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# INHIBITION OF ERYTHROCYTE δ-AMINOLEVULINIC ACID DEHYDRATASE (ALAD) ACTIVITY IN FISH FROM WATERS AFFECTED BY LEAD SMELTERS*

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**Abstract.** We assessed the effects on fish of lead (Pb) released to streams by smelters located in Trail, BC (Canada), E. Helena, MT, Herculaneum, MO, and Glover, MO. Fish were collected by electrofishing from sites located downstream of smelters and from reference sites. Blood from each fish was analyzed for δ-aminolevulinic acid dehydratase (ALAD) activity and hemoglobin (Hb), and samples of blood, liver, or carcass were analyzed for Pb, zinc (Zn), or both. Fish collected downstream of all four smelters sites had elevated Pb concentrations, decreased ALAD activity, or both relative to their respective reference sites. At E. Helena, fish from the downstream site also had lower Hb concentrations than fish from upstream. Differences among taxa were also apparent. Consistent with previous studies, ALAD activity in catostomids (Pisces: Catostomidae-northern hog sucker, *Hypentelium nigricans*; river carpsucker, *Carpiodes carpio*; largescale sucker, *Catostomus macrocheilus*; and mountain sucker, *C. platyrhynchus*) seemed more sensitive to Pb-induced ALAD inhibition than the salmonids (Pisces: Salmonidae-rainbow trout, *Oncorhynchus mykiss*; brook trout, *Salvelinus fontinalis*) or common carp (*Cyprinus carpio*). Some of these differences may have resulted from differential accumulation of Zn, which was not measured at all sites. We detected no ALAD activity in channel catfish (*Ictalurus punctatus*) from either site on the Mississippi River at Herculaneum, MO. Our findings confirmed that Pb is released to aquatic ecosystems by smelters and accumulated by fish, and we documented potentially adverse effects of Pb in fish. We recommend that Zn be measured along with Pb when ALAD activity is used as a biomarker and the collection of at least 10 fish of a species at each site to facilitate statistical analysis.

**Keywords:** ALAD, Columbia River, fish, hemoglobin, lead, metals, Mississippi River, smelters, zinc

## 1. Introduction

The smelting of sulfide minerals such as galena (PbS) releases substantial quantities of liquid, solid, and gaseous byproducts enriched in sulfur, metals, and other contaminants to the environment. Consequently, smelters have historically represented major environmental sources of sulfur dioxide and elemental contaminants



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including lead (Pb), cadmium (Cd), zinc (Zn), copper, arsenic, selenium, and thallium (U.S. EPA, 1977, 1979; Nriagu, 1978; Schmitt, 1999). Atmospheric releases from smelters are widely recognized as significant threats to human and ecological health (Baker *et al*., 1977; U.S. EPA 1977, 1979). Impacts of atmospheric emissions on terrestrial ecosystems include direct toxicity to vegetation, mostly from sulfuric acid and metals deposition, and associated shifts in animal communities (e.g., Jordan, 1975; Tyre and Barton, 1986; U.S. EPA, 1977). Effects on individual terrestrial organisms (Lower and Tsutakawa, 1978) and ecosystem processes (Watson, 1975; Jackson and Watson, 1977) have also been documented. Fallout from smelters, along with solid wastes deposited in waterways and leachates from upland solid wastes, constitute significant non-point sources of contaminants to surface waters and groundwater (e.g., Benes *et al*., 1985; Moore *et al*., 1991; Harrison and Klaverkamp, 1990; Chapman *et al*., 2001). Smelters also produce significant liquid and slurried wastes, which were historically discharged untreated directly to waterways (e.g., Bortelson *et al*., 1994) and which represent significant point-sources of metals (U.S. EPA, 1977).

Contaminant releases from smelters in North America have been reduced substantially over the last two decades. Many older, inefficient complexes have been decommissioned and subjected to environmental remediation (e.g., Woodward *et al*., 1994; Chapman *et al*., 2001). Those remaining in operation have become more efficient by recovering substantially larger proportions of byproducts formerly released as wastes, thereby reducing liquid, solid, and gaseous emissions (e.g., Tsai, 1987; Pilgrim and Hughes, 1994; Rosin, 1998). Nevertheless, Schmitt *et al*. (1993) reported elevated blood and carcass metals concentrations and lower activity of the enzyme $\delta$-aminolevulinic acid dehydratase (ALAD; E.C. 4.2.1.24) in the blood of fish collected downstream from the discharge of a modern, operational Pb smelter. ALAD catalyzes the synthesis of porphobilinogen (PBG), a hemoglobin (Hb) precursor. Erythrocyte ALAD activity is inhibited by Pb (Finelli, 1977; Hodson *et al*., 1977), and the measurement of ALAD activity in fish blood as a biomarker of environmental Pb exposure is well documented (Hodson *et al*., 1984; Dwyer *et al*., 1988; Schmitt *et al*., 1984, 1993; Burden *et al*., 1998). Previous studies have shown that ALAD activity is negatively correlated with both carcass- and blood-Pb concentrations in many fishes (Haux *et al*., 1986; Schmitt *et al*., 1984, 1993) and that the inhibitory effect of ALAD activity is specific to Pb (Hodson *et al*., 1977). Together, these biochemical and analytical methods provide widely accepted documentation of the biological availability and biological activity of Pb in aquatic ecosystems, as they do in birds (Dieter, 1979) and mammals (Wigfield *et al*., 1986) inhabiting terrestrial systems.

We report here the results of four independent studies of Pb and ALAD inhibition in fish from waters affected by smelters. Our primary objective was to obtain information on the release of biologically available Pb from smelters and its biochemical effects as a baseline against which to evaluate ongoing and future discharge restrictions and remedial activities at the sites. A secondary objective was

