin 1980). Some animals methylate arsenic to forms that are less toxic and are easily excreted. During methylation, the As(III) form undergoes enzymatic methylation to form monomethyl arsenic (MMA) and dimethyl arsenic (DMA) (Marcus and Rispin 1988). MMA may be methylated to DMA, but neither MMA nor DMA are demethylated to yield inorganic arsenic.

Arsenate given orally to fresh water brown trout (*Salmo trutta*) is converted to organic As by intestinal microflora in the gastrointestinal tract then rapidly absorbed (Tamaki and

Frankenberger 1992). Each time a methyl group is added to a compound it becomes less water soluble, more lipid soluble, and hence more cell permeable and more rapidly excreted (Simkiss 1996). It appears that toxicity occurs when the methylation capacity is not adequate to clear As(III) and prevent it from reaching the tissues. A target of As(III) in the cell is the mitochondria, which accumulates As. Arsenic inhibits dehydrogenase activity and uncouples oxidative phosphorylation, which results in reduced ATP levels and affects cellular functions (e.g., Na^+/K^+ balance, and protein synthesis).

Copper

Although the dissolved Cu concentration in water is important with respect to toxicity, there is also concern over the dietary exposure of aquatic organisms to Cu contained in contaminated sediments. The concentration of Cu in sediments usually exceeds the concentration in surface water by three to five orders of magnitude. Although the relative importance of the routes of exposure remains unclear, at such high concentrations the bioavailability of even a small fraction from the diet is important and it has been suggested that diet is a much more significant source of nutritionally essential elements such as Cu (Spry et al. 1988). In the solid phase, Cu is associated with both oxides of Fe and organic components such as humics (Luoma and Bryan 1981). It is not clear, however, whether Cu bioavailability depends on the concentration in the interstitial pore water or that bound to particles. There is some evidence that Cu uptake can occur follwing the ingestion of particles or by pinocytosis followed by desorption during contact between the particles and the intestinal epithelia (Simkiss 1996).

There is clear evidence that sediment associated biota are sensitive to Cu at concentrations $> 28\ \mu g\ g^{-1}$ (Jones and Suter1997). In caddisfly larvae and trout there are two possible ways that metals may enter the body: through the body surface and the alimentary tract (Munger and

Hare 2000). In fish the gills are a third pathway (Dalllinger et al. 1987). The gills, however, are not only the main organs of gas exchange, they are also important in the uptake of dissolved metal ions in the water. The gills of rainbow trout can show 10-fold increases in Cu accumulation within a few hours of aqueous exposure, and this occurs simultaneously with the appearance of Cu in the blood (Handy 1992, Grosell et al. 1997). In contrast to the gills, the ailimentary tract appears to be the most feasible route for the uptake of metals that are consumed during feeding. While it appears that little is known about metal uptake through the skin it is assumed that the skin is impervious to metal absorption (Dalllinger 1987).

Although trace metals dissolved in water tend to be more bioavailable than those associated with particles, dietary uptake is probably the most important source of copper-loading when copper concentrations are low in water and high in sediments (Hare 1992). Metal concentrations in food are often several orders of magnitude higher and represent a more contaminated source than the soluble forms in water.

In a study of tissue metal accumulation in rainbow trout exposed to foodborne and waterborne metals Farag et al. (1994) showed that, in general, metal accumulation in tissues was higher in gill and kidney with waterborne exposures and was higher in stomach and pyloric caeca with dietary exposure. In the absence of dissolved Cu, dietary uptake is probably the most important source of metal-loading in the fish and caddisfly larvae.

In a study on cut-throat trout, Borgman (1983) showed that decreasing the pH of the environmental water decreased the toxicity of copper. The expectation was that decreasing pH would increase the free ion concentration of Cu and, therefore, its toxicity. The observation that the opposite effect occurred was interpreted as showing that protons competed with copper for ligand binding sites in the ion transport channel in the apical membrane of exposed cells. It was proposed that the protons, considered to be highly mobile, penetrate the channel and bind to anionic sites thus obstructing the narrowest parts of the pore. Because the channel is obstructed,

fewer Cu ions can penetrate the pore and toxicity is reduced.

Copper is an essential trace element utilized as a cofactor to various enzymes involved in oxygen metabolism, such as cytochrome C (Ostrakhovitch et al. 2002) and Cu^{2+} is involved in the formation of reactive oxygen species (ROS), such as hydrogen peroxide. At toxic concentrations, Cu^{2+} or its low molecular weight complexes, may induce apoptotic cell death, which is preceded by the upregulation of Bax (Zhai 2000), the loss of mitochondrial membrane potential or permiability transition (Pourahmad 2000), and the release of cytochrom C into the cytosol. Cytochrome C is responsible for inducing apoptosis downstream from mitochondria (Liu et al 1996, Yang et al. 1997, and Kluck 1997).

CHAPTER 4

INFLUENCE OF TRACE ELEMENTS FROM MINE WASTES ON INVERTEBRATES AND FISH IN THE METHOW RIVER

INTRODUCTION

Trace element contamination from abandoned mines affect benthic invertebrates and fish in rivers in Colorado, Montana, and Idaho (Kiffney and Clements 1993, Canfield et al. 1994, Farag et al. 1998). There is now great concern that abandoned mines may be contaminating the Methow River in eastern Washington.

Numerous studies have been carried out to measure the bioavailability and potential effects of trace elements on aquatic insects and fish (Warnick and Bell 1969, Lett et al. 1976, Nehring 1976, Chapman 1978, Anderson et al. 1980, Clements and Kiffney 1994, Canivet et al. 2001, Tisler and Koncan 2002). Much of the knowledge regarding the toxicity and effects of trace elements is based either on chemical analyses, laboratory toxicity tests, tissue metal accumulation, or the effects of trace element exposure at different trophic levels, including bacteria, macroinvertebrates and fish (Gower and Darlington 1990, Canfield et al. 1994). Chemical assessment methods are used to describe the chemical exposure of aquatic biota to a suspected cause of an environmental problem.

Toxicity threshold values can vary, however, by as much as three orders of magnitude between families and by over one order of magnitude within a species (Warnick and Bell 1969, Nehring 1976, Anderson et al. 1980). In addition to differences in toxicity among taxa, toxicity is also related to the chemical characteristics of the water (e.g., including alkalinity, hardness, pH, and the presence of specific organic compounds), interactions with other metals, speciation and complexation (Chakoumakos et al. 1979, Stephenson 1983, Gauss et al. 1985).

While there is a wide range in toxicity and it is difficult to identify universal threshold values for trace elements in the environment, biological assessment methods measure directly the

effects of contaminants on aquatic communities and ecosystem health. Usually, effects on nutrient cycling or energy flow at the ecosystem level, reduced diversity or abundance at the community level, and reduced growth or increased mortality among individual members of endangered species at the population level are more relevant to resource managers and ecologists than effects at lower levels of biological organization. However, the degree to which cause and effect are related (i.e., specificity) and our knowledge of the mechanisms of toxicity is lowest at higher levels of organization (Hodson 1990, Clements 2000). At lower levels of organization, however, 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, the specificity and usefulness of electron microscopy is evident based on the ability to diagnose 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 or programmed cell death. 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 submitochondrial granules that accumulate heavy metals was also found to coincide with the toxicity data for aquatic organisms (Argese 1996). The EC50 (50% of the effective concentration) data for submitochondrial 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 submitochondrial 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. It does seem likely that the identification of metals that are contained in electron-dense submitochondrial 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.

Pollution impact studies often use inferential statistics to determine significant differences of aquatic community sample results upstream and downstream of a suspected perturbation (Darlington and Gower 1990, Gower and Darlington 1990, Kiffney and Clements 1993, Brumbaugh et al. 1994, Canfield et al. 1994, Farag et al. 1998, Goldstein et al. 2001). In this study, the above-and-below-mine approach was used to compare exposed and reference populations to five trace elements (i.e., As, Cd, Cu, Pb and Zn). Electron microscopy was used to observe the cytotoxic effects of trace element contamination and elemental analysis of submitochondrial granules by X-ray analysis was used to identify contaminants present at high

concentrations in the environment surrounding an organism, its cells and its mitochondria. Secondary effects related to reduced body weights and delayed development occurring at higher levels of biological organization were also determined.

The objectives of this study were to determine, (1) the concentrations of contaminants of potential ecological concern in sediments and water in the Methow River above and below the Alder Mine, Red Shirt Mill and Alder Mill, (2) whether *in vivo* exposure of caddisfly larvae in microcosms to trace element 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 submitochondrial granules in gut epithelial cells and hepatocytes, (4) whether the composition of the submitochondrial granules relates to exposure, and (5) the effects of trace element contamination on the growth and developments of caddisfly larvae (*Ecclesomyia spp.*) and trout (*Oncorhynchus mykiss*).

METHODS

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 7). Sample station 1 is located at the entrance to the adit at Alder Mine where ARD is discharged into a retention pond. Station 2 is a mixing zone where ARD from Alder Mine enters Alder Creek. Station 3 is a private drinking water well adjacent to the Alder Mill. Stations 4-7 are reference sites upstream from the abandoned mine sites and stations 8-11 are treatment sites located downstream from area in the Methow River impacted by subsurface flows of groundwater from the abandoned mine sites.

