Bull. Environ. Contam. Toxicol. (1993) 51:226–233


Environmental Contamination and Toxicology

# Bacterial Enumeration and Mercury Volatilization in Deep Subsurface Sediment Samples

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Many of the deep subsurface aquifers are vulnerable to anthropogenic activities including contamination by metals. Although microbes play a major role in determining the relative mobility and toxicity of pollutants, little is known concerning their activities and interactions with pollutants in the deep terrestrial subsurface. A pollutant of continuing concern is mercury (Klein and Thayer 1990). In reduced environments, such as aquatic sediments, $Hg^{2+}$ reacts with sulfides, forming highly insoluble precipitate, HgS. It was originally believed that mercury released to aquatic environments would be rapidly immobilized and maintained in an innocuous state. However, it has since been recognized that inorganic mercury is biologically transformed to more toxic organic mercury compounds such as mono- and dimethylmercury. This methylation process mobilizes mercury, returning it to the aqueous or gas phase where it may be bioaccumulated by fish and other higher-trophic level consumers. The major microbial group implicated in this process are the sulfate-reducing bacteria *Desulfovibrio* spp. (Compeau and Bartha 1985).

Other biologically-mediated mercury transformations include volatilization. The molecular basis of this mode of resistance has been extensively studied (Misra 1992; Summers 1992). The process involves the enzymatic reduction of $Hg^{2+}$ to elemental Hg. This process has also been observed for organomercurial compounds, such as phenylmercuric acetate (PMA) (Robinson and Tuovinen 1984). However, reduction of PMA to $Hg^0$ requires organomercurial lyase to cleave the Hg-C bond.

Biologically-mediated transformations play an important role in the global cycling of mercury. Some transformations may provide a detoxification mechanism which can be utilized in bioremediation programs. This concept has been proposed for As, Se, and Hg in surface materials (Sanford and Klein 1988; Karlson and Frankenberger 1989; Bisogni and Lawrence 1975).

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The scope of the present work was (i) to determine the Hg-resistance levels of two subsurface microbial communities as influenced by prior Hg exposure; (ii) to determine the ability of two subsurface microbial communities to volatilize added $^{203}Hg^{2+}$; and (iii) to identify limiting nutritional factors for the volatilization of mercury from these samples under aerobic and anaerobic conditions.

## MATERIALS AND METHODS

The Savannah River Plant (SRP) occupies 768-$km^2$ in the upper Atlantic Coastal Plain near Aiken, South Carolina. The plant was operated for the U.S. Department of Energy by the E.I. DuPont de Nemours Co. until 1989. Unconsolidated sediments extend to depths of over 400 m below the surface. These sediments, deposited since the Cretaceous period, rest on igneous and metamorphic bedrock which lies approximately 528 m below the surface. Chemical and radioactive waste generated at the plant have found their way into the underlying sediments and are affecting the groundwater. As part of a larger study of physical, chemical and biological phases, samples from 11 unique geologic formations (taken from an uncontaminated site southeast of the plant) were analyzed. Of these strata, sediments from the lower Ellenton Formation and the Cape Fear Formation were sampled for this study. The Ellenton formation lies approximately 213 meters below the surface at the drilling site. It is described as dark gray, micaceous, slightly calcareous, silty, very fine sand, and very fine sandy silt. This sample was composed of 84.4% sand, 5.1% coarse silt, 0.0 % fine silt, and 10.6% clay. The Cape Fear Formation lies approximately 437 m below the ground surface at the drilling site. The subsurface is described as dark grayish-brown, very micaceous, lignitic silt. The sediment was composed of 15.6% sand, 33.0 % coarse silt, 4.0% fine silt, and 47.4 % clay (Phelps et al. 1989). The sample procurement procedures for the DOE Deep Subsurface Microbiology Program have been previously summarized (Phelps et al. 1989). After removal from the bore hole, samples were stored under $N_2$ atmosphere for transportation; in the laboratory, samples were placed under an atmosphere of $N_2$:$CO_2$:$H_2$ (85:10:5).