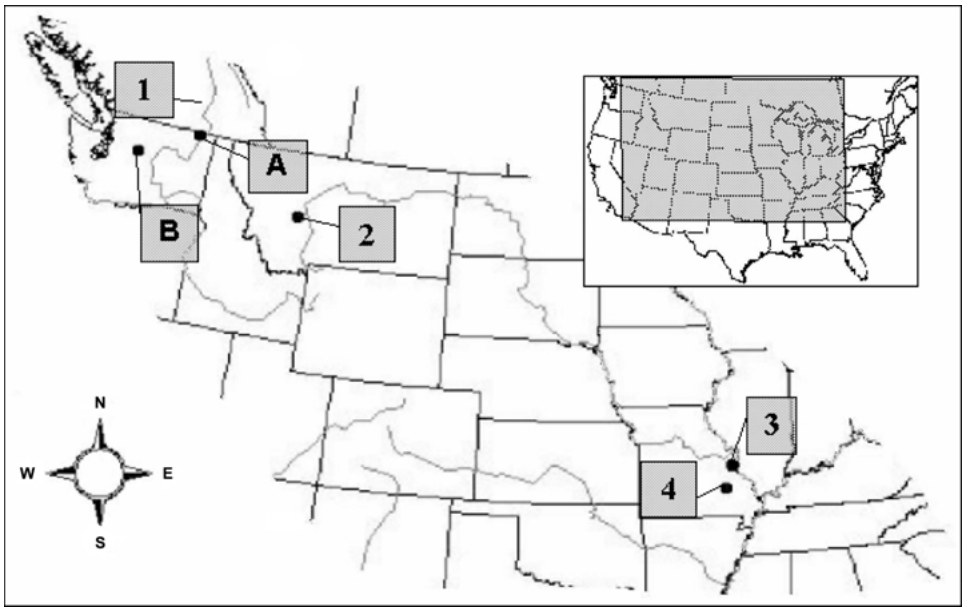


*Figure 1.* Map of the U.S. and southern Canada, with inset showing locations of smelters: **1**, Trail, BC; **2**, East Helena, MT; **3**, Herculaneum, MO; and **4**, Glover, MO. Also shown are the locations of **A**, Northport, WA, the downstream study site; and **B**, Lake Wentachee, WA, the reference site, for the Trail, BC smelter. See text and Table I for collection locations.

to further ascertain the Pb-induced inhibition of ALAD activity in fish, especially for taxa in which this effect had not been documented in field studies. Our general approach was to compare ALAD activities and Hb concentrations in fish from sites downstream of smelters and from reference sites, and to interpret these results relative to concentrations of metals in blood, liver, or carcass.

## 2. Study Sites, Methods, and Materials

### 2.1. Study sites

Fish were collected from sites on rivers and streams affected to differing degrees by smelter complexes located in Herculaneum, MO; Glover, MO; E. Helena, MT; and Trail, BC (Canada); and from appropriately situated reference sites (Figure 1, Table I). All sites were sampled during July, August, and September 1992 except for Glover, which was sampled in August 1993.

#### 2.1.1. *Trail, BC*

The Consolidated Mining and Smelting Company of Canada, Ltd. (COMINCO) smelting complex in Trail, BC is situated on the Columbia River about 16 km upstream of the international border (Figure 1). Smelting and related activities

TABLE I
Collection sites (R, reference site)

| Smelter, location, and collection site | County, state | Latitude, longitude | Distance to smelter (km) |
|---|---|---|---|
| COMINCO, Trail, BC | | | |
| Lake Wenatchee, WA (R) | Chelan, WA | 47°48′30″N, 120°43′30″W | 260 (different watershed) |
| Columbia River at Northport, WA[a] | Stevens, WA | 48°55′13″N, 117°46′32″W | 30–33[a] (below) |
| Doe Run Co., Herculaneum, MO | | | |
| Mississippi River at Bushburg, MO (R) | Jefferson, MO | 38°18′16″N, 090°22′26″W | 4 (above) |
| Mississippi River at Joachim Creek, Herculaneum | Jefferson, MO | 38°15′42″N, 090°22′16″W | 0.8 (below) |
| ASARCO, E. Helena, MT | | | |
| Prickly Pear Creek above McClellan Creek (R) | Lewis and Clark, MT | 46°32′59″N, 111°55′02″W | 4 (above) |
| Prickly Pear Creek below Riggs Street | Lewis and Clark, MT | 46°35′29″N, 111°55′14″W | 1 (below) |
| ASARCO, Glover, MO | | | |
| Big Creek at Hogan (R) | Iron, MO | 37°30′51″N, 090°41′32″W | 4 (above) |
| Big Creek at Glover | Iron, MO | 37°28′33″N, 090°41′15″W | 0.5 (below) |

[a] Fish were collected from the Columbia River at the confluences of Deep and Fivemile Creeks and from the vicinity of the Highway 25 Bridge in Northport.

have occurred at this site since the 1890s, and slag and slurry effluent containing relatively high concentrations of elemental contaminants have been discharged to the river (Bortelson *et al*., 1994). Despite recent discharge reductions, elevated concentrations of metals attributed primarily to COMINCO have been detected in sediments (Johnson *et al*., 1990; Bortelson *et al*., 1994) and fish (Hopkins *et al*., 1985; Lowe *et al*., 1985; Smith, 1987; Johnson *et al*., 1988, 1990; Serdar *et al*., 1994; Munn *et al*., 1995; Munn and Short, 1997; Schmitt *et al*., 1999) in the Northport (WA) reach of the Columbia River and as far downstream as Grand Coulee Dam, some 240 km from the international border. Toxicity to invertebrates, reduced benthic macro-invertebrate community diversity (Bortelson *et al*., 1994), and metallothionein induction in fish (Smith, 1987) have also been reported for the Columbia River near the international border.

We collected largescale sucker (*Catostomus macrocheilus*) from the Northport Reach of the Columbia River, 31–33 km downstream from the smelter and just upstream of Franklin D. Roosevelt Lake (Figure 1, Table I). Fish were collected from near the mouths of Deep and Fivemile Creeks and at the Highway 25 bridge in Northport. As a reference, largescale sucker were collected from the outlet of Lake Wenatchee, which is located in an undeveloped area of the Wenatchee National Forest in rural Chelan Co., WA, about 260 km southwest of Northport (Figure 1, Table I). All fish were analyzed for ALAD activity, Hb, and concentrations of Pb and Zn in liver.

2.1.2. *Herculaneum, MO*

Lead has been smelted since 1864 on the bank of the Mississippi River near the site of the St. Joseph Lead Company complex (now operated by the Doe Run Company) in Herculaneum, MO (U.S. EPA, 1977). Industrial-scale operations at Herculaneum commenced in the 1890s (Rosin, 1998). The smelter complex is situated at the confluence of Joachim Creek and the Mississippi River (Figure 1). Historically, liquid and solid smelter wastes, along with sanitary sewage, were discharged directly to Joachim Creek and the Mississippi River. In addition, slag containing elevated concentrations of elemental contaminants has been deposited along Joachim Creek. Although the Herculaneum facility still discharges metals, recent improvements have generally reduced the volume and the metals content of the slag and liquid wastes discharged (Rosin, 1998).

We collected river carpsucker (*Carpiodes carpio*), common carp, and channel catfish (*Ictalurus punctatus*) from the west bank of the Mississippi River above the complex (at Bushburg, MO) and at the mouth of Joachim Creek in Herculaneum (Figure 1, Table I). Blood samples were analyzed for ALAD activity and Hb, and samples of whole fish (hereafter carcass) were analyzed for Pb and Zn.