Trace Elements in Methow River Water and Sediments

Water samples were collected from Methow and Twisp River stations 6 and 7 above the

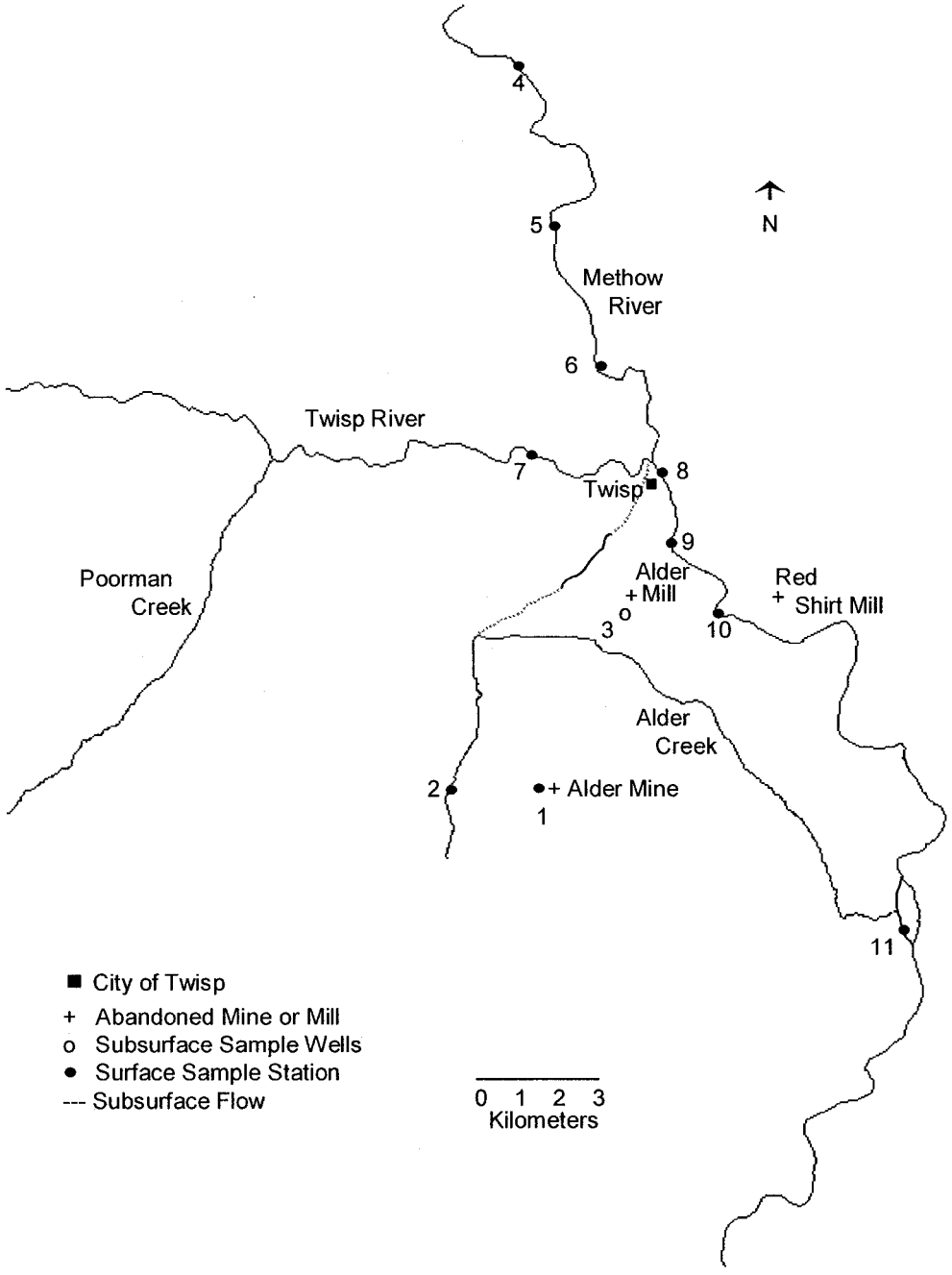

Figure 7. Map showing study area, abandoned mine and mill sites, and sample stations.

mines and from Methow River stations 9 and 11 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 0.45 µm, disposable 25 mm sterile disposable Acrodisc filter) for determination of dissolved trace element concentrations (As, Cd, Cu, Pb, and Zn). All water samples for metals analysis were preserved to pH<2 with 0.15% nitric acid and stored at less than 5°C. Samples for As analyses were frozen until analyzed. 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 and periphyton samples were dried 24 h at 60°C, ground and analyzed for metals and As. All water and sediment sampling equipment was cleaned by washing with Liquinox detergent and sequential rinses with distilled water, dilute nitric acid, and de-ionized water.

Samples of water, sediment and periphyton were analyzed for trace elements (As, Cd, Cu, Pb, and Zn) at the University of Washington, College of Forest Resources Analytical Laboratory in Seattle, Washington. Dissolved metal concentrations (0.45µm filtered) were determined by ICP atomic emission spectrophotometry (ICP-AES; Thermo Jarrell Ash ICAP 61E, EPA Method 3050). Samples were also analyzed for As by Hydride Generated Atomic Fluorescence Spectrophotometry (HG-AFS) (Corns et al., 1993).

General Water Chemistry

Water quality parameters (temperature, pH, alkalinity, dissolved oxygen) were analyzed during the caddisfly and trout exposure studies described below. A YSI model 85 meter was

employed for the measurement of dissolved-oxygen (DO) and temperature. Determination of dissolved-oxygen was also made in the field, where flow was inadequate and the YSI probe was not applicable, 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 were standardized daily before and after use.

Effects of Trace Elements 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 contamination in the Methow River from the abandoned mines resulted 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) at 4% of their body weight•day^{-1}. 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 41 trout in the pen at station 11 were weighed. Forty-one 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 expressed 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 Static Microcosms

To determine whether submitochondrial granules and apoptosis could be induced *in vivo* by exposure to trace elements from abandoned mines, caddisfly larvae were exposed to three treatments, (1) As-contaminated groundwater from station 3, (2) trace element contaminated periphyton from station 2, and (3) stream water contaminated with ARD from station 2. Thirty-one mostly 4th and 5th instar caddisfly larvae (*Ecclesomyia* spp.) were collected in June 2001

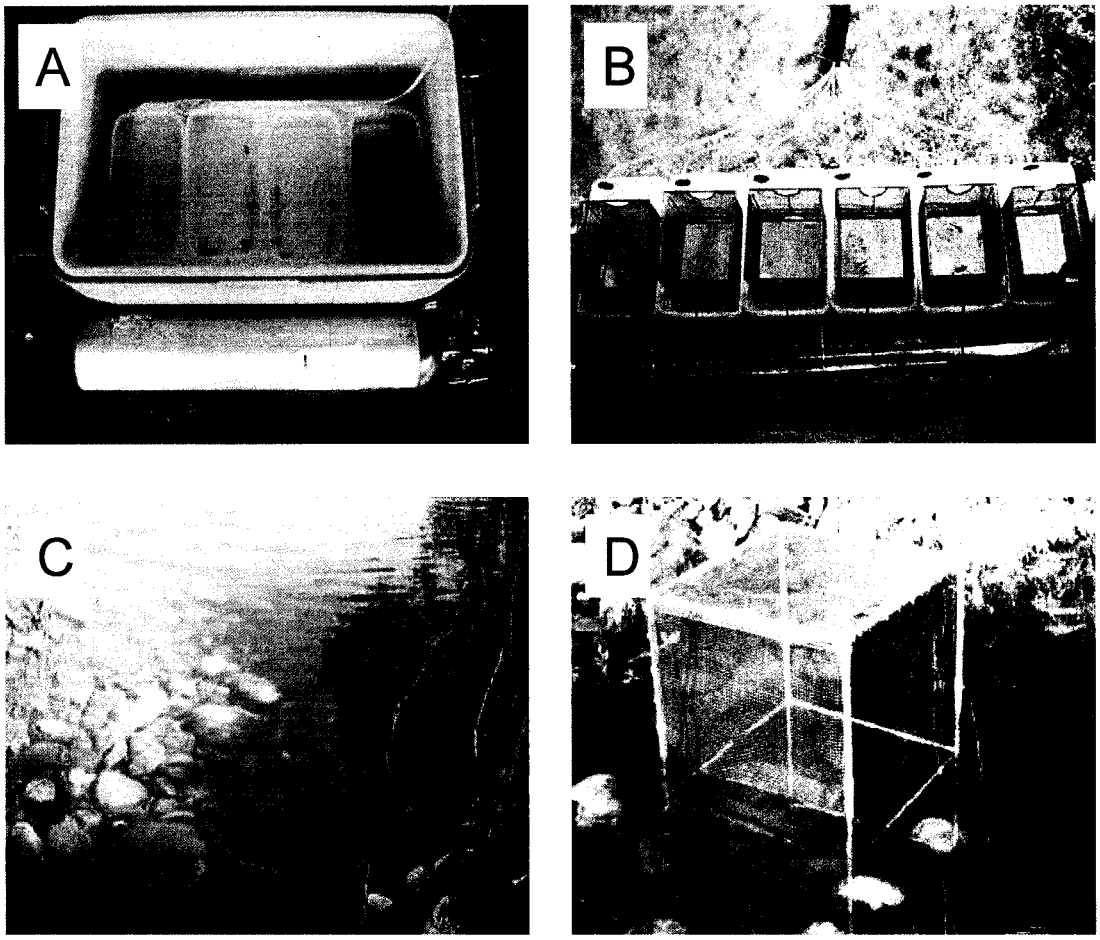

Figure 8. Microcosms used to expose caddisfly larvae and trout to contaminants from abandoned mines. (A) Static caddisfly microcosm, (B) Flow-through caddisfly microcosm, (C) *In situ* caddisfly microcosm, (D) *In situ* trout microcosm.

from sample station 7 (Figure 1) and divided into three groups of ten each. Ten larvae were added to each of three trays, 10cmW x 15cmL x 6cmD (Figure 8A).

Tray 1 contained 500 ml of As contaminated groundwater from Station 3; Tray 2 contained a 25 x 25 cm terracotta tile incubated 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 6; and Tray 3 contained 500 mL water from Alder Creek Station 2, which was contaminated by ARD from Station 1. The three trays were maintained in an insulated chest 46cmW x 76cmL x 46 cmD that contained ice approximately 20 cm deep in the bottom. The chests and trays were maintained in a shaded area in the vicinity of station 7.

The trays were 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 trays at a rate of 0.25-0.5 L min^{-1} to promote oxygenation of the water in the trays.

One water sample was collected from each microcosm chamber at the beginning of the test and from the station 2 periphyton test chamber at the end of the 24-h exposure period. Periphyton was removed from the tile in the station 2 periphyton test microcosm at the end of the exposure period using a soft nylon brush (1.5 x 0.5 cm). The periphyton was dried 24 h at 60°C, ground and analyzed for metals and As. 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 from each test chamber were dissected and samples of columnar epithelial cells from the small intestine were collected for electronmicroscopic studies. One larva from station 7 was dissected and preserved at the beginning of the study for use as a control. Tissue samples were analyzed to determine whether submitochondrial granules and apoptosis (i.e., condensation and margination of nuclear heterochromatin, and presence of nuclear vesicles) were detectable in larvae exposed

to water and periphyton contaminated by abandoned mine waste.

Caddisfly Cytotoxicity in Flow-Through Microcosms

To determine whether submitochondrial granules and apoptosis could be induced *in vivo,* caddisfly larvae were exposed to trace element-contaminated periphyton in microcosms perfused with contaminant-free water (Figure 8B). The microcosms were maintained in a shaded area in the vicinity of station 7. In June 2001, one-hundred-fifty caddisfly larvae (*Ecclesomyia* spp.) were collected from sample station 7 (Figure 1), divided into six groups of twenty-five each, added to wire baskets (30cmW x 30cmL x 30cmD) containing terracotta tiles (25 cm x 25 cm x 2 cm) incubated one-year and colonized with periphyton from Station 7.