Each bulk sediment sample was divided into four subsamples. Two subsamples were amended with 5 $\mu$g Hg $gdw^{-1}$ sediment, added as PMA or $HgCl_2$. The other two subsamples were amended with an equivalent volume of sterile distilled water as controls. All subsamples were maintained at a constant moisture content of approximately 20%, maintained by the addition of sterile water at 2-3 week intervals. The samples from the Ellenton and Cape Fear Formations were incubated anaerobically for 57 and 83 days, respectively.

The heterotrophic microbial populations of each metal-impacted sample were enumerated on PTYG media (Ghiorse and Wilson 1988) which contained ($l^{-1}$): glucose, 10 g; trypticase soy broth, 5 g; yeast extract, 10 g; peptone, 5 g; and agar, 15 g. A 1% dilution of this medium was also used to estimate the low nutrient-responsive bacteria in each sample. Mercury-containing PTYG media

were prepared with 5 $\mu$g $Hg^{2+}$ $ml^{-1}$, added as $HgCl_2$. The pH values were adjusted to 6.6 and 8.2 to match the measured pH values of the two subsurface samples. Sediment samples were blended at high speed using an Osterizer blender for 3 min, diluted in sterile water, and spread in duplicate on each of the four different types of media. Replicate sets of plates were incubated at 22-24°C under aerobic and anaerobic conditions.

Control and PMA-treated samples (0.75 g) from the Ellenton and Cape Fear formations, and a reference surface soil (Satanta loam) sample were amended in duplicate with one or all of the following nutrients ($gdw^{-1}$): glucose-C 2 mg, $NaNO_3$-N 0.08 mg, $K_2HPO_4$-P $8 \cdot 10^{-3}$ mg, $FeSO_4$-Fe $1.6 \cdot 10^{-2}$ mg. The samples which received carbon (glucose) additions initially were pulsed with a second carbon addition on the 15th day of the experiment (not repeated for the anaerobic experiments). Unamended and autoclaved samples were included as controls.

For Hg-volatilization, Hg-stock solution was prepared by mixing $^{203}HgCl_2$ (New England Nuclear, Boston, MA) with unlabelled $HgCl_2$ (50 $\mu$g $Hg^{2+}$ $ml^{-1}$) to a specific activity of 4 kBq $\mu$g $Hg^{-1}$. This solution was added to all samples to a final concentration of 5 $\mu$g $Hg^{2+}$ $gdw^{-1}$ and a final activity of 20 kBq $gdw^{-1}$. The samples were counted immediately in a Beckman Gamma 5500 scintillation counter to determine the initial level of radioactivity present in each sample. All samples were prepared in duplicate and incubated aerobically at 23-24 °C without agitation in a ventilated glove box. The entire experiment was duplicated under anaerobic conditions.

Small glass vials (4.5 ml) were modified to assess $^{203}Hg$-volatilization rates in subsurface and surface samples incubated under aerobic and anaerobic conditions. The caps were fitted with two 18 gauge needles for inlet and outlet ports. To trap volatiles, syringes (3 ml capacity) from which the tops were removed were filled with 0.8 g of activated carbon (200 mesh, Fisher Scientific, Pittsburgh, PA). The upper area was covered with a filter paper disk held in place with a lateral section of the rubber syringe plunger tip. To check the trapping efficiency of the carbon traps, secondary acid traps were used in initial experiments. No radioactivity was recovered in secondary acid traps, indicating that a single carbon trap was sufficient to capture the volatile Hg products. For the aerobic incubation, the headspace of each vial was flushed continuously with filter-sterilized air. At 2-7 day intervals, the radioactivity in carbon traps was counted. For anaerobic incubation, samples were held in an anaerobic glove box and flushed using a plastic syringe filled with 60 ml of the glove chamber atmosphere ($N_2$:$CO_2$:$H_2$) just prior to sampling (approximately every 8-9 days). A flow rate of 60 ml $min^{-1}$ was selected to approximate the continuous flow rates used in the aerobic volatilization studies. At the conclusion of the experiment, the reaction vials were counted to provide a material balance for the radioactive mercury.