2.1.3. *East Helena, MT*

Mining and related activities in the Prickly Pear Creek drainage of west-central Montana began in the 1860s. The American Smelting and Refining Co. (ASARCO) smelter complex in E. Helena has been in operation since the 1890s (Pagenkopf and Maughan, 1984). Large quantities of slag and other solid wastes are evident around Prickly Pear Creek near the smelter (Olsen *et al*., 1997). Prior to the implementation of discharge restrictions around 1970, untreated liquid wastes containing metals were also discharged directly to Prickly Pear Creek (Pagenkopf and Maughan, 1984). A decade later, concentrations of metals remained elevated in sediments and water in Prickly Pear Creek immediately below the smelter relative to upstream sites (Pagenkopf and Maughan, 1984; Crossey and La Point, 1988). By 1991, metals concentrations in Prickly Pear Creek downstream of the smelter were elevated only during high-flow conditions, and Pb concentrations in brook trout (*Salvelinus fontinalis*) and rainbow trout (*Oncorhynchus mykiss*) did not differ significantly between sites upstream and downstream of the smelter; however, concentrations in sediments and invertebrates remained elevated downstream (Olsen *et al*., 1997).

We collected mountain sucker (*Catostomus platyrhynchus*), brook trout, and rainbow trout from Prickly Pear Creek upstream (above confluence with McClellan Creek) and downstream (below Riggs Street bridge) of the smelter (Figure 1, Table I). Although mining and related activities have also occurred upstream of the reference site (Olsen *et al*., 1997) and there has been substantial atmospheric deposition metals to the watershed (Pagenkopf and Maughan, 1984), the collection sites were selected to document any additional effects of the smelter. Blood samples were analyzed for concentrations of Hb and Pb and for ALAD activity.

#### 2.1.4. *Glover, MO*

The ASARCO facility in Glover is much newer than the other smelters we studied, beginning operation in 1968 (Ryck, 1974). Situated along Big Creek in rural Iron Co., MO (Figure 1), the facility re-circulates much of its wastewater. Metals are nevertheless discharged to a small tributary of Big Creek. By 1970, several fish kills had occurred in Big Creek and benthic macro-invertebrate diversity had been reduced downstream of the smelter (Ryck, 1974). Fish (northern hog sucker, *Hypentelium nigricans*) collected from Big Creek downstream of the smelter in 1989 contained elevated concentrations of Pb, Zn, and other metals in carcass and blood, and ALAD activity was reduced relative to reference sites (Schmitt *et al*., 1993). We collected northern hog sucker from Big Creek upstream (at Hogan, MO) and immediately downstream of the smelter complex (Figure 1, Table I). Blood samples were analyzed for ALAD activity and Hb, and Pb concentrations were measured in the carcass and blood of each fish.

### 2.2. FIELD METHODS

At each site, fish were collected by electrofishing. Blood (ca. 1 mL) was obtained by caudal veinipuncture using a heparinized, disposable needle and syringe. About half of the blood was dispensed into a labeled Cryovial® and immediately frozen in liquid nitrogen for subsequent analysis of ALAD activity and Hb. These samples were stored frozen at –80 °C until analyzed. The remainder of the blood, which was analyzed for metals, was dispensed into a pre-labeled, acid-cleaned, borosilicate glass tube; chilled (0 °C) immediately; and frozen (–20 °C) upon return to the laboratory (ca. 24 hr). Following blood collection, fish were weighed (g) and measured (mm), then dissected and identified to gender. The carcasses were individually wrapped in foil, chilled (0 °C) until returned to the laboratory (ca. 24 hr), and frozen (–20 °C) until analyzed for metals. Livers to be analyzed for metals were dissected from the fish collected from the Columbia River at Northport and from Lake Wenatchee were also wrapped in foil, chilled, and frozen until they were analyzed.

### 2.3. LABORATORY METHODS

#### 2.3.1. *Metals Analyses*

The studies reported here were conducted independently, and metals concentrations were measured by several laboratories using different methods. Liver samples from the Columbia River and Lake Wenatchee were analyzed for Pb and Zn by the Washington Department of Ecology (WDE) using graphite-furnace atomic absorption spectroscopy (AAS), as described by Serdar *et al*. (1994). Carcass samples from Herculaneum and Glover and blood samples from Glover were analyzed for Pb, Zn, or both using inductively coupled plasma emission spectroscopy (ICP). These analyses were performed at contract laboratories managed by the U.S. Fish

and Wildlife Service (FWS)-Patuxent Analytical Control Facility (PACF), Laurel, MD as described by Schmitt *et al*. (1993, 1999) and Olsen *et al*. (1997). AAS analyses of blood samples from E. Helena for Pb and Zn were also performed by FWS-PACF contract laboratories following methods described by Schmitt *et al*. (1984). Analytical quality assurance was provided by the WDE and the FWS-PACF and included analyses of standard reference materials, spiked samples, and blind replicates, the results for all of which were deemed acceptable. Limits of detection (LOD) were estimated independently for Pb and Zn in each sample. Analyte concentrations were well above LOD except for blood-Pb in one mountain sucker from the upstream E. Helena site (<0.2 $\mu$g $g^{-1}$ dry-weight); and liver-Pb in largescale sucker from Lake Wenatchee, all of which were <0.2 $\mu$g $g^{-1}$ (wet-weight). Although comparatively high, we considered these detection levels acceptable given the objectives of the studies.

#### 2.3.2. *ALAD and Hb Analyses*

ALAD activity (nmol PBG $g^{-1}$ whole blood $hr^{-1}$) was determined in duplicate as described by Schmitt *et al*. (1993). Hb concentrations were measured by the cyanomethemoglobin method (Larsen and Sniesko, 1961) with bovine Hb (Sigma Chemical Co., St. Louis, MO) as the standard. The samples from each study were analyzed within 7–14 days of collection. The accuracy and precision of ALAD and Hb determinations were quantified by duplicate analyses, procedural blanks, reference samples, and standards. Precision, as the coefficient of variation [CV; (standard deviation/mean) $\times$ 100], was measured within and between assays from pooled samples of whole blood from rainbow trout and deemed acceptable if < 10% for ALAD and < 20% for Hb. Both ALAD and Hb occur in erythrocytes, so ALAD activity was also expressed per unit concentration of Hb (i.e., ALAD/Hb; nmol PBG $mg^{-1}$ Hb $hr^{-1}$; Schmitt *et al*., 1984, 1993).

