ARSENATE AND SULFATE REDUCTION IN THE SEDIMENTS OF COEUR D'ALENE LAKE, IDAHO

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Major Professor: R. Frank Rosenzweig, Ph.D.

AUTHORIZATION TO SUBMIT

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Abstract

Sediments of Lake Coeur d'Alene, Idaho (CDAL) have been extensively contaminated with iron and trace elements from mining activities in the Coeur d'Alene mining district. Previous surveys of (CDAL) sediment have suggested the majority of the metals are bound in an oxide phase, and if true, contaminated water may result if anoxic conditions occur in the water column. In contrast, data from this study indicate that the majority of the iron and trace elements are bound in a sulfidic phase. Examination of the sediment pore waters shows iron and trace element concentrations to be more than federal drinking water standards. Strongly reducing conditions and an abundant microbial community are also present, indicating that reactions that occur under reducing conditions are predominant in CDAL sediments. In particular, sulfate-reducing bacteria (SRB) are abundant in CDAL sediments. Isolates of native SRB have the ability to form sulfides from soluble metal. In such conditions, metal solubility is low, indicating that SRB may act to mitigate the effects of metal contamination in CDAL. Arsenic shows some different trends as compared with other trace elements. Arsenic solubility is highest in redox potential between the ferric to ferrous couple and the sulfate to sulfide couple. As (III) is not sorbed as well to sediment components, and hence formation of reduced forms of arsenic may encourage arsenic mobilization in anoxic sediments. Bacterial transformations of arsenic have recently been shown to occur in sediments. In this study, arsenic reducing organisms were found to be less abundant than SRB, but still numerous. Additions of soluble As (V) to sediment microcosms were reduced to As (III). This reduction was stimulated by organic acids, and was inhibited by a respiratory inhibitor of SRB. These results suggest that SRB can form metal sulfides, and also mobilize arsenic by creating a reduced, mobile form.

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Most of all, to my wife, Jennifer, whose love and good faith carried me through the thick and thin of these efforts. Thanks beyond measure.

Dedication

This thesis is dedicated to God, the author of creative power, and hence any creativity that has been embodied in this work.

This thesis is also dedicated to Jennifer Harrington, whose longsuffering efforts in her own school, as a mother, and as a wife of a crazy graduate student kept me going. I love you.

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Chapter 1:

Introduction

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Introduction

This thesis examines the activities of mesophilic sulfate-reducing bacteria (SRB) in the sediments of Coeur d'Alene Lake (CDAL), Idaho. The upper half-meter of these sediments is heavily contaminated with mine tailings. CDAL sediments can be up to 12 % iron, 2 % lead, 1% zinc, and 600 ppm arsenic by dry weight. The tailings originated in the Coeur d'Alene mining district, having been secondarily transported down the Coeur d'Alene River (CDAR) by flooding and normal spring runoff. Indeed, the entire CDAR plain is extensively contaminated, and floods and periods of high stream flow resuspend tailings such that CDAL constantly receives fresh, heavy metal-laden sediments.

The three papers that comprise this thesis progress from evaluating the extent and nature of the metal(oid) contamination in CDAL sediments, to comparing the vertical distribution of redox-active *vs.* redox-inactive metal(oid)s, to examining the role that SRB populations may play in sequestering or mobilizing the metal(oid)s. I tentatively conclude that these microorganisms have a significant impact on the biogeochemical cycling of trace elements in CDAL sediments.

Sediments of CDAL contain high concentrations of trace elements including arsenic, manganese, lead, and zinc (*1, 2, and 3*). The point source of these contaminants is the CDAR, whose banks and bars are highly enriched with tailings produced as a result of unregulated mining through much of the 20th Century (*2*). Several recent studies of CDAL sediments have raised questions as to their redox status, and suggest that the majority of trace elements are deposited in a highly labile oxide/hydroxide form (*2, 4*). Such reports support the claim that eutrophication leading to seasonal anoxia would promote release of trace

elements into the water column. Such an event would severely degrade the surface water quality of the lake.

This thesis challenges several of the assumptions upon which this scenario is based. I show that the majority of trace elements are bound as sulfidic compounds, rather than oxyhydroxides. Thus the amount of trace elements that could be released should anoxia occur may be significantly less than previously held (*2, 4*). I also show that sulfate-reducing bacteria are abundant, and that in sediment microcosms, pure cultures of SRB isolated from CDAL sediments will form metal sulfides from soluble metals. Thus, even if anoxia promoted dissolution of metal (oxy)hydroxides, trace elements might not be released. Rather, the activities of anaerobic bacteria like SRB could provide a mechanism that would favor the formation of additional metal sulfides.

The vertical profile (stratigraphy) of sediments is commonly used to infer the history of lake conditions. Examination of CDAL sediments shows Mt. St. Helens ash (ca. 1981), as well as fine layering patterns of different colored sediment in-between (*5*). In the highly contaminated CDAR Delta region, the sediment layer comprised of tailings is up to 1m in thickness. Therein, the highest concentrations of lead and zinc are found deep in the sediment ($\geq$ 0.5 m), while the maximum concentrations of arsenic and iron occur much closer to the surface ($<$ 0.1 m). While no claim is made concerning the homogeneity of ore mined in the Coeur d'Alene mining district, any heterogeneity would likely be masked by the long distance required for transport from the mining district to the CDAL delta (~ 100 river miles). The high concentrations of Pb and Zn observed are indicative of poor recovery methods utilized early in the 20th Century (*6, 7*). It seems reasonable to expect that the stratigraphic profiles for As and Fe would be the same as Pb and Zn, unless the former were undergoing diagenesis.

However, we find near-zero correlation between arsenic and either lead or zinc. This suggests that in the course of its biogeochemical cycling, arsenic goes through a diffusible intermediate.

The utilization of arsenic by microorganisms as a terminal electron acceptor has only recently been studied in any detail (*8-11*). In this reaction, microorganisms utilize arsenate as a sink for electrons gained in the oxidation of organic matter. To date, four As (V)-reducing organisms have been isolated in pure culture. Of these four, one also utilizes sulfate as a terminal electron acceptor. Given the appearance that arsenic may be undergoing diagenetic mobilization, it was reasonable to investigate the ability of CDAL sediment microbes including the sulfate reducers to perform the transformation of As (V) to As (III).

In the absence of added sulfate, I show that CDAL sediment microbes will reduce arsenate (As (V)) to arsenite (As (III)). The use of a specific inhibitor of SRB metabolism indicates that SRB populations contribute to the reduction of soluble As (V). These findings are significant because As (III) is more toxic than As (V), and because As (III) does not bind as well as As (V) to mineral surfaces found in anaerobic environments (*12-15*). Thus As (III) would be expected to be the more mobile form of arsenic under anaerobic conditions. Our data indicate that CDAL sediments below 5-10 cm are highly reduced. This transformation of arsenic to its (III) valence could provide the diffusible intermediate that has contributed to the current stratigraphy of arsenic. It is noteworthy that Cummings (*16*) has recently demonstrated iron reducers to be capable of releasing As (V) from structural iron compounds, as well as from CDAL sediments themselves. Thus, dissimilitory iron-reducing bacteria (dIRB) may act to increase arsenic solubility by reducing ferric oxyhydroxides that act as sorbents for arsenic.

The present study suggests that some SRB species may play dual roles in the cycling of arsenic in metal(oid) enriched sediments. SRB pure culture isolates were shown to form arsenic sulfides when As (V) was added in the presence of available sulfate. However, as yet unidentified SRB were shown to be capable of producing As (III) from As (V) when sulfate was not available. The control on these processes, either of arsenic solubilization by reduction, or arsenic precipitation by sulfidic precipitation, may ultimately depend on sulfate availability. It may also depend on the environmental redox status (*i.e.*, more oxidized conditions may inhibit SRB that precipitate, but not those that solubilize arsenic). It is interesting to note that Urban *et al* (*17*) showed that sulfate produced endogenously was more rate-limiting to *in situ* sulfate-reduction rates than sulfate that was exogenously supplied (*i.e.*, diffusing in from surface waters). By extension, it may be that arsenic biogeochemistry in CDAL sediments may ultimately be controlled by rates of endogenous sulfate production.

Conclusions

The overlying waters in CDAL are of high quality (*4*) indicating that there are efficient metal scavenging mechanisms in CDAL sediments. These mechanisms include biotic sulfidogenesis with the concomitant formation of insoluble metal sulfides. Bacteria that perform sulfate reduction may also contribute to the diagenetic cycling of arsenic in CDAL sediments that results in maximal arsenic abundance near the sediment-water interface. Clearly, further work is required to understand the ecology of the SRB community in CDAL sediments, particularly with regard to carbon flux to SRB, as well as sulfate production and diffusion rates. Ideally, this work would include study of the lake sediments over several annual cycles. The benefits of such an analysis would include not only a better understanding

of metal cycling and sequestration in Coeur d'Alene Lake, but also other lakes that have been similarly impacted by contamination related to mining.

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Chapter 2:

Phase Associations and Mobilization of Iron and Trace Elements in Coeur d'Alene Lake, Idaho

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Introduction

Coeur d'Alene Lake, ID: General Description-- Coeur d'Alene Lake is Idaho's second largest lake, covering about 129 km^2. Fed in the south by two rivers, the St. Joe (SJR) and the Coeur d'Alene (CDAR), and drained in the north by the Spokane River, Coeur d'Alene (CDA) Lake has become a collecting bed for sediments produced by human activities in its two major drainages (see Figure 2-1). These activities are predominantly mining and ore processing, logging, and agriculture along the CDAR, and logging and agriculture along the SJR (1). Mining and mineral processing on the South Fork of the CDAR have affected the water and sediment quality to the extent that the trace element concentrations in the sediment pore water are characterized as acutely toxic to freshwater biota, and the sediment quality has been classified as severely affected by arsenic, cadmium, lead, and zinc (1). Horowitz *et al.* (2) surveyed (~1 sample/ km^2) surficial lake sediments for trace element contamination, and concluded that the vast majority of the lake sediment surface was enriched with Ag, As, Cd, Cu, Fe, Hg, Mn, Pb, Sb, and Zn, as compared with uncontaminated sediments. This agrees with the conclusions reached by many other researchers (*e.g.*, 3 and 4).

CDA Lake appears to be variable in trophic status as inferred from total nitrogen and phosphorus levels. Based on 1991-92 data, the southern end near Lake Chatcolet was classified as mesotrophic-eutrophic, while the rest of the lake was determined to be oligotrophic-mesotrophic (1). At times during the 1970s, anoxic conditions were reported in many parts of the lake (5), suggesting eutrophication. Recent studies have reported a decrease in both nitrogen and phosphorus loading by the St. Joe (SJR) and Coeur d'Alene Rivers (CDAR) relative to that observed in the 1970s and 1980s (1, 5).

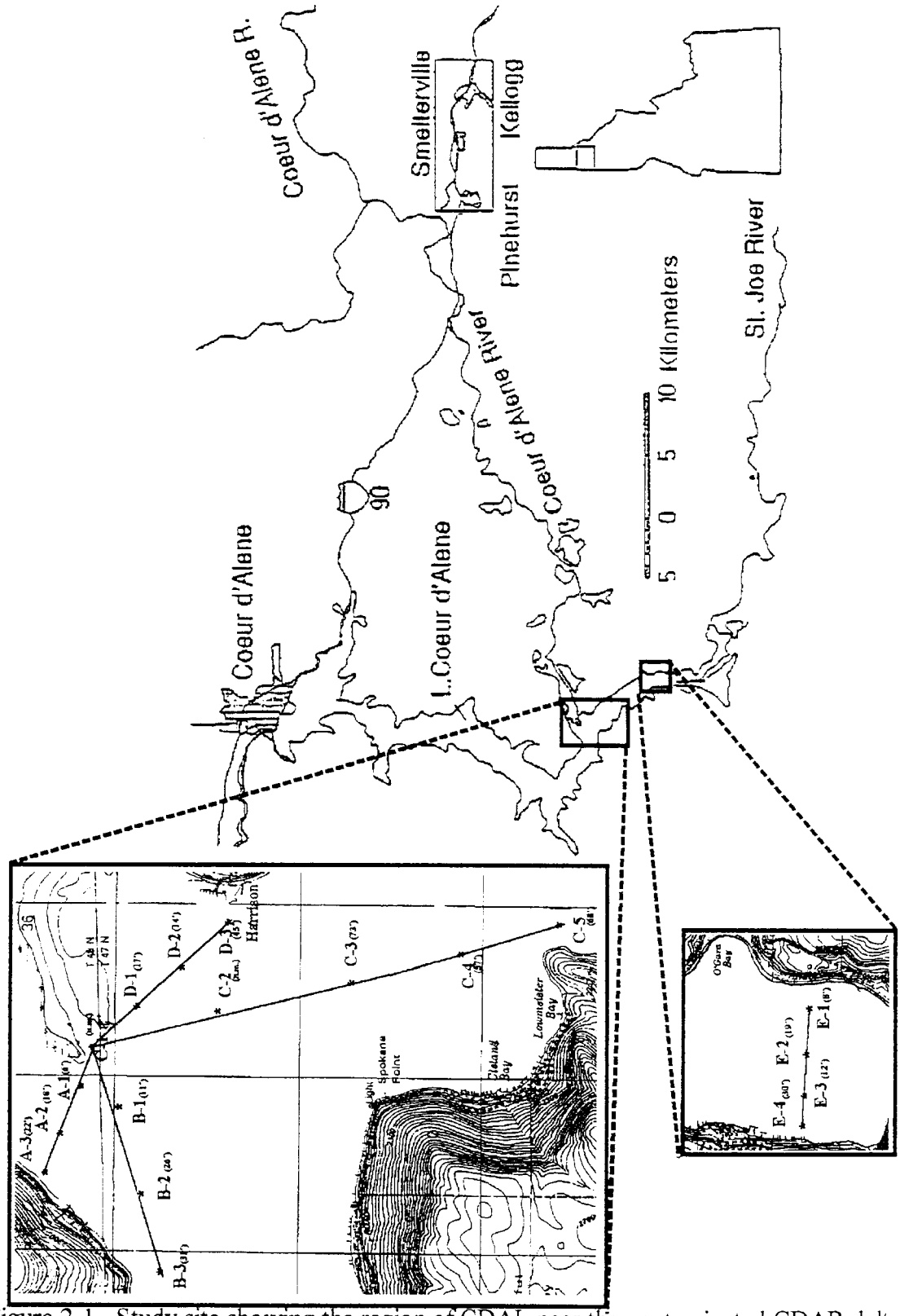

Figure 2-1. Study site showing the region of CDAL near the contaminated CDAR delta (bottom left), and CDAL near the pristine SJR delta (bottom right).

Nonetheless, many authors have raised concerns (*e.g.*, 1, 2) that eutrophication and the concomitant development of a seasonally anoxic hypolimnion could combine to significantly raise concentrations of toxic trace elements, as well as soluble N and P in CDA Lake waters. Release of such elements would severely impact aquatic organisms, and imperil the six community water supply systems that utilize CDA Lake water (1). Seasonal anoxia is not the only mechanism that might favor the release of sediment nutrients and/or toxic trace metals. Redox transformation of trace elements by metal-respiring bacteria can change the solubility and, hence, mobility of these elements within the sediment environment (6, 7, 8). Metals may also be immobilized as sulfides consequent to the release of H_2S by sulfate-reducing bacteria (SRB) (7). In addition, Baccini (9) has shown that lake sediment iron concentration strongly influences phosphorus flux from sediments. Indeed, a lake with high sediment Fe concentration and an anoxic hypolimnion may have phosphorus release into its overlying water equivalent to another lake with low Fe concentrations and an oxic hypolimnion (9). We note that to the extent that iron-reducing bacteria (IRB) control the relative abundance of oxidized iron within sediments, these organisms may indirectly affect sediment phosphorus flux. Clearly, many factors besides trophic status need to be examined in order to evaluate the risk posed by contaminated CDA Lake sediments under conditions of local or seasonal eutrophication.