The tiles in Test Baskets 4-6 were seeded with 6.5 g dry metal-oxides that coprecipitated and adsorbed trace elements from the ARD discharged at Station 1. The periphyton on tiles in Control Baskets 1-3 were not seeded with dry metal-oxide precipitates. The six baskets were maintained in plastic trays 36cmW x 46cmL x 15cmD. Each tray was perfused with metal-free water at a rate of 1.1 L min^{-1}. Larvae were monitored daily and maintained for 14-d. Temperature, pH, dissolved oxygen, and alkalinity were measured three times during the study.

One water sample was collected from each microcosm chamber at the beginning, middle and end of the test. Periphyton was removed from the tiles in each chamber at the end of the exposure period using a soft nylon brush (1.5 x 0.5 cm). The periphyton was dried 24 h at 60°C, ground and analyzed for metals and As.

At the end of the 14-d exposure period, two larvae from each of the treatment trays 4-6 were dissected and samples were collected for electronmicroscopic studies of columnar epithelial cells from the small intestine. One wild larva from station 7 and one larva from each of the reference trays 1-3 were dissected and sampled for use as controls. Periphyton from the tiles in control trays 1-3 and treatment trays 4-6 were sampled, dried, and analyzed for metals

by ICP and As by HG-AFS. The incidence of occurrence of submitochondrial granules was measured by observing 25 mitochondria per larvae and counting the number of granules per mitochondrion.

Methow River In Situ Microcosm Caddisfly Cytotoxicity

Caddisfly larvae from the Methow River, upstream from the abandoned mines were placed in microcosm cages and exposed to conditions downstream from the abandoned mines at station 9 to determine whether intramitochondrial granules and apoptosis could be induced in the larvae after exposure to ambient conditions (Figure 8C). In June 2001, twenty-five caddisfly larvae (*Ecclesomyia spp.*), collected from sample station 7 (Figure 7) was added to a 30-cm square basket made of 0.5-cm wire mesh. The basket was secured to rebar driven into the side channel in the Methow River at station 9 where water depth was approximately 10 cm. The basket contained a flat stone approximately 30 cm in diameter (4 cm thick) from the side channel covered with periphyton. Larvae were maintained for 14-d. 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 9 at the end of the exposure period using a soft nylon brush (1.5 x 0.5 cm). The periphyton was dried 24 h at 60°C, ground and analyzed for metals (ICP-AES) and As (HG-AFS). Water samples were collected once monthly for four months from January through April at station 9 and five-times in June during the *in situ* caddisfly cytotoxicity test.

Larvae were monitored daily and at the end of the 14-d exposure period five larvae were removed, dissected, and samples of small intestine were collected for cytotoxicity studies. One wild larva from station 7 and one larva from each of the reference trays 1-3 in the flow-through microcosm test were dissected and sampled for use as controls.

Wild Caddisfly Larvae Cytotoxicity

Five wild larvae from the side channel where the *in situ* microcosm caddisfly cytotoxicity test was performed (station 9) were also collected at the end of the exposure period in June 2001. One wild larva from station 7 and one larva from each of the reference trays 1-3 in the flow-through microcosm test were dissected and sampled for use as controls. The incidence of submitocondrial granules was determined by observing 25 mitochondria per larvae and counting the number of granules per mitochondrion.

Methow River In Situ Trout Cytotoxicity

At the end of the 5-week exposure period in June 2001 five juvenile trout from the pen at station 11 (Figure 8D) were euthanized (0.1% MS-222, pH 7), 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 submitocondrial 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 < 2mm 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 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 Cu grids and stained with 4% aqueous uranyl acetate for 45 min., the sections were examined and pictures taken using a Jeol JEM 1010 transmission electron microscope, located in the Zoology Department at the University of Washington, which was

operated at 80 keV. 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 (Rouiller 1960, Peachy 1964) were counted and the average number of granules per mitochondrion were calculated.

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

Tissues for the analysis of metals in the mitochondrial granules were collected on carbon-coated Ni and 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 50,000x 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 (i.e., comparative mensurative experiments) mimicking a classical treatment-control design were conducted (Hurlbert 1984, Wiens and Parker 1995). The matched pair test assumes that other environmental factors besides trace elements that may influence the response are equal among experimental units (i.e., treatment and control) and samples. It was also assumed 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 submitochondrial granules at the treatment and control locations and to provide an

objective estimate of the probability of obtaining an observed difference between a treatment and the control under the null hypothesis of no treatment effect.

Hurlbert (1984) critiqued how ecologists design and analyze field experiments and suggested it is impossible to infer causal relationships from unreplicated experiments, the repeated use of the same growth chamber, or when inferential statistics are used withought true replication. The term pseudoreplication was used to describe experimental studies where inferential statistics are applied to unreplicated or compound treatments. Recognizing that no two biological systems are identical, sampling cannot be entirely randomized and all ecological studies involve some degree of confounding and pseudoreplication. Single experimental units per treatment are common in ecology, however, when very large-scale systems (whole lakes, watersheds, rivers, etc.) are studied, when gross effects are anticipated, when only a rough estimate of the effect is required, or when the cost of replication is very great (Wiens and Parker 1995, Oksanen 2001). Inferential statistics applied to unreplicated experiments are useful to see whether there is a significant difference between treatments at two locations and to determine an objective estimate of the probability of obtaining an observed difference between a treatment and the control under the null hypothesis of no treatment effect.

In my study, chemistry data were reported as the mean concentration (i.e., m_t = downstream treatment mean for stations 9 and 11 and m_c = upstream control mean for stations 6-7) $\pm$ the standard error of the mean (SEM). The one-tailed *t*-test for the hypothesis H_o: $m_t \leq m_c$ and Ha: $m_t > m_c$ 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 8-11) compared to upstream samples (stations 4-7).

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 Ha: $m_t > m_c$ 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 evakuated using the one-tailed *t*-test for the hypothesis *Ho*: $m_t \leq m_c$ 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

Trace Elements in Methow River Water and Sediments

In the Methow River, the mean concentration of dissolved trace elements showed that the concentrations were less than the limits of detection by ICP-AES. Dissolved As was detected in a single sample at station 11 below the mines on week 5 by ICP-AES where the As concentration for the surface water sample was high at 62 $\mu g\ L^{-1}$. Arsenic concentrations in station 11 samples analyzed by HG-AFS were all <5 $\mu g\ L^{-1}$ and in the week-5 sample from station 11 the concentration was 3 $\mu g\ L^{-1}$ by HG-AFS (Table 1). Overall, the differences in As concentrations in surface water samples above and below the mines analyzed using ICP-AES and HG-AFS were not significant (p=0.48).

In contrast to surface water samples, Table 2 shows that trace element concentrations were higher in sediments at stations 8-11 below the mines compared to reference stations 4-7 above the mines ($p<0.05$). In general, the order of trace element concentrations below the mines compared to mean reference concentrations was Cu > As = Cd = Pb = Zn. Copper was also higher at all four stations whereas As, Cd, Pb and Zn concentrations were higher only at stations 9-11. The other parameters measured (pH, temperature, dissolved oxygen, alkalinity,

Table 2. Metal concentrations in sediments from the Methow River. Values are mean (standard error of mean)

		Metal Concentration (µg/g dry weight)				
Station	n	As	Cd	Cu	Pb	Zn
4	4	0 (0)	6 (2)	18 (1)	31 (2)	64 (4)
5	4	0 (0)	5 (2)	16 (1)	31 (3)	57 (4)
6	4	0 (0)	3 (1)	15 (2)	27 (2)	58 (5)
7	4	0 (0)	4 (0)	16 (2)	30 (2)	56(5)
REF[a]	16	0	5	16	30	59
8	4	0 (0)	6 (1)	24 (1)*	28 (1)	56 (2)
9	4	8 (4)*	7 (1)*	171 (37)*	45 (2)*	113 (8)*
10	4	15 (3)*	8 (1)*	79 (12)*	36 (6)*	73 (13)*
11	4	4 (2) *	6 (0)	70 (4)*	42 (3)*	87 (4)*

[a] REF = pooled value for stations 4-7.
* Indicates that a concentration is significantly greater than pooled reference (REF), using the Dunnet's one-tailed t-test, ($p<0.05$).

and total dissolved solids) are given in Table 3. Alkalinity, dissolved Ca, and total dissolved solids (TDS) were higher below the mines than above.

Abiotic Deposition of Iron and Trace Elements

The Fe concentration in sediments below the mines was significantly higher (p=0.027) than at the reference sites above by over 72% (32,152 vs. 18,655 μg g^{-1}, respectively). In Methow River sediments (stations 8-11), Fe occurred mainly in the Fe-oxide fraction (32%, 10,345 μg g^{-1}). Like Fe, the concentrations of As, Cd, Cu, and Pb were also greater below Twisp than above ($p < 0.02$). Trace elements in the exchangeable and carbonate fractions, assumed here to be the most biologically available relative to those that reside primarily in the Mn-oxide, organic, Fe-oxide and residual fractions, were approximately 14% in sediments at stations 8-11 in the Methow River below the mines. In the Fe-oxide fraction, total trace elements occupied a larger proportion at 25% (Figure 9). Copper followed a similar pattern with 18% in the exchangeable and carbonate and 43% in the Fe-oxide fractions.

Wild Caddisfly Larvae Body Weight and Instar Development

The mean live body weight of caddisfly larvae (*Ecclesiomia* spp.) was lower in the Methow River below the mine sites (stations 8-11) than upstream at stations 4-7 [2.3 $\pm$ 0.5 g (SD) vs. 1.2 $\pm$ 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 as can be seen in Figure 10, which shows that eighty-four percent 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 distribution of caddisfly larval instars were significantly different based on the Kolmogorov-Smirnov goodness of fit test for discrete data ($p<0.05$).

Table 3. General parameters for Methow River at station 11 below the mines during trout toxicity experiment compared to control site at station 7 above the mines. Calcium and Mg concentrations were included to compliment data on alkalinity and total dissolved solids.

