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Biodeterioration of Standard Reference Materials

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Biodeterioration of Standard Reference Materials

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Several National Bureau of Standards Standard Reference Materials have been examined for susceptibility to biodeterioration. Several of these materials were attacked by bacteria and fungi when stored at elevated humidity after exposure to outdoor air. Some SRMs underwent deterioration after following certificate instructions for handling. Suggestions for some certificate revisions are made.

Key words: bacteria; biodeterioration; biotransformations; deterioration of materials; element volatilization; fungi; metals; microorganisms; Standard Reference Materials.

1. Introduction

The validity and reliability of Standard Reference Materials certified for elemental composition in a given host matrix require that homogeneity of the SRM, and the concentrations of the certified elements, will remain unaltered over a reasonable shelf life. This demands that no chemical or biological process will occur that alters the homogeneity or certified concentration of the specified elements or that alters the matrix so as to reduce homogeneity or change concentrations of those elements.

Many chemical or physical processes may occur during storage or use of SRMs which can result in composition changes. These include thermal cycling, photolysis, adsorption, volatilization, contamination, atmospheric oxidation, hydrolysis, and the like. Such occurrences have been studied in certain SRMs (R. Alvarez, personal communication). A more subtle form of deterioration, which has not received adequate attention by analysts, may result from contamination by microbiota such as bacteria or fungi. Such biodeterioration may be initiated by microbiota originally present in the particular matrix employed, as with biological or environmental reference materials. Alternatively, contamination may be introduced subsequent to certification and sale to the

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user, who may introduce microbiological contamination in following inadequate instructions for use or by using conventional handling procedures without adequate sterile controls.

Perhaps the most critical factor in the metabolism of the microbial cells present in contaminated SRMs is water availability. The most useful measurement of water availability is "water activity" (a_w), defined as the ratio of the equilibrium vapor pressure of water over the material in question to that of pure water at the same temperature [1]. Growth of most microorganisms occurs only when $a_w > 0.90$, however, xerophiles, (usually yeasts and molds) which grow under relatively dry conditions ($a_w = 0.85$ to 0.60), exist [1]. Microorganisms do not grow at $a_w < 0.60$. Most biologically susceptible SRMs are freeze dried, and therefore, unless rehydration occurs, either accidentally or by design, they should not experience biological deterioration. Rehydration is possible, however, during customer use while dispensing and weighing or as a result of failure to close a bottle cap tightly. Condensation of moisture on a refrigerated SRM is very possible when it is moved to a warm laboratory.

Microbial or fungal attack on substrates containing trace (10^{-6} to 10^{-9} $g \cdot g^{-1}$) quantities of a particular element in a natural or introduced (spiked) form may well result in measurable losses or translocations of the element over relatively short periods of time. Much of the current environmental literature devotes considerable attention, for example, to the biological transformations, transport, and reaccumulation in new reservoirs (often biological) of many trace toxic elements, especially those frequently certified in needed SRMs [2, 3]. Fortunately, modern measurement techniques and methods are available which are uniquely suited to assess the formation of certain molecular species containing elements of concern even if this occurs slowly and at very low concentrations. Such methods permit diagnostic use of such element-containing metabolites as kinetic markers for use in establishing degradation rates and predicting form and trends of deterioration of suspected SRMs. Table 1 lists some examples of microbially catalyzed transformations of certain elements certified in some of the SRMs potentially susceptible to biodeterioration. Volatile products are emphasized since these could lead to greatest loss of certified trace elements from SRMs.

The present paper reports preliminary work designed to survey a group of currently produced NBS SRMs which we regard as susceptable to biodeterioration, and for which certain elements, which we know to be especially susceptible to such biotransformations [2, 3], have been certified.

Table 1. Examples of microbial transformations (especially volatile products) of elements and some SRMs certified for these elements potentially susceptible to such biodeterioration. Volatile species formed may be lost to the atmosphere resulting in alteration of certified element composition.

