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DETECTION OF MICROBIAL SULFATE-REDUCTION ASSOCIATED WITH BURIED STAINLESS STEEL COUPONS

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ABSTRACT

The objective of this study was to demonstrate applicability of an innovative radioactive isotope method for imaging microbial activity in geological materials to a comprehensive study of metal corrosion. The method was tested on a sample of stainless steel coupons that had been buried as part of a corrosion study initiated in 1970 by the National Bureau of Standards (now National Institute of Standards and Testing or NIST). The images showed evidence of microbial activity that could be mapped on a millimeter scale to coupon surfaces. A second more conventional isotope tracer method was also used to provide a quantitative measure of the same type of microbial activity in soil proximal to the buried coupons. Together the techniques offer a method for detecting low metabolic levels of microbial activity that have the potential for significant cumulative corrosion effects to metals. The methods are particularly applicable to monitoring steel components that are expected to remain buried and intact for very long periods as in nuclear waste storage applications.

Key words: Microbially induced corrosion, microbial sulfate-reduction, buried stainless steel, nuclear waste storage.

INTRODUCTION

The chemistry of solutions in geological settings is strongly influenced by slow but persistent action of microbial metabolic activities in the biosphere. These activities are known to strongly influence metal corrosion¹. An understanding of the effect of these solutions on the integrity of buried metal objects such as pipes, wires, and containers is important for estimation of their life expectancy. The same solution chemistry has a significant effect on the mobility of environmental contaminants. Microbial sulfate-reduction readily occurs in organic rich low oxygen environments where sulfate is available. It can result in significant changes to local electrochemistry, and it is often associated with metal corrosion in anaerobic environments². The net energy yielding respiration reaction is:



Although this is a simple net reaction, reduction of sulfur is a complex process of 6 electron steps and associated reaction products. The process is very sensitive to system redox potential and may play an important role in total solution chemistry. The ability to detect very low levels of sulfate-reducing activity, as demonstrated in this study, has potential applications for evaluating behavior of buried metals where burial times are intended to be very long.

Storage containers used for burial of nuclear waste are just such materials. Nuclear industry waste must be isolated to prevent spread of contamination for periods of time that are much longer than a human lifetime. The first line of defense to prevent spread of radioactivity is to place waste repositories in remote geologically stable locations that are inaccessible, and likely to remain so; ideally the location is dry or has very slow groundwater movement. The second line of defense is to immobilize materials in stable and insoluble forms, and to package them in strong corrosion resistant containers. Stainless steels that are highly resistant to corrosion are currently the preferred materials for nuclear waste storage containers.

Although far superior in resistance when compared to other ferrous metals, stainless steels are relatively new alloys, and there is no observational basis for predicting the long term integrity of containers made from them. Since its inception the NBS and NIST have acted on a mandate to provide baseline materials testing including properties of stainless steels. Test samples buried at sites across the United States in 1970 and 1971 for corrosion study by NBS have been made available to researchers at the Idaho National Laboratory (INL). Site 'D' from that NBS project, on a Coast Guard station near Wildwood, N.J., was selected for this study because of accessibility, and because corrosion processes were expected to be accelerated there due to the damp saline marine environment.

The imaging process demonstrated in this study was originally developed for mapping microbial activity on sandstone and shale cores in subsurface microbiology investigations^{3,4}. The technique results in a radiographic image of a silver foil that has been incubated in close contact with a radioactive ³⁵S-sulfate coated surface. Microbial activity on the surface produces radioactive sulfide that readily reacts to form an insoluble metal sulfide on the foil surface. Radioactivity from this fixed sulfide produces the radiographic image.

In parallel with imaging, sulfate-reduction activity in soil from within 2 cm of coupons was quantified using a standard radioactive tracer technique. Trace amounts of radioactive sulfate were added to soil slurries; sulfide produced from microbial reduction of the labeled sulfate was quantified by liquid scintillation counting. The strength of this technique relies on the assumption that the amount of labeled sulfate added to the system is small with respect to total sulfate present, so the introduction does not stimulate activity^{5,6}.

MATERIALS AND METHODS

Coupons were recovered in May 2004 and shipped to Idaho National Laboratory for analysis. Collateral analyses from this site and on these coupons are reported elsewhere⁽¹⁾.