Station 11 Below Mines

Date	Temperature Degrees C	Dissolved Oxygen (mg L^{-1})	pH	Alkalinity mg L^{-1} $CaCO_3$	Total Dissolved Solids	Ca (mg L^{-1})	Mg (mg L^{-1})
May - 7	11	10	7.3	123	115	7	2
May - 8	11	9	7.7	207	213		
May - 9	10	9	7.8	262	215	16	6
May - 10	10	8	7.6	283	269		
May - 11	12	8	7.3	272	281	18	7
May - 12	14	11	7.5	268	289		
May - 13	14	9	7.3	287	293	16	6
May - 14	12	10	7.1	151	145		
May - 21	12	8	7.4	286	293	21	6
June - 4	8	8	6.8	241	154		
mean	11	9	7.4	238	227	16	5

Station 7 Reference Above Mines

Date	Temperature Degrees C	Dissolved Oxygen (mg L^{-1})	pH	Alkalinity mg L^{-1} $CaCO_3$	Total Dissolved Solids	Ca (mg L^{-1})	Mg (mg L^{-1})
May - 7	10	10	6.8	106	105	12	3
May - 8	11	11	7.0	87	99		
May - 9	10	10	6.7	103	96	5	1
May - 10	11	10	7.3	86	83		
May - 11	11	10	6.3	95	84	7	1
May - 12	12	10	7.5	95	81		
May - 13	13	10	7.6	91	80	7	1
May - 14	13	10	6.6	89	82		
May - 21	12	9	6.9	75	77	7	1
June - 4	12	10	6.9	68	76		
mean	12	10*	7.0*	89.5*	86.3*	7.6*	1
t-Test, *p*=	0.88	0.02	0.02	0.00	0.00	0.03	0.09

* Indicates parameter below mines is significantly greater than reference using the t-test, ($p<0.05$).

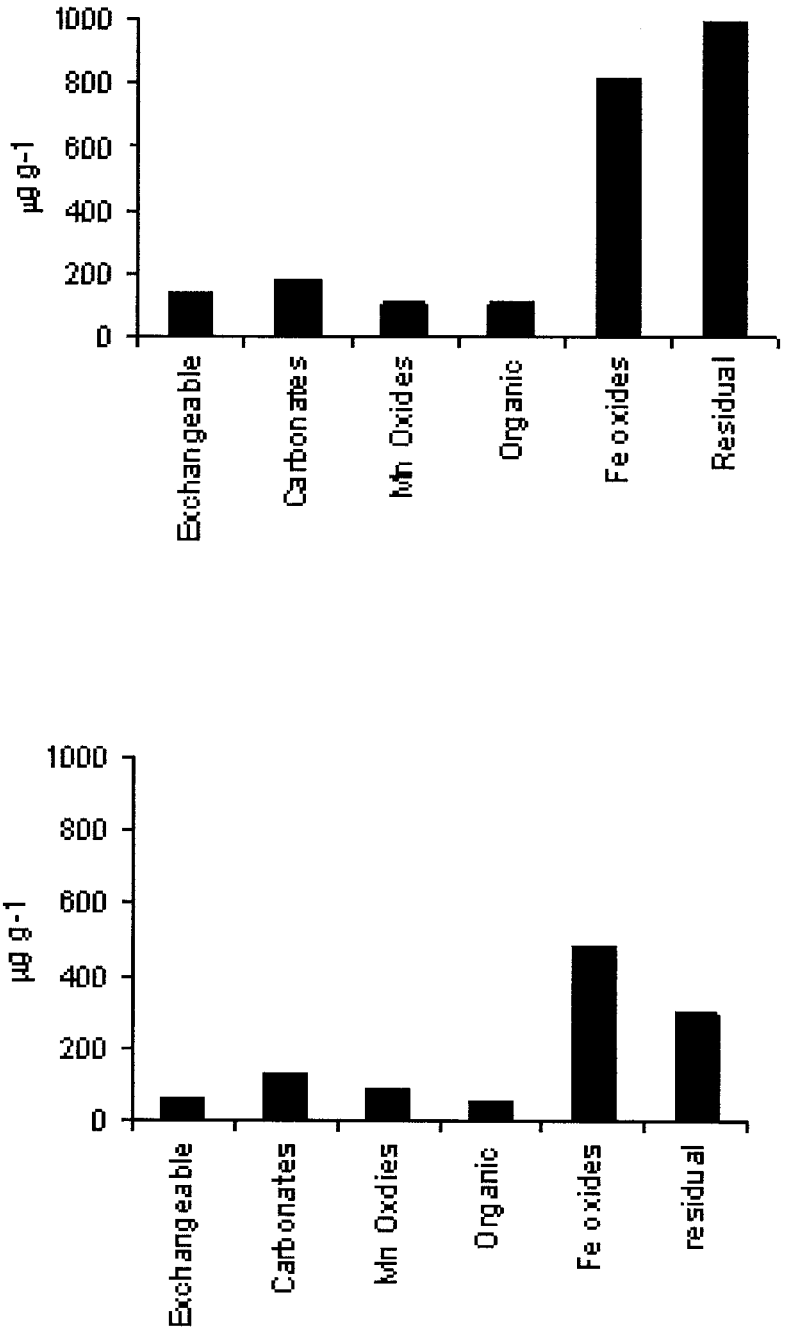

Figure 9. Sequential extraction results for total trace elements *(A)* and copper concentrations in Methow River sediments in mg g^{-1} *(B)*.

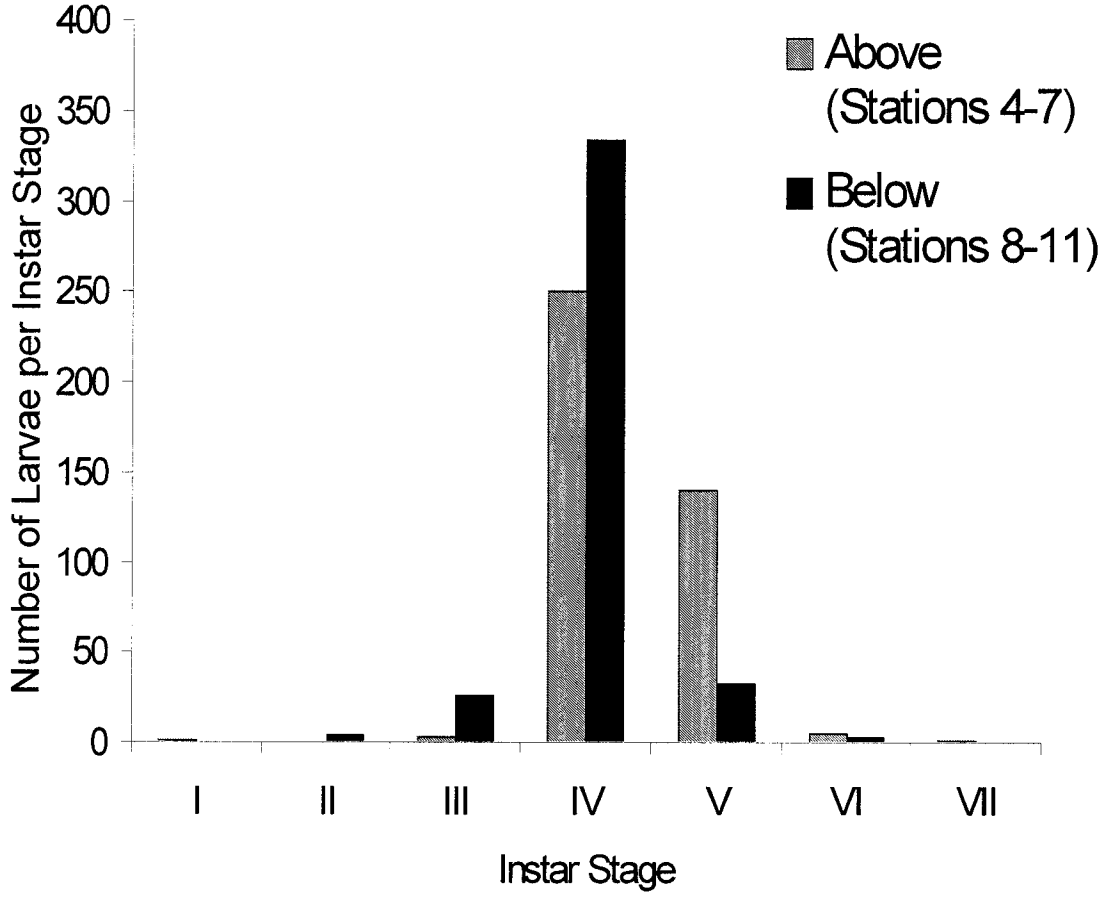

Figure 10. Frequency distribution of caddisfly larvae (*Ecclesomyia spp.*) instars in the Methow River above and below the abandoned mine sites.

Effects of Trace Elements in the Methow River 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 $\pm$ 10 (SD) vs. 71 $\pm$ 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 compared to no deaths in the control group. Two dead indigenous Coho parr were also encountered at station 15 during the study period.

Caddisfly Cytotoxicity in Static Microcosms

Treatment Condition. In tray 1 the station-3 well water treatment contained 234 $\mu g\ L^{-1}$ dissolved As but no Cu. In tray 2, the Alder Creek station-2 surface water treatment contained 28 $\mu g\ kg^{-1}$ dissolved Cu but no As. In tray 3, the station-2 periphyton treatment contained 292 $\mu g\ kg^{-1}$ Cu and 70 $\mu g\ kg^{-1}$ As. Neither Cu nor As were 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 mg L^{-1}. In all trays the other parameters measured (i.e., pH 8.6, temperature $\leq$9°C, dissolved oxygen $\geq$ 17 mg L^{-1} and alkalinity 219 mg L^{-1} CaCO3) were within the ranges tolerated by aquatic insects (Ward 1992, Merritt and Cummins 1996).