The Nature of Trace Element Enrichment in Coeur d'Alene Lake Sediments-- There is a consensus in the literature that CDA Lake sediments are contaminated, and that the CDAR continues to act as the principal source for trace element-rich sediments. Although tailings ponds have been constructed in the mining district, influx of metal into the lake from the CDAR continues as a result of resuspension of tailings-rich stream sediments (2, 3, 10). Information about the phase partitioning of metals within these sediments is limited to two studies. Mok and Wai (11) measured dithionite-reducible iron in CDAR sediments in order to assess their potential for binding arsenic. They reported an average of 2500 mg Fe/ kg

sediment in that phase. Iron extractable by dithionite is usually present as Fe (III) oxyhydroxides, compounds that have been shown to have high affinities for As (11). Horowitz *et al.* (2) performed sequential dissolution on four grab samples from surface sediments of CDA Lake. A 0.25 M hydroxylamine hydrochloride extraction was used to estimate iron oxide-associated metals, and an H_2O_2 - HNO_3 extraction was used to estimate metal bound to organic/sulfidic phases. These workers determined that ~95% Pb, 90% Cd, 80% Zn, 75% As, and 55% Cu were released by the first extraction. Horowitz *et al.* (2), therefore, concluded that Fe and Mn oxides represented the dominant hosts for trace metals in surface sediments.

The results presented by Horowitz *et al.* (2) have been criticized by Pedersen (12) because sample processing was conducted under aerobic conditions. Pedersen (12) also criticizes the two-step procedure utilized by Horowitz on the basis that it was designed for aerobic sediments, not anaerobic sediments. Furthermore, inasmuch as carbonates were not removed prior to oxide dissolution, the selectivity of the extraction procedure is questionable (12, 13). Hence, these results must be interpreted with caution. Sequential extraction of sediments below the top 2 cm was not performed by Horowitz *et al.* (14) because evidence of oxidation was observed in the cores shipped back to the laboratory.

To date, only visual or inferential evidence (*e.g.*, color, vertical metal distribution) has been offered to assess the redox status of CDA Lake sediments (14, 15). Furthermore, detailed phase-partitioning of the sediments has not been performed (3, 10, 14). Reduction potential measurements and phase partitioning analyses are necessary prerequisites to establishing whether trace elements are likely to remain permanently buried or subject to post-depositional remobilization. Such data would also help disclose whether oxide formation in CDA Lake sediments is authigenic, or whether these oxides have been formed primarily on the CDAR plain and banks, as suggested by Horowitz *et al.* (2, 14). If measurement of these parameters reveals metals to be bound predominantly in reduced phases, such as metal

sulfides, the threat of metal release postulated under eutrophic or seasonally anoxic conditions may require reevaluation.

In this study, we seek to extend and clarify understanding of the phases and associations of trace elements in CDA Lake, and to help provide a rational basis for predicting their interactions within the sediments and overlying waters. Sequential dissolution analyses, redox and pH measurements, estimates of bacterial densities, as well as correlation analyses of trace element abundance by depth are combined with previous data to describe phase associations and mobilities of trace elements in CDA Lake sediments. Based on these findings we speculate on the relative potentials for trace metal burial or resolubilization within these environments.

Materials and Methods

Sediment Sampling. Deep sediment cores (x = 46 cm $\pm$ 9 (SD)) were removed from CDA Lake (Figure 1) using a gravity coring device (16). Sediments processed for measurement of total trace element abundance and sequential dissolution analysis were frozen immediately in dry ice, then stored in the laboratory at -80^{0}C (March 1994). Sediment cores were cut in half while frozen solid; one side was used for total metal analyses, the other for sequential dissolution analyses. Cores were subsampled from homogenized 4 cm intervals for total metal analysis, and homogenized 8 cm intervals for sequential dissolution analysis. Care was taken not to sample from the cut surfaces of the cores. Sediment cores collected for Eh/pH measurements and estimates of bacterial density were stored vertically at 4^{0}C, and purged continuously with $N_{2(g)}$ (March 1995). Cores were assayed within 12 hours of collection.

Estimates of pH, reduction potential and microbial abundance. Redox potentials were measured by platinum electrode on cores freshly extruded in a positive pressure nitrogen atmosphere, and adjusted to the standard hydrogen electrode (SHE). pH measurements were

performed on these same cores with a portable pH probe and meter (Cole Palmer). Bacterial densities were estimated by epifluorescence microscopy using acridine orange (17).

Analytical techniques. Total iron and trace element abundance was determined by ICP (inductively coupled plasma) analysis of aqua regia-digested sediment. Metal content of operationally defined phases obtained by selective dissolution was also determined by ICP. Every twentieth sample was duplicated, and less than 10% error between samples was detected.

Sequential Extraction Analysis. Selective extractions are common procedures that provide useful information on trace element partitioning within sediment (18, 19, 20). One should note that sequential extractions are based on operational definitions, and the values generated by such procedures should be interpreted with caution (21, 22). The use of sequential extractions for trace-element partitioning in soils and sediments has drawbacks owing to readsorption of metals onto other sediment fractions (19, 23, 24, 25, 26). However, the effects of readsorption may not be as problematic as once thought (26). Thus, sequential extractions do allow for a useful comparison between materials, and provide the opportunity to study large numbers of samples, unlike many spectroscopic or microscopic methods.

A sequential extraction procedure was employed that utilized sodium pyrophosphate to remove an organic phase, ammonium oxalate (run in the dark) to remove a phase associated with amorphous inorganic materials such as hydrous iron and manganese compounds, citrate-bicarbonate-dithionite to remove a phase associated with crystalline iron and manganese (hydr)oxides, and perchloric acid-boiling nitric acid to dissolve remaining sulfidic compounds. Total metal recovery from the sum of the four fractions was 96% $\pm$ 8.2% (S.D.) compared to the metals measured by aqua regia digest.

Pyrophosphate extraction. This first extraction (27) was performed on sediment samples from which pore water had been extruded using 1 atm positive pressure $N_{2(g)}$. Fifty mL of 0.1 M sodium pyrophospate were added to 1 g (wet weight) samples. The resulting

suspensions were placed in 50 mL conical centrifuge tubes, oriented at 45^0, then shaken at room temperature in the dark for 12 h at 150 rpm. One drop Superfloc7® was added, and the suspensions were allowed to settle for 1 h. Centrifugation at 3560 x g for 15 min resulted in separation of liquid and solid phases. The liquid phase was refrigerated at 4^0 C until ICP analysis.

Oxalate fraction. To the solid phase obtained above, 200 mL of 0.2 M ammonium oxalate (adjusted to pH 3.0 with conc. HCl) were added. Sediment suspensions were placed in aluminum foil-wrapped tubes, and shaken at 150 rpm for 2 h (20). Liquid and solid phases were separated by centrifugation at 3560 x g for 15 min, and the supernatant was refrigerated at 4^0 C until ICP analysis.

CBD fraction. To the remaining sediment, 20 mL of 0.3 M sodium citrate and 2.5 mL of 1 M sodium bicarbonate were added. Suspensions were vortexed vigorously, then heated in a water bath to 80^0C. 0.5 g sodium dithionite was added as a powder, and the solution stirred intermittently while held at 80^0C for 10 min. The solution was then centrifuged at 3560 x g for 15 min. This process was repeated, beginning with the addition of sodium citrate. After the second extraction, a room temperature wash with citrate-bicarbonate was performed. All 3 fractions were combined, acidified with 1 mL conc. HCl, and diluted to 250 mL (28). In using sodium dithionite to extract metals associated with crystalline iron oxides, it is noteworthy that available dithionite reagent contains metal impurities, particularly zinc, that can be as high as 400 ppm (29).

Sulfidic fraction. To the remaining sediment 1 g of $KClO_3$ was added, followed by 20 mL conc. HCl (30). This mixture was incubated at 25^0 C for 30 min, whereafter 20 mL glass distilled H_2O were added. The mixture was vortexed, centrifuged at 3560 x g for 15 min., and the supernatant separated into a second flask. To the pellet, 20 mL 4 *N* HNO_3 were added, and the mixture boiled for 20 min in a water bath. Twenty mL of a 0.5 g/L KH_2PO_4 solution were then added, and the mixture was allowed to stand at room temperature for 10

min. with occasional vortexing. The sample was then centrifuged at 3560 x g for 15 min., and the supernatant was mixed with the previous supernatant and diluted to 200 mL. All extracts were filtered through 0.2μm nylon filters before ICP analysis.

Results and Discussion

CDA Lake sediments near the mining-impacted CDAR are highly enriched in Fe, Mn, As, Pb, Zn, and other trace elements compared to the relatively pristine sediments obtained near the SJR (see Table 2-1, and Table 2-2). *Mean* total metal concentrations (in mg/kg dry weight sediment) for Fe, Mn, As, Pb, and Zn in contaminated sediments are 82486, 5953, 201, 3820 and 2995, respectively. Mean values (mg/kg sediment) for Fe, Mn, As, Pb, and Zn in tailings-free sediments are 27642, 349, 11, 78 and 751, respectively. *Maximum* total metal concentrations detected in contaminated sediments are Fe - 123200, Mn - 9200, As - 568, Pb - 21493, and Zn - 11169.

Examination of the vertical profile of total metal abundance in CDA Lake sediments reveals that different elements have characteristic patterns of distribution. For example, peak abundance of As and Fe is consistently bimodal, whereas Zn and Pb typically show single peaks (Figure 2-2a and 2-2b). Enrichment of As within 15 cm of the sediment-water interface is evident in nearly every core examined. By contrast, maximal values for Zn and Pb are consistently observed at depths $\geq$ 30 cm. About half of the cores taken were not deep enough to reveal maxima for Pb and Zn (compared to Horowitz *et al.* (14)). Not unexpectedly, cores with deeper Pb and Zn peak values were extracted nearer the CDAR delta, where past sampling has shown a much thicker tailings layer (3, 14).

TABLE 2-1. Mean Values (mg/kg) of Selected Elements in CDA Lake and SJR Delta Cores.

		Pb ± SE, (range)	Zn ± SE (range)	Mn ± SE, (range)	Fe ± SE, (range)	As ± SE, (range)	P ± SE, (range)
SJR delta cores	N= 42	78 ± 15 (294-20)	751 ± 41 (1,744-100)	349 ± 32 (618-208)	27,642 ± 364 (3,560-2,260)	12 ± 3 (17-3)	681 ± 92 (1290-510)
CDA Lake cores	N= 206	3820 ± 241 (21,413-42)	2,995 ± 125 (11,169-103)	5953 ± 173 (9,208-409)	82,486 ± 1,694 (123,200-21,500)	201 ± 11 (568-2)	560 ± 18 (1,320-190)
Fold increase		49	4	17	3	17	0.8
CDA Lake cores below contaminated zone*	N= 19	124 ± 14 (200-2)	156 ± 22 (342-2)	781 ± 48 (1,018-142)	37,618 ± 457 (55,670-22,100)	10 ± 1 (17-1)	1,074 ± 121 (1610-670)

* Refers to a sediment depth where lead concentrations are at or below 200 ppm.

Table 2-2. Mean (± std. error) metal content (mg/kg) of deep sediment cores retrieved from CDA Lake (A_1-O_3) and the SJR Delta (E_1-E_4).

Core	N*	Depth (cm)	Lead	Zinc	Iron	Manganese	Arsenic
A-1	14	56	5,085 ± 1,239	3,154 ± 459	79,685 ± 7,253	6,109 ± 741	172 ± 37
A-2	11	44	3,890 ± 362	3,209 ± 156	99,945 ± 3,109	7,970 ± 829	284 ± 38
A-3	11	44	3,470 ± 243	3,138 ± 135	103,081 ± 3,099	8,084 ± 667	349 ± 36
B-1	12	48	3,794 ± 127	3,792 ± 63	85,808 ± 1,647	6,617 ± 590	174 ± 5
B-2	14	56	3,059 ± 181	2,948 ± 128	98,657 ± 4,395	7,598 ± 1,291	354 ±40
B-3	15	60	3,365 ± 220	3,035 ± 118	91,035 ± 3,784	7,087 ± 1,190	275 ±40
C-1	9	36	3,261 ± 153	3,163 ± 118	100,856 ± 3,662	7,736 ± 251	286 ± 34
C-2	16	64	6,340 ± 1,461	4,197 ± 840	74,768 ± 7,437	5,614 ± 809	105 ± 23
C-3	15	60	3,468 ± 1,166	2,222 ± 608	58,153 ± 4,843	3,435 ± 697	58 ± 15
C-4	14	56	2,768 ± 986	1,802 ± 516	51,814 ± 4,300	2,761 ± 596	40 ± 10
C-5	13	52	1,993 ± 816	1,550 ± 483	51,777 ± 3,322	2,227 ± 514	37 ± 10
D-1	9	36	3,538 ± 323	3,174 ± 82	91,333 ±3,690	6,839 ± 271	205 ± 34
D-2	9	36	3,153 ± 285	2,857 ± 119	101,025 ± 4,955	7,476 ± 583	307 ± 43
D-3	14	56	8,096 ± 1,574	4,938 ± 766	85,692 ± 6,355	6,576 ± 217	136 ± 25
D-4	12	48	3,767 ± 262	3,149 ± 119	91,664 ± 2,838	6,763 ± 295	251 ± 27
O-1 **	16	64	3,271 ± 153	3,036 ± 141	104,031 ± 3789	7,603 ± 278	391 ± 33
O-2 **	11	44	2,809 ± 118	2,935 ± 157	98,527 ± 4848	7,538 ± 402	334 ± 35
O-3 **	10	40	1,287 ± 514	1,329 ± 422	43,210 ± 2,179	1,299 ± 266	20 ± 6
E-1	10	40	82 ± 19	1,009 ± 156	27,590 ± 1215	442 ± 34	9 ± 2
E-2	10	40	132 ± 27	1,014 ± 186	29,691 ± 1310	322 ± 21	11 ± 2
E-3	11	44	53 ± 3	590 ± 55	26,694 ± 519	293 ± 8	12 ± 1
E-4	11	44	52 ± 4	455 ± 42	27,102 ± 606	345 ± 21	13 ± 2

* N = number of 4 cm samples measured per core.
** "O" cores were taken within the CDA Lake delta, but not within a transect.

Fe shows a bimodal distribution. The major Fe peak generally coincides with the As maximum found near the sediment-water interface, while the minor peak occasionally coincides with Pb and Zn peaks found in deeper sediments.

In the contaminated sediments, Fe correlates strongly (Table 2-3a) by depth with Mn and As (r= 0.97 Fe:Mn, r= 0.82 Fe:As). These values are in agreement with Horowitz *et al.* (14). Zinc and Pb also correlate strongly with each other by depth (r= 0.90). In contrast with the findings of Horowitz *et al.* (14), our data indicate that Fe correlates weakly with Pb (r= 0.22) and Zn (r = 0.46), and that As correlates very poorly with both Pb (r = -0.13) and Zn (r = 0.07).

