Contract No:

This document was prepared in conjunction with work accomplished under Contract No. DE-AC09-08SR22470 with the U.S. Department of Energy.

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Microbiological and Chemical Test Results on an Unknown Material from the L-Area Spent Fuel Basin

5/18/2012

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Revision Summary

Revision	Description	Date
0	Initial Issue	5/18/2012

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Summary

Two sampling events were completed to obtain cobweb-like material from the L-Area basin to determine what the material is and any potential impacts the material may have on the basin. The first sampling indicated that the material has a biological component but the low amount of material obtained from sampling was insufficient for full microbial and chemical analysis. The amount from the second sampling was more, approximately 50 ml. The material consisted of microorganisms, trace metals, and crystalline materials. The microorganisms are mostly, if not exclusively bacteria and this result indicates that the material and formation was mostly likely the result of biological activities. The highest concentration metals measured in the basin material include silica, aluminum, titanium, and iron. This microbiological matrix was dominated by many different types of heterotrophic bacteria, meaning that they are dependent upon external supplies of organic carbon and nutrients. Without these external inputs the bacteria cannot sustain themselves. Inferred from the measured levels of microbial diversity measured these requirements are currently being met. Further study of the water chemistry, examination of the cobweb-like material using scanning electron microscopy (SEM), and visual inspection of the fuel storage materials are recommended to further assess the mechanisms the microbial materials use for growth and assess the impact, if any, the material has on the underlying aluminum spent fuel. Additional testing and mitigation options include additional monitoring with strategically placed coupons, targeted testing at SRNL, further analysis of the microbiological matter, and removal of the material from the basin.

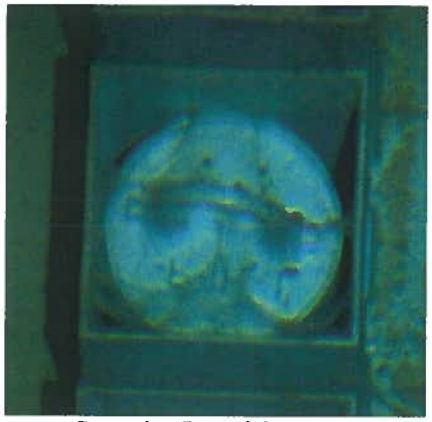
Background

The unknown material was noted by operations personnel in October 2011. The material was described as "cobwebs" due to its physical similarity to these structures, see Figure 1. An initial sampling was performed using brushes and cloth wipes to sample the material. During sampling a small amount of material was obtained and initial test results indicated the material could contain microbiological components. A visual inventory of the basin was also conducted in 2011 and this inventory indicated that the cobweb like material was observed in 7% of the basin and on 40% of the fuel (Jesee, 2011). Figure 1 shows five levels of severity used during the basin inventory. The levels were assigned to basin storage locations based on a visual comparison to the pictures shown in Figure 1. The report concluded that there were no clear correlations between the material and the type of fuel stored, the amount of material and the amount of light, or the radioactivity of the fuel where the material was observed.

A recent review of video footage indicated that similar structures, at much lower density, were observed in the basin in 2004. A greenish hue was observed in the L Basin in 2004 and was attributed to underwater lighting being used in the basin. The operators of CANDU reactors in Canada reported that their fuel is often completely obscured by microbial growth and they have had to use hydrogen peroxide on a monthly basis to retain visibility in their spent fuel basin and keep their deionizer and filter units from clogging (personal communication). Three Mile Island operators published a report in 1989 describing microbial growth that impeded visibility. They treated their basin with hydrogen peroxide to mitigate the effects (Hofstetter and Ausmus, 1989).

Severity Level 1

Severity Level 2



Severity Level 3

Severity Level 4

Severity Level 5

Figure 1. Severity Classifications of the unknown material on a 0 to 5 Scale

Historical data obtained from microbial analysis of the L Area spent fuel basin did not indicate any large changes that correlate with the appearance of the cob-web like material on surfaces in the basin. Water sample results show general trends, in which all measured microbial parameters have decreased or stayed relatively low. Over the past ten years the metabolic diversity of planktonic microorganisms in the water samples has decreased as has the density of planktonic bacteria.

Initial Sampling

Initial sampling of the L Basin material was performed using fuel handling tools with brushes and cloth wipe tied to the ends of the tools. When attempting to sample the material most of the cobweb-like structure broke apart and dispersed when touched with the brushes or cloth wipes. Most of the material that was collected washed away while the tools were moved from the fuel racks to the surface. The small amount of L Basin 'cobweb' material was shipped to SRNL for further characterization. Microbiological characterization was done by conventional examination using microscopy, culturing the material, performing Biolog identification, and using DNA sequencing on the cultured material.

