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4.3 History of Development

The Oak Ridge Research Institute has addressed the question of whether sulfate-reducing bacteria can be used in the remediation of heavy metal contamination. We have performed experiments in which mercury, lead and cadmium have been removed from liquid media in which sulfate-reducing bacteria were growing. It is clear that heavy metals can be precipitated from solution by sulfate-reducers. We believe that the activity of these organisms can be enhanced to help stabilize mercury and other metals in waste holding pond sludges, sediments and in contaminated soils.

In situ treatment of contaminated soils, sediments and sludges requires that the microbes be there, that nutrient levels be adequate, and that the products formed have the desired properties. We have assessed in laboratory studies the ability of soil samples taken from a metal-contaminated stream flood plain to support sulfate respiration. We found that all samples tested contained sulfate-reducing bacteria and adequate carbon sources, but in every case the concentration of endogenous sulfate was too low to allow the significant production of hydrogen sulfide. When sulfate was added to these samples, sulfate reduction began. This finding is important, not only because it demonstrates the presence of sulfate-reducers, but for an additional reason. It has recently been shown that sulfate-reducing bacteria growing in low concentrations of sulfate appear to be the major producers of methyl mercury released from estuarine sediments through metabolic reactions that do not occur when sulfate concentrations are high enough to support active respiration (Compeau and Bartha, 1984; 1985; 1987). If this observation also applies to terrestrial soils as well, it means that there is the potential for formation of methyl mercury throughout the flood plain of East Fork Poplar Creek. Recent studies at this institute have confirmed the observations of Compeau and Bartha. Tables A-6a and A-6b show that soil samples from the flood plain contain small amounts of methyl mercury. In preliminary laboratory experiments we have also observed in vitro production of methyl mercury in incubations containing these soil samples, with lower levels produced in the presence of additional sulfate.

Table A.6a. Concentration of methyl mercury in EFPC flood plain soil.

<u>Sites</u>	<u>depth</u> <u>(cm)</u>	<u>MeHgI</u> <u>(ug/g)</u>	<u>total Hg</u> <u>(ug/g)</u>	<u>% MeHgI</u> <u>of total</u>
X1000	0-5	0.058	316	0.018
	15-20	0.085	1274	0.007
	25-30	< 0.01	46.8	< 0.02
X1100	0-5	< 0.01	109	< 0.009
	15-20	< 0.02	226	< 0.009
	25-30	< 0.01	2314	< 0.0004

Soil was collected from two sites at the depths shown and assayed for total Hg using the cold vapor technique, following complete acid digestion. Methyl mercury (MeHgI) was assayed by gas chromatography of benzene extracts of the soil samples prepared as described in the standard AGAC Methods manual. This involved an initial extraction into toluene, back extraction into aqueous thiosulfate containing ethanol, and finally into benzene. Control experiments demonstrated that this extraction procedure gave quantitative recovery of methyl mercury from spiked soil samples.

TABLE A-6b
ANALYSIS OF METHYL MERCURY IN SOIL SAMPLES
FROM THE EAST FORK POPLAR CREEK FLOOD PLAIN

Total mercury and methyl mercury concentrations were determined as described in the Methods section. The sample location (creek mile) was determined with reference to a TVA map of the creek. Concentrations are given on a dry weight basis. In most cases, the results reflect the lower detection limit of the method under the conditions used (10 ppb).