⁽¹⁾ M. K. Adler Flitton and T. S. Yoder. Long-term underground corrosion of stainless steels. NACE Corrosion Expo 2007. Paper # 07595

NBS Methods

The NBS experimental design for the buried coupon study included several different treatments for each metal type. Treatments were designed to sensitize or otherwise compromise some coupons to simulate the results of container fabrication and/or to accelerate the effects of long-term burial. Four coupons representing 3 stainless steel (SS) types were selected for the present microbiology study. This group consisted of untreated coupons of types 301, 304 and 316 SS and one of type 316 sensitized. Sensitization consisted of heating to 1200° F for 2 hours, followed by cooling in ambient air, de-scaling in sodium hydroxide, and passivating in concentrated nitric acid to establish a thin surface oxidation layer⁷.

Site Description

At inception of the study in 1970, the burial site near Wildwood, NJ was described as coastal dune environment with strong marine influence. At the time coupons were recovered in 2004, the site was a densely vegetated back-dune environment sheltered from direct ocean exposure. Mean annual rainfall was 104 cm, and temperature ranged between -1 and 27° C. The predominant vegetation now consists of woody shrubs that present a nearly complete 3 m over-story. A moderate under-story of mixed vegetation is also present. The soil is a light sandy loam.

Collection and Handling of Coupons

The 20 x 30 cm coupons were buried 76 cm deep in parallel rows of 20 coupons 30 cm apart. Coupons for microbiological study were given special care during handling to prevent contamination. Surgical gloves were worn during the last stages of uncovering, removal and packaging. Coupons were immediately placed in sterile plastic bags, double wrapped in heavy gage polyethylene bags, placed in a 4° C cooler, and immediately shipped to INL. On arrival at the laboratory coupons were placed in storage at 4° C in an oxygen free nitrogen atmosphere. Experiments were initiated within 10 days of arrival.

Silver Foil Method

Ten cm squares (100 cm²) of silver foil used for imaging were prepared for sulfide capture by cleaning with successive washes of ethanol, acetone and hexane, and their surfaces were sensitized for reactivity to sulfide by treatment with concentrated nitric acid then rinsed repeatedly with filter-sterilized de-ionized water.

Four coupons that showed visible signs of beginning corrosion were selected for imaging. These coupons were stainless steel types 301, 304, 316, and 316 sensitized. Ten cm square areas of interest on coupons were cleaned with a soft bristle ¾ inch diameter camel's hair brush⁽²⁾ and blown with dry filtered nitrogen gas to remove loose soil particles. These areas were then coated with a solution containing Na³⁵SO₄ and 1 mM sodium lactate solution sufficient to wet the area of interest without run-off. Final sulfate concentration on the coupon was 0.05 mM with a net ³⁵S activity at the start of the experiment of 2 µCi per foil. Sensitized silver foils backed by foam blocks cut to match were placed on the coupons and

⁽²⁾ Fisher Scientific model 03-661

fastened securely in place. Coupons were maintained under humid anaerobic (N₂ atmosphere) conditions in a sealed plastic container and incubated in the dark at 20° C for 2 months.

Positive and Negative Control Experiments

A separate set of imaging experiments were conducted as positive controls and to provide baseline data for estimating the rate at which steel coupons may be colonized by sulfate-reducing microorganisms. In these experiments sets of coupons recently obtained from a metals supplier were prepared at INL according to the procedures described by NBS for preparation of the original buried materials. They were then immersed and incubated in an active sulfate-reducing bacterial culture. Three sets of coupons for three time points of observation were immersed in the culture. The sets consisted of the same metal types as the original 4 types used in the main treatment of this study with the exception that no type 316 stainless steel could be found, so type 316L coupons were used instead. Each set consisted of types 301, 304, 316L, and 316L sensitized coupons. In addition, 3 mild steel coupons were included to provide a metal that rapidly reacts with products of microbial sulfate-reduction.

Coupons to be used in the control experiments were washed in acetone, ethanol, and sterilized de-ionized water before immersion in the sulfate-reducing microbial culture. The bacterial strain used for the positive control sulfate-reducing microorganism was *Desulfovibrio thiooxidans* from INL laboratory stock; growth medium consisted of a dilute minimal salts solution that was developed in this laboratory to be used in studies of microbial interactions with buried steel waste canisters⁸. Salt concentration in the medium was below drinking water standards; lactate and sulfate were added as energy source and electron acceptor respectively (Table 2).

Incubation of coupons in the sulfate-reducing culture was conducted at room temperature. Evidence of bacterial sulfate-reduction was indicated by production of black metal sulfides visible in the culture vessel (Figures 1A and 1B).

Negative control experiments consisted of steam sterilized foils and coupons that were coated as described above with sterile lactate and labeled sulfate solutions and incubated for 2 months.

