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Quarterly Report

Microbial Recovery of Metals from Spent Coal
Liquefaction Catalysts

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Quarterly Report, January-March 1994

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Summary

Mo release from spent coal liquefaction catalysts has been shown to be dependent upon many parameters, but release is dominated by microbial growth. The microbial Mo release is a rapid process requiring less than one week for 90% of the releaseable Mo to be solubilized from whole washed (THF) catalyst. It could be expected that the rates would be even greater with crushed catalyst. Efforts are now centering on optimizing the parameters that stimulate microbial growth and action.

Project Objective

The project objectives outlined in the previous reports involved defining conditions and cultures best suited to achieve the most effective metal release from spent coal liquefaction catalysts by microbial processes. Various microbiological and physiochemical parameters which may be important in microbial release of metals from spent coal liquefaction catalysts continue to be investigated so that this technology can be optimized.

Introduction

The work initiated in the first quarter of 1993 was continued and expanded using solvent extraction systems defined by the multiple solvent tests used for washing the catalysts. Several of the solvents had desirable characteristics and would provide an extracted test catalyst which would respond well to the leaching ability of the cultures. To reduce the number of solvent systems, the data were examined and tetrahydrofuran (THF) was selected as a representative system following consideration of attributes including cost, ease of handling, and extractive capabilities. In the present simplified extractive system, the catalysts are washed with the solvent with simple agitation to prevent catalyst breakage. This approach of using unbroken catalyst was considered feasible since the controlled breakage and particle sizing would involve extensive grinding and sieving to obtain selective and reproducible samples having similar surface areas. It has been shown that the greater the surface area, the greater and more rapid will be the metals release. Thus, it can be expected and demonstrated that positive results with unbroken catalysts can be enhanced by increasing surface area. Studies on the effect of catalyst surface area will be continued in conjunction with the progressive development of the optimized microbial metal releasing system. Since THF has proven to be a superior solvent system as judged by metal release, research has concentrated on using THF as the solvent system allowing work to proceed rapidly. However, additional work will still be directed toward defining any solvent system superior to THF in terms of aiding microbiological release of metals and economic feasibility. These studies include measurements of metal release at various catalyst-media ratios. The importance of the catalyst-media ratios is assuming a greater importance since it is desired to reduce the media volume to the lowest level possible while still retaining

good metals release. To maintain continuity and demonstrate the rationale for the experiments performed in the last quarter, the results from last report are included herein followed by the results for this quarter starting with test number 20.

Methodology

The solvent washed catalysts were examined in a series of controlled studies of microbial growth curve data versus observed metal release using a carbon source selected from the previously tested multiple fermentative carbon sources. Previous work has shown that many carbon sources can be used to enhance the metals releasing capabilities of the denitrifying cultures. Thus, the selection of the carbon source would be governed by a series of parameters involving cost and availability considerations in addition to increased growth efficiency. The carbon source selected was citrate because it results in microbial growth enhancement and its ability as a chelating agent. The effect of metabolizable chelating sources in the growth media was studied and their impact on the observed molybdenum and nickel release determined. It has been found that chelators (such as citrate and EDTA) will cause initial rapid release of the metals. This chelating effect confers an added advantage to the biological releasing system and is being more fully investigated and in the last quarter two additional chelating agents were tested, nitrilotriacetate (NTA) and diethylenetriaminepentaacetic acid (DTPA).

Multiple microbial metal releasing experiments with a series of comparative growth systems have been established. The basic growth medium (Table 1) was supplemented with various carbon sources and catalyst loadings as well as a trace metal solution (Table 2). All cultures were incubated at 30° C. The growth studies were conducted with THF solvent extracted catalyst and with various concentrations of media additions and include various tests of catalyst-media ratios. Metals released at shorter time periods (every two days) were determined and comparative responses related to microbial growth (turbidity), pH changes, and gas production.

TABLE 1
MEDIUM B

1.0g KH₂PO₄
1.8g Na₂HPO₄
10.0g NH₄NO₃
5.0g Na₃ Citrate
1.0ml SL-4 trace metals (See Table 2)
1L dH₂O

Use for growing bacteria in the presence of catalyst

NOTE: For lactate and isopropanol growth medium, substitute 5g Na Lactate or 5 ml isopropanol for Na Citrate and experiments in the last quarter employed medium B in which the citrate concentration and ammonium nitrate concentration were elevated to 1 and 1.5% respectively.

TABLE 2
SL-4 Trace Metals (500X)

Chemical Component	mg/l
EDTA, di Na salt	500
FeCl ₂ •5H ₂ O	100
MnCl ₂ •4H ₂ O	25
CuCl ₂ •2H ₂ O	10
CoCl ₂ •6H ₂ O	20
NiCl ₂ •6H ₂ O	20
Na ₂ MoO ₄ •2H ₂ O	30
Na ₂ WO ₄	5
Na ₂ SeO ₃	5
H ₃ BO ₃	25

Thermophilic pure cultures have been obtained from the American Type Culture Collection (*Bacillus stearothermophilus* ATCC 12016) and the cultures are being grown at 60°C for initial metal releasing studies. It was later noted that higher levels of catalyst were inhibitory for growth of this thermophile, therefore strain development for tolerance to catalyst is being undertaken. Strain tolerance has not been successfully developed yet. A culture of *Metallosphaera sedula* was obtained from Dr. Thomas Clark (DOE-PETC) in order to determine if this microorganism had the capability of anaerobic growth under denitrifying conditions. We have not been able to grow *Metallosphaera sedula* under denitrifying conditions and have

had difficulties reviving this culture under aerobic conditions. We hope to obtain the thermophilic denitrifying strains FV-A and ITI 382-H-90 from Dr. Jacob Kristjansson (Institute of Biology, University of Iceland) in order to test the metal releasing capabilities of these bacteria isolated from Icelandic hot springs and we will be obtaining a sampling permit from the U.S. Park Service so that we can start enrichment cultures for thermophilic denitrifiers using Yellowstone National Park hot spring algal-bacterial mat material as a source of inocula. In addition, Dr. Pieter Visscher, USGS, Menlo Park, CA has kindly consented to supplying us with a culture of his unique marine denitrifier (ASN-1) which degrades alkyl sulfides so that this organism can be tested for its ability to release metals from spent coal liquefaction catalysts.