### 2.4. Statistical analyses

For statistical analyses, reporting, and comparisons with other investigations, dry-weight carcass-Pb and -Zn and blood-Pb concentrations were converted to wet weight values using gravimetrically determined moisture content (%) obtained from this and other investigations (Schmitt *et al*., 1993, 1999; Burden *et al*., 1998; Brigham *et al*., 1998). A value of 50% LOD was substituted for the one censored blood-Pb value from E. Helena; however, the liver-Pb data from the Trail, BC study were not analyzed statistically because all 16 values from Lake Wenatchee were below the LOD. ALAD in channel catfish from Herculaneum was also not analyzed statistically because no activity was detected in any sample ($n$ = 4). All other data were analyzed by analysis of variance (AOV) treating collection sites and species as fixed effects. A one-way AOV was employed for Trail and Glover, where only one species was collected. For E. Helena and Herculaneum, where three species were collected, a two-way AOV was used and differences between

TABLE II

Arithmetic means and (standard errors) by species and collection each site. All Pb and Zn results are reported as wet-weight concentrations

| Smelter location, species and collection site | ALAD (nmol PBG $g^{-1}$ $hr^{-1}$) | ALAD/Hb (nmol PBG $mg^{-1}$ $hr^{-1}$) | Hb (g $L^{-1}$) | Blood Pb ($\mu g$ $g^{-1}$) | Carcass- or liver-(L) Pb ($\mu g$ $g^{-1}$) | Carcass- or liver-(L) Zn ($\mu g$ $g^{-1}$) |
|---|---|---|---|---|---|---|
| Trail, BC | | | | | | |
| Largescale sucker | | | | | | |
| Lake Wenatchee (R)[a] | 369.1 (34.9)[a] | 3.7 (0.3)[a] | 98.1 (5.8) | nm[c] | (L) < 0.2 (0) | (L) 49.7 (3.8) |
| Northport | 165.8 (22.2)[a] | 1.6 (0.2)[a] | 104.7 (0.4) | nm | (L) 1.34 (0.31) | (L) 54.1 (4.85) |
| Herculaneum, MO | | | | | | |
| River carpsucker | | | | | | |
| Above smelter (R) | 272.4 (131.7) | 3.1 (1.3) | 86.4 (5.5) | nm | 0.27 (0.03) | 13.2 (0.04) |
| Below smelter | 199.0 (97.7) | 2.0 (0.8) | 96.2 (8.1) | nm | 2.17 (1.75) | 15.9 (1.70) |
| Common carp | | | | | | |
| Above smelter (R) | 455.5 (128.9) | 4.6 (0.6) | 97.6 (1.6) | nm | 0.14 (0.012)[a] | 49.5 (10.10) |
| Below smelter | 444.6 (73.3) | 5.2 (0.6) | 86.0 (1.3) | nm | 4.39 (3.09)[a] | 59.3 (1.64) |
| Channel catfish | | | | | | |
| Above smelter (R) | nd | nd | 102.4 (4.0) | nm | 0.17 (0.03)[a] | 15.3 (0.2) |
| Below smelter | nd | nd | 94.8 (0.1) | nm | 1.22 (0.14)[a] | 17.7 (1.0) |
| E. Helena, MT | | | | | | |
| Mountain sucker | | | | | | |
| Above smelter (R) | 637.8 (152.4)[a] | 6.78 (1.72) | 94.5 (1.5)[a] | 0.03 (0.01)[a] | nm | nm |
| Below smelter | 422.1 (33.3)[a] | 5.88 (0.17) | 71.8 (5.1)[a] | 0.06 (0.02)[a] | nm | nm |
| Rainbow trout | | | | | | |
| Above smelter (R) | 812.2 (39.2) | 6.59 (0.45) | 124.0 (5.2) | 0.22 (0.02)[b] | 0.81 (0.21–3.69)[c] | 39.5 (32.1–53.3)[c] |
| Below smelter | 762.4 (33.1) | 6.40 (0.41) | 118.8 (6.1) | 0.39 (0.05)[b] | 0.85 (0.15–7.22)[c] | 42.5 (29.1–63.5)[c] |
| Brook trout | | | | | | |
| Above smelter (R) | 828.1 (55.0) | 8.58 (0.48) | 96.7 (5.7) | 0.10 (0.05)[a,b] | 0.31 (< 0.14–0.68)[c] | 55.8 (50.8–62.6)[c] |
| Below smelter | 767.1 (23.4) | 8.09 (0.35) | 95.3 (2.4) | 0.39 (0.14)[a,b] | 0.13 (< 0.14–0.20)[c] | 44.0 (29.6–61.2)[a] |
| Glover, MO | | | | | | |
| Northern hog sucker | | | | | | |
| Above smelter (R) | 369.0 (37.6) | 5.48 (0.70) | 68.3 (5.0) | 0.15 (0.06)[a] | 0.48 (0.07)[a] | nm |
| Below smelter | 318.5 (41.6) | 4.03 (0.42) | 78.4 (2.70) | 1.25 (0.21)[a] | 3.95 (0.77)[a] | nm |

[a] Wet-weight concentrations converted from dry-weight using moisture (87.5%) for catostomid blood from Schmitt *et al*. (1993).

[b] Wet-weight concentrations converted from dry-weight using moisture (78%) for rainbow trout blood from Burden *et al*. (1998).

[c] Mean and range of wet-weight concentrations converted from dry-weight data of Olsen *et al*. (1997) using moisture (71.8%) for rainbow trout from Schmitt *et al*. (1999). Carcass Pb concentrations in rainbow trout and brook trout did not differ significantly between sites ($p > 0.05$, Mann-Whitney U-test; Olsen *et al*., 1997).

pairs of least-squares means (i.e., between collection sites, same species) were tested using Fisher's protected LSD. For Glover and E. Helena, we also computed product-moment correlation coefficients to evaluate relationships between pairs of variables. All statistical analyses were conducted with $\log_{10}$-transformed data to better approximate normality and equality of variances, and a nominal significance level of $\alpha \leq 0.05$ was used in most tests. More rigorous exploratory statistical analyses were precluded by the censored data from Lake Wenatchee and small numbers of samples from the other sites.