TRANSECT	CREEK MILE	SAMPLE	TOTAL Hg (ppm)	METHYL Hg (ppb)	% Hg as MethylHg
1	10.4	E 1-A	19	< 10	< 0.05
		1-B		< 10	
		2-A	57	< 10	< 0.02
		2-B		< 10	
		3-A	100	19.6	0.02
		3-B	192	< 10	< 0.005
		4-A	142	< 10	< 0.007
		4-B	152	< 10	< 0.007
		5-A	406	16.8	0.004
		5-B	37	< 10	< 0.027
		6-A	24	< 10	< 0.042
		6-B	15	< 10	< 0.067
2	10.8	E 1-A	46	< 10	< 0.02
		2-A	56	< 10	< 0.02
		3-A	184	< 10	< 0.005
		3-B	194	< 10	< 0.005
		4-A	176	< 10	< 0.013
		4-B	213	< 10	< 0.005
		5-A	172	< 10	< 0.006
		5-B	380	< 10	< 0.003
		6-A	187	< 10	< 0.005
		6-B	411	< 10	< 0.002
3	11.2	B 1-A	287	< 10	< 0.003
		1-B	166	< 10	< 0.006
		2-A	255	< 10	< 0.004
		2-B	78	< 10	< 0.012
		3-A	728	< 10	< 0.001
		3-B	126	< 10	< 0.008
		4-A	114	< 10	< 0.008
		4-B	369	< 10	< 0.003
		5-A	85	< 10	< 0.012
		5-B	148	< 10	< 0.007
		6-A	122	< 10	< 0.008
		6-B	179	< 10	< 0.006

TRANSECT	CREEK MILE	SAMPLE	TOTAL Hg (ppm)	METHYL Hg (ppb)	% Hg as MethylHg
4	13.5	D 1-A	94	< 10	< 0.01
		1-B	98	< 10	< 0.01
		2-A	222	< 10	< 0.005
		2-B	5	< 10	< 0.20
		3-A	46	< 10	< 0.02
		3-B	8	< 10	< 0.13
		4-A	200	< 10	< 0.005
		4-B	286	< 10	< 0.003
		5-A	247	< 10	< 0.004
		5-B	288	< 10	< 0.003
		6-A	610	14.5	0.002
		6-B	190	9.6	0.005
		X-A	88	24.8	0.028
		X-B	103	< 10	< 0.010
5	13.7	A 1-A	116	< 10	< 0.009
		1-B	27	< 10	< 0.04
		2-A	248	< 10	< 0.004
		2-B	126	< 10	< 0.008
		3-A	329	< 10	< 0.003
		3-B	78	< 10	< 0.012
		4-A	117	< 10	< 0.009
		4-B	196	< 10	< 0.005
		5-A	144	14.7	0.01
		5-B	144	< 10	< 0.007
		6-A	110	< 10	< 0.009
		6-B	27	< 10	< 0.04
6	14.4	F 1-A	3	< 10	< 0.33
		1-B	5	< 10	< 0.2
		2-A	6	< 10	< 0.17
		2-B	6	< 10	< 0.17
		3-A	35	33.4	0.095
		4-A	1.5	< 10	< 0.67
		4-B	1.6	< 10	< 0.63
		5-A	2.3	< 10	< 0.43
		5-B	0.5	< 10	< 2.00
		6-A	1.7	< 10	< 0.59
		6-B	0.82	< 10	< 1.22
		X-A	0.62	< 10	< 1.61
		X-B	1.3	< 10	< 0.77

As a laboratory test of the ability of the sulfate amendment of soil to provide sulfate at reasonable depth without adversely affecting soil pH or the leaching of heavy metals, we have constructed columns containing soil from the flood plain of East Fork Poplar Creek. These columns were treated with sulfate in various ways and leached with water, and various parameters of the leachate were determined. The results suggested that addition of sulfate to the soil could provide sufficient sulfate to depths where contaminating metals were found, and that the amendment did not alter soil pH or increase the leaching rate

of the metals (Figure A.2). In fact, a reduction in the concentration of soluble mercury in the leachate occurred in sulfate-treated columns as compared to untreated columns during the initial weeks of the experiment. The next step in the development of this technology was an experimental test of sulfate addition on the contaminated flood plain of East Fork Poplar Creek. Porous-cup lysimeters were installed into plots on the flood plain and a pre-treatment sampling carried out in November, 1986. The plots were treated with two levels of gypsum addition (0.02 kg m^{-2} and 0.2 kg m^{-2}) in December and some plots were left untreated as controls. Water in the lysimeters was sampled monthly and analyzed for mercury, sulfate, conductivity and pH. Consistent significant differences in the concentrations of mercury have not been observed among sulfate treatments, although sulfate levels have increased dramatically in the highest treatment (Figure A.3 and A.4). Addition of gypsum caused essentially no change in soil water pH (Figure A.5). Values for conductivity exhibited a large peak in the high level treatment and only a small increase in the low level treatment as compared to the control plot values (Figure A.6).