Silver Foil Imaging

Images were created using a phosphorescence imaging system⁽³⁾. After incubation, foils were detached from coupons, thoroughly rinsed with sterile de-ionized water to remove residual radioactive sulfate, and sealed in sterile plastic bags. The foil containing bags were exposed face down on the phosphor imager plate. Foils were placed on and removed from the plate surface by use of the sample exposure platform that allows for transfer without light exposure of the phosphor coated surface. Exposure times were standardized to 24 hours after comparing image densities from different exposure durations. Digital images of foils from each coupon were created on the system imaging platform (Figures 4 A-D).

⁽³⁾ Molecular Imaging System (Bio-Rad, Hercules, CA), including a Sample Exposure Platform and Model GS-525 Molecular Imager

Soil Activity Serum Vial Experiments

Soil samples for measurement of microbial activity that were collected at the same time as coupon recovery, were handled in the same manner as described above for steel coupons. Samples were taken from areas central to the flat surface of and within 2 cm distant from coupons.

Sulfate-reduction activity in four soil samples was quantified by measuring production of radioactive sulfide from $^{35}\text{SO}_4$. One soil sample was tested from locations adjacent to the types 301, 304, and 316 coupons used in the imaging experiments. A fourth sample was tested that came from adjacent to a type 301 sensitized coupon which at the time of unearthing was observed to be coated with "corrosion products" consisting of friable and discolored material. For each of those 4 soil samples, 6 crimp-top serum vials were inoculated each with 2 g of soil plus 4 ml liquid medium formulated for culture of sulfate-reducing organisms ⁽⁴⁾. Experiments were started by addition of ^{35}S stock solution to give a final sulfate concentration of 0.0125 mM and activity of 2 μCi . Microbial activity in vials was sequentially stopped, by addition of concentrated base, after elapsed times of 0, 1, 2, 4, 8 and 18 hours. Sulfide present from microbial sulfate-reduction was driven from solution by acidification, flushed from headspace by a stream of ultra high purity (99.9995%) nitrogen, and captured by bubbling into 1 M NaOH. The radioactive sulfide containing NaOH was placed in scintillation cocktail ⁽⁵⁾ (2 ml per 10 ml) and quantified by liquid scintillation counting ⁽⁶⁾. Vials were counted for 30 minutes each. Quench standards used were low energy ^{14}C , and sample disintegrations per minute (DPM) were converted to ^{35}S concentration by comparison to standards prepared from the original isotope stock.

RESULTS

Coupon images and corresponding foil phosphor images are shown in Figures 2A-D. Phosphor images of the foils from 3 of the 4 metal coupons showed localized very low rates of sulfate-reduction. It appeared that only a tiny fraction of the 2 μCi of $^{35}\text{SO}_4$ spread on these plates was reduced to sulfide. Based on correlation of the phosphor images with the sites of their incubation, activity on the coupons appears to have been associated with dark stained streaks residual from organic material in the ground, and did not correspond to any signs of incipient corrosion such as pitting. Faint darkening visible in the foil image from the fourth coupon, the un-treated type 316 SS (Figure 2C), may have been an artifact of residual un-reacted sulfate.

Serum vial experiments also indicated very low sulfate-reduction activity (Table 3). Of 28 enrichments from 4 soils in the ^{35}S rate experiment, 6 showed measurable activity. Of the 6, based on standards and correcting for decay (half life = 88 days), sulfate-reduction rate ranged from 0.5 to 55 pmol/g sediment/day. Another 8 enrichments had activity around experimental detection limit; these enrichments had sulfate-reduction activity less than 0.5 pmol/g/day. Enrichments had a seemingly random distribution of activity, with some series having a positive response in earlier time points and none in later time points, the reverse of what might be expected. There was no evidence of a time series with increasing response over time. No correlation between soil activity and coupon metal type was noticeable. Positive

⁽⁴⁾ C & S Laboratories Inc., Tulsa, OK

⁽⁵⁾ Ultima Gold AF

⁽⁶⁾ Perkin Elmer Tri-Carb 3170 TR/SL, Downers Grove, IL

controls of laboratory sulfate-reducing strains showed robust activity, and time zero enrichments and negative controls consisting of autoclaved soil did not show activity. One possible conclusion from this seemingly random distribution of soil sulfate-reduction activity is that positive enrichments reflect random distribution of “single cells” from the original sample. If these data are treated as replicates in a most probable number distribution (MPN), a method for estimating microbial numbers in environmental samples⁹, analysis suggests 1-3 active colony forming units per 10 g of soil. These are indeed low numbers.