TABLE III

Results of analysis-of-variance (all $\log_{10}$-transformed) as $F$-values, degrees-of-freedom (df), error mean squares, and coefficients of determination ($R^2$). * $p < 0.05$; ** $p < 0.01$

| Smelter, source of variation, and (df) | ALAD | Hb | ALAD/Hb | Blood-Pb (wet-weight) | Carcass or liver (L) Pb | Carcass or liver (L) Zn |
|---|---|---|---|---|---|---|
| Trail, BC | | | | | | |
| Site (1) | 20.13** | 1.01 | 29.05** | nm[a] | na[a] | 0.25 (L) |
| Error (31) | 0.0604 | 0.0091 | 0.0495 | | | 0.0220 |
| $R^2$ | 0.39 | 0.03 | 0.48 | | | < 0.01 |
| Herculaneum, MO | | | | | | |
| Site (1) | 0.15 | 0.14 | 0.20 | | 20.98** | 4.78 ($p = 0.07$) |
| Species (2) | 3.46 ($p = 0.14$) | 0.43 | 5.47 ($p = 0.08$) | | 0.19 | 108.06** |
| Site* Species (2) | 0.15 | 0.69 | 0.70 | | 0.96 | 0.04 |
| Error (6) | 0.0641 | 0.0041 | 0.0412 | | 0.1320 | 0.0036 |
| $R^2$ | 0.48 | 0.28 | 0.61 | | 0.80 | 0.97 |
| E. Helena, MT | | | | | | |
| Site (1) | 9.45** | 7.36* | 1.08 | 7.90* | nm* | nm* |
| Species (2)[b] | 22.54** | 28.19** | 9.53** | 12.69** | | |
| Site* Species (2)[b] | 3.07 ($p = 0.07$) | 3.63* | 0.18 | 1.160 | | |
| Error (18) | 0.0032 | 0.0017 | 0.0036 | 0.0978 | | |
| $R^2$ | 0.81 | 0.82 | 0.58 | 0.68 | | |
| Glover, MO | | | | | | |
| Site (1) | 0.88 | 3.47 ($p = 0.11$) | 3.64 ($p = 0.11$) | 33.91** | 54.85** | nm* |
| Error (6)[c] | 0.0108 | 0.0023 | 0.0095 | 0.0464 | 0.0295 | |
| $R^2$ | 0.13 | 0.37 | 0.38 | 0.87 | 0.90 | |

[a] nm, not measured; na, not analyzed statistically because all values from Lake Wenatchee were < 0.2 $\mu$g g$^{-1}$.
[b] df = 1 for ALAD and ALAD/Hb (channel catfish excluded from analysis; see text for explanation).
[c] df = 5 for Blood-Pb.

## 3. Results

ALAD activity was generally, but not uniformly, lower and Pb concentrations were higher downstream of all the smelters than at their respective reference sites (Tables II and III). At reference sites, Pb concentrations in fish were comparatively low. Smelter influences on Pb concentrations and ALAD activity were more evident and consistent than effects on Zn concentrations and Hb. Substantial among-species differences were also evident as were some inconsistencies in the ALAD response to Pb.

### 3.1. TRAIL, BC

Liver-Pb concentrations in largescale sucker from the Columbia River at Northport were elevated at least 6-fold relative to Lake Wenatchee, where liver-Pb was < 0.2 $\mu$g g$^{-1}$ in all 16 fish analyzed (Table II). ALAD activity differed significantly between the two sites but Hb did not (Table III); ALAD activity was 55% lower (56% as ALAD/Hb) at Northport than at Lake Wenatchee (Table II). In contrast, liver-Zn at Northport and Lake Wenatchee did not differ significantly (Tables II and III).

### 3.2. HERCULANEUM, MO

Only carcass-Pb differed significantly between the Mississippi River sites above and below the Herculaneum smelter (Tables II and III). Differences among species were also significant for carcass Zn, and were marginally significant for ALAD and ALAD/Hb (Table III), all of which were greatest in common carp (Table II). No ALAD activity was detected in channel catfish from either site (Table II). Relative to the upstream site, carcass-Pb concentrations downstream of the smelter were 7-fold greater in channel catfish, 8-fold greater in river carpsucker, and 31-fold greater in common carp. The differences for common carp and channel catfish were statistically significant but those for river carpsucker were only marginally significant (Tables II and III). ALAD activity was 27% lower (35% as ALAD/Hb) in river carpsucker below the smelter than above, but neither these differences nor any others tested in this species were statistically significant (Tables II and III). Carcass-Zn concentrations were 16–17% greater in all species at the downstream site relative to upstream (Table II), but these differences were also only marginally significant (Table III).

### 3.3. HELENA, MT

Relative to the upstream site on Prickly Pear Creek, blood Pb concentrations (wet-weight) were elevated 1.8-fold in rainbow trout, 2-fold in mountain sucker, and 3.9-fold in brook trout collected below the ASARCO complex; however, only the difference for brook trout was statistically significant (Tables II and III). On a dry weight basis, however, the differences were significant in all species ($p < 0.05$, data not shown). ALAD activity was significantly lower (44%) in mountain sucker but not brook trout or rainbow trout; in the salmonids ALAD activity was reduced only 7–8% relative to the upstream site (Tables II and II). Hb concentrations were also significantly lower in mountain sucker from the downstream site. Consequently, ALAD/Hb was only 24% lower, which was not statistically significant. All variables differed significantly among species (Tables II and III); both blood-Pb concentrations and ALAD activity were lower in mountain sucker than in either salmonid, but Hb concentrations were greater in rainbow trout than in either brook trout or mountain sucker (Table II). In brook trout, ALAD activity was weakly correlated (negative) with blood-Pb ($r = –0.44$, $p = 0.21$), but the negative correlation between ALAD/Hb and blood-Pb was stronger ($r = –0.57$, $p = 0.09$). Conversely, in rainbow trout the negative correlation between blood-Pb and ALAD ($r = –0.76$, $p = 0.05$) was stronger than that between ALAD/Hb and blood-Pb ($r = –0.53$, $p = 0.17$). In mountain sucker, ALAD was positively correlated with Hb ($r = 0.78$, $p = 0.07$) but was not correlated with blood-Pb ($r > –0.12$, $p > 0.8$).

### 3.4. GLOVER, MO

Relative to the upstream site, carcass-Pb was elevated by 9-fold and blood-Pb by 8-fold in northern hog sucker from Big Creek below the ASARCO smelter; both differences were statistically significantly (Tables II and III). ALAD activity was lower by 14% and ALAD/Hb by 26% at the downstream site, but only the ALAD/Hb difference approached statistical significance (Tables II and III). Hb concentrations were 13% greater downstream, but this difference was also not significant. ALAD was only weakly correlated with blood-Pb ($r = –0.41$, $p = 0.36$) and carcass Pb ($r = –0.37$, $p = 0.34$); however, ALAD/Hb was more strongly correlated (negative) with both carcass-Pb ($r = –0.69$, $p = 0.06$) and blood-Pb ($r = –0.58$, $p = 0.17$).