All current work will center on development of a low cost process system having optimum metal (molybdenum) releasing capabilities for initiation of preliminary economic evaluation studies.

The effectiveness of the heterotrophic denitrifying bacteria to remove the nickel and molybdenum at high rates and for the system to operate efficiently at small volumes is being further defined to develop a practical and cost effective processing system for coal liquefaction catalysts.

Data from several of the tests conducted since the last quarterly report are summarized below in the text and in some cases are reported in tabular form. Each test was designed to provide insights into several parameters that might be important for metals release and are numbered in chronological order. Tabular data will be presented for molybdenum and nickel release. Except where noted, tests employed 150 ml of medium contained in capped prescription bottles and solubilized metals were analyzed by atomic adsorption after media ultrafiltration to remove cell biomass and catalyst debris.

In this last quarter a new culture transfer/rejuvenation procedure was adopted wherein mixed denitrifying cultures were transferred every 3 to 4 days into fresh medium of a composition which duplicated the culture conditions which would probably be used in future specific tests. This rejuvenation medium always contained 1.5 g of spent THF washed catalyst. This new procedure has led to faster and greater levels of Mo release than has been seen in the past.

Test Results

It was found that initial and rapid metal release was due to the chelating power of the citrate medium followed by increased release due to microbial growth. Metal release by microbial growth was slower and a preferential release of the molybdenum was evident. Tests involving the ability of cultures which have been refortified with rejuvenated nutrient supplementation to release more metals are in progress. In addition, the effects of non chelating carbon sources such as lactate to

enhance microbial growth, but which have no chelating power, are being compared to comparable growth media employing citrate.

Data from a representative, newly initiated experiment (only several days old) is provided in Table 3 below as an illustration of the current efforts to define optimum economic operational parameters.

TABLE 3
Comparative Mo Release in Presence of Isopropanol
and THF Washed Catalysts
% Mo Released After 11 Days

Sample	Isopropanol Washed Shell 324	THF Washed Shell 324
1	18.5	24.6
2A	31.4	48.5
2B	27.2	47.2
3	36.4	62.6
4	33.7	56.8
5	24.1	34.0
6	28.7	40.0
7	27.2	39.7
8	29.0	36.7

Sample

- 1 3.0g cat in 2 H₂O
- 2A 3.0g cat in citrate media
- 2B Same as 2A, but media rejuvenated at day 7
- 3 1.5g cat in citrate media
- 4 5.0g cat in citrate media
- 5 3.0g cat in isopropanol media
- 6 3.0g cat in lactate media
- 7 1.5g cat in thermophilic culture at 60°C
- 8 3.0g cat in thermophilic culture at 60°C

Test 1 Results

Test 1 was designed primarily to determine if the alteration of components of the medium employed (see Table 1), or changing the levels of catalyst treated, affected metals release. It is evident from the data presented in Table 4 that several variables affect the release of Mo from the catalyst (see Table 4 below). In general, the greatest percentage of Mo was released when the catalyst/media ratio was lowest and citrate levels were higher than the medium recipe outlined previously in Table 1. Varying the levels of nitrate in the medium had little effect for the release of Mo

in these cultures and overall best release for Mo and Ni from the catalyst occurred in the culture employing 1.0% citrate and 1.5% nitrate. Bottles were monitored for metals release for up to 24 days after inoculation. While higher percentages of Mo were released at 24 days, the increase in percentage Mo released was never more than 10% higher (data not shown) than after 10 days. Therefore, a 10 day incubation after inoculation was chosen as an appropriate sample time frame for examining incubation parameters.

TABLE 4
Test 1
% Mo Released After 10 Days Under Various Culture Conditions

Sample	Media	Grams Catalyst	% Citrate	% NH ₄ NO ₃	% Mo Released
1	Medium B	1.5	-	-	55
2	Medium B	3.0	-	-	46
3	Medium B	5.0	-	-	43
4	Medium B	10.0	-	-	39
5	Altered Medium B*	3.0	0.25	1.0	38
6	Altered Medium B	3.0	0.75	1.0	47
7	Altered Medium B	3.0	1.0	1.0	47
8	Altered Medium B	3.0	0.5	1.5	43
9	Altered Medium B	3.0	0.5	0.5	43
10	Altered Medium B	3.0	0.25	1.5	34
11	Altered Medium B	3.0	0.25	0.5	38
12	Altered Medium B	3.0	1.0	1.5	47

* Medium B altered by changing levels of catalyst, citrate and NH₃NO₃

Test 2 Results

One parameter investigated in Test 2 was particularly revealing because it showed that (see Table 5 below) more Mo is leached from the spent catalyst as a result of bacterial growth versus an uninoculated blank control. Also, more Mo was released when growth occurred in the presence of nitrate as opposed to the chloride ion suggesting that denitrifying growth promotes Mo release. In general, higher turbidity readings correlated with increased Mo release, whereas, it was surprising that bacterial growth conferred no added Ni release suggesting that Ni release is preferentially influenced chemically. Ni release was no greater for bottles exhibiting good growth and no increase in Ni release was observed after three days (data not shown). These data support the idea that Ni is preferentially chemically leached. These results confirm previous experiments that Mo release paralleled bacterial growth, and the majority of the Mo release occurred within 10 days.