Cytotoxicity. The mitochondria and nuclei of small intestine epithelial cells from the control larva generally appeared to be normal in appearance and little variation was found in their shape and size (Figure 11A and B). 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 As-

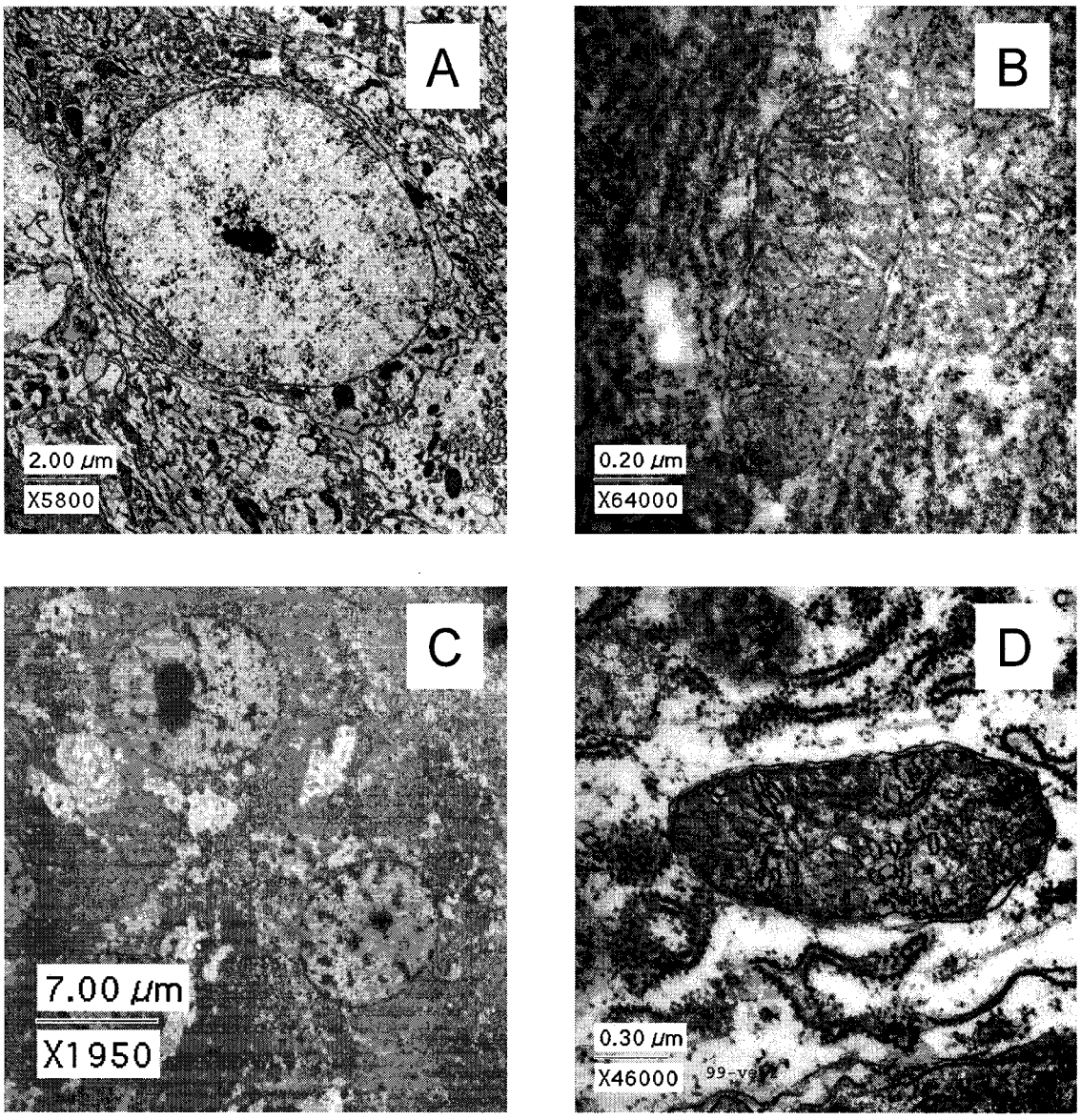

Figure 11. Normal nuclei and mitochondria in caddisfly small intestine epithelial cells and trout hepatocytes . (A) Caddisfly larva normal nucleus from station 7. X5800, (B) Caddisfly larva normal mitochondrion from station 7. X64000, (C) Normal nucleus from juvenile triploid trout hepatocyte from station 11(Figure 1). X1950, (D) Normal mitochoncrion from Juvenile triploid trout hepatocyte from station 11. X46000.

contaminated groundwater (tray 1) also appeared to be normal in appearance (Figure 12A). 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 caddisfly larvae exposed to As-contaminated groundwater (Figure 12B). In some nuclei showing chromatin compaction, some chromatin-free nuclear vesicles, which evolved from the nuclear envelope and had no chromatin, were expelled from apoptotic nuclei (Figure 12C). An enlargement of Figure 12C shows the membranes of the apoptotic bodies were also a bilayer derived from the nuclear membrane (Figure 12D). Numerous objects that appeared to be apoptotic bodies containing compacted chromatin and chromatin-free nuclear vesicles were also observed (Figure 12E). 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 12F).

Caddisfly Cytotoxicity in Flow-Through Microcosm

Treatment Condition. The periphyton Cu concentration in the treatment microcosm (i.e. 606 mg kg^{-1}) was over 40-times the concentration found in the control periphyton (15 mg kg^{-1}) and 20-times the concentration of Cu (30 mg kg^{-1}) in the Methow River periphyton at station 9. Copper was not detectable in the water at the input but dissolved Cu concentrations averaged 15 µg L^{-1} during the exposure period. Arsenic was not detected in either the periphyton or the water. The other parameters measured (i.e., pH 7.5, temperature $\leq$14°C, dissolved oxygen $\geq$ 6 mg L^{-1} and alkalinity 156 mg L^{-1} $CaCO_3$) were within the ranges normally tolerated by aquatic insects (Ward 1992, Merritt and Cummins 1996).

Cytotoxicity. Numerous electron-dense granules were detected in the mitochondria of small intestine epithelial cells from caddisfly larvae exposed to periphyton containing 606 mg Cu

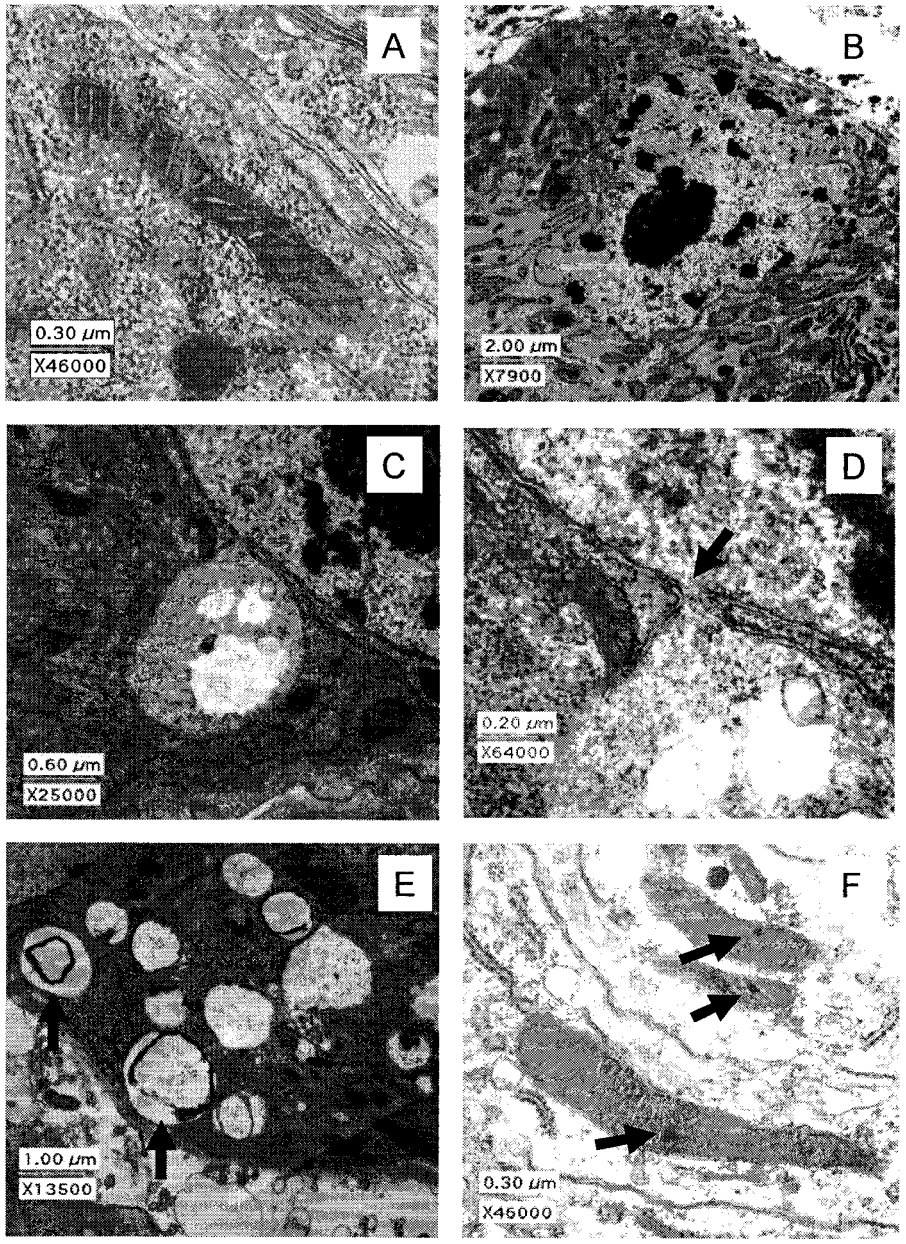

Figure 12. Mitochondria and nuclei in caddisfly larvae small intestine columnar epithelial cells from static microcosm caddisfly cytotoxicity test. (A-E) After exposure to water containing arsenic, (A) Mitochondria with no apparent effects, (B) Chromatin in the nucleus showing condensation and margination, (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.

kg^{-1} (Figure 13A). The mean number of granules per mitochondrion, which was greater than the means for the control larvae in six replicates (Table 4A), was significant at the 10% level (p=0.09) but not at the 5% level of significance.

Methow River In Situ Microcosm Caddisfly Cytotoxicity

Treatment Condition. The concentrations of As and Cu (i.e., 55 and 294 mg kg^{-1}, respectively) in periphyton from Methow River below the Red Shirt Mill at station 9 was 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$17°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 (Ward 1992, Merritt and Cummins 1996).

Cytotoxicity. Submitochondrial granules were induced in five caddisfly larvae that were translocated from reference station 7 and maintained in the microcosm at station 9 below the Red Shirt Mill for 14-days (Figure 13B). The mean number of electron-dense granules was significantly greater than in the control at both the 10 and 5% levels of significance (Table 4B).

Wild Caddisfly Larvae Cytotoxicity

Submitochondrial granules were induced in the seven wild larvae from Station 9 that were analyzed to determine whether matrix granules were being formed under natural conditions (Figure 13C). The mean number of electron-dense granules was significantly greater than in the control at both the 10 and 5% levels of significance (Table 4C).