## 4. Discussion

### 4.1. REFERENCE SITES

Most Pb concentrations (blood, liver, or carcass) at the reference sites were consistent with previously reported levels in fish from relatively uncontaminated areas. Mean carcass-Pb concentrations at the Herculaneum and Glover reference sites were 0.14–0.48 $\mu g\ g^{-1}$, which lie within the range of 1986–87 concentrations for most U.S. freshwater fish (Schmitt *et al*., 1999) and reference areas in Missouri (Schmitt *et al*., 1984, 1993). Similarly, blood-Pb averaged 0.15 $\mu g\ g^{-1}$ in northern hog sucker from the upstream site on Big Creek, which is also consistent with previously reported reference values (Schmitt *et al*., 1993). Liver Pb concentrations in largescale sucker from Lake Wenatchee were uniformly below the LOD of 0.2 $\mu g\ g^{-1}$ wet-weight (Table II), a censoring level at least 10-fold greater than measured concentrations reported for reference areas in other investigations (Harrison and Klaverkamp, 1990; Munn *et al*., 1995; Goldstein and DeWeese, 1999). Because of these high detection limits, the values cannot be compared directly; however, Haux *et al*. (1986) reported liver-Pb concentrations of $< 1.0\ \mu g\ g^{-1}$ dry-weight (about 0.2 $\mu g\ g^{-1}$ wet-weight) in whitefish (*Coregonus* spp.) from an uncontaminated lake in Norway, which agrees with our findings for Lake Wenatchee. Blood-Pb concentrations were also generally low (means 0.03–0.22 $\mu g\ g^{-1}$), but variable, relative to other studies (Schmitt *et al*., 1984, 1993) in all species collected at the upstream site on Prickly Pear Creek in E. Helena (Table II). Variably elevated carcass-Pb concentrations in rainbow trout from the upstream site have also been reported (Olsen *et al*., 1997). Collectively, these results suggest that the reference sites were not substantially contaminated by Pb from the smelters or other sources, with the possibly exception of Prickly Pear Creek where there is a history of upstream mining and related activities (Olsen *et al*., 1997) and atmospheric deposition from the E. Helena smelter (Pagenkopf and Maughan, 1984). Regardless,

concentrations should not be considered 'background' at any of these sites because of the pervasive nature of anthropogenic Pb pollution (Settle and Patterson, 1980).

### 4.2. AFFECTED SITES

Relative to Lake Wenatchee, ALAD activity was 55% lower in largescale sucker from the Columbia River at Northport, where liver-Pb averaged 1.3 $\mu$g g$^{-1}$ wet-weight (Table II). These results are similar to those of Haux *et al*. (1986), who reported ALAD inhibition of 87–88% in whitefish with wet-weight liver-Pb concentrations of 1.4–1.6 $\mu$g g$^{-1}$ (converted from dry-weight using the average moisture content for fish livers of 78% given by Brigham *et al*., 1998) from Pb-contaminated lakes in Norway. Additionally, carcass-Pb concentrations were 12 $\mu$g g$^{-1}$ in largescale sucker collected at Northport one year after our study (Serdar *et al*., 1994). These carcass concentrations are about the same as those in other catostomids (several spp.) from tailings-contaminated sites in Missouri with similarly reduced ALAD activity (Schmitt *et al*., 1993). Collectively, these findings indicate that substantial amounts of bioavailable Pb are present in the Northport reach of the Columbia River.

Results were more variable at the other sites, and statistical analysis was problematic due to small sample sizes. At Herculaneum and E. Helena, responses also varied among species. At Glover, ALAD activity (as ALAD/Hb) in northern hog sucker was only marginally lower ($p = 0.11$; Tables II and III) below the smelter than upstream; however, when these results were analyzed as part of a larger data set spanning more sites, with correspondingly more degrees-of-freedom, the difference between these two sites were highly significant ($p < 0.01$; Schmitt and Caldwell 1997). In addition, ALAD activity in the fish from the upstream site was lower than expected. In an earlier study of ALAD activity in northern hog sucker that utilized identical methods (Schmitt *et al*., 1993), ALAD activity at references sites was typically about 900 nmol PBG g$^{-1}$ hr$^{-1}$ whereas it was 300–500 nmol PBG g$^{-1}$ hr$^{-1}$ at Pb-contaminated sites (including a site on Big Creek). The latter levels of activity are similar to what we found at both Big Creek sites despite low blood-Pb concentrations upstream (Table II). We have no explanation for these seemingly low values. The samples from both sites were analyzed in random order, and the levels of ALAD activity in most other samples appeared normal given the Pb burdens of the fish. Moreover, ALAD/Hb was negatively correlated with both blood- and carcass-Pb concentrations (albeit weakly for blood-Pb) despite the low levels of ALAD activity at the upstream site.

### 4.3. DIFFERENCES AMONG FISH TAXA

Previous studies have consistently shown that ALAD activity varies among fishes. Hodson *et al*. (1977) reported that activity in goldfish (*Carassius auratus*), a species closely related to common carp, was 75% of that in brook trout and rainbow trout; and that activity in pumpkinseed (*Lepomis gibbosus*, a centrarchid) was

lower still. Similarly, Schmitt *et al*. (1993) found that ALAD activity in yellow bullhead (*Ictalurus natalis*), a channel catfish congener, was 61–90% of that in catostomids and other fishes collected from the same sites. Unfortunately, comparisons among studies are difficult because assay conditions vary. In addition, ALAD activity in laboratory studies is typically expressed per unit volume of red blood cells (based on hematocrit) whereas in field studies hematocrit is not always measured and results are reported per unit of blood homogenate (weight or volume). Results of ALAD studies are therefore reported and compared as proportional differences among species and between control (laboratory) or reference (field) conditions.

Despite the difficulties noted, our findings and those of previous investigations indicate that ALAD sensitivity to Pb also differs among taxa, and that catostomids are more sensitive than many other fishes. ALAD activity in some of the fishes collected downstream from the smelters we investigated was lower than at the reference sites, but the degree and magnitude of the response varied considerably among species (Table II). In contrast, laboratory exposure of salmonids to Pb has consistently resulted in dose-dependent decreases in erythrocyte ALAD activity (e.g., Hodson *et al*., 1977; Johansson-Sjobeck and Larsson, 1979). The latter reported 21 and 74% reductions in ALAD activity following a 30-day exposure to 0.01 and 0.08 mg $L^{-1}$ of Pb in water, respectively. In common carp exposed to $PbNO_3$ in the laboratory, ALAD activity was reduced by more than 60% and was negatively correlated with blood-Pb; 50% ALAD inhibition was associated with blood-Pb of about 0.1 mg $L^{-1}$ (Nakagawa *et al*., 1995). Hodson *et al*. (1977) also found that ALAD activity was reduced in goldfish and pumpkinseed exposed to waterborne Pb in the laboratory despite their lower overall enzyme activity. We did not observe a dose-dependent response in common carp collected from the Mississippi River near the Herculaneum smelter even though carcass-Pb concentrations at the downstream site were 31-fold greater than upstream and more than 4-fold greater than concentrations previously associated with 50% ALAD inhibition in catostomids (Schmitt *et al*., 1993).