Element	SRM Certified for Element	Microbial Transformations of Element	Typical Environments Where Transformation Occurs
As	1571, 1648	$(CH_3)_3As$, AsH_3, $(CH_3)_2AsH$ as gases; AsO_2^- in solution or solid	seawater [4], sewage [5], sediments [6,7]
Hg	2672, 1645	Hg^o, $(CH_3)_2Hg$ as gases; CH_3Hg^+ in solution or solids	tissue and urine [8], sediments [9-12], feces [13], water [14,15]
Cr	1569, 1570, 1571	Cr^{+++} in solution	waters and sediments [16,17]
S	1621	H_2S, $(CH_3)_2S$, CH_3SH, CS_2, $(CH_3)_2S_2$ as gases	soils [18], manure [19], waters and sediments [20,21]
Se	1567	$(CH_3)_2Se$, $(CH_3)_2Se_2$, $(CH_3)_2Se_2$, Se^o (all volatile)	soils and sewage [22], waters [23]
Pb	1571	$(CH_3)_4Pb$ (volatile), organoleads	bogs [24], sediments [25]
Sn	2672*	$(CH_3)_2SnH_2$, $(CH_3)_4Sn$, other methyltin compounds (most are volatile)	waters and sediments [26,27,28]

*Certified for total Hg not Sn.

2. Experimental Procedures

2.1 Enumeration of Microorganisms

Microorganisms in SRMs were enumerated by surface or pour plating [29] using trypticase soy agar or a modified iron-peptone agar [30] with glucose (0.5 g L^{-1}) substituted for gluconate. Incubation was at room temperature (about 22 °C) or 37 °C.

2.2 Elemental Analysis

Mercury in urine was quantified using a manual cold vapor procedure [31] employing a flameless atomic absorption spectrophotometer (Mercometer, Anti-Pollution Control Technology Corporation). Volatile mercury was detected and identified using a tandem gas chromatograph-atomic absorption system [32]. Chromium was determined using a Perkin-Elmer model 360 atomic absorption spectrophotometer equipped with a graphite furnace. Chromium volatilization experiments were performed using a dual tube absorption system [33] shown in figure 1.