Our data indicate that, regardless of depth, metals are predominantly associated with an operationally defined sulfidic phase. For Pb and Zn this fraction represents 49.3% $\pm$ 1.4 (SE) and 63.3% $\pm$ 1.2 (SE) of the total sediment metal content; for Fe and As this fraction comprises 67.7% $\pm$ 1.4 and 73.3% $\pm$ 2.3 (see Figure 2-3 and Table 2-4). The next largest fraction of Pb associates with the organic phase (~27.8% $\pm$ 1.9 (SE)), in contrast with As, Fe, and Zn whose next largest fractions partition with the amorphous oxide phase (10.3% $\pm$ 1.5, 16.6% $\pm$1.2, and 17.2% $\pm$ 0.9, respectively). These results are not directly comparable to those published by Horowitz *et al.* (2, 14) inasmuch as those workers employed a two-step procedure wherein iron oxides were removed prior to the removal of any organic phase. Our method consisted of a five-step procedure wherein the organic phase was removed as the first step in sediment dissolution. Additionally, our samples were frozen in an anaerobic environment, and stored at -80 ^{0}C until sequential extractions were performed.

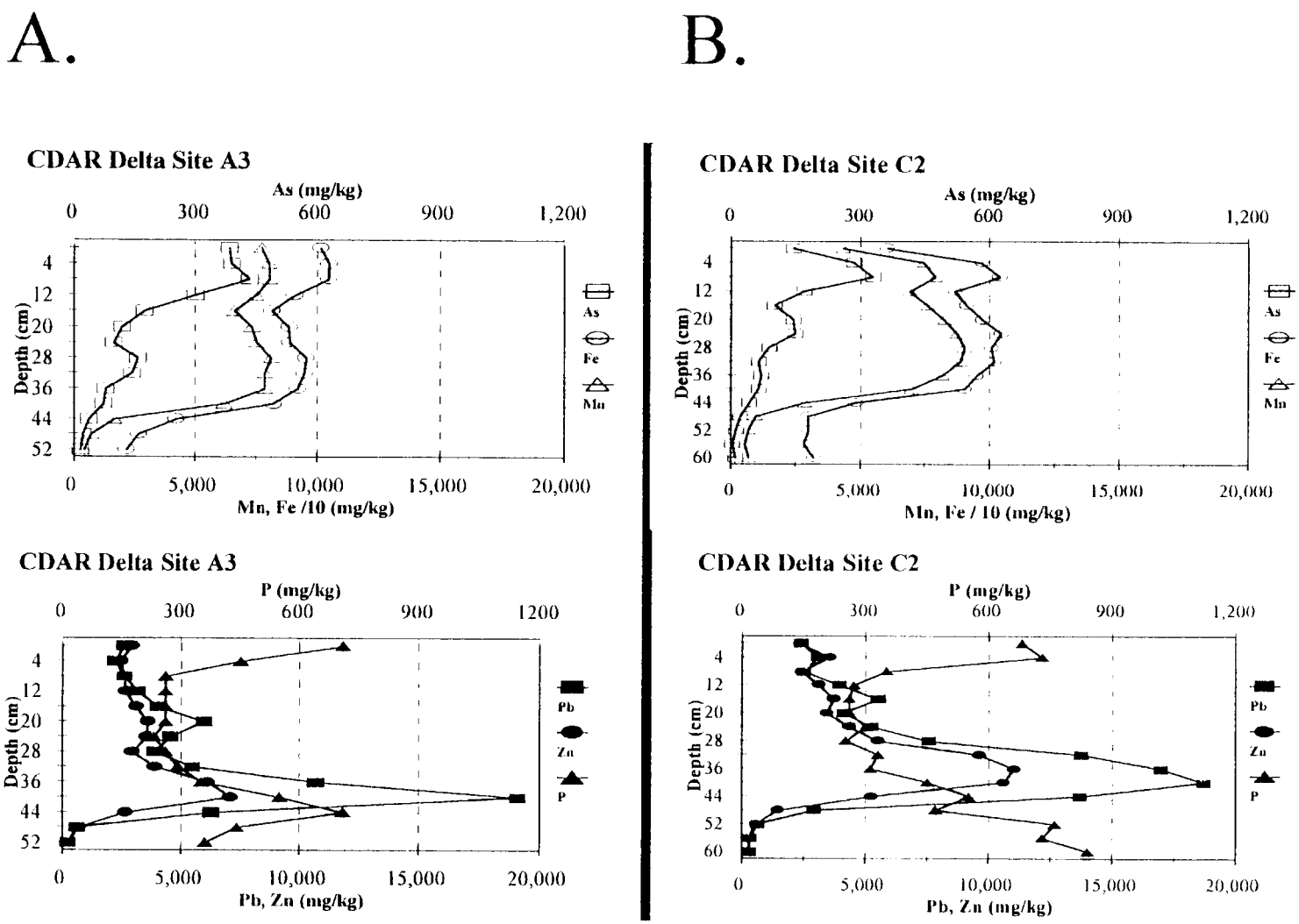

Figure 2-2. Sediment cores extracted from CDAR Delta, showing vertical metal profiles.

TABLE 2-3 A. Correlation (r) Between Selected Elements in CDA Lake Cores (A_1-O_3).

	Pb	Zn	Mn	Fe	As	P
Pb	1					
Zn	0.90	1				
Mn	0.30	0.55	1			
Fe	0.22	0.46	0.97	1		
As	-0.13	0.07	0.74	0.82	1	
P	-0.21	-0.30	-0.53	-0.48	-0.33	1

N = 206. Includes only values where metal contamination exceeds SJR metal concentrations.

TABLE 2-3 B. Correlation (r) Between Selected Elements in SJR Delta Cores (E_1-E_4).

	Pb	Zn	Mn	Fe	As	P
Pb	1					
Zn	0.69	1				
Mn	0.18	0.55	1			
Fe	0.68	0.71	0.53	1		
As	0.06	-0.10	-0.24	0.01	1	
P	0.78	0.69	0.48	0.83	-0.15	1

N = 42.

Table 2-4. Mean (± std. error) over 8 cm depth intervals of percentage metal partitioning in sequentially extracted phases of CDA Lake Cores A_1 and B_3.

	Sulfidic	Crystalline	Amorphous	Organic
As	73.2 ± 2.3	8.6 ± 1.4	10.3 ± 1.5	7.9 ± 0.9
Fe	67.7 ± 1.4	14.2 ± 0.7	16.6 ± 1.2	1.5 ± 0.1
Pb	49.3 ± 1.4	17.1 ± 1.2	5.8 ± 0.4	27.8 ± 1.9
Zn	63.3 ± 1.2	11.8 ± 0.6	17.2 ± 0.9	7.8 ± 0.4

N=12.

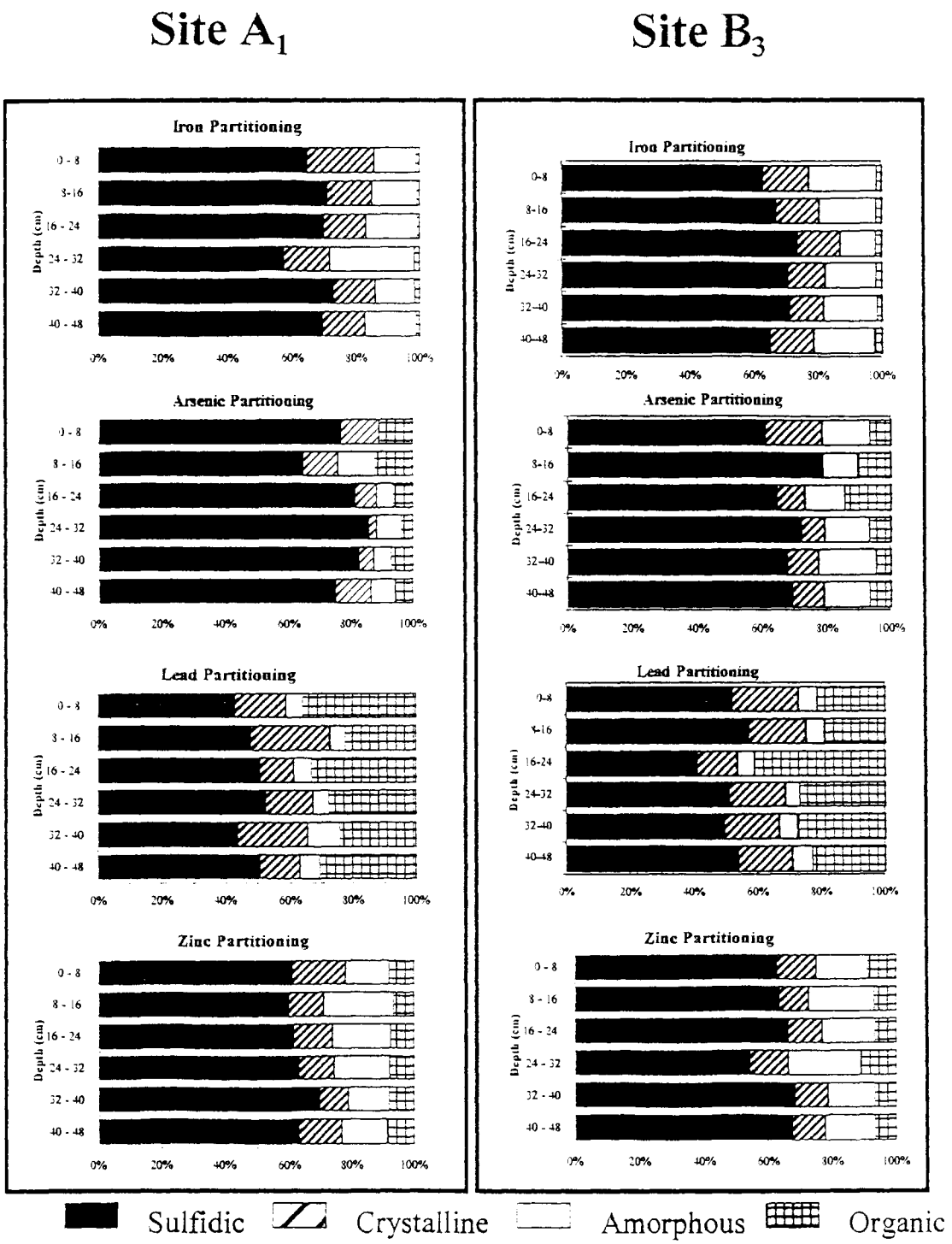

Figure 2-3. Sequential extraction of two sediment cores from the CDAR Delta.

CDA Lake sediments near the SJR and CDAR contain abundant bacterial populations. Direct counts reveal a gradient in estimated density from ~10^8 cells/g (wet weight) in the surficial sediments to ~10^6 cells/g (wet weight) in deeper sediments (Table 2-5). Our estimates are similar to those observed in other freshwater lake environments (31). The average pH of the sediments we examined was generally lower at the surface (pH 5.5), and increased with increasing depth (pH 6.4--Table 2-5). Bacterial sulfate reduction directly raises pH (7). Moreover, utilization of organic acids as electron donors in metal reduction would also serve to increase pH in deeper sediments.

In general, we found CDA Lake sediments to be highly reduced, with most redox potentials measured well below 0 mV (relative to the SHE; Table 2-5). At sites distant from the mouth of the CDAR, sediment redox potentials were consistently below zero - even in the uppermost 2 cm. In sediments close to the CDAR, the Eh gradient was less steep. However, no redox potential was recorded higher than +100 mV.

Table 2-5. Microbial abundance (cells /g wet wt. sediment), and mean (± std. error) pH and Eh in CDA Lake sediments.

Depth (cm)	Direct Microbial Count*	pH**	Eh**
0	2.8 e+8	5.57 ± 0.08	-2 ± 32
5	2.8 e+8	5.64 ± 0.02	-167 ± 16
10	1.6 e+7	5.93 ± 0.05	-125 ± 11
15	7.6 e+7	6.05 ± 0.07	-85 ± 14
20	9.1 e+7	6.09 ± 0.06	-123 ± 38
25	6.7 e+7	6.17 ± 0.11	-104 ± 36
30	4.6 e+7	6.19 ± 0.08	-122 ± 22
35	2.3 e+7	6.21 ± 0.12	-87 ± 14
40	2.0 e+7	6.26 ± 0.13	-135 ± 11
45	1.3 e+7	6.34 ± 0.15	-150 ± 14
50	9.7 e+6	6.34 ± 0.07	-140 ± 19
55	8.8 e+6	6.24 ± 0.03	-130 ± 9
60	7.8 e+6	6.27 ± 0.06	-110 ± 16

* Core D_3

** Cores A_1,A_2, B_1, B_2, C_1, C_3, D_1 and D_2.

Gross Metal Deposition. The horizontal deposition patterns of trace elements within the CDAR delta are similar to those described by Maxfield *et al.* (3) and Horowitz *et al.* (2, 14). In addition, we observe that the thickness of contaminated sediments decreases along a transect running from the CDAR toward the SJR (transect "C" in Figure 1 and Table 2-2). The zone of metal contamination at site C-2 extends to approximately 52 cm, 32 cm at site C-3, 24 cm at site C-4, and 20 cm at site C-5.

We observed two distinct subsurface maxima for iron and trace elements: one characteristic of As, Fe, and Mn, another characteristic of Pb and Zn. Generally, maximal abundance of the redox-active elements (As, Fe, Mn) occurs near the sediment-water interface in the region of the redox boundary (2-6 cm). This pattern suggests diagenetic cycling of these elements by oxidation-reduction reactions. It has been noted that large quantities of iron oxides are not required to bind substantial quantities of As or P (9, 11). Moreover, As solubility should be highest where Fe is reduced and sulfate reduction is not active (32). The reduction potentials we recorded (Table 2-5) indicate that these conditions likely exist just below the top few centimeters. Indeed, oxidized iron has only been reported to occur in the top 2 cm of CDA Lake sediments (2). Altogether, the accumulation of As and P near the sediment-water interface is to be expected given the occurrence of a redox boundary in the uppermost region of these sediments.

Iron and phosphorus are negatively correlated in CDA Lake sediments where lead or zinc concentrations are elevated (at least more than twice the SJR delta sediment concentrations). Oxidized Fe in freshwater lake sediments typically acts as a phosphate sink (9). Our correlation data therefore suggest that Fe (and necessarily, Mn) in these regions is reduced enough so that phosphate or arsenate are not bound efficiently. Thus, we believe that

the > 80% correlation of As and Fe abundance, as well as the high As in the sequential extraction sulfide phase, can be explained by postulating that the majority of As is present as an Fe-As sulfide (*e.g.*, FeAsS).

The >90% correlation between Pb and Zn suggests similar patterns of deposition, as well as similar patterns of cycling (or lack thereof). Interestingly, sequential extraction analyses show that of the metals examined, Pb and Zn have the smallest fraction bound as sulfides. Our data indicate that significant amounts of both elements partition with the operationally defined organic phase. This is consistent with other studies which have demonstrated that Pb and Zn are easily sequestered by a variety of complex organic compounds including bacterial exo-polysaccharides (21) and humic acids (33). While Zn has been described as capable of diagenetic re-mobilization (23), the vertical profiles we describe within the sediment column indicate that remobilization by this process has not occurred.

In order of absolute magnitude, Fe>Mn>Pb>Zn>As are the trace elements most enriched within contaminated regions of CDA Lake (see Table 2-1). CDA sediments collected below the zone of metal contamination, however, do not differ significantly from pristine SJR delta sediments in Pb or As content. Sediments collected near the SJR appear to have higher Zn, and lower P content than sediments found below the zone of metal pollution near the CDAR. Low P concentrations in the contaminated zone suggest that recent levels of phosphorus loading (per unit sediment) by the CDAR may be lower than levels that existed prior to the onset of mining.