Mercury-tolerant colonies (colonies which grew in the presence of 5 $\mu$g $Hg^{2+}$ $ml^{-1}$ PTYG and 1% PTYG) were isolated for determination of the minimal inhibitory

$Hg^{2+}$ concentrations (MIC). Forty-two aerobic mercury-resistant isolates and 34 mercury-resistant anaerobic isolates were tested on PTYG plates containing 5, 10, 25, 50, 100, and 200 $\mu$g $Hg^{2+}$ $ml^{-1}$, added as $HgCl_2$. The MIC for each isolate was defined as the highest $Hg^{2+}$ concentration that completely inhibited growth on solid PTYG plates.

## RESULTS AND DISCUSSION

Bacteria were enumerated under aerobic and anaerobic conditions following sediment incubation under 85% $N_2$:10% $CO_2$:5% $H_2$. The aerobic plate counts ranged between 3.2 and 4.0 log CFU $gdw^{-1}$ for the Ellenton sample and between 4.4 and 4.9 log CFU $gdw^{-1}$ for the Cape Fear sample (Table 1). In both instances the dilute formulation of PTYG yielded higher plate counts. Except for the Ellenton sample, the plate counts remained below the level of detection in media amended with 5 $\mu$g $Hg^{2+}$ $ml^{-1}$ (Table 1).

The plate counts for the Ellenton sediment samples that had been previously treated with either PMA or $HgCl_2$ were slightly increased in PTYG and dilute PTYG media. The respective plate counts in Hg-amended PTYG media were uninfluenced by the pretreatment. In dilute Hg-amended PTYG media the plate counts were not recovered (Table 1). The lack of counts may reflect a difference in the bioavailability of mercury in the two media. The PTYG medium contains a higher concentration of organic constituents which may chelate a portion of the added $Hg^{2+}$ ion, thereby decreasing its toxicity to cells. In the Cape Fear sediment samples, prior Hg treatment resulted in decreased plate counts on PTYG and dilute PTYG media (Table 1). No growth was observed on media amended with mercury.

The anaerobic PTYG plate counts for the Ellenton control sample were higher than the respective aerobic plate counts (Table 1). Bacteria were not recovered on the dilute PTYG media. For the Cape Fear sample, the aerobic and anaerobic PTYG plate counts were similar, whereas the anaerobic plate counts on dilute PTYG media were lower than those obtained under aerobic conditions.

Prior treatment with mercury had no effect on bacterial recovery from the Ellenton samples under anaerobic conditions (Table 1). For the Cape Fear samples, bacteria were not recovered, suggesting that the bacteria were more Hg-sensitive or mercury was more available when compared with the Ellenton sample.

A total of 72 colonies were isolated for testing Hg-sensitivity on PTYG media. About 15% of the isolates were able to grow on media that contained the highest mercury level, 200 $\mu$g $Hg^{2+}$ $ml^{-1}$ (Table 2). These levels of resistance appear to be higher than those typically reported for bacteria from surface soils and aquatic environments. However, a comparison with previously published data would be invalid because MIC testing for metal resistance is medium-specific.

Table 1. Bacterial plate counts of sediment samples. The numbers in parentheses represent one standard deviation.

| Sample | Plate Counts (log CFU $gdw^{-1}$) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PTYG | | | 1% PTYG | | | Hg-PTYG | | | Hg-1% PTYG | | |
| | Control | PMA | $HgCl_2$ | Control | PMA | $HgCl_2$ | Control | PMA | $HgCl_2$ | Control | PMA | $HgCl_2$ |
| *Aerobic incubation* | | | | | | | | | | | | |
| Ellenton | 3.2 (0.4) | 4.0 (0.2) | 3.9 (0.2) | 4.0 (0.1) | 5.0 (0.4) | 4.8 (0.3) | 3.6 (0.4) | 3.8 (0.5) | 4.0 (0.1) | NG | NG | NG |
| Cape Fear | 4.4 (0.3) | 2.4 (0.4) | 2.6 (0.4) | 4.9 (0.3) | 2.9 (0.3) | 3.4 (0.4) | NG | NG | NG | NG | NG | NG |
| *Anaerobic incubation* | | | | | | | | | | | | |
| Ellenton | 4.7 (0.1) | 5.1 (0.1) | 4.9 (0.1) | NG | NG | NG | NG | 5.0 (0.1) | 5.0 (0.1) | NG | NG | NG |
| Cape Fear | 4.2 (0.1) | NG[a] | NG | 3.5 (0.3) | NG | NG | NG | NG | NG | NG | NG | NG |

[a]NG, No growth (log CFU $<2.0$ $gdw^{-1}$).