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 11C, 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

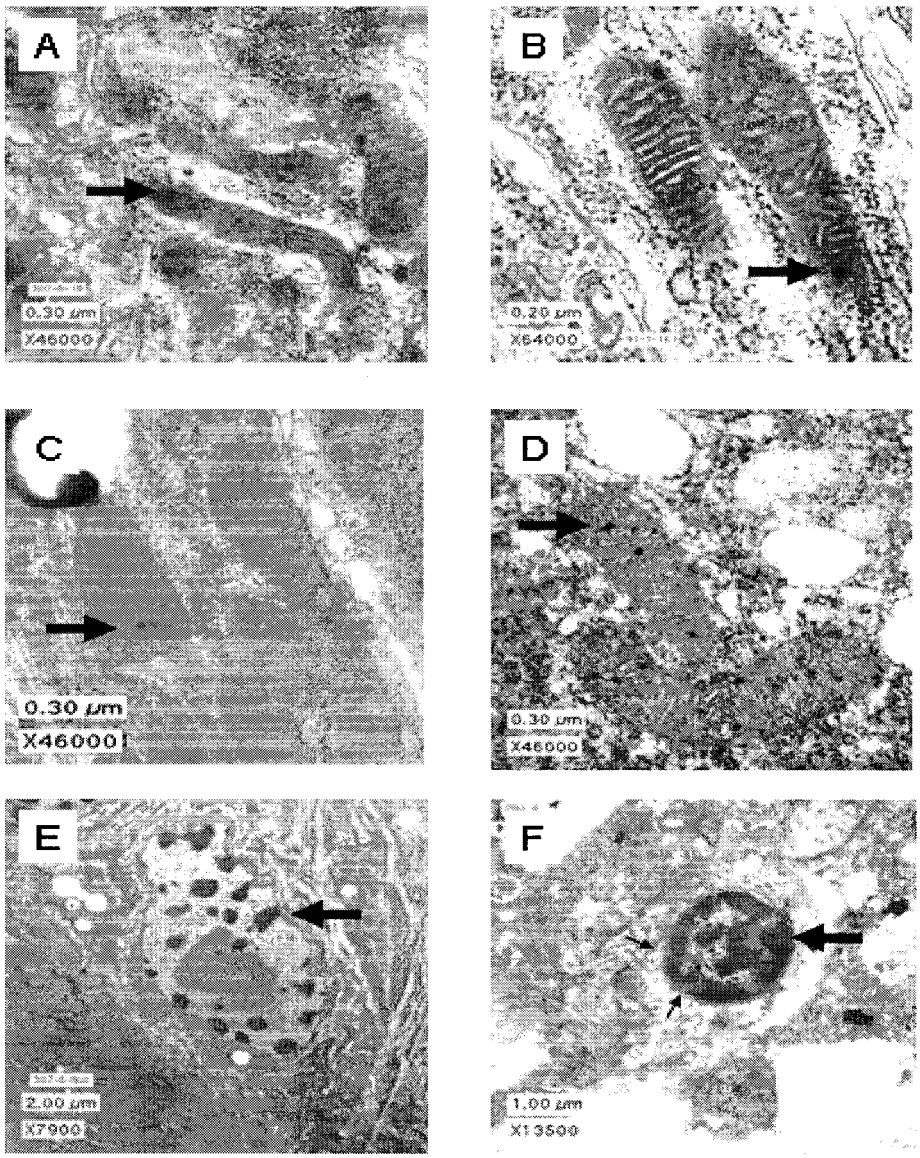

Figure 13. Mitochondria and nuclei from caddisfly larvae and trout exposed to Cu in sediments. *(A)* Submitochondrial granules (arrow) in caddisfly from flow-through microcosm caddisfly cytotoxicity test. *(B)* Submitochondrial granules (arrow) from caddisfly caged at station 9 below the mines. *(C)* Submitochondrial granules (arrow) in wild larvae from station 9 below the mines. *(D)* Submitochondrial granules in hepatocyte mitochondria (arrow) from control trout at station 11 below the mines. *(E)* Chromatin condensation and margination of apoptotic nucleus (arrow) in wild caddisfly from station 9 below the mines. *(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 4. 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 $m_{test} > m_{control}$.

A. Caddisfly Flow-through microcosm Test: Algae from Station 7 seeded with FeOOH from Station 1.

	Mean Number of Granules/Mitochondrion	
Replicate	Test	Control
1	1.28	0.00
2	1.04	0.39
3	0.64	0.06
4	0.16	0.29
5	0.08	
6	0.00	
Mean	0.53	0.19
p=	0.09	
Conclusion:	H_0(5%)	Accept
	H_0(10%)	Reject

B. Caddisfly I*n Situ* Microcosm Exposure Test: Larvae from Station 7 in cage at station 9.

	Mean Number of Granules/Mitochondrion	
Replicate	Test	Control
1	0.72	0.00
2	1.04	0.39
3	0.64	0.06
4	0.84	0.29
5	0.08	
Mean	0.66	0.19
p=	0.04	
Conclusion:	H_0(5%)	Reject
	H_0(10%)	Reject

(Continued)

Table 4. (continued)

C. Wild caddisfly larvae from station 9.

	Mean Number of Granules/Mitochondrion	
Replicate	Test	Control
1	0.12	0.00
2	0.92	0.39
3	0.28	0.06
4	0.72	0.29
5	0.84	
6	0.16	
7	1.04	
Mean	0.58	0.19
p=	0.00	
Conclusion:	H_0(5%)	Reject
	H_0(10%)	Reject

D. Trout Exposed Group from Station 11 compared tocontrol Group from Station 7.

	Mean Number of Granules/Mitochondrion	
Replicate	Test	Control
1	2.00	0.00
2	1.32	0.00
3	1.24	0.00
4	2.20	0.00
5	1.72	
Mean	1.70	0.00
p=	0.00	
Conclusion:	H_0(5%)	Reject
	H_0(10%)	Reject

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 was significantly greater than in the control at the 5% level of significance (Table 4D). 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 13F).

X-ray Analysis of Metals in Submitochondrial Granules

The EDS-spectra for intramitochondrial granules are shown in Figure 14. Figure 14A 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. After elements that are found in the system background spectra (Figure 14A) and the Ni grid material are taken into consideration, submitochondrial granules in trout and wild caddisfly larva tissue were found to contained Cu (Figures 14B).

DISCUSSION

Trace Elements in Methow River Water and Sediments

The concentrations of trace elements in surface water samples from the Methow River above and below the mines were not detectable. Although these results differed from Kiffney and Clements (Arkansas River, Colorado, 1993) and Farag et al. (Coeur d'Alene River, Idaho, 1998) where dissolved metals were detected, the concentrations of trace elements in Methow River sediments were similar. The mean sediment copper 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

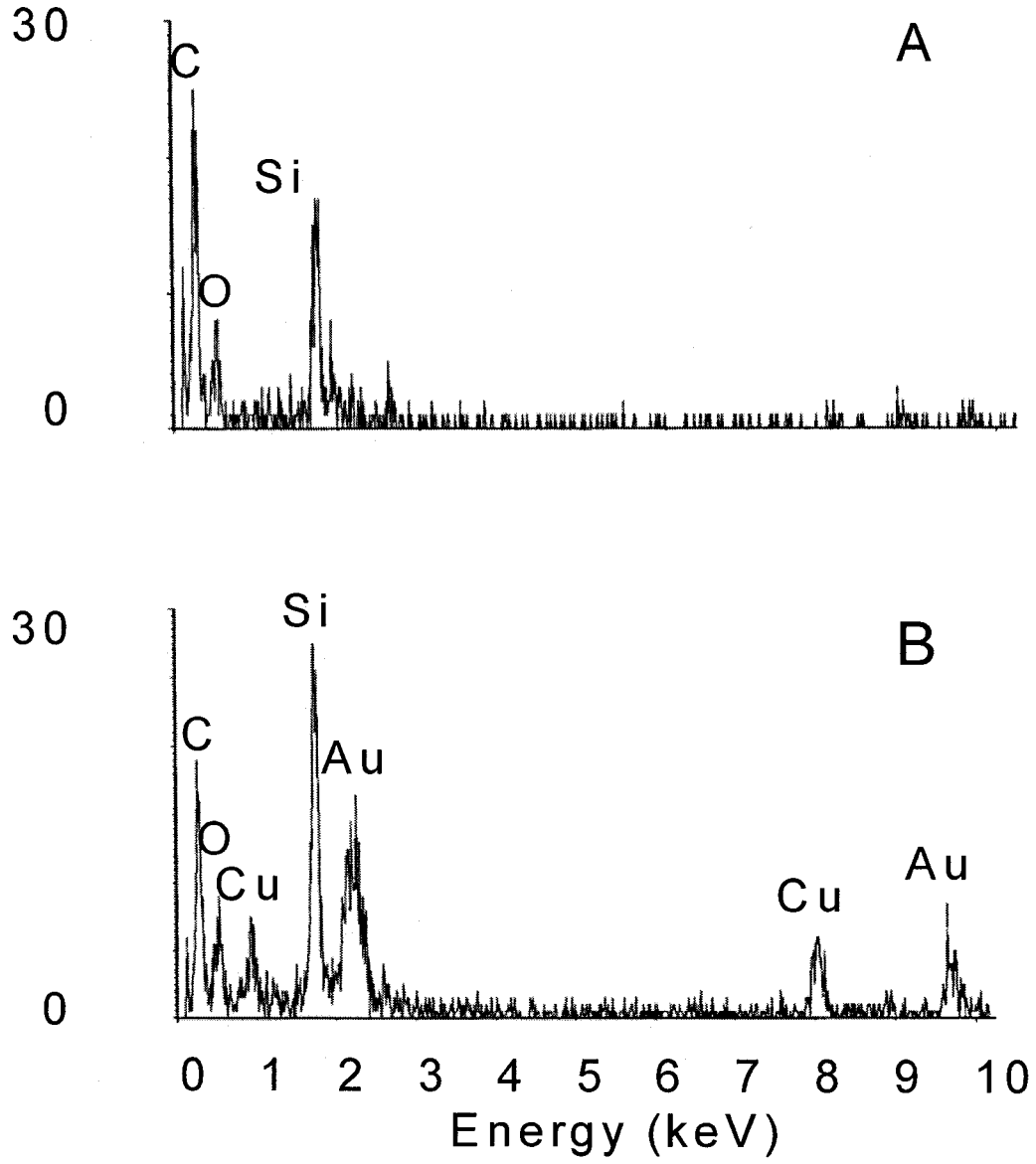

Figure 14. Energy dispersive X-Ray analysis (EDX) spectra showing composition of electron-dense spheres in matrix of caddisfly and trout mitochondria . *(A)* System background is typical spectra from the analysis of the mitochondrial matrix not including the grid material or matrix granule, *(B)* Spectrum showing Cu was sequestered by matrix granules in mitochondria of caddisfly and trout in the Methow River. Background included Au from grid.