Metal Phase Partitioning. In contaminated sediments, 60-70% of all Fe is associated with an operationally defined sulfidic phase, while much of the remainder is split between the amorphous and crystalline (hydr)oxide phases. The organic phase contains the least amount

of Fe, typically less than 10%. In contrast, two divalent metals, Pb and Zn, partitioned comparatively less with the sulfidic phase (Pb, 40 -50% and Zn, 55-65%), and more with the organic phase (Pb, 20-40% and Zn, 8-12%). More Zn than Pb appears to be sequestered in the amorphous (hydr)oxide phase, a finding which may help explain why this trace element has been released into the overlying waters (1). Zn in this phase could be readily solubilized consequent to iron reduction. Arsenic also shows variable levels of binding to the amorphous oxide phase, with core A-1 half the levels observed for core B-3.

We may infer from the relative quantities of metal bound as sulfides that bacterial sulfate reduction, particularly below the top 4 cm, may be an important mechanism for trace element sequestration. The inference of sulfate-reduction activity is also supported by the low redox potentials registered at these depths (Table 2-5), the relatively high pH values observed in these zones (pH 6.25), as well as most probable number (MPN) estimates (34) that confirm the presence of cultivable SRB in densities ranging from 10^6 to 10^4 cells/g wet weight sediment (Harrington *et al. Unpublished results*).

SRB can participate in metal reducing reactions, and by their production of sulfides, in trace element sequestration (7). The activities of SRB were once thought to be constrained to strictly anaerobic environments. Bacterial communities in lake sediments have been postulated to be stratified with respect to dominant community members. In this scenario SRB, were believed to predominate beneath dissimilatory iron-reducing bacteria (DIRB) (35). However, recent work has shown that SRB numbers can be high in more aerobic regions of the sediments, and that iron and sulfate reduction can occur at the same depth and time in a sediment (36). Further, it has also been shown that, as a group, SRB can support growth using a variety of terminal electron acceptors, including Fe(III), U(VI), Se(VI), and As(V)

(7). SRB may therefore be active in the upper sediments, perhaps as high as 4 cm from the sediment-water interface.

Our data indicate that reducing conditions prevail in CDA Lake sediments, and that trace elements are chiefly associated with an operationally defined sulfidic phase. Spring runoff and flooding within the CDAR Valley continues to introduce iron and trace elements into CDA Lake. Since these materials are transported as resuspended streambank sediments, it has been proposed that they are introduced into CDA Lake in a predominantly oxidized state (2, 14). Our findings suggest that, if this is indeed the case, considerable metal reduction and sequestration takes place within the lake sediments secondary to deposition. We also observe that the fraction of trace elements bound by organic matter is significant, particularly with respect to Pb and Zn. Thus we concur with Pedersen (12) that the risk of release of Pb and Zn into overlying waters under eutrophic conditions may be exaggerated. The biogeochemical cycling of As in CDA Lake requires further study in regard to bacterial formation and dissolution of arsenic sulfides.

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Chapter 3

Biotic Generation of Arsenic (III) in Metal(oid)-Contaminated Freshwater Lake Sediments

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Introduction

During the first two-thirds of the 20th Century, mining in northern Idaho produced over one billion troy ounces of silver and eight million tons of lead (*1*). Much of this activity took place along the South Fork of the Coeur d'Alene River (CDAR), a region that came to be known as the Silver Valley. As a result, mine tailings containing high concentrations of lead, zinc, arsenic and other trace elements accumulated in stream banks and bars throughout the lower CDAR Valley. Although tailings ponds were constructed in the late 1960s, spring run-off and periodic flooding have continued to transport these materials downstream, heavily contaminating Coeur d'Alene Lake (CDAL). CDAL is a natural lake of glacial origin covering ca. 130 km^2 and having an average depth of roughly 64 m. CDAL is dimictic, regularly undergoing two periods of thermal stratification and a fall and spring overturn. The lake appears to be variable in trophic status as inferred from total nitrogen and phosphorus levels. Based on 1991-92 data, the southern end near Lake Chatcolet is mesotrophic-eutrophic, while the rest of the lake is oligotrophic-mesotrophic (*2*).

On the basis of a surface sediment survey (*3*), 85% of CDAL sediments appears to be enriched with Ag, As, Cd, Cu, Fe, Hg, Mn, Pb, Sb and Zn. Field measurements of sediment Eh consistently reveal a steep Eh gradient and the presence of a redox boundary within 10 cm of sediment-water interface (*4*). Total metal analysis of deep sediment cores reveals that iron and trace elements in CDAL exhibit two distinct patterns of distribution (*4*). Maxima for total abundance of redox-active elements such as iron and manganese are observed near the sediment-water interface (<10 cm), whereas maxima for less redox-active elements such as lead occur more deeply (>25 cm). We have postulated that biotic and abiotic transformations of arsenic, iron and sulfur account in large measure for these patterns. In this communication, we demonstrate

that the capacity for microbially-mediated redox transformation of arsenic exists in CDAL sediments.

Arsenic is toxic to both plants and animals, and its trivalent form is considered to be much more toxic than its pentavalent form (*5*). Both forms readily accumulate in living tissues owing to their affinity for proteins, lipids, and other cellular components (*6*). In many aqueous systems arsenite (As (III)) has been shown to predominate under reducing conditions (*7, 8*). Moreover, compared to arsenate (As (V)), arsenite demonstrates greater mobility in both sediment and groundwater systems (*6-10*). This difference in mobility has been attributed to the high affinity of As(V) for insoluble species such as hydrous ferric or manganese oxides (*10*). Some researchers postulate that arsenite has a lower affinity for these compounds to the extent that binding only occurs consequent to the oxidation of As (III) to As (V). This transformation is thought to be mediated either by the iron or manganese oxyhydroxides themselves (*10, 11*), or by microorganisms (*12*). Alternatively, the greater apparent mobility of As (III) under reducing conditions in neutral or slightly acidic sediments may result from the fact that these conditions favor reduction of both iron and manganese (hydr)oxide sorbents, as well as reduction of As (V). In fact, some recent studies suggest that at circumneutral pH, As (III) may actually have a higher affinity for iron (oxyhydr)oxides than As (V) (*13*).

Accumulation of arsenic at the redox boundary has been repeatedly observed in both stratified water columns and sediments (*9, 14, 15*). Under the highly reduced, neutral pH conditions characteristic of deeper strata, arsenic can be precipitated by sulfide (*14, 16*). Under the oxidizing conditions that prevail nearer the surface, scavenging and precipitation of arsenic by iron and manganese oxyhydroxides limits upward diffusion (*10, 11, 17*). Near the redox boundary, however, arsenic is more likely to occur as As (III) and appears to be more mobile (*7,*

8, 9, 17). Over time, diffusion of soluble arsenic released by chemical or bacterial activity leads to its accumulation within this layer of the sediment.

Detailed examination of the vertical profile of As (V) *vs.* As (III) in stratified sediments or water columns, however, has sometimes revealed unexpected speciation profiles. Where strongly reducing conditions exist, As (III) should be favored over As (V) from thermodynamic considerations alone. However, in several systems, such as the meromictic Lake Pavin in France, as well as lakes in the Aberjona watershed, As (V) is found at unexpectedly high levels in permanently anaerobic regions of the lake (*14, 18*). In other systems, As (III) is found to predominate in aerobic regions of lakes (*11, 15*) even though the thermodynamically favored As (V) species should be prevalent. These anomalies have been variously interpreted as being caused by the presence or absence of diagenetic forms of iron or manganese, sulfide, or key microbial transformants.

Microorganisms capable of direct As (III) oxidation and direct As (V) reduction have been described (*12, 19- 22*). In particular, two physiologically defined taxa, the dissimilatory iron-reducing bacteria (DIRB) and the sulfate-reducing bacteria (SRB), contain representatives which can either directly transform arsenic, or act upon inorganic compounds that transform arsenic. In an iron-rich environment, iron oxyhydroxides bind arsenic (*10*). However, DIRB can utilize iron oxyhydroxides as terminal electron acceptors, releasing bound trace elements. In addition, certain DIRB have also been shown to reduce As (V) (*20*). Production of aqueous sulfide by SRB can directly reduce both arsenate and iron hydroxides (*17, 22*). When aqueous sulfide is not in excess these reactions can increase arsenic solubility; where sufficient levels of soluble sulfide exist, arsenic sulfides can form (*17*). It is therefore reasonable to suppose that in

freshwater sediments DIRB and SRB play essential roles in the cycling of arsenic under low redox conditions.

We have hypothesized (*4*), based on sequential extraction data, redox profiles, and the strength of correlation between arsenic and other contaminant metals in CDAL, that arsenic is being mobilized toward the sediment-water interface while, in contrast, lead and zinc remain buried in deeper strata. In this communication, we report that the microbial elements capable of performing the necessary transformations of As, Fe, and S to account for this phenomenon are present in CDAL sediments. We also demonstrate the occurrence of significant biotic As (V) reduction in replicate sediment microcosms.

Materials and Methods

Sediment core removal and sampling. Cores were retrieved from sediments along a transect extending from one edge of the Coeur d'Alene River Delta toward the town of Harrison, ID (Figure 3-1). At the times of sampling the overlying water column in this region ranged between 1 and 2 m. Capped 3 m sections of 1-inch (I.D.) PVC pipe were inserted into the water column until the end of the pipe touched the sediment, at which point the cap was removed, and the PVC was pressed into the sediment ~60 cm. The cap was then replaced, the core removed, and the pipe cut in a manner that left 10 cm of lake water overlying sediments. Pipe ends were sealed, and the cores were transported to the laboratory and stored upright in a plastic container. The container was kept in a 4^0C cooler and flushed continuously with N_2 gas.

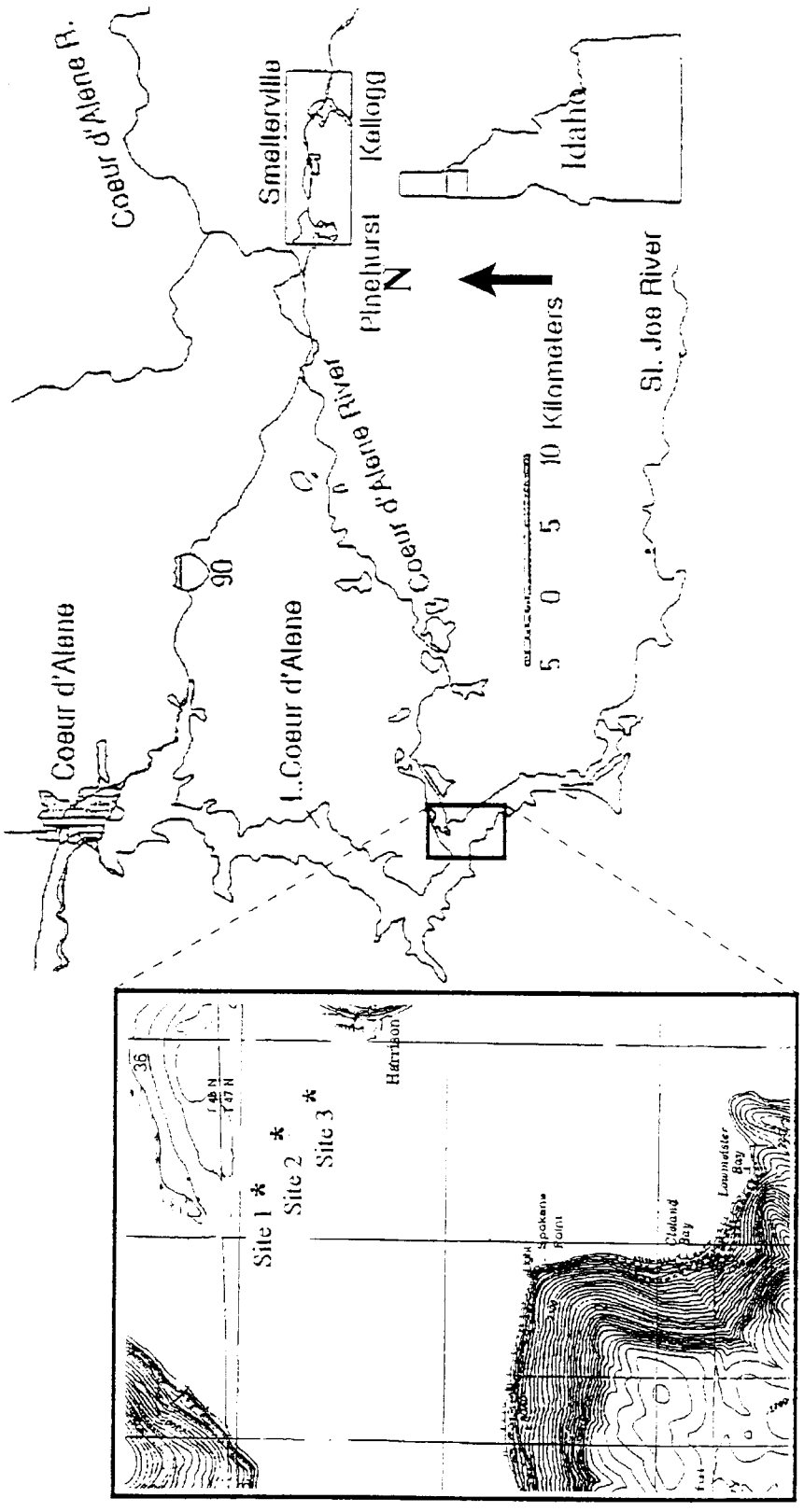

Figure 3-1. Coeur d'Alene Lake, Idaho, with focus on CDAR Delta region.

Sediment cores were extruded from the bottom up onto a waxed paper-coated processing board and immediately transferred into an anaerobic chamber containing pre-mixed N_2:CO_2:H_2 gas in a ratio of 80:15:5. Pore water was expelled from subsamples of 8 cm core sections in an acid-washed, plastic-lined compression chamber at 0.5 P N_2 (g), and passed through 0.2 μm nylon filters.

Analysis of iron and trace elements. Trace element abundance was determined using a Thermo Jerell Ash (Franklin, MA) IRIS ICP. Analyses were performed on aqua regia-digested sediments and pore water extracted as described above. Every twentieth sample was duplicated, and less than 10% error was detected. As(III) was analyzed by hydride gas generation followed by ICP spectrometry. The hydride gas generation method of Glaubid and Goldberg (*23*) was modified by an addition of oxalic acid/sodium oxalate buffer (to obtain a pH of 4.5) and 0.35% sodium borohydride/0.30% sodium hydroxide solution (*24*), and utilized a gas-liquid separator (Bausch and Lomb ARL341) hydride gas generation unit.

Most Probable Number (MPN) Analysis. Abundance of cultivable microbes belonging to one or more physiologically defined taxa was estimated by a set of replicate terminal dilution enrichments. Sample processing for microbiological analyses was conducted entirely within an anaerobic chamber containing a N_2:CO_2:H_2 atmosphere in a ratio of 80:15:5. One gram of sediment was removed from the center of the extruded core at depth intervals of 2, 18, and 35 cm. Sediments were placed into 10 mL serum vials containing 9 mL 1/2X Ringers solution (per liter: 4.0 g NaCl, 0.5 g $NaHCO_3$, 0.125 g KCl, and 0.13 g $CaCl_2$) (*25*), then sealed in the chamber by crimping. 0.01% Cetyl-trimethyl ammonium bromide (CTAB) was added to promote formation of uniform sediment suspensions (*26*). Vials were shaken at 200 rpm on a

gyrorotary platform maintained at room temperature for 1-2 h. Sterile, 96-well BioBlock containers (Rainin, Woburn, MA) were used for evaluating MPN of cultivable bacteria. Each of the ninety-six 2-mL wells was filled with 0.9 mL of media described below.