TABLE 5
Test 2
Comparison of Metal Release and Microbial Growth
% Mo and Ni Released After 10 Days* and Turbidity

<u>Sample</u>	<u>Media</u>	<u>Aerobic/ Anaerobic</u>	<u>% Mo Release</u>	<u>% Ni Release</u>	<u>Turbidity</u>
1	Citrate B	Aerobic	47.9	13.1	0.72
2	Citrate B - Uninoculated	Aerobic	30.5	29.1	0.04
3	Lactate B	Aerobic	19.0	3.2	0.15
4	Lactate B - Uninoculated	Aerobic	30.0	11.6	0.02
5	Water Blank	Aerobic	28.3	11.6	0.01
6	Citrate B NH ₄ Cl (as N Source)	Aerobic	27.9	13.3	0.36
7	Citrate B NH ₄ Cl - Uninoculated	Aerobic	37.4	27.6	0.04
8	Citrate B	Anaerobic	46.0	14.5	0.36
9	Citrate B - Uninoculated	Anaerobic	28.0	27.5	0.04
10	Lactate B	Anaerobic	29.7	12.9	0.11
11	Lactate B - Uninoculated	Anaerobic	29.6	11.5	0.05
12	Water Blank	Anaerobic	30.1	11.6	0.05
13	Citrate B NH ₄ Cl (as N Source)	Anaerobic	30.7	13.8	0.24
14	Citrate B 0.25% Citrate	Anaerobic	39.6	13.5	0.44
15	Citrate B 0.25% Citrate Uninoculated	Anaerobic	37.4	11.9	0.04
16	Citrate B 0.75% Citrate	Anaerobic	38.8	27.3	0.78
17	Citrate B 0.75% Citrate Uninoculated	Anaerobic	29.1	31.3	0.06
18	Citrate B 1.0% Citrate	Anaerobic	29.4	32.4	0.50
19	Citrate B 1.0% Citrate Uninoculated	Anaerobic	19.6	29.1	0.04

* All bottles received 150 ml media or water and 1.5 grams catalyst

Test 3 and 4

Test 3 and 4 confirmed the findings in test 2 that all Ni release occurred by day three and in some cases uninoculated controls exhibited more Ni in solution than in bottles where good growth had occurred (data not shown). It is possible that more Ni was released from the spent catalyst in cases of good growth, but was not revealed in our analysis procedure because some of the solubilized Ni might have been incorporated into bacterial enzyme complexes. Experiments are now in progress to investigate this possibility and preliminary results indicate that the Ni is associated with the cell biomass.

Test 5

In this experiment, the thermophilic culture *B. stearothermophilus* (ATCC 12016) was grown at 60° C in a simple defined minimal salts medium and no Mo or Ni release above an uninoculated control could be demonstrated. This minimal salts medium contained MgSO₄ such that, during growth, the sulfate instead of the metal sulfides were used as a source of nutritional sulfur. It was noted previously that higher levels of the spent catalyst were toxic for growth of *B. stearothermophilus* in the absence of MgSO₄ and attempts are currently underway to develop catalyst tolerant strains. While a previous experiment did show thermophilic metals release (Table 3), that experiment was conducted in a rich undefined medium and was not compared to a parallel uninoculated control.

Test 6

The experiments in test 6 were designed to reconfirm the differences in the kinetics of metals release between Mo and Ni as well as to compare release between aerobically and anaerobically grown cultures (Table 6). Test 6 also showed the metal releasing capabilities of enrichment cultures developed in this laboratory. These enrichment cultures were started using soil from near our laboratory as a source of enrichment inoculum. The cultures were enriched using citrate B as the enrichment medium and reduced metal sulfides (Cu, Mo Fe and Ni sulfides) as the only source of nutritional sulfur. Earlier test were confirmed that crushed catalyst gave the greatest percentage Mo release. Also, while Mo release continued with time, levels of solubilized Ni actually decreased with time especially in aerobically grown cultures

TABLE 6
% Mo and Ni Released After 4 and 7 Days

<u>Sample</u>	<u>Inocula</u>	<u>Conditions</u>	<u>Mo 3 Days</u>	<u>Mo 7 Days</u>	<u>Ni 3 Days</u>	<u>Ni 7 Days</u>
1	Mixed Denitrifiers	Capped +	10.7	19.2	10.0	4.4
2	Uninoculated	Capped +	4.2	18.2	2.4	16.3
3	Mixed Denitrifiers	Capped + Crushed Catalyst	34.3	49.0	21.4	18.2
4	Mixed Denitrifiers	Anaerobic	28.2	33.6	9.3	7.3
5	Mixed Denitrifiers	Aerobic / Not Agitated	23.6	37.8	9.2	0.4
6	Enrichment Culture	Anaerobic	28.0	33.2	20.8	16.8
7	Enrichment Culture	Aerobic	27.8	40.3	20.3	5.9

* All bottles received 150 ml liquid, 1.5 grams catalyst.

+ Bottle closed but not air tight.

Test 7

Experiments conducted in test 7 investigated some further medium parameters that were thought might influence metals release (Table 7). The standard medium, citrate B (see Table 1) was modified to contain different levels of citrate, nitrate, or a medium component not normally present, EDTA. Again it can be seen that growth (versus uninoculated controls) generally promoted good Mo release, and the presence of EDTA or higher levels of citrate showed higher levels of solubilized Ni after ten days of incubation.

TABLE 7
Effect of Media Composition and Chelating Agents on Metal Release
% Mo and Ni Released After 10 Days

<u>Sample</u>	<u>Conditions</u> ^o	<u>Metal Release</u>	
		<u>% Mo</u>	<u>% Ni</u>
1	Citrate B ⁺ 0.1% EDTA Combined Denitrifiers *	32.3	32.7
2	0.1% EDTA only - NO Inoculum	5.1	35.9
3	1.0% Citrate, 1.5% NO ₃ Combined Denitrifiers	31.6	32.2
4	1.0% Citrate 1.5% NO ₃ No Inoculum	14.3	36.2
5	Like Condition 3, but 0.1% EDTA	31.6	35.6
6	Like Condition 4, but 0.1% EDTA	13.2	33.0
7	Citrate B, but 0.25% Citrate	27.5	14.0
8	Like Condition 7, but No Inoculum	26.7	13.9
9	Citrate B, but 0.75% Citrate	35.8	13.2
10	Like Condition 9, but No Inoculum	17.9	35.4
11	Citrate B, but 1.0% Citrate	32.0	31.1
12	Like Condition 11, but No Inoculum	13.4	35.4

^o All bottles capped (facultatively anaerobic).

⁺ See Table 1.

* Previous denitrifiers combined with enrichment cultures.