Although mercury levels were not consistently lower in the sulfate treated plots soil mercury levels in all plots were relatively high. The levels of mercury in soil isolated from areas where the lysimeters were placed ranged from 129 to 1054 ppm, whereas mercury in water from the lysimeters never exceeded 0.95 ppb. This observation suggests that mercury in the soil is not mobile. This suggestion is supported by results shown in Table A.7. As shown, less than 0.001% of the total soil mercury was soluble using the EPA EP toxicity procedure.

Table A.7 EP Toxicity test for Mercury (RCRA MCL 200 ug/L)

Sites EFPC	Soil and Sediment mg/kg	EP Toxicity Test ug/L
X900 ₁	1.97 ± 0.6	2 ± 1.5*
X900 ₂	12.19 ± 1.3	3 ± 1.8*
X900 ₃	507.55 ± 40.1	17 ± 2.6
X1000 ₁	974.83 ± 63.6	18 ± 3.4
X1000 ₂	49.03 ± 5.9	10 ± 1.3
X1000 ₃	97.57 ± 8.6	8 ± 1.8
X1090 ₁	61.06 ± 7.1	9 ± 0.9
X1090 ₂	16.16 ± 2.6	4 ± 1.1*
X1090 ₃	48.50 ± 1.7	8 ± 0.6
X1100 ₁	1799.36 ± 88.9	33 ± 5.2
X1100 ₂	17.91 ± 3.2	5 ± 0.6
X1100 ₃	987.94 ± 70.6	23 ± 4
X1200 ₁	43.52 ± 5.1	7 ± 2
X1200 ₂	33.18 ± 5.4	3 ± 1.6*
X1200 ₃	220.85 ± 33.6	13 ± 0.8
X1300 ₁	78.84 ± 7.9	9 ± 1.4
X1300 ₂	13.33 ± 0.5	3 ± 1.4*
X1300 ₃	65.43 ± 4.1	5 ± 1.0
X1400 ₁	22.79 ± 3.6	7 ± 1.9
X1400 ₂	12.51 ± 1.3	7 ± 2.3
X1400 ₃	6.13 ± 0.6	4 ± 1.6
X1500 ₁	11.23 ± 1.1	7 ± 1.3
X1500 ₂	3.47 ± 0.6	6 ± 0.9
X1500 ₃	34.62 ± 2.1	9 ± 2
X1600 ₁	6.61 ± 1.3	0
X1600 ₂	0.77 ± 0.06	0
X1600 ₃	2.64 ± 0.93	3 ± 2*
X1700 ₁	0.59 ± 0.09	0
X1700 ₂	0.72 ± 0.10	0
X1700 ₃	1.3 ± 1.7	0

* These values were obtained at the limit of detection.

Soil samples were obtained from East Fork Poplar Creek (EFPC). The numbers 1, 2, and 3 refer to samples isolated from the left flood plain, sediment, and right flood plain, respectively.

The results shown above suggest that mercury in soil is either tightly bound to soil and/or in an insoluble form. Several experiments were performed to determine which of these factors are responsible for the low solubility of soil mercury. In the first experiment radioactive mercury was added to soil and incubated for 5 days. Samples were then extracted with deionized water, nitric acid or sodium sulfide (Na_2S). Results show that mercury bound to soil was extracted only with nitric acid (Table A.8). Sodium sulfide was used in these experiments because it is recommended as a solvent for mercuric sulfide (HgS) in the Handbook of Chemistry and Physics. That Na_2S and water did not solubilize the radioactive mercury in the soil samples suggest that the mercury had become tightly bound to soil. This observation was expanded by extracting mercury from soil using the above solvents. Table A.9 shows the results of these experiments. In contrast to results shown in Table A.8, Na_2S extracted 83 to 98% of the total mercury. In samples with mercury levels of 1000 ppm or greater nitric acid only extracted 12% of the total mercury. The amount extracted was greater using nitric acid when soil mercury levels were less than 160 ppm. These results suggest that, of the total mercury present, 90% is in the form of mercuric sulfide. Since the solubility constant of mercuric sulfide in water is 3×10^{-53} , and low in nitric acid we suggest that the solubility data discussed above may be explained by the chemical form (HgS) of mercury in soil from the flood plain.