Abundance of SRB was estimated using Pfennig medium modified by the addition of 0.93 g/L lactate and 7.33 mg/L $FeSO_4$; the media contained all recommended electron donors except palmitic acid (*26*). Abundance of cultivable arsenate-tolerant SRB was estimated using modified Pfennig media amended with 1 mM sodium arsenate (0.312 g/L final conc.). Abundance of arsenic reducers was estimated using Pfennig media further modified by replacing sulfate with 10 mM sodium arsenate. The addition of dithionite and sodium sulfide to Pfennig media was eliminated in this last determination in order to avoid abiotic reduction of arsenate. One mL of sediment suspension was removed in the anaerobic chamber using a 1 mL syringe fitted with a 19-gauge needle; 0.1 mL were added to each of the first 4 wells of the first row of the appropriately marked BioBlock. These suspensions were taken through six successive ten-fold serial dilutions using a multichannel pipettor; the final well thus represented a 10^{-7} dilution. Sediment dilutions were incubated in the dark at room temperature for 30 d before they were scored for blackening indicative of sulfidogenesis. All MPNs were calculated using techniques and tables of the American Public Health Association (*27*). MPN for As-reducers was calculated after cultures were assayed for As (III) formation using hydride-generation ICP (measured sensitivity <100 ppb). Wells were scored positive for As (V) reduction if As (III) levels exceeded the 95% confidence interval around the mean of the abiotic controls.

Arsenic Reduction Assays. Three sets of microcosm experiments were initiated on the basis of their position relative to the sediment-water interface. Sediments taken from the 2 cm depth were representative of the region containing the surficial As peak, whereas sediments from 18 cm and 35

cm were representative of those regions containing the principal Fe peak, and the maxima for Pb and Zn, respectively (see Figure 3-2). A three-tenths milliliter aliquot of the same sediment suspension used to initiate the MPN was added to 9 mL of 10 mM (sodium) arsenate-containing buffer in 10 mL serum vials. This phosphate-based buffer (*20*) consisted (per liter) of 0.34 g of K_2HPO_4, 0.34 g of KH_2PO_4, 0.46 g of NaCl, 0.12 g of $MgCl_2 \cdot 6H_2O$, and 0.06g of $CaCl_2 \cdot 2H_2O$. Sediments were subjected to six treatments run in triplicate for each depth assayed: Pfennig organic acids (final conc. per liter, 2 g sodium acetate, 0.7 g propionic acid, 0.8 g butyric acid, 0.5 g benzoic acid, 0.93 g lactic acid), Pfennig organic acids + Na-molybdate (10 mM final conc.), organic acids + formaldehyde (1% final conc.), molybdate alone, formaldehyde alone, and no amendment. The total volume of all vials was adjusted to 10 mL with additional anaerobic phosphate buffer (pH 7.2), whereupon vials were sealed and crimped. All manipulations were carried out anaerobically. Serum vials were incubated at room temperature in the dark for 30 d. after which time reactions were terminated by the addition of 0.1 mL 10% w/v ascorbic acid (*28*). Serum vials were stored at 4^0C until ICP analysis.

Results and Discussion

Coeur d'Alene Lake sediments in areas that experience relatively low flow from the CDAR show maximum arsenic abundance within 10 cm of the sediment-water interface. Iron is extremely abundant throughout CDAL sediments; its maximum abundance generally occurs at depths $\leq$ 25 cm. By contrast, the maxima for lead and zinc occur at depths > 25 cm (Figure 3-2). These patterns hold across all three sites, and are consistent with previously reported results (*4*).

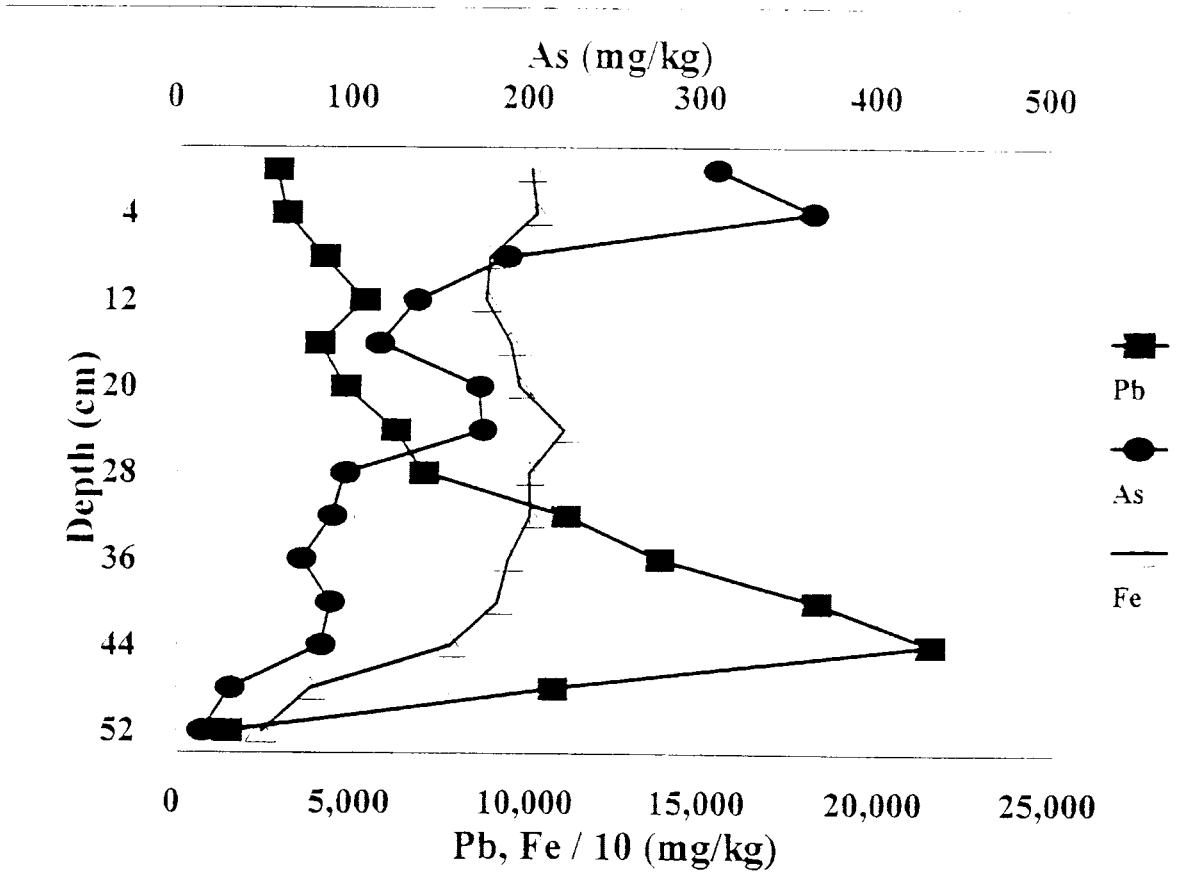

Figure 3-2. Vertical profile of total metal abundance in a core from Site 3 (Figure 3-1), CDAL delta. Note a surficial arsenic peak, and the deeper lead peak.

Using sequential extraction methods, we have established that regardless of depth ~70% of total arsenic in CDAL sediments is associated with an operationally defined sulfidic phase (*4*). Approximately 15-20% of solid phase As is bound to amorphous or crystalline Fe/Mn (hydr)oxides. Arsenic in the $MgCl_2$-exchangeable fraction ranges from 0 to 6 mg/L (*29*), while As concentrations in sediment pore waters range from 0.35 mg/L to below detection limit (Table 3-1, and Table 3-2). Since sequential extraction information is based on operational definitions, these values should be interpreted with caution. Nevertheless, metal(loid)s found in $MgCl_2$-exchangeable and pore water fractions are considered to be most bioavailable, as well as most easily transported from sediments into overlying waters (*7*).

Iron is extraordinarily abundant throughout CDAL sediments (5-10% by dry weight), and more than 70% of Fe occurs as iron sulfide (*4*). However, iron also occurs in forms that are more readily bioavailable. Iron in the $MgCl_2$-exchangeable fraction ranges from approximately 800 mg/kg (dry weight) in surficial sediments to 300 mg/kg at depths below 30 cm (*29*). Iron pore water concentrations range from approximately 2 mg/L to greater than 40 mg/L (Table 3-1, and Table 3-2).

Previous work has shown that total microbial abundance in CDAL sediments ranges from 10^5 to 10^8 cells/g wet weight sediment (*4*). Our MPN estimates indicate that cultivable SRB range in abundance from 10^3 up to 10^6 cells/g wet wt sediment (Figure 3-3). Because we have imperfect knowledge of the substrate preferences and micronutrient requirements of all SRB, these numbers necessarily provide a minimum estimate of SRB abundance. Our estimates of 10^6 cells/g in zones of peak SRB abundance rank among the highest reported recoveries of this taxon in the literature (*30, 31*).

TABLE 3-1: Iron and Trace Element Abundance in CDAL Waters

ELEMENT (µg/L)	As	Fe	Pb	Zn
Lake Waters				
Concentration range[1]	<1 - 1	NA	< 1 - 41	<10 - 390
Median	<1	NA	3	99
No. samples (near surface & near bottom waters)	145	NA	145	146
Sediment Pore Waters				
Concentration range in CDAL pore water[2]	40 - 350	1,880 - 40,330	40- 1,540	300 - 1,550
Median	180	4,270	250	490
Mean, ± Std dev (N= 12)	160 ± 98	7,687 ± 10,755	185 ± 104	560 ± 346
Federal Water Quality Stds				
Drinking water standards[3]	50	300	50	5000
Concentrations considered chronically toxic to aquatic FW life[3]	342	NA	2000	25,000

Sources: [1] (*38*). [2] This study. [3] (*4*).

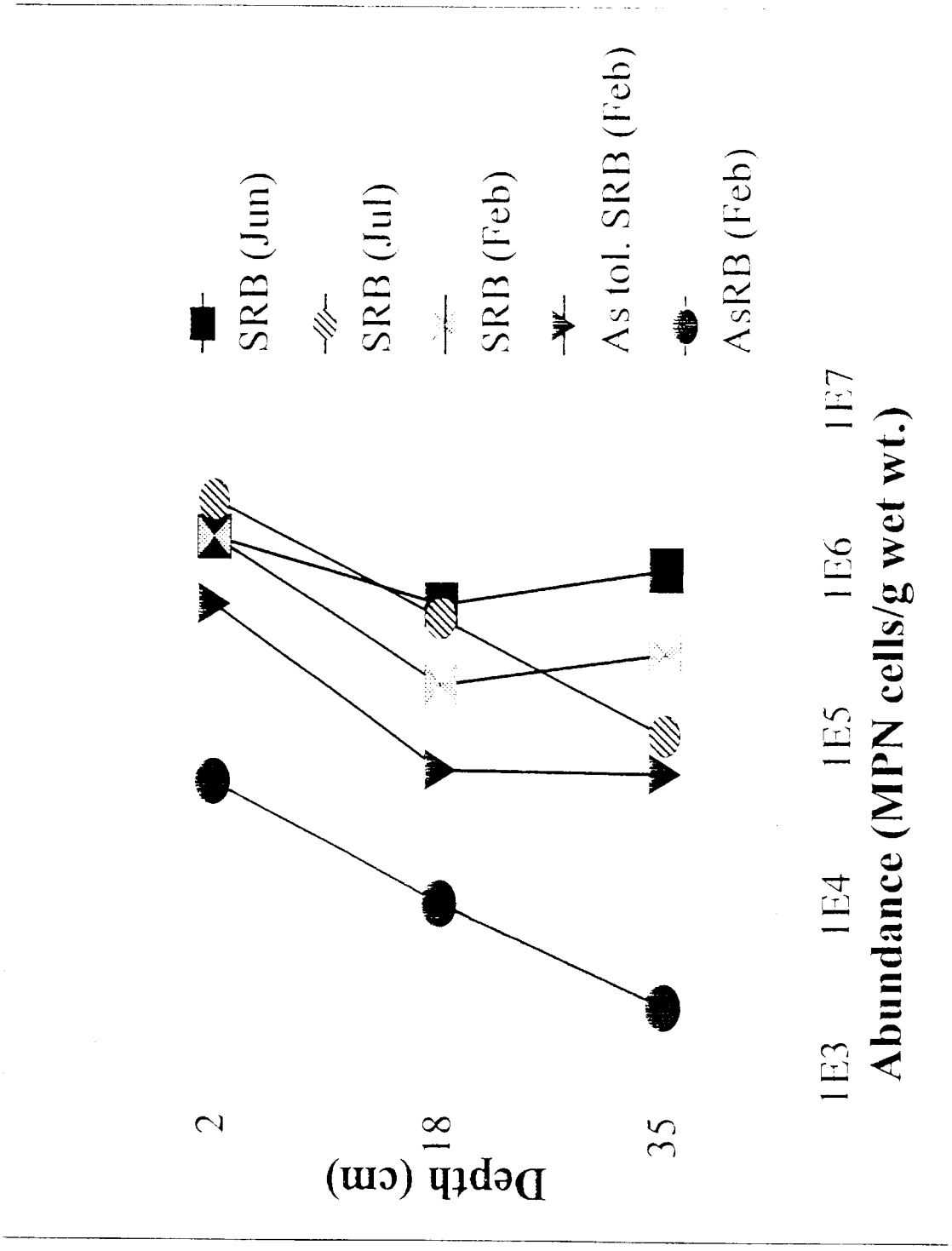

Figure 3-3. MPN of arsenate and sulfate reducing bacteria at three different time points.

Approximately 10% of the Pfennig media incubations which contained 1 mM sodium arsenate were sulfidogenic. Thus, a significant fraction of the CDAL sulfate reducer community may be described as arsenic-tolerant (Figure 3-3).

Dissimilatory arsenic-reducing organisms have been recently isolated in pure culture (*19-22*). The physiology and 16S-ribosomal subunit phylogeny of one such organism places it within the sulfate reducer group (*22*). We therefore designed a medium to estimate the MPN of arsenate reducers that could also be sulfate reducers. The basal medium used in this assay supports growth by all currently recognized SRB genera (*26*), but replaces sulfate with arsenate, an alternate terminal electron acceptor whose structural similarity may enable it to behave as a metabolic analogue. Using this medium we estimate that the abundance of cultivable As (V)-reducers in CDAL sediments ranges between 10^3 and 10^5 cells/g wet weight sediment (Figure 3-3).

Work in progress on CDAL sediments provides evidence for an active dissimilatory iron-reducing bacterial (DIRB) community (*29*). These studies show that profiles of reduced iron begin at approximately 550 mg/kg at the sediment-water interface and increase with depth to levels exceeding 2800 mg/kg. Incubation of anaerobic sediment microcosms for 39 d with 2 mM sodium acetate (final conc. 0.16 g/L) stimulates ferrous iron production, and serial dilutions incubated under iron-reducing conditions have blackened iron gel media to dilutions of 10^{-5}. Altogether, these observations indicate that certain strata of CDAL sediments support active and abundant DIRB populations.