Test 8

Experiments were conducted in test 8 to determine the abiotic release of Mo and Ni under conditions of no microbial growth, but carried out in Citrate Bm medium testing pH regimes from pH 5 to 9 (Table 8). This test demonstrated greater levels of Mo and Ni release correlated with more acidic conditions or lower pH. However, this pH test is currently being repeated in water only because preliminary findings from a currently run nitric acid leach indicate that the citrate present in the citrate Bm medium used in this test probably contributed to metals release.

Test 9

Test 9 was conducted to determine if varying levels of citrate at constant pH contributed to abiotic metals release. Generally, it appeared that higher levels of citrate led to greater Ni release, but led to a much greater decrease in Mo release. This latter result, the decrease in Mo release with increasing citrate levels, can not presently be adequately explained.

TABLE 8
Effect of pH on Abiotic^a Metals Release
% Mo and Ni Released After 21 Days

		Metal Release	
Sample	pH	% Mo	% Ni
1	5	51.36	72.12
2	6	45.9	72.61
3	7	39.4	59.3
4	8	42.2	57.55
5	9	40.0	41.3

- a Test was conducted in standard Citrate Bm Medium with azide added to prevent growth

TABLE 9
Effect of Varying Citrate Levels on Abiotic^a Metal Release
% Mo and Ni Released After 21 Days

		Metal Release	
Sample	% Citrate	% Mo	% Ni
1	0.5	40.0	63.0
2	1.0	30.0	67.0
3	2.5	10.0	68.0
4	5.0	10.0	68.5
5	10.0	15.0	69.0

- a Test was conducted in standard Citrate Bm with varying levels of citrate and azide added to prevent growth

Test 10

Test 10 was conducted to determine if sulfate (as a sulfur source in the medium), and three days pregrowth had an effect on metals release from spent, THF washed Shell 324 catalyst. A very interesting result (which has been noted

previously) was found for Ni release in conditions of microbial growth versus conditions of no microbial growth due to the inhibitory effects of azide. In looking at the data (see Table 10), it would appear that either azide promotes Ni release or that microbial growth inhibits Ni release as measured on day 21. However, earlier measurements (data not shown) at day 2, 5, 7 etc. indicated a rapid Ni release followed by a gradual decline in measured levels of Ni to the levels seen at day 21 shown in Table 10. This same phenomenon has been seen in prior tests also. We are currently running some mass balance studies to determine the fate of the released Ni in systems where microbial growth occurs. One possible explanation of the Ni disappearance involves Ni uptake by the denitrifiers or cell wall sorption such that upon sample sub-micron filtration prior to sample analysis, the Ni is retained by the filter. This is currently being investigated. Again it can be seen that greater Mo release occurs in cases of microbial growth, and, in this experiment, higher levels of citrate led to greater levels of Mo release in both growth and non growth conditions. No conclusions about pre-growth or the effect of sulfate on metals release can be made at this time and further experiments are being planned to address these issues.

Test 11

Since it is now known that denitrification can occur in the presence of oxygen and that denitrification can occur at the same time as aerobic respiration, an experiment was set up wherein air was not actively excluded (i.e. uncapped) from the culture vessel. This test was conducted to determine if oxygen had an effect on metals release. The data show (see Table 11) that after 21 days 92.6 % of the Mo originally on the spent, THF washed Shell 324 was released under conditions where air was not excluded from the culture vessel. This is the highest level of Mo release noted to date for uncrushed catalyst. All cultures in this test were inoculated with our combined, mixed heterotrophic denitrifiers. Again, as noted earlier above, the percentage Ni released shown at day 21 is not a true representation of the Ni removed from the catalyst. As seen repeatedly in past experiments, and also seen in this test, microbial growth promoted greater Mo release than non-growth controls containing azide. Catalyst from sample #1 from this test as well as catalyst from some of the previous tests reported above was saved for media rejuvenation experiments reported below.

TABLE 10
Effect of Sulfate and 3 Days Pregrowth^a on Metal Release
% Mo and Ni Released After 21 Days

Sample	Condition	Metal Release	
		% Mo	% Ni
1	Citrate A ^b	51.67	14.03
2	Citrate A & Azide	41.0	43.0
3	Double Citrate A ^c	62.87	38.06
4	Double Citrate A & Azide	59.0	63.0

- a Samples 1 and 3 were grown for 3 days prior to 1.5g of catalyst being introduced. Samples 2 and 4 were also started at that time.
- b Citrate A is the same medium as Citrate Bm but supplemented with 0.5% Na₂SO₄.
- c Double Citrate A contained twice the level of all media components as Citrate A.

TABLE 11
Effect of Aerobic Versus Facultatively Grown Cultures on Metal Release
% Mo and Ni Released After 21 Days

Sample	Condition	Metal Release	
		% Mo	% Ni
1	Aerobic ^a	92.6	15.42
2	Aerobic & Azide	36.0	60.8
3	Facultative ^b	68.3	66.3
4	Facultative & Azide	52.0	65.2

- a 150 ml of Citrate Bm in 1 liter Erlenmeyer flasks, no closure, no agitation.
- b 150 ml of Citrate Bm in 200 ml bottles, loosely capped.

Test 12

Test 12 made use of catalyst previously subjected to experiments to test metals release and these catalyst samples were then tested for additional metals release after old media had been decanted and fresh media added. Table 12 identifies the samples from previous tests that were used by test number (in parentheses) followed by specific sample number. All samples received new Citrate Bm medium and were grown facultatively. Column 2 (First) represents metals release after the first medium rejuvenation measured after 3 days and column 3 (Second) represents metals release measured 13 days after a second round of medium rejuvenation.

Some very interesting and notable findings resulted from this test. Most importantly, it can be seen (Table 12) that 100% of the Mo was released from the spent catalyst for sample number 6. This is the first time we have released all of the Mo from whole uncrushed spent catalyst. It is also notable that decreasing levels of Mo were released after each rejuvenation and poor growth occurred during the final rejuvenation suggesting a limiting growth factor. After the second rejuvenation and after final measurements of metals release were made, these cultures were used to inoculate Citrate Bm subcultures that contained thiosulfate whereupon good growth occurred suggesting that during the second rejuvenation the cultures had become starved for a source of nutritional sulfur.