Additional studies were performed to determine the distribution of HgS in soil isolated from the affected flood plain. A total of 36 samples were collected, and total and Na_2S extractable mercury were determined. Of the total mercury present 76 to 100% was Na_2S extractable (Table A.10). Based on these results we suggest that mercury in soil from the above identified flood plain is in an immobilized form requiring no further remedial action other than periodic monitoring for mercuric sulfide. We therefore infer from these results that mercury immobilization by sulfate reducing bacteria had already taken place.

TABLE A.8
The extraction of radioactive mercury from soil
using deionized water, nitric acid or sodium sulfide

Sites	Radioactive	Water soluble	Nitric Acid	Sodium Sulfide
	Mercury			
	dpn/gram dry			
	weight soil			
	Total mercury extracted from a gram of soil			
	dpn/gram dry weight soil			
X1000	24,000	240	22,892	316
		(1.0 %)	(95 %)	(1.3 %)
X1100	24,000	256	23,356	232
		(1.0 %)	(97 %)	(1.0 %)

An aqueous solution of radioactive mercuric chloride (24,000 dpm of ^{203}Hg) was added to soil (1 gram) from the above sites and incubated at room temperature for 5 days. After incubation, samples were centrifuged and the supernatant removed. The above extraction solutions were added to the soil samples and this mixture was incubated overnight. After incubation, the samples were centrifuged and the supernatant was removed and filtered and the radioactivity determined in the pellet and filtrate. The counts observed in the supernatant of the first incubation were less than 1% of the total counts added. Results are the mean of three experiments.

TABLE A.9 Solubility of mercury in soil into deionized water,
nitric acid or sodium sulfide

Sites	Soil Depth cm	Total Mercury in Soil Digest	Water Soluble	Nitric Acid Soluble	Sodium Sulfide Soluble
ug Hg/gram dry weight soil					
X1000					
	0-5	156	0.032 (144)	124 (60)	132 (33)
	5-20	1,003	0.028 (988)	118 (896)	838 (201)
	20-35	1,379	0.132 (1,420)	186 (1,111)	1,140 (90)
X1100					
	0-5	137	0.030 (144)	86 (58)	100 (13)
	5-20	1,716	0.120 (1,801)	219 (1,479)	1,678 (101)
	20-35	1,859	0.132 (1,930)	244 (1,699)	1,656 (193)

Soil samples (1 gram) were isolated from the sites shown above and added to 4 ml of the above extraction solutions and mixed overnight. After incubation and centrifugation, the 4 ml extraction solutions were removed and the soil was washed with 2 ml of the respective extraction solutions. The two extracts were combined and filtered, and mercury was determined by the cold vapor technique. In parentheses are the digested soil mercury levels remaining in each soil after it had been treated with H_2O , HNO_3 , or Na_2S (soil digest). Results are the mean of 3 experiments.

Table A.10 Sodium sulfide extractable mercury in soil: a comparison of sodium sulfide extractable mercury in soil to mercury levels in a soil digest.

Sites	Sample Number	Total Mercury in Soil Digest ug Hg/gram dry weight soil	Total Mercury in Na ₂ S Extract	Percent Extracted %
X1000				
	1	170	135	79
	2	138	116	84
	3	158	148	94
	4	1,097	885	81
	5	909	656	72
	6	1,003	970	97
	7	1,485	1,228	83
	8	1,273	1,146	90
	9	1,380	1,052	76
	10	19	20	100
	11	641	622	97
	12	421	438	100
	13	460	495	100
	14	60	63	100
	15	2,920	2,970	100
			MEAN	88±7.1
X1100				
	1	116	115	99
	2	181	149	82
	3	114	130	100
	4	1,410	1,914	100
	5	2,461	1,680	68
	6	1,289	1,442	100
	7	2,013	1,273	63
	8	1,996	2,006	100
	9	1,563	1,306	84
	10	1,685	1,273	76
	11	2,961	3,030	100
	12	2,200	2,015	92
	13	98	122	100
	14	2,562	2,492	97
	15	3,003	3,772	100
			MEAN	92±11.8
X1200				
	1	45	40	89
	2	9	11	100
	3	43	39	91
	4	1.8	2.4	100
	5	2,339	1,757	75
	6	1,797	1,856	100
			MEAN	93±10