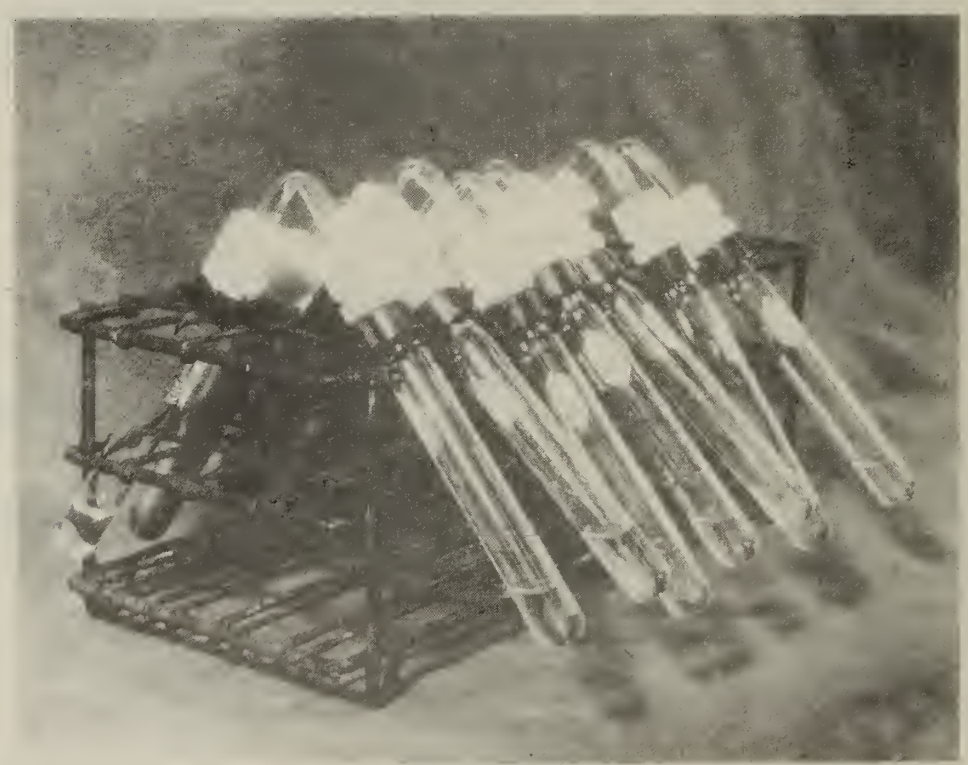

Figure 1. Dual growth tube/absorbing system for measurement of microbial metabolites. One tube contains sterile, deionized water, the other contains brewers yeast (SRM 1569) and contaminating fungal spores. Volatile metal-containing metabolites can be measured by atomic absorption after partitioning into the sterile water.

2.3 Exposure and Storage of SRMs

Some of the SRMs were exposed to outdoor air (NBS grounds) for periods of 30 to 60 min on successive days (total exposure 3.5 hr), then were stored loosely capped at relative humidities of 100, 90, 70, and 49 percent. The higher humidities were maintained using dessicator jars containing calcium chloride solutions [34], and the lowest humidity represented the laboratory ambient relative humidity which averaged 49 percent and ranged from 33 to 73 percent (April to June, 1980).

2.4 Mercury Binding

Mercury absorbed to cells growing in urine certified for mercury (low level, SRM 2672) was quantified by passing 5.0 mL of contaminated urine through a 25 mm, 0.45 μm membrane filter (Millipore Corp.) to trap cells. After a 5.0 mL deionized water rinse, filters were digested in 1.5 mL plastic centrifuge tubes with 0.2 mL HNO_3 at 80 °C for 30 min to solubilize Hg. The volume was made to 1.0 mL with deionized water and an aliquot of the digest was analyzed for total mercury [31]. Dry weights of cell material were determined by filtration through 0.45 μm filters, rinsing as before, followed by drying to constant weight at 105 °C. Duplicate bottles were run in these experiments, and duplicate mercury determinations from each bottle were performed. Duplicate bottles rehydrated with sterile water and analyzed as above served as controls.

2.5 Chromium Studies

Possible chromium volatilization from brewers yeast and orchard leaves (SRMs 1569 and 1571), both certified for chromium, was investigated using a dual tube apparatus (fig. 1). The two SRMs were placed in a 100 percent relative humidity chamber for three days, then 3 g portions were added to 12 x 100 mm glass screw cap tubes which were connected to an identical tube (containing 3.0 mL deionized water) via a thin glass tube through the caps. The SRMs were inoculated with fungal spores taken from contaminated brewers yeast and orchard leaves stored at high humidity. Aliquots of the deionized water were analyzed at intervals for total chromium using atomic absorption spectrophotometry.

3. Results and Discussion

All SRMs were found to be sterile as received except for the urban particulate SRM 1648 which contained more than 10^4 viable microorganisms per gram (table 2). As a comparison, total counts of bacteria in soil range from 10^5 per gram in poor soil to 10^8 per gram in rich soil [35]. Freeze dried urine (SRMs 2671 and 2672) was sterile in the dehydrated form. Rehydration, however, with non-sterile water (though it may meet the purity requirement of < 0.1 μg L^{-1} Hg set forth in the certificate) resulted in microbial contamination of the material (fig. 2). NBS

Table 2. Numbers of viable microorganisms in several as received, urban particulate (SRM 1648) vials. Numbers indicate organisms per g.