Table 3-2. Iron and trace element abundance in CDAL porewaters (mg/kg)

Depth (cm)	As	As	Fe	Fe	Pb	Pb	Zn	Zn
	Site 1	Site 3	Site 1	Site 3	Site 1	Site 3	Site 1	Site 3
0-8	0.04	0.04	4.27	1.83	0.07	0.07	0.70	0.34
8-16	0.24	0.06	8.68	5.53	0.19	1.54	0.50	0.42
16-24	0.35	0.09	12.78	2.70	0.05	0.25	0.76	0.34
24-32	0.25	0.18	4.38	4.11	0.30	0.31	0.39	0.49
32-40	0.18	0.09	3.43	1.88	0.30	0.22	0.60	0.30
40-48	0.16	0.24	40.38	2.41	0.04	0.30	1.55	0.35

The total arsenic load delivered to CDAL sediments over the last century has been estimated to exceed 1,500 tons (*32*). Examining the potential for dissimilatory As reduction in this environment has special relevance in light of this fact; moreover, we have observed that total As concentration can exceed 500 mg/kg within 10 cm of the sediment-water interface (*4*) (Figure 3-2). Currently, Federal drinking water standards restrict acceptable As concentrations to 50 μg/L (*5*). Pore water As concentrations an order of magnitude greater than these limits can be found within 20 cm of the water column (Table 2-1 and Table 2-2).

Arsenic (V) and As (III) differ in their toxicity, as well as in their respective solubilities in reduced environments such as the CDAL benthos (*7*). Our MPN estimates indicate the presence of $\sim 10^4$ As (V)-reducing organisms per gram wet weight sediment. We therefore sought to establish the overall capacity of CDAL sediments for biological As (V) reduction. Our experiments were designed to investigate the fate of As (V) imported by sediment deposition, or released by desorption consequent to the reduction of hydrous ferric oxides. We further sought to establish the potential for As (III) release into overlying waters given the presence or absence of additional electron donors in the form of organic acids. Additionally, we sought to establish the contribution of specific taxa to As (V) bioreduction. Thus, 10 mM molybdate (2.06 g/L final conc.) was added as an inhibitor of SRB metabolism (*33*). To evaluate the capacity for abiotic As (V) reduction, formaldehyde was added to sediment microcosms run with and without organic acid amendment.

Arsenic (V) reduction was most pronounced in uninhibited microcosms amended with organic acids (Figure 3-4). The capacity to reduce As (V) also decreased with increasing depth. This observation can be partly attributed to the presence of fewer As (V)-reducing bacteria at lower depths (see Figure 3-4). Considering that As accumulates in uppermost sediments, it is

possible that the microbial community at 2 cm was simply more tolerant of the 10 mM arsenate amendment. Arsenic (III) has been shown to be toxic to many microbes at concentrations as low as 100 μM (*34*). It is noteworthy that each microcosm not treated with formaldehyde produced As (III) well in excess of 100 μM (Figure 3-4).

At every depth arsenic reduction in electron donor-unamended microcosms was two to four-fold greater than either abiotic control ($p << 0.001$). These results demonstrate that sufficient nutrients are present in CDAL sediments to support biological reduction of As (V). The amount of abiotic As (III) production observed (up to 10 mg/L in 30 d) likely results from reactions with reduced inorganic components (*e.g.*, sulfides) of the sediment that we have reported previously (*4*). On the basis of published rate data for arsenate reduction by aqueous sulfide (*22*), dissolved sulfide concentrations of greater than 100 μM would be required to reduce even 10% of the added arsenate over a 30 d period clearly unrealistic. Thus, biological rather than chemical pathways are required to account for the extent of arsenate reduction, a point supported by the formaldehyde-treated microcosms.

Amendment of sediment microcosms with organic acids resulted in a two to three-fold increase in As (V) reduction compared to unamended microcosms ($p << 0.001$). This observation indicates that additional carbon input to CDAL sediments would likely favor increased biological As (V) reduction. Depending on the speciation of iron in surficial sediments, the fate of the As (III) produced could include release into overlying waters, as has occurred in the Lake Ohakuri and Aberjona watersheds (*18, 19, 35, 36*).

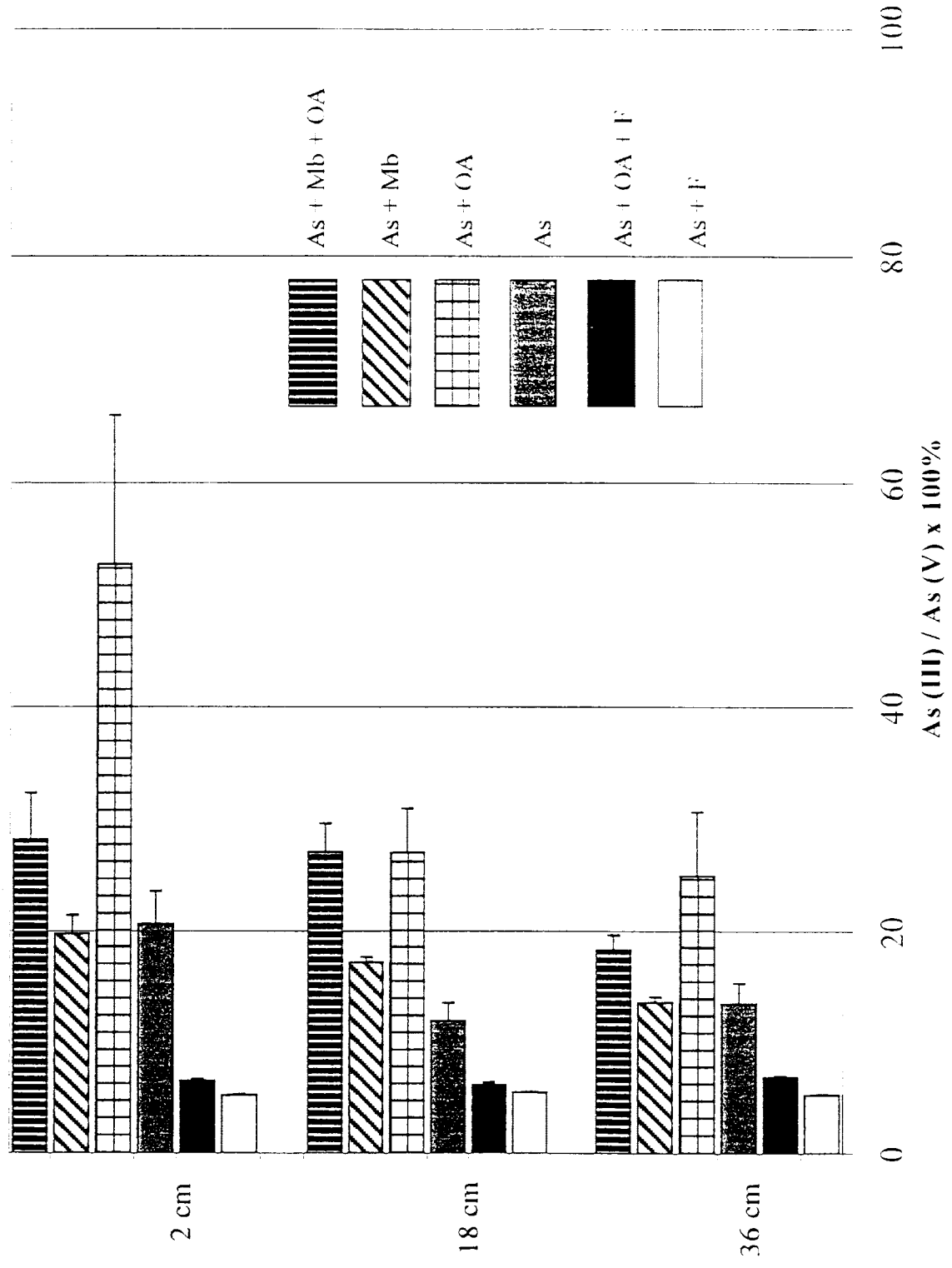

Figure 3-4. Microcosms containing CDAL sediments, arsenate, organic acids, and inhibitors.

At some strata within the CDAL sediments, organic acid-amended microcosms showed diminished capacity to reduce As (V) when treated with molybdate (Figure 3-4). Because molybdate inhibits SRB metabolism (*34*), these results indicate that SRB populations may be partly responsible for biological As (V) reduction in CDAL sediments. It is also possible that some non-sulfate-reducing arsenic-reducers might be molybdate-sensitive; however, in other anoxic sediments molybdate has not been found to inhibit the rate or extent of As (V) reduction (*37*). We found no significant difference in As (III) production between molybdate-treated microcosms and those to which no organic acids were added ($p = 0.056$). However, when organic acids were added to molybdate-treated microcosms, significantly more reduction occurred than in microcosms amended with arsenic and molybdate only ($p < 0.02$). We therefore conclude that the capacity to reduce As (V) is neither limited to, nor exclusive to the SRB.

Metal-contaminated sediments in CDAL support an abundant and diverse microbial community. Elements of this community have the capacity to reduce arsenic. Previous studies have suggested that bacterially-mediated As (V) reduction contributes to As accumulation near redox boundaries in sediments and water columns (*35, 36*). We should also bear in mind that CDAL sediments are highly enriched with iron; thus, transformations of this element are likely to significantly influence sediment biogeochemistry. We hypothesize that in CDAL sediments As is initially mobilized by direct As (V) reduction and/or the release of sorbed As consequent to Fe (III) reduction. Arsenic then diffuses until it reaches the redox boundary, whereupon it is resorbed by hydrous ferric oxides in oxidized surficial sediments.

The capacity for arsenic reduction and mobilization in Coeur d'Alene Lake sediments must be understood in order to predict the conditions under which As levels could increase in surface waters. Coeur d'Alene Lake serves six community water supply systems, and provides a

place for outdoor recreation to thousands of people annually (*2*). Presently, these waters comply with Federal drinking water standards (Table 3-1). However, given that bacterial As reduction can be demonstrated in CDAL sediments, it is reasonable to suppose that certain conditions might favor an increase in surficial As concentration. SRB and DIRB are both present and active in these environments. Furthermore, such organisms can be expected to engage in reactions that directly reduce arsenate, and favor the release of bound arsenic compounds consequent to the reduction of ferric and manganese (hydr)oxides. Because these reactions are favored under anaerobic conditions, and the reverse reactions favored under aerobic conditions, we recommend management practices for CDAL that will maintain the lake hypolimnion in an aerobic state.

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Chapter 4:

Sulfate-Reducing Bacterial Interactions with Heavy Metals in the Sediments of Lake Coeur d'Alene, Idaho.

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Introduction

The ecology of sulfate-reducing bacteria (SRB) continues to be a subject of great interest among scientists interested both in environmental and pollutant management as well as in the diversity of microbial species and physiology. Recent advances in the study of SRB metabolism have revealed that these organisms can utilize a range of carbon sources (*1*), and are variably tolerant of oxygen. As a group, SRB are capable of using diverse terminal electron acceptors, including many metals, metalloid anions, and diverse carbon compounds (*2*). Their physiological diversity make this group a promising source of organisms for bioremediation, particularly for anaerobic degradation of organic or halogenated organic compounds, as well as for anaerobic stabilization of metal(oid)-containing effluents, soil, or sediments.

Coeur d'Alene Lake, Idaho (CDAL) sediments contain high levels of iron and trace elements (*3*, *4*). The point source for this contamination is the Coeur d'Alene River (CDAR) which over the last 100 years has secondarily transported tailings-laden sediments from the Coeur d'Alene mining district (Figure 1). Public concern has been raised regarding the fate of the metals in CDAL bottom sediments (*5*). Particular concern has been expressed that should the lake become seasonally anoxic, higher concentrations of trace elements in CDAL waters would result from iron oxide dissolution and subsequent release of bound trace elements. We have subsequently shown that trace elements in CDAL sediments are predominantly bound in a sulfidic phase (*3*), though reducible forms of iron and other trace elements are found in significant quantities as deep as 48 cm from the sediment-water interface. The question remains unanswered as to what relative percent of these sulfides are generated within CDAL (authigenic), as opposed to being transported in an un-oxidized state from the mining district. To address this question the activities of SRB within these sediments must be studied.

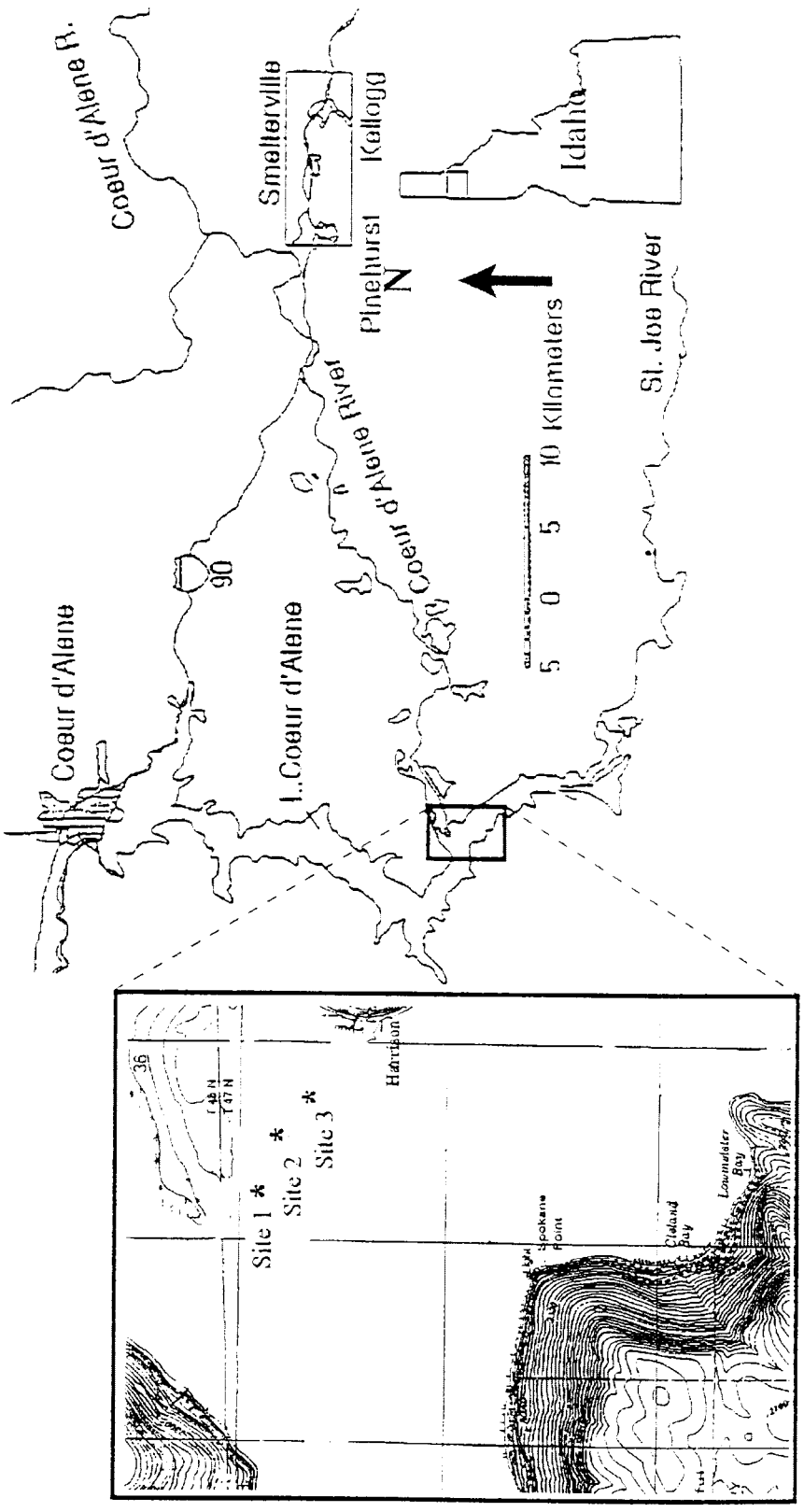


Figure 4-1. Coeur d'Alene Lake, with focus on CDAR Delta region.