Soil samples were collected from the above sites and mixed; part of the sample was digested and the other part was extracted with Na₂S. Mercury was determined in the soil digest and the Na₂S extract. Results in the percent extraction column are given as the mean ±SD. The coefficients of variation between samples were X-1000, 8.4, X-1100, 12.9 and X-1200, 11.

TABLE A.11 The Sequential Extraction of Mercury from Soil using
Nitric Acid and Sodium Sulfide

Site	First Extraction Nitric Acid	Second Extraction Sodium Sulfide	Total Mercury Extracted	Total Mercury in the Soil Digest
	ug Hg/gram dry weight soil			
X1000				
	53	198	251	264
	50	255	295	315
	11	102	113	124
	373	983	1,356	1,410
	27	86	113	100

TABLE A.11b

Site	First Extraction Sodium Sulfide	Second Extraction Nitric Acid	Total Mercury Extracted	Total Mercury in the Soil Digest
	ug Hg/gram dry weight soil			
X1100				
	477	44	521	611
	189	66	254	304
	313	28	341	319
	1,529	140	1,669	1,883
	159	45	204	209

Soil samples were collected from the above sites, mixed, and divided in half. Half of each sample was digested and the other half was sequentially extracted with nitric acid and then sodium sulfide (or the reverse). After extracting for 24 h. samples were centrifuged and the supernatant was removed and filtered. Mercury was determined in the filtrate and soil digest by Atomic Absorption (cold vapor technique). Results are the mean of 3 experiments.

Most compounds of mercury including methyl mercury are soluble in nitric acid. Since in some samples we were only able to extract 76% of the total mercury using Na_2S we decided that sequential extraction may provide a better method for isolating the various compounds of mercury in soil. In Table A.11 a and b are results from sequential extraction experiments with soil. Samples of soil were extracted first with nitric acid, then with Na_2S or the reverse. Using this procedure we were able to recover approximately 100% of total mercury present in soil. Nitric acid extracted approximately 20% (range was 10 to 28%) of the total mercury and the balance was extracted with Na_2S (Table A.11a). Using the reverse procedure Na_2S extracted approximately 86% (range was 74 to 92%) of the total mercury and the balance was extracted with nitric acid (Table A. 11b). These results agree well with data described above. In conclusion we suggest that of the total mercury present in this flood plain 80 to 90% is present in the immobilized mercuric sulfide form. Furthermore, it is reasonable to assume that sulfate-reducing bacteria were responsible for the formation of this compound which would support the application of the above discussed technology.

A.4. Applications to hazardous waste site cleanup

The technology described is intended to be used to immobilize heavy metals, particularly toxic metals, as insoluble sulfides in a soil or sludge pond environment. This technology is also likely to be applicable to emergency situations where hazardous wastes containing heavy metals are spilled onto soil, and the natural binding capacity of soils for metals is exceeded. Heavy metal waste is generated by manufacturers of chemicals, cleaning agents and cosmetics, metals and metal plating, pesticides and textiles and plastics. In addition, motor freight terminals, laboratories, printing industries and vehicle maintenance businesses may also be sources of environmental heavy metal pollution (U.S.E.P.A., 1986).

The proposed technology is versatile in its application to the extent that a fairly wide array of toxic heavy metals may be immobilized simultaneously. Little or no complicated machinery or extensive manpower is required. The proposed technology would be especially useful when hazardous spills occur in regions that are remote or otherwise difficult to reach with more sophisticated equipment. It is probable that gypsum treatment of soils and sludge ponds would also be advantageous at sites where complete cleanup could not begin for an extended period of time. This would permit further stabilization of metals at a site until complete removal of the soil, or other treatments, could begin.