vial 1	4.00×10^4
2	3.45×10^4
3	3.20×10^4
4	2.05×10^4

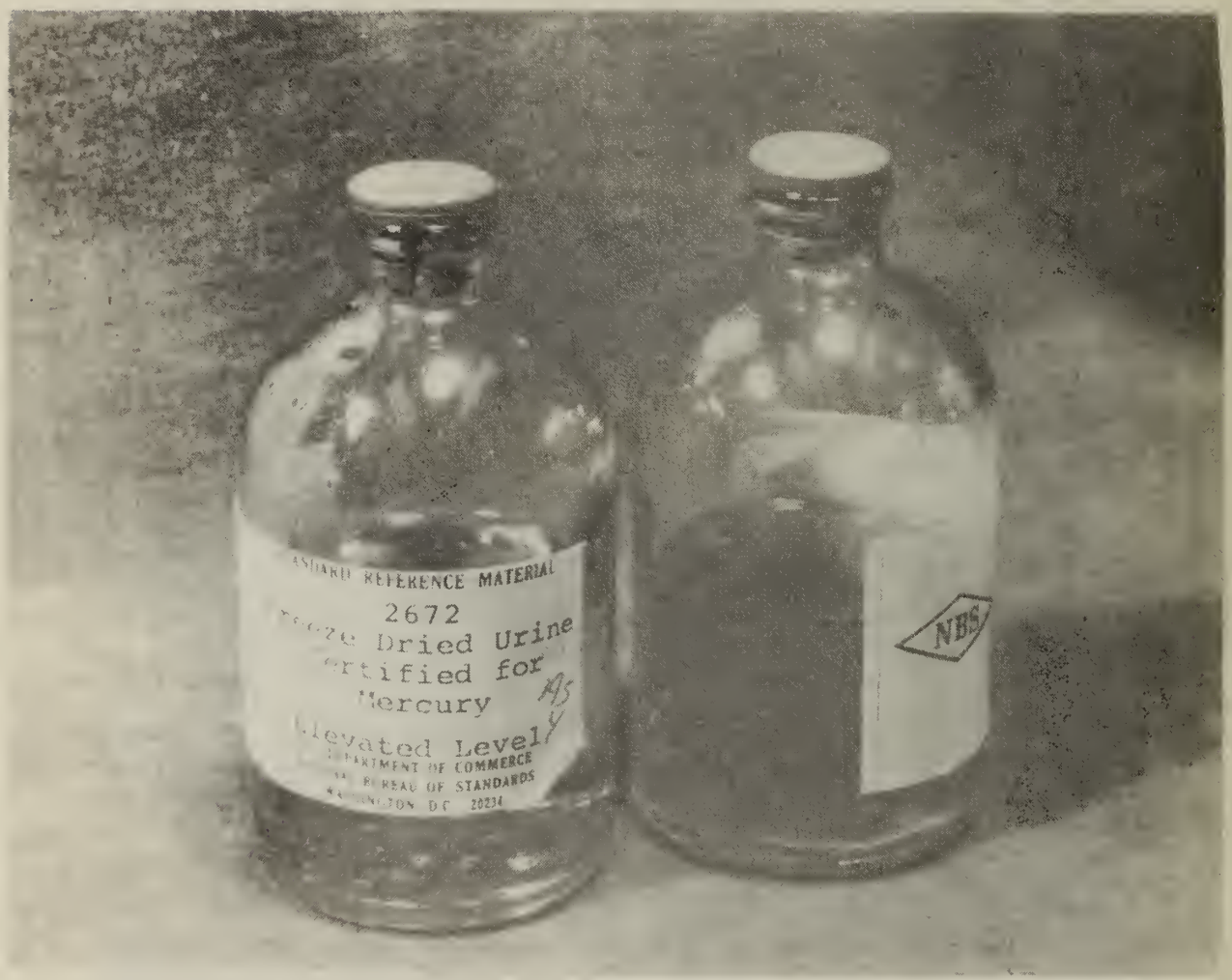

Figure 2. Freeze dried urine rehydrated with sterile (left) and non-sterile (right) distilled water.