Hodson *et al*. (1977) noted that brook trout and rainbow trout differed in sensitivity of ALAD to waterborne Pb, and presumed that the differences reflected differential gill permeability. We were unable to corroborate this observation; ALAD activity was reduced by only 7–8% in brook trout and rainbow trout from E. Helena despite 4-fold greater blood-Pb concentrations in rainbow trout from the downstream site relative to upstream. Nevertheless, ALAD activity was negatively correlated with blood-Pb in both salmonids.

In a previous study, ALAD activity in three catostomid species responded similarly to Pb in a stream contaminated by tailings (Schmitt *et al*., 1984), and catastomids were selected for a subsequent larger-scale investigation (Schmitt *et al*., 1993). We found that ALAD activity was reduced to a greater extent in catostomids (largescale sucker, river carpsucker, mountain sucker, and northern hog sucker) than in brook trout, rainbow trout, or common carp despite greater Pb burdens in

the latter taxa at some sites. At E. Helena, blood-Pb in mountain sucker was consistently lower than in either brook trout or rainbow trout, but ALAD activity was reduced to a greater extent in mountain sucker than in either salmonid. In addition, and in contrast to the two salmonids, ALAD activity in mountain sucker was not correlated with blood-Pb. We consequently suspect that blood-Pb concentrations at the upstream site were above the threshold for ALAD inhibition in this species. We also failed to detect ALAD activity in channel catfish from the Mississippi River even though activity has been documented (in the liver) in this species (Conner and Fowler, 1994) and was detected in the other two species from both Herculaneum sites.

Catastomids are predominantly benthivorous, foraging on the bottom for a variety of plants and animals. Consequently, they come into frequent contact with and often ingest sediments and sediment-associated contaminants, which makes them ideal for the assessment of environmental Pb contamination. Nevertheless, common carp and, to a lesser extent, channel catfish share similar food habits and behavior. The common carp from Herculaneum had greater Pb burdens, but there was no evidence of ALAD inhibition. Channel catfish had lower Pb burdens than either common carp or river carpsucker, but no detectable ALAD activity; perhaps they are more sensitive to Pb. We are not aware of other field studies of ALAD activity in the blood of siluriform fishes against which to compare this finding; however, Conner and Fowler (1994) reported that hepatic ALAD activity of channel catfish was 40-fold more sensitive than that of rats to inhibition by Pb. Many factors, including differential susceptibility to Pb as well as methodological inconsistencies (e.g., pH, temperature) among investigations could be involved in these differences. In addition, we did not analyze any centrarchids, which may be more sensitive still. As noted earlier, Hodson *et al*. (1977) reported lower levels of activity in pumpkinseed than in common carp, brook trout, or rainbow trout. Nevertheless, Dwyer *et al*. (1988) reported 48% ALAD inhibition in longear sunfish (*Lepomis megalotis*) with blood-Pb of only 0.43 $\mu$g mL$^{-1}$ and carcass-Pb of 0.2 $\mu$g g$^{-1}$. Collectively, these findings indicate that there are substantial biochemical differences among fishes, as there are between warm-blooded vertebrates and fish (Conner and Fowler, 1994) and among avian species (Henny *et al*., 2000).

### 4.4. Possible effects of zinc on ALAD activity in fish

Differences in Zn burdens may be partly responsible for inconsistencies in our findings and between previous field studies and laboratory results documenting the effects of Pb on ALAD activity in fish. As demonstrated by the data from Herculaneum, and in agreement with the findings of other studies (e.g., Schmitt *et al*., 1999), Zn concentrations in wild common carp are typically at least twice those of most other fishes. Previous field studies have documented an apparent ameliorative effect of Zn on ALAD inhibition by Pb in fish (Schmitt *et al*., 1984, 1993). Schmitt *et al*. (1993) hypothesized that this was caused by displacement of Pb by Zn and

re-activation of the enzyme. In mammals, ALAD requires Zn as a cofactor and reactivation has been demonstrated (Hutton, 1983; Wigfield *et al*., 1986). It was further hypothesized (Schmitt *et al*., 1993) that blood-Zn could thereby provide some protection from the harmful effects of Pb on heme synthesis, as it does in humans (Joselow, 1980). Subsequent research has shown that fish ALAD may not require Zn as a cofactor (Gonzalez *et al*., 1987; Rodrigues *et al*., 1989; Conner and Fowler, 1994). Our results from Herculaneum nevertheless indicate that the characteristically high Zn burden of wild common carp may afford this species some protection from the harmful effects of Pb.

Our findings for Zn at Northport and Herculaneum were also consistent with previous studies (Schmitt *et al*., 1993) that have shown Zn to vary much less in response to environmental concentrations than Pb. At Northport, liver-Pb concentrations in largescale sucker were elevated by at least 6-fold relative to Lake Wenatchee whereas liver-Zn concentrations were low (about 50 $\mu$g $g^{-1}$) and not significantly different (Table II). Liver-Zn concentrations at both sites were greater than those reported by Munn *et al*. (1995) for rainbow trout (mean = 31.3 $\mu$g $g^{-1}$) and walleye (*Stizostedion vitreum*, mean = 17.0 $\mu$g $g^{-1}$) from the Columbia River and Franklin D. Roosevelt Lake near Northport. At Herculaneum, the downstream Zn concentrations exceeded those upstream in all species, but all were within the previously reported ranges for catostomids, common carp, and channel catfish in U.S. rivers (Schmitt *et al*., 1984, 1993, 1999). Moreover, concentrations of Pb in fish were elevated to a far greater extent than Zn despite abundant Zn in sediments downstream of the smelter (FWS, Rock Island, IL; unpublished data). Although all samples were not analyzed for Zn, our results (and those of the studies we cite) nevertheless support the hypothesis that some of the inconsistency observed was related to Zn differences among sites and species. It also reinforces previous recommendations (Schmitt *et al*., 1984, 1993) that Zn should be measured together with ALAD and Pb for an accurate assessment of Pb and its effects. In addition, and despite the problems noted, the overall relationships we observed between Pb, Zn, and ALAD activity are consistent with the results of previous field studies (Schmitt *et al*., 1984, 1993; Dwyer *et al*., 1988).