The rates reported here for microbial sulfate-reduction on and near the coupons are comparable to values reported from deep subsurface nutrient-poor environments^{10,11}, whereas they are a tiny fraction of rates reported in shallow marine sediments where sulfate-reduction may be the dominant electron accepting reaction¹². These observations imply sulfate-reduction potential was minimal at burial depth at the Wildwood site, and was not likely to be a significant player in the soil around the buried coupons. Judging from the high water table (Figure 3), from deterioration of the wooden stakes (Figure 4), and from the report that workers detected H₂S (rotten egg odor) while unearthing the coupons, microbial sulfate-reduction was taking place in some soil horizons at this location. Additional evidence was provided by dark banding in soil layers as shown in Figure 3. Based on the appearance of the wooden stakes, microbial sulfate-reduction was probably restricted to shallower “microaerophilic” soil layers above coupon burial depth.

CONCLUSIONS

Activity reported in the present study may be evidence of scattered microbial populations that survive at depth in microhabitats. Under changing soil conditions, for example with annual water table fluctuations and with incursion of marine influenced groundwater high in sulfate, these isolated types may blossom. This work demonstrates a method for visualizing and quantifying such microbial activity on metal surfaces, even at these very low levels. The techniques are ideally suited for quantification of the progress of potentially detrimental bacterial colonization of surfaces. If combined with methods for enumerating types and numbers of bacteria, the techniques presented here can provide quantitative information for prediction of potential for microbial activity at sites for burial of metal containers, conduits, and wires.

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**TABLE 1
TYPICAL WATER AND SOIL CHEMISTRY FROM STUDY SITE.**

	Nutrient (mg/L)								
	<u>Na</u>	<u>Ca</u>	<u>K</u>	<u>SO₄</u>	<u>Fe</u>	<u>NO₃</u>	<u>Org C</u>	<u>HCO₃</u>	<u>pH</u>
Water	34.1	2.42	1.66	BDL	48.5	0.25	63	BDL	4.84
Soil	44.8	869	160	BDL	1820	BDL	2200	BDL	6.04

BDL – below detection limit

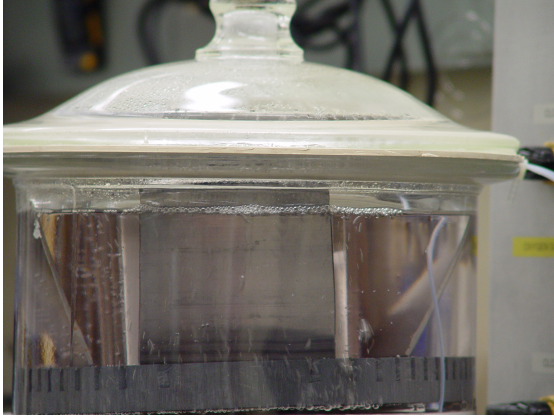
**TABLE 2
BASIC MINIMAL SALTS MEDIUM FOR GROWTH OF *Desulfovibrio thiooxidans***

Component	quantity (g/L)
Sodium chloride	1
Ammonium chloride	1
potassium dibasic phosphate	1
yeast extr.	0.5
Sodium lactate	0.65
Magnesium sulfate heptahydrate	2
Resazurine	0.001
<u>Poising solution (added after completion):</u>	
Thioglycolic acid	0.01
L-ascorbic acid	0.01

**TABLE 3
SUMMARY OF SOIL SULFATE-REDUCTION ACTIVITY ASSOCIATED WITH COUPONS**

Coupon type closest to location of soil sample:	Time points of vials showing high (>0.5 pmol/g sediment/day) or low (<0.5 pmol/g sediment/day) activity (hr)	
	High	Low
55D20/316 SS-sensitized	6, 72	
52D20/301 SS	2, 72	1, 6, 10, 48
53D20/301 SS-sensitized**	1	24
59D20/316 SS	1	6, 48, 72