Test 13

It had been hypothesized that better Mo release might occur if the THF washed catalyst could be pretreated with a good chelating agent to remove Ni, a metal which is known to quite toxic to some microorganisms. In order to test this idea samples of spent THF washed catalyst were pretreated with either nitrilotriacetic acid (NTA) or Diethylenetriaminepentaacetic acid, two good chelating agents. While the chelating agents appeared to promote Ni release (See Table 13), a generally poor release of Mo occurred, and this was attributed to the poor growth that occurred in this test which might have been caused by the toxicity of any remaining chelating agent. It is notable that sample number 1, which served as a control and had received no pretreatment with chelating agents, exhibited the best Mo release in this test. The above hypothesis will again be tested in the future, but a different means of Ni removal will be employed.

TABLE 12
Effect of Selective Media Rejuvenations on Metal Release
% Mo and Ni Release

Sample	Condition ^a	Metal Release					
		Initial ^b		First ^c		Second ^d	
		% Mo	% Ni	% Mo	% Ni	% Mo	% Ni
1	(8) 1	51.36	72.12	66.43	81.89	73.8	85.53
2	(8) 2	45.9	72.61	63.71	83.25	68.77	86.1
3	(8) 4	42.2	57.55	58.53	71.28	61.62	73.85
4	(10) 1	51.67	14.03	64.84	45.33	69.25	46.75
5	(10) 3	62.87	38.06	73.72	56.62	77.55	64.28
6	(11) 1	92.64	15.42	100.77	28.65	N ^e	NT
7	Control ^f	---	---	41.47	51.66	50.86	61.67

- ^a Supernatant from previous tests (8,10,11) poured off and 150 ml new citrate B (only) added. Condition = test number followed by that specific test sample number.
- ^b % Metal Release after 21 days in tests 8, 10, 11.
- ^c % Total Metal Release after 1st media rejuvenation after 3 days.
- ^d % Total Metal Release after 2nd media rejuvenation 13 days after 2nd rejuvenation.
- ^e NT reading not taken as 100% Mo release had already occurred.
- ^f Control started at time zero start of test 12.

TABLE 13
Effect of Chelators on Metal Release
% Mo and Ni Released After 8 and 16 Days

Sample	Condition a	Condition b	<u>8^a Day Release</u>		<u>16^b Day Release</u>	
			% Mo	% Ni	% Mo	% Ni
1	H ₂ O Only	Citrate Bm	17.0	21.0	34.0	41.0
2	H ₂ O & DTPA	Citrate Bm	11.0	41.2	21.0	49.0
3	H ₂ O & NTA	Citrate Bm	9.0	49.0	20.0	54.0
4	Citrate Bm Only	Citrate Bm	21.0	44.0	30.0	53.0
5	Citrate Bm & DTPA	Citrate Bm	14.0	50.0	20.0	54.0
6	Citrate Bm NTA	Citrate Bm	16.0	53.0	21.0	57.0

- a 1st 8 days no inoculum, DTPA and NTA at 1.0% concentration. Only Sample 4 received azide.
- b At day 8, supernatants were decanted and all samples rejuvenated with 150ml fresh, sterile Citrate Bm and inoculated with mixed denitrifiers. Metal release read after 8 more days.

Test 14

The high levels of Mo release seen in one condition in test 11 needed to be reconfirmed so test 14 was conducted to determine if we could duplicate those results. Again, it can be seen (Table 14) that the greatest Mo release occurred in the culture condition where air was not actively excluded. Interestingly, more Mo release occurred in the aerobic azide control (no growth) than in sample 3 which was grown facultatively suggesting that not microbial growth contributes to Mo release, but also that air somehow contributes to Mo release. As noted above previously, the low levels of released Ni measured at day 7 probably do not reflect the true amount of Ni released from the spent catalyst.

TABLE 14
Effect of Aerobic Versus Facultatively Conditions on Metal Release
% Mo and Ni Released After 7 Days

Sample	Condition	Metal Release	
		% Mo	% Ni
1	Aerobic ^a	71.0	6.81
2	Aerobic & Azide	57.55	64.63
3	Facultative ^b	44.34	46.60
4	Facultative & Azide	22.56	56.22

- a 150 ml of Citrate Bm in 0.5L Erlenmeyer flasks, no closure, no agitation.
- b 150 ml of Citrate Bm in 200 ml bottles, loosely capped.

Test 15

Test 15 was conducted to try and relate growth conditions, pH, turbidity and different sets of inocula to metals release. Table 15 gives tabular results for Mo release at day 7 and day 14 and tabular results for Ni release at day 7 only. Figures 1 through 9 give a more detailed graphic view showing measurements made for Mo release, pH and turbidity for each sample condition. Interestingly, there was not a great deal of difference in the measured levels of Mo release between the different sample conditions except for the no growth azide controls. Generally, Mo release lagged behind growth (turbidity) and the pH increased over time except for the "N" series. It is not known why the absence of active air exclusion (aerobic series) did not lead to greater Mo release in this experiment.

TABLE 15
Effect of Different Inocula and Culture Conditions on Metal Release
% Mo and Ni Release

Sample ^a	Condition	Day 7		Day 14	
		% Mo	% Ni	% Mo	% Ni
1A	Aerobic ^b	54.22	24.69	54.67	ND*
2A	Aerobic ^b	54.47	24.49	54.29	ND
3A	Aerobic ^b	56.05	39.59	58.83	ND
4A	Aerobic ^b	57.67	30.52	56.53	ND
5A	Aerobic ^b	56.35	27.92	56.50	ND
6A	Aerobic ^b	28.73	43.56	29.95	ND
1F	Facultative ^c	49.52	29.04	55.77	ND
2F	Facultative ^c	50.31	30.33	59.86	ND
3F	Facultative ^c	43.97	35.51	54.63	ND
4F	Facultative ^c	52.13	30.30	56.08	ND
5F	Facultative ^c	48.65	32.74	49.91	ND
6F	Facultative ^c	24.49	38.09	24.95	ND
1N	Anaerobic ^d	43.80	42.08	52.20	ND
2N	Anaerobic ^d	38.60	39.92	47.28	ND
3N	Anaerobic ^d	40.15	39.75	51.83	ND
4N	Anaerobic ^d	42.47	39.89	50.29	ND
5N	Anaerobic ^d	39.94	39.61	49.43	ND
6N	Anaerobic ^d	25.98	37.92	35.32	ND