(i.e., 16±1 mg kg^{-1}) and at stations 9-11, sediment Cu concentrations 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 Gurrieri (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. This finding was supported in my study by high levels (43%) of Cu associated with the amorphous Fe oxide fraction. Trace metal adsorbed and depostion by Fe oxides was also evident in the sequential extraction data for Cd and Pb. Results from sequential extractions were used procedurally to suggest the percentage of total metals which were associated with particular solid phases and the percentage of total metals that may be labile. Considerable data exist for As, Cu and Pb in sediments and there is a relatively high degree of confidence in the toxicity benchmark values that are considered to be highly reliable predictors of the threshold for effects (Jones and Suter 1997). A relatively large amount of data also exists for Cd, including spiked-sediment toxicity tests and EqP-based assessments (MacDonald et al. 1994).

Possible Exposure Pathways

Exact exposure pathways for metal uptake by caddisfly and fish could not be determined, but there are at least three possibilities. The body surface and the alimentary tract in caddisfly larvae and fish are two possible ways that metals may enter the body. In fish the gills are a third pathway (Pickering 1981, Munger and Hare 2000, Roberts 2001). However, the gills are important only in the uptake of dissolved metal ions in the water and not from sediments (Pickering 1981, Jobling 1995, Roberts 2001). Since little is known about metal uptake through the skin it is assumed that the skin has been impervious to metal absorption (Dallinger 1987). However, since the concentrations of trace elements in sediments below the mine exceeded

estimated background levels and since the concentrations of dissolved trace elements in the Methow River were less than the detection limits, it is likely that oral uptake was an important source of metal-loading.

Although the concentration of dissolved trace elements in water is important with respect to toxicity, concern over the oral uptake of trace elements contained in contaminated sediments and food is also significant (Spry et al. 1988). It is generally believed that the uptake of adsorbed trace elements is significantly less than the absorption of dissolved forms (Tamaki and Fankenberger 1992). While the relative importance of the routes of exposure remains unclear, at high concentrations the bioavailability of even a small fraction of adsorbed trace elements from the diet would be important. It has also been suggested that diet is a significant route of exposure (Dallinger 1987, Hare 1992). Woodward et al. (1994) showed that rainbow trout (*Oncorhyncys mykiss*) fed invertebrates that contained elevated concentrations of As, Cd, Cu and Pb were more important than water-borne metals in reducing survival and growth. Other investigations of metal contamination where concentrations were low in water by elevated in sediments and benthic macroinvertebrates also indicated that diet was an important source of metal accumulation in fish (Dallinger and Kautzky 1985, Lanno et al. 1987, Farag et al. 1994, Mount 1994).

Where there is a "food chain effect" from metals contamination biomagnification has not been observed and bioconcentration is small (Woodward et al. 1994). However, the amount of metal transferred from food can be high enough to be biologically harmful (Dallinger et al. 1987). Once in the lumen of fish, metals are then absorbed into gut tissue, where they are distributed to other organs such as liver, kidney, and muscle (Dallinger and Kautzky 1985). Woodward et al. (1994) showed that the livers from fish fed benthic macroinvertebrates that contained elevated levels of As, Cd, and Cu exhibited degenerative changes, increased cytoplasmic granularity, and degeneration of individual hepatocytes. Metals-induced degeneration of hepatocytes can result in death in severe cases and can divert energy from

growth and metabolism duringsublethal exposure (Hodson 1988).

The uptake of metals from the ailimentary tract occurs through the apical epithelial cell membrane, which is the organism's interface with the external environment in the gastrointestinal tract. Metals in the diet enter apical cell membranes through either the lipid, aqueous, or endocytic route (Simkiss 1996). According to this model, the greatest flux of dissolved cations would occur through the aqueous route, however, cations adsorbed to inorganic particles or bacteria also have the potential of entering the cell through the endocytic route.

In the aqueous route the lipid membrane is selectively permeable to cations, which is explained by the presence of aqueous pores that are formed by proteins that loop in and out of the membrane (Luecke et al. 1999). Selectivity is based on the interaction between the hydration and dehydration of the ion and on the binding and release of the cation from the ligands in the channel wall. Some channels are selectively permeable to cations (Simkiss 1996). Membrane-bound enzymes that split adenosine triphosphate (ATP) can use the chemical energy released to drive ions against their electrochemical gradients.

In the endocytic route, trace element contaminants associated with bacteria in particulate form may be preferentially processed into the digestive system as a nutrient source and sorted by well defined mechanisms so that they are phagocytosed into specific digestive cells (Simkiss 1996). This phenomenon includes processes that range from specific receptor-mediated events to the simple assimilation of food particles. These are fundamental cellular processes that link the endocytosis of food particles to intracellular lysosomal digestion. The uptake of iron is one example of a very specific type of endocytosis.

After uptake, toxicity begins as a reaction between the chemical and an organism at the molecular level. Iinitial chemical reactions result in secondary and tertiary responses at the cellular and tissue level that ultimately affect organisms at higher levels of biological organization (Hodson 1990). Toxicity is generally based on studies at the population level that measure the

96-hour LC50 (96-LC50), however, the degree to which cause and effect are related (i.e., specificity) and our knowledge of the mechanisms of toxicity is low at this level of organization (Hodson 1990, Clements 2000).

Uncertainty factors are sometimes applied to 96-LC50 values to account for uncontrolled environmental effects and variations in sensitivity among taxa. The 96-LC50 threshold values for aquatic invertebrates can vary by as much as three orders of magnitude between families and by over one order of magnitude within a species. Nehring (1976) reported a 96-LC50 for *Pteronarcys californica* (stonefly) that was between 10,000 and 13,900 μg-Cu L^{-1}, whereas the 96-LC50 for *Hydropshche belteni* (Caddisfly) was 8,300 μg-Cu L^{-1} (Warnick and Bell 1969) and for *Tanytarsus dissimilis* (Midge) it was 16 μg-Cu L^{-1} (Anderson et al. 1980). The dissolved Cu observed to be toxic for *Salmo gairdneri* ranged from 17 to 680 μg L^{-1} (Lett et al. 1976, Chapman 1978).

In addition to differences in toxicity between taxa, toxicity is also related to the chemical characteristics of the water (e.g., including alkalinity, hardness, pH, and the presence of specific organic compounds), interactions with other metals, speciation and complexation (Chakoumakos et al. 1979, Stephenson 1983, Gauss et al. 1985). While chemical assessments are useful to describe the potential cause of an environmental problem, water quality alone is not adequate to characterize the biological integrity of an aquatic ecosystem. As a result of the uncertainty associated with chemical assessments, biological assessments appear to be the most direct method of measuring biological integrity (Davis and Simon 1995).

Cytotoxicity of Caddisfly Larvae and Trout

The storage of metals in granules is one mechanism that cells have for the immobilization of excess concentrations of the metals entering from the environment. Metal containing granules have been reported in the cytosol of rainbow trout (*Salmo gairdneri*) and all major invertebrate

phyla (Walker 1977, Brown 1982, Woodward and Bergeron 1984, Pirie et al. 1985, Adams and Shorey 1998, Vesk and Byrne 1999). The only report on metal-containing granules in aquatic larvae of an insect is that of Darlington and Gower (1990). This is the first account that granules occur in the mitochondria of aquatic insect larvae. There are no known reports on the potential for a loss of mitochondrial function or the induction of apoptosis in invertebrates as a result of metal toxicity.

The homeostatic regulation of normal tissue mass is affected by the cyclic production of growth and death factors, which induce mitosis and apoptosis (the cell death program), respectively (Kerr and Harmon1991). Mitochondria, in addition to generating ATP, are critical in regulating the complex survival signals that determine whether cells live or die (Kamp 2002). It is not clear whether the processes that occur in caddisfly larvae and fish are the same. However, since the intestinal epithelia in the larvae and hepatocytes in trout are both aerobically poised and energetically demanding and mitochondrial densities are high in both, the loss of mitochondrial function could be injurious due not only to the loss of ATP but also to the release of cytochrome C, which is responsible for the initiation of apoptosis (Zhao 2001).

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 (Liu et al 1996, Yang 1997, and Kluck 1997, Dragan 2001, 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. 2002). At toxic concentrations Cu^{2+}, or its low molecular weight complexes, causes the formation of reactive oxygen species (ROS), such as hydrogen peroxide and induces apoptotic cell death. This is preceded by the upregulation of Bax (Zhai 2000), the loss of mitochondrial membrane potential or permiability transition (Pourahmad and O'Brian 2000), and the release of cytochrom C into the cytosol.

The precipitation of metals in the matrix of mitochondria are potential indicators 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 called 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.

Results from the static microcosm caddisfly cytotoxicity test showed that the *in vivo* exposure of caddisfly larvae (*Ecclesomyia* spp.) to trace elements in water and periphyton induces nuclear apoptosis and the formation of electron-dense granules in the matrix of caddisfly gut epithelial cell mitochondria. Caddisfly larvae exposed *in vivo* in static microcosm chambers contained periphyton contaminated with Cu at 292 $\mu g\ g^{-1}$ and As at 70 $\mu g\ g^{-1}$. Both Cu and As exceeded the reported threshold concentration for these elements in sediments. However, it is not clear whether the concentration of Cu in the dissolved fraction (i.e., 26 $\mu g\ L^{-1}$), apparently leached from the periphyton, contributed to the formation of submitochondrial granules and apoptotic bodies.

Much of the knowledge regarding acute toxicity and the effects of dissolved As in the aquatic environment is based primarily on laboratory studies (Anderson et al. 1980, Canivet 2001, Jones and Suter 1997, Remwoldt et al. 1972, Tamaki and Frankenberger 1992, Tisler 2002). Arsenic toxicity, although highly species specific, generally occurs at concentrations exceeding 17 $mg\ kg^{-1}$ in sediments and at least 200 $\mu g\ L^{-1}$ in the dissolved fraction (Canivet 2001, Tisler 2002). It is generally believed that the uptake of adsorbed As is significantly less than the absorption of dissolved As and that to enter cells As must eventually be in the dissolved

form (Tamaki and Frankenberger 1992). Similar 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 mg L^{-1} (Anderson et al. 1980). Gower and Darlington (1990) observed a positive linear relationship between the concentration of Cu in caddisfly larvae (*Plectrocnemia conspersa*) and the amount of dissolved Cu in water up to a concentration of 320 µg L^{-1}. Some caddisfly larvae appear, however, capable of tolerating dissolved Cu at concentrations as high as 6200 µg L^{-1} (Remwoldt et al. 1972) and there is clear evidence that benthic organisms tolerate sediment Cu concentrations up to 28 µg g^{-1} (Jones and Suter 1997).