Relatively few studies of SRB in metal-enriched environments have been published (*6-8*). Nevertheless, several treatment schemes for metal-contaminated water produced by acid mine drainage (AMD) have been published. In these designs, SRB reform metal(oid) sulfides through the addition of sufficient quantities of electron donors (*e.g., 9, 10*). Herlihy and Mills (*11*) noted increased rates of sulfate reduction in the sediments of a lake receiving AMD, and demonstrated high levels of acid volatile sulfides (AVS) in those lake sediments. AVS is now considered to be a key parameter predicting metal toxicity and bioavailability in anaerobic sediments (*12*). Jackson (*13*) noted that in sediments with ample sulfide more efficient binding and stabilization of many metals such as zinc, cadmium, and copper resulted, and that sulfidic sediments were much more effective at suppressing re-mobilization of metals than were organic sorbants. SRB appear then to be able to stabilize metal(oid)s in lake sediments.

Given optimal conditions, SRB in lake sediments can produce highly insoluble (K_{sp}< 10^{-18}) metal(oid) sulfides (*13, 14*). These conditions include a sufficiently reducing environment, ample sulfate (as compared stoichiometrically with precipitable metal(oids)), ample electron donors, and an abundant SRB population. While it has now been established that even "strictly anaerobic" SRB can be active in the presence of oxygen (*e.g., 15*), sulfide production will not precipitate metals in the presence of oxygen because aqueous sulfide is highly reactive with oxygen. While exogenous sulfate sources to sediments have been shown to stimulate AVS production (*e.g., 11*), endogenous sulfate sources from re-oxidation of sulfur-compounds within the sediments themselves are often the major supplies of sulfate for SRB (*16*). Even with re-oxidation reactions, sulfate concentrations, not electron donors, are most often the limiting factor controlling sulfate-reduction rates in sediments (*16*).

Measuring the activities and abundance of SRB in sediments is often a complicated task. Nucleic acid-based techniques have shown promise for the enumeration and identification of SRB in ocean water columns (*17*), biofilms (*18*), mine effluent water (*19*), and subsurface groundwater (*20, 21*). However, repeated efforts to isolate quantitative

amounts of nucleic acids from metal-rich CDAL sediments or to probe them with oligonucleotide probes have met with mixed results at best (*22*). Bak and Pfennig (*23*) observed that in freshwater sediments, with appropriately designed media, relatively high percentages of SRB can be cultivated, and their abundance measured by most probable number (MPN) analysis. Isolates from these MPNs showed interesting carbon-utilization patterns that were not strictly dependent on the carbon sources used in their enrichment. Subsequently, other authors have used MPN analysis to enumerate and isolate SRB in water columns, sediments, and soils (*6, 17, 24, 25*).

We describe in this study the isolation and analysis of two SRB isolates from terminal dilution enrichments from previously published MPN analysis (*26*). We sought to establish the capacity of these isolates to tolerate and grow in high concentrations of heavy metals, that were many times excess than the soluble metal concentrations observed in CDAL sediment pore waters. We hypothesize that it is the ability of SRB to precipitate metals in the CDAL sediments that is, in part, responsible for keeping the overlying lake waters relatively pristine (*5*).

Materials and Methods

Sediment core removal and sampling. Sediments were removed from CDAL near the delta of CDAR (see Figure 1). Capped 3 m sections of 1 inch (I.D.) PVC pipe were inserted through the water column into the sediments through holes augered in the ice (February, 1997 sampling). The cap was simultaneously removed as the pipe was forced into the sediment to a depth of 1 m. The cap was then replaced, the core removed, and the pipe was cut so as to leave ~ 10 cm lake water overlying the removed sediment. In June and July (1996) 1.5 m sections were inserted directly into the sediments by hand, capped, and removed. Pipe ends were sealed, and the cores transported back to the lab stored upright in a 5 mil plastic container that was maintained at $4^{\circ}C$ and flushed continuously with N_2 (g). Sediment cores

were extruded from the bottom up onto a waxed paper coated processing board and were placed immediately into an anaerobic Labconco chamber containing an atmosphere of N_2:CO_2:H_2 gas in a ratio of 80:15:5.

Terminal Dilution Enrichments. Sample processing for microbial analysis was conducted entirely within the Labconco anaerobic chamber. One gram of sediment was removed from the middle of extruded core at depths of 2, 18, and 35 cm. Sediments samples were placed into 10 mL serum vials containing 9 mL ½x Ringers solution (68 mM sodium chloride, 5.9 mM sodium bicarbonate, 1.7 mM potassium chloride, and 1.2 mM calcium chloride (*27*), and sealed in the chamber by crimping. Cetyltrimethylammonium bromide (0.1%) was added to promote uniform dispersion of cells and sediments (*28*). Vials were shaken on a rotary shaker at 200 rpm at room temperature. Sterile, 96 x 2 mL well plates (BioBlocks, Rainin Inc., Woburn, MA) were used to make a 10^7 fold dilution series of the sediment. This dilution was performed with the media of Pfennig (as described in *28*), modified by the addition of 5 mM lactate, 7.33 mg/L $FeSO_4$, and containing all the recommended electron donors except palmitic acid. A MPN was determined after a 30 d incubation at room temperature. From these MPNs, the terminal dilutions showing blackening (indicative of sulfate-reduction) was again serially diluted to 10^{-7} dilution and incubated an additional 30 d. This procedure was repeated once more. The terminal dilution from the third series was inoculated (0.5 ml /10 ml) into modified Pfennig media containing 1% agar in rubber capped tubes at 40°C. After 30 d incubation individual black colonies were removed by withdrawal through a 19 gauge needle, flushed into the syringe by withdrawal of 0.5 mL modified Pfennig media, and inoculated into 10 mL modified Pfennig media (in a 30 mL rubber-capped tube, headspace 80:20 N_2:CO_2). This tube was incubated at room temperature for 30 d, at which point two successive terminal dilution series were performed in the modified Pfennig media.

Electron Donor Assays. Isolates were characterized for their ability to utilize particular electron donors by incubation in modified Pfennig media containing *singly* either 10 mM

acetate, benzoate, butyrate, ethanol, formate, lactate, or propionate. Each tube was transferred into fresh media after 30 d incubation. Individual tubes were held for 60 d (total) for positive readings.

Electron Acceptor Assays. Isolates were assayed for their ability to utilize arsenate, ferric iron, or nitrate. Inocula were made into sulfate-free modified Pfennig media to which were added prepared anaerobic stock solutions of ferric pyrophosphate, sodium arsenate, or potassium nitrate. Final concentration of each electron acceptor was 10 mM. Initial inoculation was at $2 \times 10^5 \pm 2 \times 10^4$ cells per mL (final concentration); tubes were read as positive if $> 1 \times 10^6 \pm 1 \times 10^5$ cells were counted after incubation for 30 days.

Metal Tolerance Assays. Tolerance to arsenate, zinc, and lead was assayed by inoculation of 10^6 cells /mL (final concentration) into modified Pfennig media supplemented with 1, 10, or 100 mg /L sodium arsenate, lead nitrate, or zinc chloride. Pre-reduction by sodium sulfide was eliminated, and replaced by reduction with sodium dithionite until the pink resazurin (0.0001 %) in the medium just turned clear. Isolates were deemed metal tolerant if they met all of the following criteria: 1) precipitate was formed or color changed from white to black, 2) if cell numbers more than doubled, and 3) if regrowth in fresh, metal-free (except for the metal carried over in the 10% inocula) media occurred within 30 days of transfer.

Other Physiological Characteristics. Desulfoviridin was assayed by a method modified from Postgate (*29*), with *Desulfovibrio desulfuricans* (ATCC 7757) used for a positive control and *Desulfoarculus baarsi* (ATCC 29494) used as a negative control. Ten mL of culture (>14 days old) was pelleted by centrifugation at 10,000 x g, and resuspended in 100 µL water and added to an ELISA plate. 100 µL NaOH (3 M) was added to each well and allowed to react for 5 min. The plate was read under long wave (365 nm) light and assayed for a positive, brick red color. Motility was determined microscopically at 3 days after transfer into fresh media. Morphological determination was performed on mature cells by

scanning electron microscopy. Gram stains were performed on mature (> 30 d) cells using the Fishers Diagnostics Gram Stain Set (Fisher, Pittsburg, PA) protocols.

Epifluorescence Microscopy. Bacterial counts were estimated by epifluorescence microscopy of DAPI-stained cells using a Zeiss Axioscop microscope equipped with a Plan Neofluor 100x objective and UV light source. Details of this method are described in Schallenberg *et al.* (*30*).

Scanning Electron Microscopy. Mature (>30 d) cells were fixed by adding gluteraldehyde to a final concentration of 0.75 % and held for 2 h. Cells were harvested by centrifugation, washed with 0.2 M sodium cacodylate buffer, and then dehydrated with a gradient series of ethanol. Fixed cells were then dehydrated onto glass cover slips that were coated with gold-palladium (60:40), and then re-coated with the gold-palladium mixture. The cover slip was then mounted on an aluminum-tin stub. SEM was performed with an AMRAY model 1830 scanning electron microscope. SEM-EDX samples were mounted instead on a carbon stub. SEM-EDX allows for elemental analysis of very small (< 1 μm diameter) particles (see Heldal *et al.*, 1985).

Rep-PCR Analysis. Isolated strains were grown to saturation (minimum 14 days) and 1 mL harvested by centrifugation and resuspended in 100 μL distilled water. Whole cell rep-PCR was performed on 2-5 μL of these resuspended cells per 25 μL total reaction size using the method of Versalovic (*31*) for 35 cycles using the BOXA1R primer (sequence = 5'-CTACGGCAAGGCGACGCTGACG-3'). Products were separated on a 7.5% polyacrylamide gel at 250 v for 4 hours.

16s rDNA sequencing. Cells were harvested as done for rep-PCR. 2 μL resuspended cells were added to PCR tubes containing 5 μL 10X PCR buffer (Gibco), 0.5 μL Taq Polymerase (Gibco), 0.4 μL 25 mM dNTP's, 0.25 μL each of an 8 F universal primer (sequence =5'-AGAGTTTGATCCTGGCTCAG-3') and a 1492 R primer (sequence = 5'-GTTACCTTGTTACGACTT-3'), 50 pM, 0.4 μL BSA (10 mg/mL), 3 μL 100% DMSO, and

water to make 50 mL total volume. PCR was performed as follows: 5 cycles at 65°C annealing, 72°C extension, and 94°C denaturation, 5 cycles of 60°C annealing, 72°C extension, and 94°C denaturation, and 25 cycles 55°C annealing, 72°C extension, and 94°C denaturation. PCR products were assayed for bands on a 1.5% agarose gel (1 x TAE). Products were purified with Promega PCR product cleanup kit, and sequenced using an ABI Prism model 377 XL sequencing machine (University of Chicago Cancer Research Center).

Sequence Alignment and Phylogenetic Analysis. An alignment was performed using the Wisconsin Package Version 9.1 (Genetics Computer Group (GCG), Madison, Wisc.) using the "Pileup" program. Alignments were checked to eliminate gaps in highly conserved areas of the 16s rRNA. Phylogenetic trees were constructed using the maximum likelihood method (PAUP).

Results and Discussion

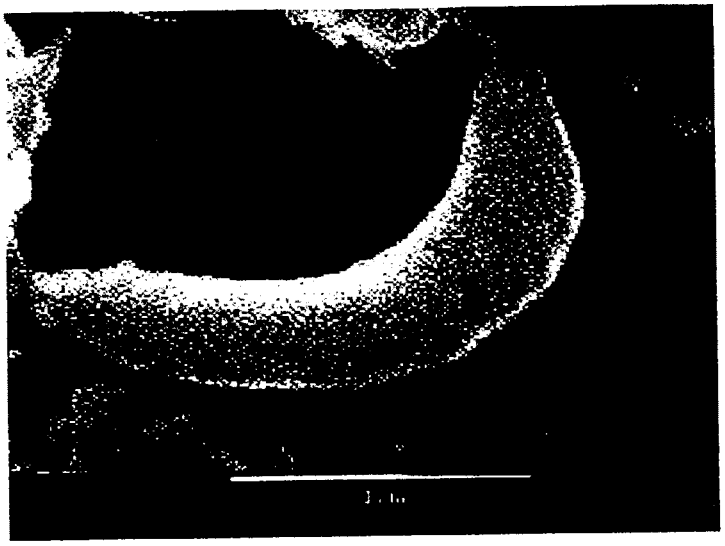
The sediments of Coeur d'Alene Lake, ID, are highly enriched in iron and other trace elements (*4*). Selective chemical extraction reveals that much of this metal, irrespective of depth, is associated with an operationally defined sulfidic phase (*3*). These sediments are also highly reduced (*3*). Taken together, these observations raise the possibility that metal sulfides can be formed authigenically in CDAL sediments. If indeed sulfidogenesis is actively occurring in these sediments, the threat of metal release postulated under anaerobic conditions (*4, 5*) might be exaggerated.

We have previously shown that there exists within these sediments an abundant population of sulfate-reducing bacteria (*26*). We now report that these sediments contain new taxa of SRB. These organisms are tolerant of several metals that are abundant in CDAL sediments, and that their characteristic sulfidogenesis results in metal precipitation under laboratory conditions in defined media.

Isolate Characterization. Two isolates were selected from 24 strains that were carried through the isolation procedures. The isolates were chosen for further analysis because they presented minimal ambiguity in their 16s rDNA sequence indicating that they were indeed pure cultures.

Strain CDA 6-18-5 present short, plump, curved motile rods (1/2 μm x 1.5 μm Figure 4-2a), and appear to be most similar phylogenetically to *Desulfoarculus baarsi* (91%). CDA 6-18-5 was desulfoviridin negative, gram negative, and grew on acetate, ethanol, formate, and lactate (Table 2). CDA 6-18-5 was fast growing at room temperature 22°C (doubling time = 0.6 days). This organism showed clumping behavior in liquid media; DAPI stains showed a cloudy mass with bright cells inside the mass. This cloudy mass may be composed of exo-polysaccharides. Maximum densities of approximately 2×10^7 were obtained after 30 d incubation in Pfennig media.

A.



B.

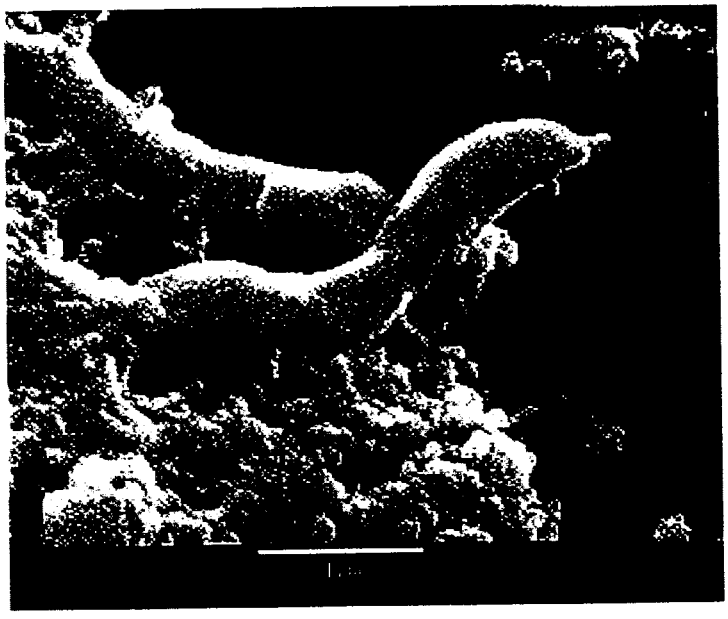


Figure 4-2. SRB isolates from CDAL sediments. A. CDA 6-5-18. B. CDA 7-2-4.