- ^a All sample "1" inoculated newly enriched mixed denitrifiers.
Sample "2" inoculated with a mix of all heterotrophic denitrifiers.
Sample "3" inoculated with old, stored heterotrophic denitrifiers.
Sample "4" as Sample "1", but pregrown on a spent catalyst other than shell 324.
Sample "5" Inoculum was equal mix of all of above.
Sample "6" No inoculum, azide control.
- ^b 150 ml of Citrate Bm in 250 ml Erlenmeyer flasks, no closure, no agitation.
- ^c 150 ml of Citrate Bm in 200 ml bottles, loosely capped.
- ^d 150 ml of Citrate Bm in 200 ml bottles, prepared in an anaerobic chamber and tightly sealed.
- * Not Determined

Test 16

This test was conducted to determine if the mixed heterotrophic denitrifiers could grow in the presence of unwashed, spent Shell 324 and promote metals release. Interesting, all the cultures grew well, but Mo and Ni release was very poor (data not shown). A possible explanation for this is provided below.

Test 17

Test 17 was designed to help define the inhibitory effects of coal liquids on Mo release as test 16 had shown very poor metals release from unwashed catalyst. Sample conditions compared a control (Sample 1-Table16) which was THF washed catalyst inoculated with mixed, heterotrophic denitrifiers incubated facultatively to various other conditions. As has been noted many times previously, microbial growth (Sample 1) showed much greater Mo release than the no growth azide control (Sample 2). Interestingly, a culture vessel which had added coal liquids (Sample 3) showed relatively good Mo release even though the amount of coal liquids added was more than would contained in a culture vessel having unwashed catalyst which was done in parallel sample number 6. Therefore, while it appears that coal liquids added to THF washed catalyst slightly inhibited Mo release, coal liquids already on or within spent catalyst greatly inhibited Mo release.

Test 18

Test 18 was designed to determine if delayed inoculation along with media rejuvenation affected Mo release. This test again showed that microbial growth promoted Mo release over no growth controls and suggested that delayed inoculation led to less Mo release (Table 17).

Table 16
Effect of Added Coal Liquids on Metal Release
% Mo Released After 14 Days

Sample	Condition ^a	% Mo Release
1	Control (with denitrifier growth)	49.55
2	Azide	25.62
3	0.1 ml Coal Liquids	40.56
4	0.1 ml Coal Liquids-No Added Catalyst	0.81
5	No Citrate	6.73
6	Non THF Washed Catalyst	6.78
7	Non THF Washed Catalyst & Azide	7.05

- ^a All bottles contained 150ml Citrate Bm with 5 ml inoculum of combined mixed heterotrophic denitrifiers, and 1.5g THF washed catalyst (except for sample 6 & 7 which had ion washed catalyst). Sample 4 contained no catalyst.

Table 17
Effect of Media Rejuvenation and Delayed Inoculation on Metal Release
% Mo Released After 14 Days

Sample	Condition ^a	Metal Release % Mo
1	Control (with denitrifier growth)	52.49
2	Reinoculated & New Media After 2 Days	43.17
3	Not Inoculated & Azide	24.97
4	Inoculated After 2 Days & New Media After 2 Days	40.09
5	Never Inoculated & New Media After 2 Days	6.73

- ^a All bottles contained 150ml Citrate Bm and 1.5g THF Washed Catalyst.

Test 19

It had been suggested the no growth conditions achieved by adding azide might lead to erroneous levels of Mo released by some unknown chemical interaction of the catalyst and the azide. Therefore, test 19 was performed to determine if another means of achieving sterility, autoclaving, could be used for no growth controls. It can be seen (Table 18) that unautoclaved catalyst released more Mo than catalyst which had been previously autoclaved and slightly less Mo was released in the autoclaved azide control compared to the unautoclaved azide control.

Table 18
Effect of Autoclaving Catalyst on Metal Release
% Mo Released After 14 Days

Sample	Condition ^a	Metal Release % Mo
1	Autoclaved, Cooled, Inoculated	43.05
2	Autoclaved, Cooled, Inoculated & Azide	25.37
3	Unautoclaved, Inoculated	51.83
4	Unautoclaved, Inoculated & Azide	26.36

^a All bottles contained 150ml Citrate Bm, 1.5g THF Washed Catalyst. All were inoculated with 5 ml of combined, mixed, heterotrophic denitrifiers.

Test 20

Test 20 was designed to determine if microbial growth per se is a requisite condition for solubilization of Mo and Ni from spent Shell 324 catalyst. Controls were compared to conditions where cells had been pregrown in the presence of catalyst or without catalyst plus thiosulfate and the resulting turbid supernatant was ultra filtered to render it cell free and challenged with fresh catalyst. Table 19 (below) shows the results of this experiment. As has been noted numerous times in past work Mo release is promoted by microbial growth and cell-free supernatant has the ability to release metals. However, while pregrowth on thiosulfate had little effect on nickel release, it appeared that pregrowth on catalyst resulted in a cell-free supernatant that released Mo better than the corresponding thiosulfate pregrowth supernatant. It again appeared that microbial growth (sample 4) retarded Ni release as noted in past work, but this was later defined in mass balance experiments as a redeposition of solubilized Ni back onto the catalyst matrix.

Table 19
Activity of Cell-Free Supernatant for Metal Release
% Mo and Ni Released After 13 Days

Sample	Condition	% Mo Release	% Ni release
1	pregrown thiosulfate	37.23	32.88
2	pregrown catalyst	53.64	36.82
3	control azide	22.30	45.11
4	control with growth	45.82	11.35

Test 21

Test 21 further investigated the inhibitory effects of thiosulfate on metals release during microbial growth in the presence of catalyst as well as an additional test of metals release on crushed versus whole catalyst. After 8 days of growth, crushed and whole catalyst yielded 70% and 38% Mo release respectively. In the presence of thiosulfate, Mo release was retarded by about 10%. Interestingly, thiosulfate appeared to have little effect on Ni release and Ni redeposition on the catalyst matrix (to be discussed later) did not occur using crushed catalyst (see Table 20 below).