house distilled water used in the reconstitution of the urine did not contain detectable mercury (< 0.1 μg L^{-1} by cold vapor atomic absorption, see reference 31), but contained more than 500 viable microorganisms per mL. A sample of distilled water from a microbiology laboratory at the University of Maryland contained 1600 viable microorganisms per mL. At 1 °C the cells do not grow in the urine, however if stored at room temperature these cells utilized nutrients in the urine and grew well, numbering greater than 10^8 cells per mL in a few days (table 3). Consequently, depending on the number and kinds of microorganisms in the water used to reconstitute the urine, the SRM composition could be significantly altered. For example, volatilization of mercury by the cells (see reviews 2, 3) or binding of mercury to the cells [36] could alter concentrations and homogeneity of distribution of mercury. To date we have not found the microflora of the NBS house distilled water capable of volatilizing elemental mercury (Hg^0) from urine certified for mercury. Microorganisms present in University of Maryland distilled water, however, volatilized Hg^0. After three days 0.43 ng Hg^0 per mL was found in the headspace of the container rehydrated with non-sterile water. A sterile control showed no volatilization of mercury. No other volatile mercury containing metabolites (table 1) were detected. The possibility exists that microorganisms in other distilled water systems can also volatilize mercury.

Table 3. Numbers of viable microorganisms in freeze-dried urine (SRMs 2671 and 2672) reconsitiuted with NBS distilled water. Storage was at 1 °C or 22 °C. Numbers are organisms per mL.

	Time of storage (1 °C)			
Urine certified	0	1 day	6 days	12 days
Hg - elevated level	6.5×10^2	6.0×10^2	5.1×10^2	4.8×10^2
- low level	5.8×10^2	6.9×10^2	3.7×10^2	3.6×10^2
F - elevated level	9.6×10^2	9.4×10^2	8.1×10^2	8.2×10^2
- low level	7.5×10^2	7.5×10^2	3.6×10^2	3.5×10^2

	Time of storage (22 °C)		
	0	1 day	6 days
Hg - elevated level	-	4.3×10^2	$>10^8$
- low level	-	6.5×10^2	$>10^8$
F - elevated level	-	1.02×10^2	$>10^8$
- low level	-	5.2×10^2	$>10^8$

Microorganisms which grew in the mercury-certified urine SRM bound a significant percentage of the mercury. After six days 21.4 to 24.2 percent of the mercury was bound to cells (cells were removed from solution by filtration) in the low level mercury in urine SRM, representating a concentration factor of 1514 to 3300 by the microorganisms, which generally formed a sediment in the bottom of the container (table 4). Cells occurring in other distilled water systems could have a higher or lower binding affinity for mercury which could influence how quickly significant alterations in SRM composition may occur.

Table 4. Binding of mercury to cells growing in urine certified for mercury (low level)--SRM 2672. Values listed are means of duplicate runs. Bottles 1 and 2 were rehydrated with NBS distilled water, bottle 3 with University of Maryland distilled water.

Bottle	Hg bound (ng/mg cells)	Hg bound (%)	Concentration Factor
1	67.16	24.2	1774
2	59.39	21.4	1514
3	101.88	22.9	3300

River sediment (SRM 1645), urban particulate (SRM 1648), brewers yeast (SRM 1569), orchard leaves (SRM 1571), and oyster tissue (SRM 1566) were examined over a range of humidities for susceptibility to attack by airborne contaminants. Visual and microscopic examination showed severe fungal deterioration of brewers yeast, orchard leaves, and oyster tissue after exposure to outdoor air for short periods of time on successive days (3.5 hr total) followed by incubation at room temperature and 90 or 100 percent relative humidity. Fungal growth on oyster tissue stored at 70 percent relative humidity was also noted. Plate counts of these high-humidity exposed materials were not possible due to tremendous fungal growth which literally consumed the SRMs. Plate counts were possible only with urban particulate. Data (table 5) indicate that storage at elevated relative humidity (90 to 100 percent) induces growth of microorganisms in this SRM, however at lower humidities cell numbers are no greater than those observed for previously unopened urban particulate (tables 2 and 5). Other SRMs exposed to outdoor air then stored loosely capped at ambient laboratory relative humidity (average 49 percent, range 33 to 73 percent) did not show development of microbial populations visually, microscopically or by plate counting after six months of storage.