### 4.5. Significance of ALAD inhibition in fish

In human medicine, blood-Pb concentrations of 1.0–1.5 mg $L^{-1}$ are considered a cause of concern (Mushak *et al*., 1989). Avian wildlife is considered Pb-poisoned at blood-Pb levels of 0.2–0.6 mg $L^{-1}$ (Anderson and Havera, 1985) and when ALAD activity is inhibited by 50% relative to control or reference animals (Dieter, 1979). In contrast to other contaminants, Pb does not bioaccumulate (Settle and Patterson, 1980) and there seems to be little risk to piscivorous wildlife from Pb in fish (Henny *et al*., 1994). Effects on heme synthesis and bone strength have been detected in fish at carcass Pb concentrations as low as about 1.0 $\mu$g $g^{-1}$ and blood-Pb concentrations of about 0.5 mg $L^{-1}$ (Dwyer *et al*., 1988; Schmitt *et al*.,

1984, 1993). 'Black tail', a symptom that precedes spinal deformity, was associated with blood-Pb of 1.7 mg $L^{-1}$ and ALAD inhibition of 74% in laboratory-exposed rainbow trout (Hodson *et al*., 1979). Stippled erythrocytes and spinal deformities have also been detected in common carp exposed to high Pb concentrations in the laboratory (Holcombe *et al*., 1976; Beretic *et al*., 1980), as have additional sublethal effects in other fishes (Johansson-Sjobeck and Larsson, 1979; Webber *et al*., 1991).

In erythrocytes (and other types of cells), ALAD catalyzes the formation of PBG, a Hb precursor, from $\delta$-aminolevulinc acid. Pb inhibits ALAD activity, thereby decreasing PBG and presumably Hb levels, and also affects other points in the heme synthesis pathway (Finelli, 1977; Beretic *et al*., 1980). Laboratory results have been inconsistent in terms of effects on Hb. Exposure of rainbow trout to 0.3 mg $L^{-1}$ of Pb in water resulted in lower ALAD activity and Hb concentrations (Haux and Larsson 1982) whereas similar Pb concentrations inhibited ALAD activity but produced no hematological changes in eels (*Anguilla anguilla*; Santos and Hall 1990). In the gray mullet (*Mugil auratus*), both Hb and ALAD activity were reduce by exposure to Pb (Krajnovic-Ozreticc and Ozretic, 1980). Previous field studies that have documented ALAD inhibition in fish have also been inconsistent in terms of effects on Hb. In Missouri streams contaminated by mine tailings, no effects on Hb were noted despite 50–60% ALAD inhibition relative to reference sites in several catostomids (Schmitt *et al*., 1984, 1993) and in longear sunfish (Dwyer *et al*., 1988). Haux *et al*. (1986) reported both greater and lower Hb concentrations in coregonines from Pb-contaminated lakes in Norway despite the fish having only 12–13% of normal ALAD activity.

Our Hb results were likewise inconsistent. At Trail and Herculaneum, Hb concentrations did not differ significantly between sites affected by smelters and reference sites. In contrast, Hb concentrations were lower in mountain sucker, rainbow trout, and brook trout from Prickly Pear Creek below the E. Helena smelter than above (Table II), and in mountain sucker ALAD and Hb were positively correlated. In contrast, Hb concentrations in northern hog sucker were slightly higher at the site below the Glover smelter than upstream. It should be noted that these inconsistencies could reflect differences in erythrocyte count, mean corpuscular hemoglobin (which is derived from the erythrocyte count), or both, and we measured only Hb concentrations.

## 5. Conclusions and Recommendations

Primary smelters release biologically available Pb to aquatic ecosystems. Despite the variety of fishes collected, the different endpoints used to assess Pb exposure, and the age and historical impacts of the four smelters, this conclusion was generally the same for all and would probably apply to others. Fish from below the smelters typically had greater Pb burdens and some had lower ALAD activity

than fish from corresponding reference sites, thus demonstrating both bioavailability and biochemical activity. Although some of these results were probably caused by residual material deposited historically by the older complexes (Trail, E. Helena, Herculaneum), our findings at the modern facility in Glover, MO indicate continuing inputs of biologically available metals to aquatic ecosystems by smelters.

Lead and other metals discharged to aquatic ecosystems become available for direct uptake from water by fish, and may also become incorporated into the aquatic food chain (Vighi, 1981). Lower trophic level fishes associated with sediments, such as catostomids, appear to be particularly vulnerable to Pb, which tends to decrease in concentration with trophic position (Settle and Patterson, 1980). In addition, our findings suggest that ALAD in catostomids is more sensitive to inhibition by Pb than in other fishes. The bioavailability and biochemical activity of Pb released from smelters to waterways was further confirmed by our findings of increased Pb concentrations (blood, carcass, or liver) and reduced ALAD activity relative to reference sites. We recommend that the ameliorative effect of Zn on Pb-induced ALAD inactivation be considered in the design of future studies, and that Zn concentrations be measured in the same tissue (e.g., blood, liver) as Pb when ALAD is used to assess environmental Pb contamination. We also suggest that at least 10 fish be collected from each site to facilitate statistical analyses by techniques such as multiple linear regression and analysis of covariance, the methods most appropriate for dealing with such inter-related variables.

Changes in ALAD activity and corresponding hematological effects have been used to determine the degree of Pb exposure in humans (Marcus and Schwartz, 1987), birds (Dieter, 1979; Gonzales and Tejedor, 1992; Henny *et al*., 1994), and mammals (Wigfield *et al*., 1986), but in fish hematological changes associated with reduced ALAD activity remain poorly defined and inconsistent. Since Hodson *et al*. (1977) first proposed ALAD activity as an indicator of Pb exposure in fish, reduced enzyme activity has not consistently resulted in either adverse hematological effects or effects at higher levels of biological organization. We found reduced Hb concentrations only in fish collected below the E. Helena smelter (Table II). Other correlates of reduced ALAD activity in fish remain restricted to effects of Pb on bone strength and composition (Holcombe *et al*., 1976; Dwyer *et al*., 1988) and, at very high Pb levels, stippled (and presumably dysfunctional) erythrocytes (Beretic *et al*., 1980) and behavioral effects (Webber *et al*., 1991). Other effects on individual fish, fish populations, or aquatic communities have not been documented. Reduced ALAD activity in fish therefore appears to be similar in many respects to other biomarkers; it represents a reliable and extremely sensitive method for the detection of biologically available Pb in the aquatic environment at the biochemical level. Although such documentation is more useful than just a measurement of environmental Pb concentrations, further research should determine if additional higher-level effects on fish and aquatic ecosystems are associated with Pb exposure

and ALAD inhibition, and laboratory studies should be conducted to determine the role of Zn in heme synthesis in fish.

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