Table 20
Effect of Thiosulfate on Metals Release
% Metals Release After 8 Days of Growth

Sample	Condition	% Mo Release	% Ni release
1	Control	44.63	9.44
2	Azide Control	19.90	40.10
3	0.5% Thiosulfate(TS)	33.09	28.50
4	0.5% TS + Azide	23.74	37.24
5	1.5% TS	32.89	24.19
6	1.5% TS +Azide	26.83	33.63
7	Crushed	70.27	55.54
8	Crushed + Azide	37.87	57.75

Test 22

It was reasoned that better biosolubilization of metals by microbial growth might occur if the THF Shell 324 catalyst was pretreated by nitric acid because the acid per se might leach some metals as well as alter the oxidation state of the metals in the catalyst matrix leading to better metals release during subsequent microbial growth. In this initial test, better leaching appeared to occur (data not shown), but the data was hard to interpret as the nitric acid solubilized a great deal of the aluminum support matrix which led to interference in Mo release measurements. A method for accurately measuring Mo release despite the presence of aluminum was developed and this experiment was later repeated (see below).

Test 23

Test 23 was a mass balance experiment that measured levels of Mo and Ni released after 7 days of microbial growth, either aerobically or facultatively, and also measured metals retained on the catalyst in an effort to account for all the metals. No metals were detected that might have been retained with cell biomass after micro filtration of sample aliquots prior to AA analysis. An Inorganic Ventures procedure was used to analyze the filters to prepare samples of culture supernatants for testing and was also used to analyze total metals content of the catalyst after the 7 days of microbial growth. It can be seen (Table 21) that in all cases close to 100% of the metals could be accounted for. It had been noted in the past that during the course of an experiment, solubilized levels of Ni in the supernatant rose then declined during the period of microbial growth. From the data gathered in this experiment, it now appears that solubilized Ni is redeposited upon the catalyst matrix during the course of microbial growth.

Table 21
% Metals Solubilized or Retained
in Catalyst After 7 Days of growth

Sample	Condition	% Mo		% Ni	
		Released	On Catalyst	Released	On Catalyst
1	Aerobic	27.77	71.72	16.27	80.40
2	Aerobic + Azide	14.76	79.15	61.73	45.64
3	Facultative	27.64	71.62	25.75	68.82
4	Facultative + Azide	12.82	78.75	48.16	52.83

Test 24

Since it had been previously noted in certain experiments that more Mo was solubilized in growing cultures where air was not actively excluded (aerobic conditions), an experiment was performed to compare Mo and Ni release using cultures that were grown facultatively, aerobically and aerobically with active aeration. It can be seen (Table 22) that active aeration seemed to retard Mo release and much lower levels of solubilized Ni were present in the supernatant of the actively aerated culture analyzed at day 8. pH data is also included for this experiment (Table 23) and to illustrate the Ni redeposition phenomenon (discussed above) Ni analyses in ppm are provided for days 3, 6, and 8 of microbial growth (Table 24).

Table 22
Effect of Active Aeration on Metals Released
% Metals Released on Day 8

Sample	Condition	% Mo Released	% Ni Released
1	Facultative	50.41	36.15
2	Facultative + Azide	24.07	34.18
3	Aerobic	52.25	16.11
4	Aerobic + Azide	23.11	38.56
5	Aerated	41.02	4.40
6	Aerated + Azide	29.61	51.99

Table 23
pH Data for Test 24

Sample	Day 0	Day 3	Day 6	Day 8
1	6.89	6.95	7.04	7.28
2	6.89	6.99	7.08	7.16
3	6.89	7.20	7.55	7.12
4	6.89	6.97	7.05	7.10
5	6.89	8.65	7.54	7.12
6	6.89	6.95	7.05	7.00

Table 24
Solubilized Ni in ppm

Sample	Day 3	Day 6	Day 8
1	59	80	88
2	52	57	84
3	68	78	39
4	64	87	105
5	58	19	16
6	90	133	176

Test 25

Test 25 was essentially a rejuvenation experiment wherein catalyst was subjected to two rounds of microbial growth; however this test employed unwashed Shell 324 catalyst. Unwashed catalyst was used because it was thought that microbial action in the first round of growth might remove enough of the coal liquids to allow biosolubilization of the Mo and Ni during the subsequent round of growth where the spent medium was decanted and fresh media added. Both citrate Bm medium and a citrate Bm medium with low citrate levels (0.25%) were employed, the idea being that limited carbon levels would increase the pressure on the microbial consortium to utilize the coal liquids as a carbon source. There were no marked differences in metals release between these two incubation conditions (data not shown) and metals release was generally less than 10% total after both rounds of growth. It appears that coal liquids removal is essential for subsequent microbial biosolubilization.

Test 26

Test 26 was set up to study the effect of pH alone in sterile citrate Bm media as it had been noted (see Test 24 above) that the pH of the cultures varies with time. A variety pH regimes were tested and pH was found to affect Ni release, but different pH regimes had little if no affect upon Mo release (see Table 25 below).

Table 25
Effect of pH on % Metals Release

pH	% Mo Release	% Ni Release
4	22.60	16.79
5	22.86	14.64
6	23.90	13.35
7	26.87	7.75
8	24.93	1.25

Test 27

Test 27 was a quite an extensive test designed to determine if a Ni or Mo releasing "factor" is produced during microbial growth, and, if so, would it function as a metal releasing agent in the absence of cells. This test also investigated if the putative metal releasing factor (MRF) is induced and produced in the culture to a greater extent if cells are grown in the presence of catalyst without an added sulfur source versus cells grown without catalyst with thiosulfate as a sulfur source. Except for the one control (sample A), all the metals release in this experiment was mediated by cell-free supernatants and secondary growth was precluded in the already grown cultures by centrifugation to obtain the cell-free supernatant, ultra microfiltration of the supernatant, and finally the addition of sodium azide. All experiments utilized citrate Bm medium. The following key provides the conditions of the 11 samples listed in Table 26 (see below). Metals release was followed daily for 3 days after challenging supernatant with catalyst.