Table 5. Growth of microorganisms in SRMs exposed to outdoor air then stored at varying relative humidity for six months.

SRM	Relative Humidity Stored			
	AMB[a]	70	90	100
orchard leaves	-	-	+	+
oyster tissue	-	+	+	+
brewers yeast	-	+	+	+
river sediment	-	-	-	-
urban particulate[b]	2.64×10^4	4.9×10^3	1.05×10^5	1.62×10^6

(-) Values indicate no colonies on plates.
(+) Values indicate dense growth on plates (severe biodeterioration).

a Range 33 to 73 percent, mean = 49 percent, NBS general purpose laboratories

b Urban particulate contains microorganisms initially, however, counts after storage at ambient and 70 percent relative humidity were either the same or lower than previously unopened bottles (see table 2). Values listed are numbers of viable microorganisms per g.

Although exposure to outdoor air and incubation at elevated humidities probably represent "worst" case conditions in SRM handling it was necessary to quickly identify conditions leading to SRM biodeterioration in this preliminary survey. Microorganisms present in outdoor air can be taken inside by ventilation systems or open windows. Elevated laboratory humidities may occur during warm, humid weather (especially with the advent of the energy crisis and resultant higher thermostat settings) or as a result of steam or hot water use (autoclaving, glassware washing) even in laboratories which are served by controlled humidity ventilation systems. Future studies to measure SRM water activity after different exposure times at various humidities will be instructive in determining biodeterioration potential.

No volatile chromium production has been detected from contaminated brewers yeast or orchard leaves. These studies are continuing and will be expanded to cover additional elements and SRMs listed in table 1.

4. Conclusions and Recommendations

Improper storage of certain NBS Standard Reference Materials will result in severe microbiological deterioration. The relative humidity

of storage and contamination by outside microorganisms are the two most critical factors in deterioration of many of the SRMs examined. Urban particulate is already laden with microorganisms which will grow at elevated relative humidity. Rehydration of freeze dried urine will result in contamination and microbial growth unless the urine is rehydrated aseptically.

Under proper handling procedures set forth in most of the SRM certificates, microbial deterioration of SRMs should not occur. Exposure to airborne microbial contaminants and elevated humidity either deliberately (during weighing or removal from bottle) or accidentally (not closing the lid) could result in SRM biodeterioration. Microbial cultures, obtained by air sampling in several laboratories, have been isolated and are being tested for ability to cause deterioration of selected SRMs. How long an SRM must be exposed to a given relative humidity for deterioration is also the subject of current investigations.

At present, however, we can recommend certain certificate modifications. For example, freeze dried urine (SRM 2671 and 2672) should be rehydrated with sterile, mercury-free water. If sterile water is not available, the rehydrated SRM must be refrigerated immediately and should be used promptly. We recommend that rehydrated urine be discarded after 24 hr [37], to ensure microbial contaminants will not grow to any extent in the SRM.

Studies designed to examine certified element composition changes due to biodeterioration in other on the shelf and proposed SRMs (table 1) are underway. These and other results will be the subject of subsequent reports.

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Certain commercial equipment, instruments, and materials are identified in this paper in order to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that these items are necessarily the best available for the purpose.

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Several National Bureau of Standards Standard Reference Materials have been examined for susceptibility to biodeterioration. Several of these materials were attacked by bacteria and fungi when stored at elevated humidity after exposure to outdoor air. Some SRMs underwent deterioration after following certificate instructions for handling. Suggestions for some certificate revisions are made.

12. KEY WORDS (Six to twelve entries; alphabetical order; capitalize only proper names; and separate key words by semicolons)

bacteria; biodeterioration; biotransformations; deterioration of materials; element volatilization; fungi; metals; microorganisms; Standard Reference Materials.

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