In my study, caddisfly larvae exposed to surface water contaminated with AMD and exposed to only 26 µg L^{-1} dissolved Cu, both submitochondiral granules and evidence of apoptosis were detected indicating these cytological responses are occurring at the lower end of the toxicity range for caddisfly larvae (Anderson et al. 1980). When caddisfly larvae were exposed to water containing only dissolved As at 234 µg L^{-1} evidence of apoptosis was detected, but the mitochondria appeared normal compared to the control and no submitochondrial granules were detected.

The caddisfly larvae exposed to water containing only As did not form granules in their mitochondria because As^{3+} and As^{5+} are characterized by the formation of oxyanionic species (e.g., arsenite [$As(III)O_3^{3-}$], arsenate [$As(V)O_3^{3-}$] that do not compete with cations for active transport through membrane channels (Simkiss 1996). Mitochondria actively transport Ca^{2+} but they are only moderately selective to divalent cations that have a similar charge and radius (ionic potential). These ions enter cells and mitochondria by way of the active transport mechanisms that regulate transcellular Ca^{2+} transport and form spherical granules *in vitro* that are comprised of elements corresponding to the metals in the cytosol and medium surrounding the cell (Rouiller 1960, Peachy 1964, Simkiss 1996). Submitochondrial granules occur when organisms, cells

and mitochondrial membranes are exposed to excess concentrations of divalent cations like Cu^{2+}, which have ionic potentials similar to Ca^{2+}.

In the treated flow-through microcosm, caddisfly larvae were exposed to 606 mg kg^{-1} Cu, almost twice the amount compared to the static microcosm experiment, no As, and approximately half the amount of Cu in the surface water. Caddisfly larvae in both the treatment and control microcosms were also exposed to dissolved Cu concentrations that averaged approximately 15 µg L^{-1}, which was less than the 16-6200 µg L^{-1} range in toxicity threshold values for aquatic insect larvae reported by Remwoldt et al. (1972) and Anderson et al. (1980). Control microcosms also contained Cu in the periphyton (i.e., 15 mg kg^{-1}), which was approximately one-half the threshold concentration for toxicity reported by Jones and Suter (1997). It appears that the exposure of caddisfly in the reference microcosm chambers resulted in a background incidence of submitochondrial granules in the control larvae (i.e., 0.19 granules per mitochondrion). The difference in presence of submitochondrial granules in the treatment group (i.e., 0.53 granules per mitochondrion) was significantly higher than the control group at the 10% level of significance but not at the 5% level.

Caddisfly larvae and trout exposed *in situ* to ambient concentrations of Cu and As in Methow River sediments and periphyton also resulted in electron-dense granules and apoptosis. The lack of submitochondrial granules in samples from the trout reference population compared to an observed background level in the caddisfly reference group could be due to increased exposure of caddisfly larvae. This resulted from differences in feeding habits or differences resulting from the pelagic behavior of trout compared to the benthic mode of caddisfly larvae (Ward 1992, Jobling 1995). Kiffney and Clements (1993) showed that the mayfly *Baetis* spp., which feeds on periphyton and detritus, accumulated significantly more trace elements than other taxa and that, in general, organisms directly or indirectly associated with aufwuchs bioaccumulated more metals than predators.

X-ray Analysis of Submitochondrial Granules

Since experimental studies suggest that submitochondrial 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 Fe and 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-three instars while upriver stage-four instars dominated suggests that metal toxicity at the mitochondrial level delayed caddisfly development by at least one-month based on differences in life-history histograms for *Ecclesiomyia spp* (Merrit and Cummins 1996, Irons 1987). Furthermore, the lower body weights of microcosm trout and wild caddisfly larvae (*Ecclesiomyia spp*) downstream from the mine sites in the Methow River suggests that reduced ATP production, and mitochondrial collapse associated with apoptotic cell death, is resulting in a diversion of energy from growth to tissue repair (Ishak and Sharp 1987).

Although alkalinity, dissolved Ca, and total dissolved solids (TDS) are higher below the mines than above it is unlikely that they are factors that could reduce caddisfly and trout body weight and development. It is difficult to separate alkalinity, Ca, TDS, and pH in waters charged with calcium bicarbonate, however, aquatic insects as a group appear to be rather indifferent to normal ranges of water hardness (< 24 mg L^{-1} Ca) (Ward 1992). Egglishaw and Morgan (1965) showed there was a positive correlation between the densities and weights of Trichoptera with alkalinity. It may be that to reside in soft water of low total ionic content requires well-developed osmoregulatory mechanisms, which may account for the absence of

some species from dilute aquatic environments. In a subsequent study, Egglishaw (1968) established a positive corelation between the Ca content of stream water and the rate of decomposition of plant detritus. Water chemistry may also exerted its control indirectly on the benthos by regulating decomposition processes. Also, trace metals are generally less toxic to aquatic biota in hard than in soft waters so the observed effects are not likely to be due to differences in metal toxicity.

It also appears unlikely that the small differences in temperature, dissolved oxygen and pH could affect caddisfly and trout body weight and development. There is an inverse relationship between oxygen solubility and temperature and a positive correlation between an organisms oxygen requirement and temperature. In this study, temperatures were much lower than the upper limits and the DO content far exceeded minimum requirements. The optimum temperature range for *Salmo gairdneri* (rainbow trout) is 10-22 ^{0}C (Elliott 1981) and the minimum DO required is 5-6 mg L^{-1} and no advantage or effect on growth of rainbow trout reared at DO above the minimum, (7, 10, and 14 mg L^{-1}) occurs (Smart 1981). In regards to acidification, osmoregulatory effects are not generally observed above pH 5 (Eddy 1981).

CONCLUSION

In this study, I 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.

- Five trace elements (i.e., As, Cd, Cu, Pb, and Zn) which were significantly higher in Methow River sediments below the mines compared to the reference area above the mines. No effects from mine waste contamination on dissolved metal concentrations in the Methow River were observed.

- Submitochondrial granules are induced in the mitochondria of live caddisfly larvae and trout exposed *in vivo* to abandoned mine waste contamination in stream water, sediments, and periphyton.
- The incidence of submitochondrial granules was significantly higher in caddisfly and trout exposed to abandoned mine waste in both controlled microcosms and to contaminants *in situ* in the Methow River below abandoned mines.
- Elemental analysis of submitochondrial granules by X-ray analysis suggest that Cu is are present at high concentrations and bioavailable in the environment surrounding the organism, its cells and the mitochondria and in small intestine epithelial cells and hepatocytes 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 and As suggest both apoptosis and mitochondrial failure are occurring.
- Food chain effects resulting from mitochondrial collapse and the diversion of energy is causing reduced growth and development in caddisfly larvae and trout in the Methow River.
- Tentative conclusions were drawn from inferential statistics applied to unreplicated experiments that make it necessary to replicate these experiments with the same design in different areas using meta-analysis to achieve greater rigor.

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APPENDIX

QUALITY ASSURANCE AND QUALITY CONTROL

QUALITY ASSURANCE

Selection of study sites depended on the goals and objectives of the study. Access, location of contaminant sources, mixing zones, and the dilution of pollutants were considered. Reference sites were as similar as possible to the study site. Sites were identified that were as similar as possible in terms of gradient, depth, substrate size and heterogeneity, and canopy cover.. Water and sediment samples were collected from the same general vicinity as the biological samples. Samples were collected for water-quality analyses in the order of the parameters' decreasing sensitivity. At least three field replicates were collected at each sample site.

QUALITY CONTROL

In regards to biological samples, all macroinvertebrate samples were replicated in the field. In regards to chemical samples, laboratory standards were run with test samples and compared to the certified concentration. In the field, standard samples were handled following the procedure for the collection of field samples and submitted with the test samples to the laboratory for analysis. The measured concentration standard samples were required to fall within 10% of the theoretical concentration. Laboratory and field blanks were submitted with the test samples to the laboratory for analysis. Measured concentrations of blanksshould be less than detection limits.

Several hundred samples were analyzed during the performance of this and other studies between 1986 and 2002. Each sample was given a sequential log number. All laboratory and field blanks that were analyzed during this period and considered. Although the following deviations were noted, none were assoicated with the data used in this study.

Sample

Log No.

#61: Fe and Mn were detected in field blank. Were both were < 1% over field spike. Lab blank was 0 and lab spike was <1% below theoretical value therefore deviation must be due to handling. All other results were within specifications. Fe and Mn results from these samples were not used. These deviations had no impact on the test results.

#53: Cu and Zn were detected in field blanks. Both were <3% over field spike. Lab blanks were 0 for both. Lab spikes were <3% below theoretical value. Copper result was used in calculating average concentrations in well water but they were less than drinking water standards. Also used in calculating average concentration in Methow R. surface water but were less than background. Deviations had no impact on test results.

#38: Cu was detected in field blank. Cu was 5% below field spike. Lab blank was 0 and no results were reported for the lab spike. Copper result was used in calculating average concentrations in well water but they were less than drinking water standards. Also used in calculating average concentration in Methow R. surface water but were less than background.

#29: Cd was 2.242 mg L^{-1} and exceded field spike by more than 10% (i.e., 12%). Field blank and laboratory blanks were 0. Cd results were used in calculating average concentrations in well water but they were less than drinking water standards. Also used in calculating average concentration in Methow R. surface water but were less than background.

#20 and 21: Cd, Pb, and Zn exceded field spike by more than 10% (i.e., 11-15%). Cu and Pb was also detected in the field blank for sample group #20. Lab blanks were 0 and lab spikes were <1% below theoretical value. Cd, Pb, and Zn results were used in calculating average concentrations in well water but they were less than drinking water standards. Also used in calculating average concentrations in Methow R. surface water. Although 15% deviation could have affected individual results the effect on average and the deviation did not justify rejection of the results.

VITA

Daniel Peplow was born in Tacoma, Washington, and attended public schools in Ellensburg, Washington. After earning a Bachelor of Science degree in Bacteriology and Public Health he lived and worked in Ecuador, Texas, California and Puerto Rico. He earned a second Bachelor of Science degree in Zoology at the University of Washington in 1997 and a Master of Science in Forestry in 1999. In 2003 he earned a Doctor of Philosophy at the University of Washington in Forestry .