Key

<u>Sample</u>	<u>Pre-Test Growth Conditions</u>
1, 2, 3	Grown for 2, 3, and 4 days respectively with thiosulfate, no catalyst present ^a
4, 5, 6	Grown for 2, 3, and 4 days respectively with catalyst ^b
7, 8, 9	Grown for 2, 3, and 4 days respectively with catalyst ^c
A	Positive control, i.e. regular microbial growth
B	Negative control, i.e. inoculated but no growth due to azide addition
a	Supernatant challenged with fresh THF washed catalyst
b	Supernatant challenged with catalyst with which the cells had been grown
c	Supernatant challenged with fresh THF washed catalyst

Table 26
Test for Metals Releasing Factor

Sample	Mo ppm				% Total		Ni ppm		% Total	
	Day 1	Day 2	Day 3	Day 3	Day 1	Day 2	Day 2	Day 3	Day 3	Day 3
1	192	209	198	24.77	35	45	54	22.11		
2	209	225	235	29.23	33	41	48	19.69		
3	79	119	225	27.08	17	21	40	16.08		
4	127	142	181	22.24	49	51	57	23.56		
5	186	235	243	27.12	60	68	72	29.33		
6	248	257	260	32.44	63	68	71	29.43		
7	239	247	352	29.14	82	91	98	18.56		
8	260	436	443	36.24	90	95	102	15.11		
9	302	359	381	25.89	74	84	87	8.04		
A	63	95	186	22.36	39	48	53	21.82		
B	61	74	99	12.11	44	60	66	27.11		

It is important to note that both Mo and Ni contained in the cell-free supernatants that were released by the pre-growth prior to challenging the supernatant with catalyst have been accounted for (subtracted) so that the values shown in the columns in Table 26 represent additional metals release. The results suggest that the cell-free supernatant contains a factor, (a metal releasing factor) MRF, that biosolubilizes Ni and especially Mo within a very short time frame (24 hours) as evidenced by comparing samples 1-9 to control samples. Work is ongoing in an effort to characterize/identify this factor.

Test 28

Test 28 was a simple straightforward test designed to determine if the mixed heterotrophic denitrifying cultures we were using routinely relied on the reduced sulfur present in the catalyst matrix as a nutritional sulfur source. Good growth occurred in citrate Bm medium if the catalyst was present whereas very poor growth occurred in citrate Bm medium in the absence of catalyst; however good growth could later be induced in this later condition by the addition of thiosulfate alone suggesting that the cultures were relying upon the catalyst as a source of nutritional sulfur.

Test 29

Test 29 was a repeat of test 27 using crushed Shell 324 catalyst and again cell-free supernatant released all of the biosolubilized metals within 24 hours. Sample 1 was crushed catalyst and sample 2 employed whole catalyst (see Table 27).

Table 27
Effect of Supernatant on Crushed Versus Whole Catalyst

Sample	ppm Mo			ppm Ni		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
1	738	589	589	194	192	200
2	561	430	453	143	142	142

Test 30

Test 30 tested the idea of pretreating the catalyst for 3 days with 1 molar nitric acid prior to biosolubilization by microbial growth. It was expected that the acid treatment would chemically leach a considerable amount of Ni and might alter the Mo oxidation state of the Mo remaining in the catalyst matrix leading to better biosolubilization. After three days of nitric acid treatment about 75% of the Ni and 25% of the Mo was removed from the catalyst matrix whereas much lower levels were removed in water controls (data not shown). However, after pre-treatment and 8 days of microbial growth up to 90% of the Ni and 100% of the Mo had been solubilized (see Table 28 below).

Table 28
Effect of Nitric Acid Pre-Treatment

Sample	Condition ^a	% Release Mo	% Release Ni
1	H ₂ O	47.78	34.46
2	H ₂ O + Azide	25.21	37.85
3	Acid	98.84	90.51
4	Acid + Thiosulfate	118.16	83.91

^a Catalyst was either soaked in water or 1 M nitric acid for three days and the acid or water poured off and replaced with citrate Bm medium and inoculated with mixed denitrifiers and incubated for 8 days.

Test 31

Since nitrite is transiently produced by denitrifying bacteria respiring with nitrate, it was thought that nitrite could be the metals releasing factor (MRF) discussed above. A straight chemical test comparing nitrate to nitrite showed that nitrite is not the MRF discussed above (data not shown).

Test 32

Test 32 was a straight chemical test designed to determine if various levels of the sodium ion had different effects upon Mo and Ni release. High levels of the sodium ion appear to retard Ni release, but had little effect upon Mo release (data not shown)

Test 33

Test 33 was a repeat of Test 27, the cell-free supernatant experiment, except it involved only two conditions. One condition tested supernatant along with added nitrite (1%), while the second condition tested supernatant alone (sample 2). Some differences can be noted and this experiment may have to be repeated. See Table 29 below for results.

Table 29
Effect of Supernatant Plus Added Nitrite

	ppm Mo			ppm Ni			% Mo Release	% Ni Release
Sample	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 3	Day 3
1	517	742	784	125	156	167	50.28	28.08
2	517	698	687	66	83	151	39.4	19.08

Future Research

The release of Mo is dependent upon many parameters, but is dominated by microbial growth. Such microbial Mo release is a rapid process usually requiring less than 1 week for 90% of the releasable Mo to be solubilized. Efforts will center on optimizing the parameters that stimulate microbial growth and action. We will continue to pursue those medium components and incubation conditions which best promote the microbial release of Mo. Since Ni is toxic to many microorganisms, we will investigate the possibility of promoting Mo release microbially by first leaching Ni (using leaching techniques other than chemical chelation) and then subjecting the preleached catalyst to microbial Mo release. Also we will be performing experiments to determine if the products of microbial growth rather than microbial growth per se is important in this Mo release system and we will continue efforts at determining the role of air (oxygen) in its suggested importance in furthering Mo release. Mass balance studies will be performed to account for the fate of released metals.

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