The addition of gypsum to fields is an accepted agricultural practice and the potential environmental problems caused by this treatment are small (see section A.8).

The proposed technology could be utilized in batch treatment of soil in large containers, but is intended primarily as an on-site in situ method for the treatment of metal sludge and holding ponds and, under proper conditions, polluted natural soils. Because it would be unnecessary to disturb natural ground cover during the treatment with

1. Development of other Technologies

a. Bacterial Sulfide Precipitation of Heavy Metals

The dissimilatory sulfate-reducing bacteria oxidize sulfur compounds and release hydrogen sulfide during their metabolism (Postgate, 1984; Siegel, 1975). Since many metal sulfides are not soluble in water, it could be expected that sulfate reduction in metal-contaminated liquid media should lead to the formation of insoluble metal sulfides (Ehrlich, 1982; Norris and Kelly, 1979). The precipitation of heavy metals from waste waters by sulfide generation has been proposed in the past, and studies of its feasibility have met with varying degrees of success. The addition of sulfide ion to waste water has been shown to decrease the concentration of dissolved mercury (Perry, 1974), although sulfide generation by bacteria apparently results in greater metal precipitation per mole of sulfide added to the reaction (Postgate, 1984).

We have been working to develop systems for the removal of heavy metals from waste water using the microbial production of sulfide by a mixed culture including sulfate-reducing bacteria. If the ions of metals whose sulfides are insoluble in water are present in solution with actively growing sulfate-reducing bacteria, those metals will be precipitated as sulfides and can be removed from the medium by centrifugation or filtration.

We have carried out a series of experiments to characterize the precipitation of heavy metals by sulfate-reducing bacteria. Growth media for these bacteria typically contains lactate and sulfate, along with ferrous ion, which reacts with the sulfide generated to form a black precipitate in the medium. This precipitate is thus an indicator of sulfate reduction. Many other metal sulfides are insoluble, notably those of mercury, cadmium, and lead. Therefore, it seems probable that those metals should be removed from solution by the growth of sulfate-reducing bacteria. To test this hypothesis, we added radioactive HgCl_2 to growth medium to which a culture of sulfate-reducing bacteria was then inoculated. After various periods of growth, the medium was filtered and the amount of soluble mercury remaining was determined. Figure B.1 shows the removal rate of soluble mercury from solution to background levels, about 0.05 ppb in the experiment of Figure B.1b. Table B.1 shows that several other heavy metals commonly found in industrial wastes can also be removed by this process. We have also treated samples of waste water from the U.S.D.O.E. Y-12 plant in this way and reduced the level of soluble mercury below the limits of detection as measured by atomic absorption spectroscopy (Table B.2).

Table B.1

The Removal Metal Ions from solution by
Sulfate-Reducing Bacteria

Metal	Concentration (ppm)		% Remaining
	Initial	Final	
Cadmium	0.5	0.02	4.0
	500	5.0	1.0
Lead	10,000	620	6.2
Mercury	5.0	0.06	1.2
	100	0.5	0.5
Nickel	10,000	2400	2.4
Zinc	1000	100	10.0

Table B.2

Waste Water Samples from Y-12 Plant

Source	Mercury conc. (ppb)	
	Untreated	Treated
Storm drain	9.6	<0.1
Coal pile run-off	18-20	<0.1
Settling pond	3-8	<0.1

The sulfides are formed at the cell surface or in the near vicinity of the cell, and are not tightly bound to the cell. Therefore they do not form deposits that poison the cell or physically block the flow of metabolites into or out of the cell. In fact, the local precipitation of metals seems to be an important factor in rendering these organisms relatively resistant to the toxic effects of heavy metals. We have observed the growth of sulfate-reducing bacteria in media containing 500 ppm Hg^{++} or 50 ppm Cd^{++} , with concomitant removal of the majority of the heavy metals from solution. We believe that these organisms can be utilized in large-scale waste treatment systems to reduce the concentrations of a variety of heavy metal pollutants to acceptable levels.