** Corrosion products This coupon was not imaged with the silver foil technique.



A



B

FIGURE 1 – Positive control coupons

(A) at the start of incubation (B) after 2 weeks

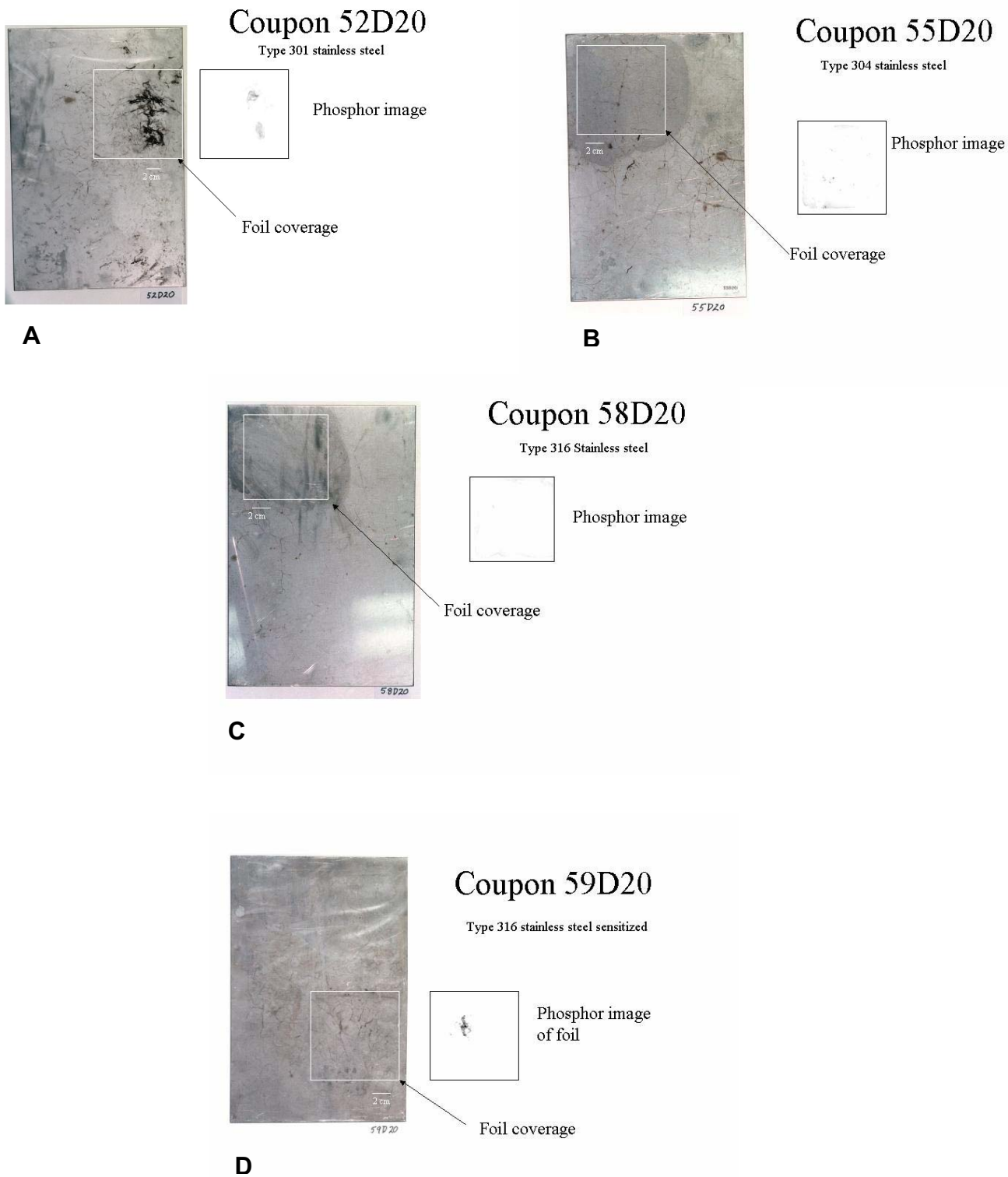


FIGURE 2 - Coupon images and corresponding foil phosphor images

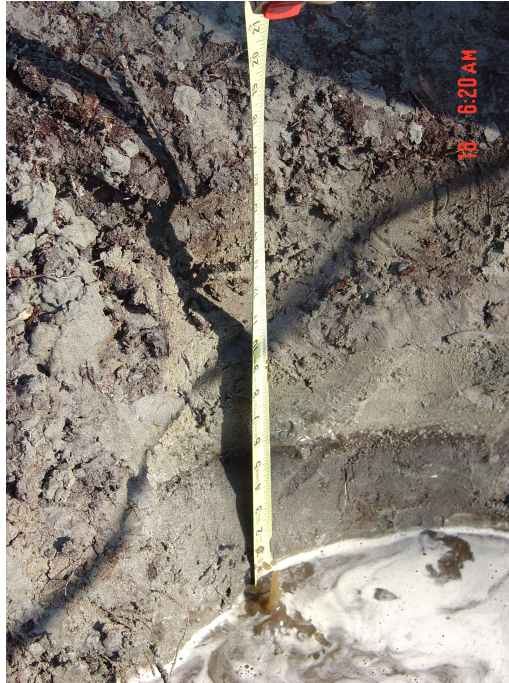


FIGURE 3 - Excavation showing dark banding



FIGURE 4 - Excavated posts showing deterioration