Table 1. Some physiological characteristics of isolates from CDAL sediments.

Strain Name*	Morphology	Size	Desulfoviridin	Motility	Gram Reaction
CDA 6-18-5	Curved Rods	1-2 μm	No	Yes	neg.
CDA 7-2-4	Curved, Tapered Rods	1-2 μm	No	Yes	neg.

The name given reflects the month isolated, the depth within the sediment (in cm), and the initial sediment dilution from which the isolate was obtained.

Table 2. Electron Donors and Acceptors Utilized for Growth.

Strain Name	Ac	Bz	Bu	Et	Fo	La	Pr	Nitrate	Fe (III)	As (V)
CDA 6-18-5	+			+	+	+		(-) *	(-) *	(-) *
CDA 7-2-4	+				+			(-) *	(-) *	(-) *

* negative results with acetate as electron donor.

Strain CDA 7-2-4 present short, plump, curved or S-shaped motile rods (1/3 μm x 1 μm, Figure 4-2b), and appear to also be most similar phylogenetically to *Desulfoarculsu baarsi* (90%). CDA 7-2-4 was also desulfoviridin negative and gram negative. Of the seven electron donors tested, CDA 7-2-4 grew on acetate and formate only (Table 2). This strain grew more slowly (doubling time =1.2 d), and attained moderate densities of 8.0×10^6 after 30 d incubation in modified Pfennig media. CDA 7-2-4 showed considerable variability in cell shape and size, with some cells appearing to be tapered, straight rods, others curved, and when longer, S-shaped. It is possible that the S-shaped rods are undergoing division. Clumping, cloudy masses were also seen in liquid cultures under DAPI staining.

Because of the relatively high degree of similarity of these strains with the Geobacteriaceae (< 85%), we assayed their capacity to grow in anaerobic ferric

pyrophosphate media. Both CDA 6-18-5 and CDA 7-2-4 were unable to grow under these conditions using acetate at sole carbon source.

The strains we have isolated are related only distantly to previously described SRB; indeed, they may even be new genera by the criteria < 3% dissimilarity define a new species, < 10% dissimilarity equal new genera (*e.g., 33*). Evaluation of these strains with respect to gram negative status, common acetate and formate utilization, desulfoviridin assay, and cell morphology suggests a high degree of similarity to *Desulfoarculus* strains (*29, 34*). Phylogenetic analysis indicates that these strains are each other's closest relative (94.3 % identity over 1189 bases). The closest known relative of these strains is *Desulfoarculus baarsii* (89% identity over 1189 bases). Other phylogenetically similar strains include *Desulfomonile tiedjei* (88%), *Desulfonema magnum* (86%), and *Desulfosarcina variabilis* (86%), all of which are considered outliers of the better characterized families of mesophilic SRB, the *Desulfovibrionaceae* and *Desulfobacteriaceae* (Figure 4-3).

Rep-PCR reactions show promise for differentiating among and between SRB isolates (*31*). CDAL isolates share many bands with each other in many instances, while few common bands appear between CDA strains and 11 standard strains (Figure 4-4). Among these standard strains, there are few common bands except among members of the same species. For instance, *Desulfotomaculum orientis* strains DSM 8445 and DSM 8323 show by comparison more than ten common bands. Comparison of CDA 6-18-5 and CDA 7-2-4 with other isolates from these same sediments also reveals that they are more similar to each other than to standard strains (Figure 4-4). We have found that use of the BOXA1R primer results in a sufficient number of variably sized bands to effectively fingerprint novel SRB isolates. The ease of the reaction and the rapid turn-around from isolate to fingerprint may make this an attractive way to catalog a large number of isolates for similarity to each other or to well-described strains.

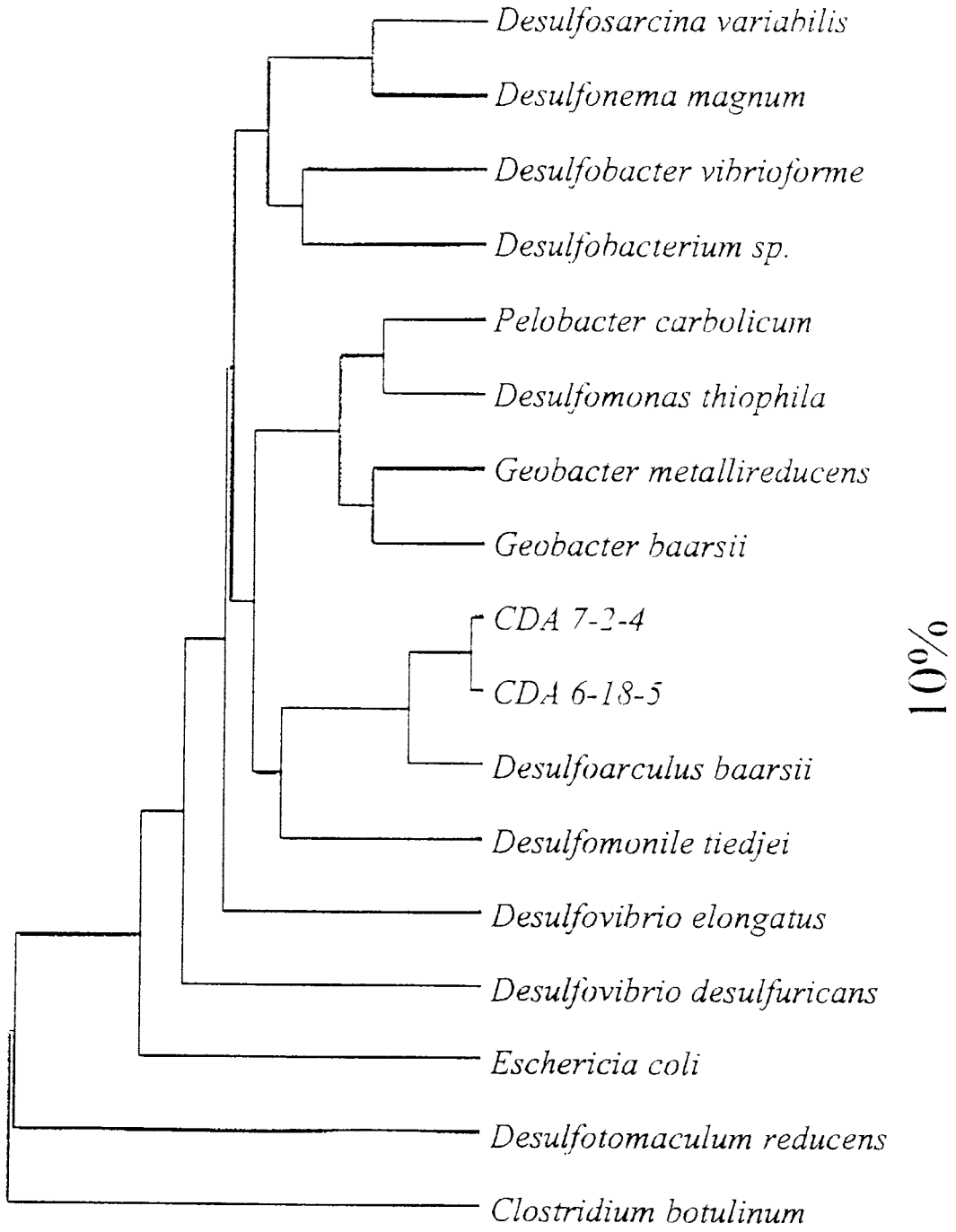


Figure 4-3. Phylogenetic tree of sulfate and iron reducing bacteria, with two CDAL SRB isolates.

Because sulfate-reducers we describe here were isolated from heavy metal enriched sediments, we examined the capacity for these isolates to survive and grow in modified Pfennig media amended with 1, 10, and 100 ppm of arsenic, lead, or zinc. The lead, added as a soluble lead nitrate, quickly formed an amorphous white precipitate. Zinc, added as the soluble zinc chloride, also quickly formed an amorphous white precipitate. Since the media contained a small amount of phosphate as well as a carbonate buffer, these ions likely contributed to precipitate formation. Calculations with MINETAQA2, a computer program that calculates thermodynamic equilibrium states (*35*), showed that metal phosphates were the dominant form of the insoluble metal. Notwithstanding the precipitate formed, calculation using MINETAQA2 (*35*) shows that high levels of soluble metals were still available (Table 4-3). It should be also noted that these tests were run with dithionite as the reductant (instead of the standard sodium sulfide solution) to allow for assay of sulfidogenesis in the media.

Table 4-3. Metal Tolerance and Precipitation for Two CDAL Isolates.

Strain	As	Inhibitory Conc.	Pb	Inhibitory Conc.	Zn	Inhibitory Conc.
CDA 6-18-5	P	>100 ppm	Pc	>100 ppm	Pc	> 1 ppm
CDA 7-2-4	P	>100 ppm	Pc	>100 ppm	Pc	> 1 ppm

Key: Np = no precipitate formed, P = precipitate formed, Pc = precipitate changed. >1 ppm inhibition means 10 ppm was inhibitory, while 1 ppm was not.

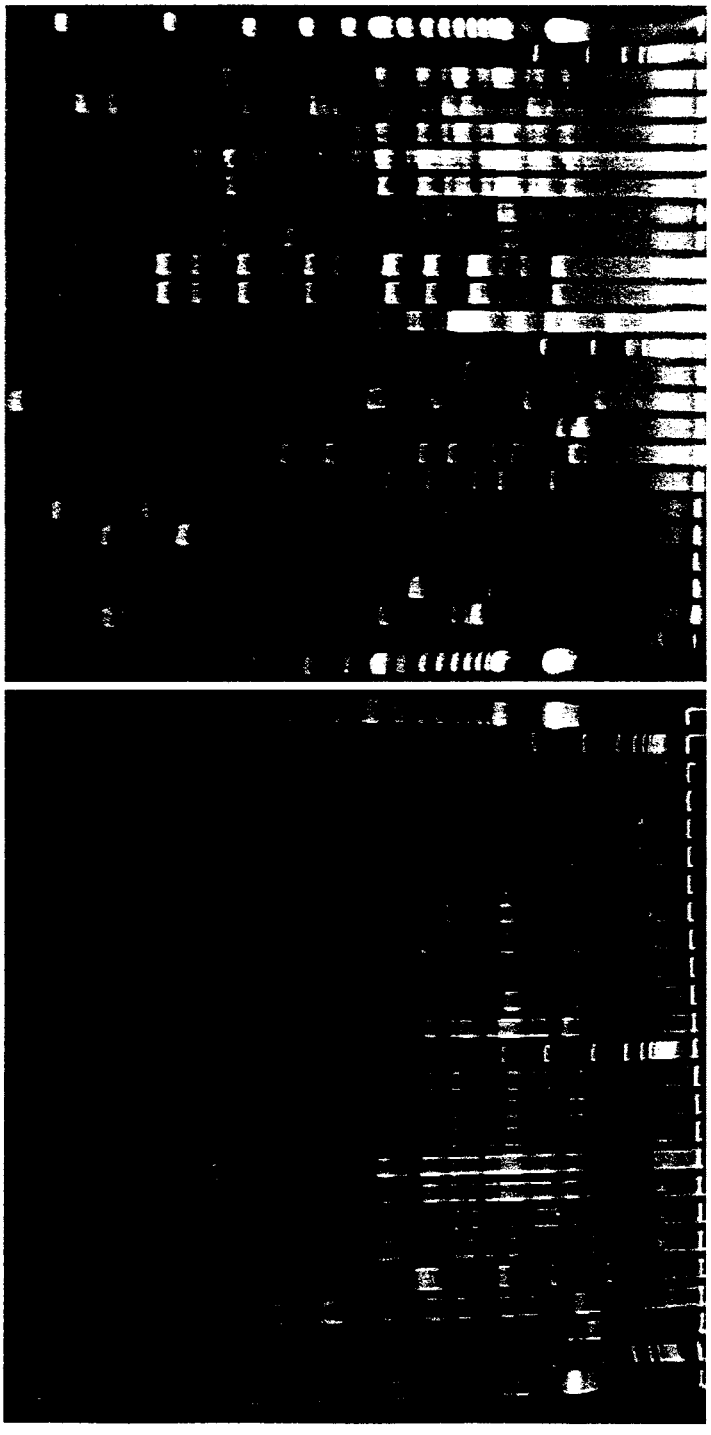


Figure 4-4. rep-PCR banding pattern for SRB isolates and standard strains using BOXA1R primer

We found that zinc was the substance most inhibitory to these SRB, and that both arsenic and lead were much less inhibitory (Table 4-3). In the presence of both sulfate and arsenate, CDA 6-18-5 and CDA 7-2-4 created a greenish-brown precipitate similar to the color of realgar and orpiment. Analysis by SEM-EDX showed that this compound contained S, As, and Fe in molar ratios of 53:45:3. When both lead and sulfate were available, the whitish precipitate changed to a gray and then a black precipitate. This black precipitate was demonstrated to be a lead sulfide by SEM-EDX, containing molar ratios of 3:2 Pb/S. CDA 6-18-5 and CDA 7-2-4 both showed the capacity to form these lead sulfides, generating black precipitates even in the 100 ppm lead cultures. With zinc and sulfate available, CDA 6-18-5 and CDA 7-2-4 changed the loose white zinc precipitate into a dense greenish precipitate. This occurred only in the 1 ppm test culture. This precipitate was a zinc sulfide with molar ratios found by SEM-EDX of 2:3 Zn/S. Test for regrowth after exposure to the metals showed that transfer cultures into metal-free media after 30 days incubation in all cases formed blackened sulfide precipitates, indicating the cultures' viability.

Strains CDA 6-18-5 and CDA 7-2-4 are tolerant of metal concentrations typical for other SRB (*36*). These concentartions are also in range of typical concentrations of metals measured in acidic mine drainage. Moreover, their ability to form metal sulfides from aqueous metals and to use cheap carbon sources such as acetate may make them excellent candidates for use in bioreactors designed to remove toxic metals from solution. Their capacity to produce arsenical sulfides seems particularly attractive in this regard, since arsenic sulfides are so highly insoluble (*37*), and remain so if reoxidation is prevented.

As a group, SRB can produce aqueous sulfide by the reduction of sulfate and the concomitant oxidation of organic carbon. Under anaerobic conditions, aqueous sulfide will rapidly react with aqueous metals to form insoluble metal sulfides, with the most insoluble sulfides being removed from solution first. The production of insoluble metal sulfides depends, therefore, on the availability of sulfate and organic carbon, as well as having

abundant SRB that are tolerant of ambient metal concentrations. We report here that sufficient quantities of these SRB are present and that these organisms can form metal sulfides. Overlying lakewater contains some low levels of sulfate (1-7 ppm, *5*). However, Urban *et al.* (*16*) have shown that in freshwater sediments autochthonous sulfate production may be the principal supply of sulfate for sulfate reduction. In any case, there is sufficient carbon and sulfate in CDAL sediments to support SRB in the $10^5 - 10^6$ range in CDAL sediments (*26*).

Eutrophication by increased nutrient loading would likely stimulate such an abundant SRB community. We suggest that given eutrophic conditions SRB activities would increase, and that metal sulfide formation *in situ* would also increase. However, metal-reducing organisms such as DIRB and arsenic-reducing bacteria would be active under anoxic conditions, and could promote metal release before SRB activities become sufficiently high. Clearly, understanding the interactions between these groups of microorganisms is critical to being able to make rational predictions concerning metal biogeochemistry in the sediments of CDAL.

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