Table 2. Hg-resistance of bacterial isolates from the sediment samples.

| Incubation | Number of Isolates | | | | | |
|---|---|---|---|---|---|---|
| | Hg Concentration ($\mu g\ ml^{-1}$) | | | | | |
| | 5 | 10 | 25 | 50 | 100 | 200 |
| Aerobic | 15 | 7 | 0 | 7 | 3 | 10 |
| Anaerobic | 18 | 8 | 3 | 2 | 1 | 2 |

The control and Hg-pretreated sediment samples and a surface soil sample were used to measure mercury volatilization. After addition of $^{203}Hg$, the samples were incubated with various nutrient amendments under aerobic and anaerobic conditions. The miniaturized apparatus developed as part of this study to assess mercury volatilization with limited size samples made it possible to conduct replicated experiments with a wide range of variables. The amount of mercury volatilized at the conclusion of each incubation is reported in Table 3.

Under aerobic incubation of the surface soil samples, the addition of 2 mg $g^{-1}$ carbon (as glucose) elicited the greatest response of the nutrient amedments tested, but there was considerable loss of Hg observed also in the sterile control. In the unamended soil, 18% of the added Hg was volatilized after 18 days. Addition of either N, P, Fe, or all nutrients had little effect on the volatilization rate relative to the unamended control.

With the Ellenton sediment sample, phosphate elicited the greatest response, with 14% of the added mercury volatilized by the conclusion of the experiment (Table 3). Some volatilization occurred also in sterile control samples. The mechanism of the abiotic volatilization is not known but the data suggested that it was sample-specific. Mercury volatilization in the Cape Fear sediments was negligible. Previous incubation of sediment samples with PMA had no effect on the subsequent volatilization of Hg.

In general, there was negligible mercury volatilization under anaerobic conditions (Table 3). These results are consistent with previous findings that reduction, followed by volatilization of $Hg^0$, occurs more readily under aerobic conditions (Steffan et al. 1988). Methylation is the primary route of Hg transformation under anaerobic conditions but it does not generally lead to the formation of volatile species.

Acknowledgements. The authors thank Dr. L. Fraley for his assistance with research design and acquisition of $^{203}Hg$, and to Dr. T.J. Logan, Dr. O.H. Tuovinen, and S.J. Traina for review of the manuscript. Subsurface samples were acquired as a part of the DOE Subsurface Science Program (FGO2-87ER-60585). Consultations and assistance with sample acquisition by Dr. F.J. Wobber and T.J. Phelps are greatly appreciated.

Table 3. Volatilization of mercury from sediment and surface soil samples.

| Sample | Pretreatment | Incubation (days) | % Hg Volatilized | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Sample Amendment | | | | |
| | | | Sterile Control | None | C | N | P | Fe | All Amendments |
| *Aerobic incubation* | | | | | | | | | |
| Surface soil | None | 18 | 27 | 18 | 41 | 17 | 19 | 22 | 20 |
| Ellenton | None | 18 | 6 | 9 | 4 | 6 | 14 | 6 | 9 |
| | PMA | 18 | 7 | 7 | 8 | 10 | 13 | 10 | 14 |
| Cape Fear | None | 54 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | PMA | 54 | 2 | 2 | 0 | 0 | 1 | 0 | 0 |
| *Anaerobic incubation* | | | | | | | | | |
| Surface soil | None | 64 | 1 | 2 | 1 | 7 | 3 | 2 | 4 |
| Ellenton | None | 64 | 0 | 0 | 0 | ND[a] | ND | ND | ND |
| | PMA | 64 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cape Fear | None | 64 | 0 | 0 | 3 | 1 | 2 | ND | ND |
| | PMA | 64 | 0 | 2 | 1 | 1 | 1 | 1 | 1 |

[a]ND, Not determined.

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Received December 4, 1992; accepted February 6, 1993.