Initially, fluorescent staining was done on the material using Fluorescein isothiocyanate (FITC) and examined using epi-fluorescent microscopy. This examination showed clear bacterial structures in the sampled material. The material was also streaked onto R2A petri plates in an effort to culture and grow the material. Once growth was observed, L Basin bacteria were streaked to isolation on R2A plating medium. Pure colonies were grown on GENIII plates and analyzed using OmniLog using standard protocols for Biolog plate analyses. The results are shown in Table 1.

Pure colonies were suspended in a 5% Chelex 100 resin solution and boiled for 10 min. to lyse bacterial cells. Cell debris was pelleted by centrifugation (6,000 x g, 1 min) and DNA quality and quantity was evaluated using a NanoDrop spectrophotometer. The 16S ribosomal RNA subunit gene was polymerase chain reaction (PCR) amplified using universal bacterial primers (27G/1492R, *E. coli* numbering) and conventional thermal cycling. Amplification accuracy and specificity was assessed by gel electrophoresis and products were purified by alcohol precipitation in high salt. Purified PCR were suspended in 10 µL of ddH2O and delivered to the University of Georgia's Genomics Facility for DNA sequencing. Sequences were trimmed to remove weak and ambiguous base calls in Sequencher (v 4.8) and the remaining >400bp partial rrn gene sequences were queried against the NCBI and RDP databases for putative identification. All IDs were confirmed by having >99% sequence identity to authentic gene sequences available in both sequence databases.

Table 1. Microbial IDs of Cultures Grown on Petri Plates

Strain	Partial 16S rRNA gene sequence	BIOLOG Phenotype Array
LB-1	*Ralstonia* sp.	*Ralstonia* sp.
LB-2	*Delftia* sp./*Comamonas* sp.	*Delftia* sp.
LB-4	*Delftia* sp.	*Delftia* sp.
LB-5	*Ralstonia* sp.	*Ralstonia* sp.

L Basin bacterial isolate LB6 failed to sequence cleanly and an LB3 isolate was not available for analysis.

All partial gene sequences for different L Basin isolates belong to the β Proteobacteria, in the order *Burkholderiales*. In general, these bacteria are ubiquitous in soils and sediments and many well-known representatives have been studied for their ability to effectively degrade a full spectrum of organic pollutants and contaminants as well as metabolizing heavy metals and radionuclide species.

Second Sampling

A second sampling was done to obtain more material for characterization. A collection and filter apparatus was constructed and tested to pump material from fuel rack surfaces up to the surface. Material was collected using 1 micron filters. The filtered material from the second sampling event was characterized chemically and microbiologically. The material was collected in autoclaved filter bags during L-Basin sampling. Approximately 50 ml of material was obtained during sampling. This material was used for the chemical and microbiological analyses and stored for future uses. The chemical analyses included inductively coupled plasma emission spectrometry, X-ray diffraction, and total carbon analysis. Microbiological analyses used PCR technology to increase the genomic signal of the DNA that was extracted, using commercial kits, from the material pulled from the filters. The analyses and results are described below.

ICP

Inductively coupled plasma emission spectrometry (ICPES) was used to analyze filtered material from the L-Basin obtained during the second sampling event. Two digestion methods were used to prepare the material for ICPES analysis. The material was digested using aqua regia and peroxide fusion. Table 2 below shows the combined results obtained from analysis. ICPES is an analytical technique used for the detection of trace metals. Major elements measured included silicon, aluminum, titanium, and iron, shown in yellow in Table 2. The presence of silicon aluminum, titanium and iron are not unusual due to the presence of the sand filter in the basin and the large scale use of aluminum materials in the basin. The source of titanium could be from paints and the sand from the filter. The presence of iron in the basin is from steel materials in the basin. The total mass of trace metals measured using ICPES was 2.5% of the total sample mass.

Table 2. Inductively Coupled Mass Spectrometry Results: Combine results of two digestions

Element	ug/g		Element	ug/g
Ag	BDL		Mn	98.5
Al	6590		Mo	BDL
B	BDL		Na	45.1
Ba	99.9		Ni	12.7
Be	BDL		P	267
Ca	355		Pb	41.6
Cd	BDL		S	182
Ce	BDL		Sb	BDL
Co	42.9		Si	7960
Cr	43.1		Sn	BDL
Cu	37.8		Sr	4.26
Fe	4110		Th	BDL
Gd	BDL		Ti	1060
K	89.7		U	32.9
La	1.1		V	1.04
Li	72.4		Zn	268
Mg	110		Zr	0.698

BDL= Below Detection Limit

XRD

X-ray diffraction (XRD) analysis was done on the filtered material from the L-Basin. XRD reveals information about the crystal structure, chemical composition, and physical properties of materials and thin films. These techniques are based on observing the scattered intensity of an X-ray beam hitting a sample as a function of incident and scattered angle, polarization, and wavelength or energy. Figure 2 shows the XRD spectra of the sample material. The major peaks identified include, rutile, quartz, bayerite, and gibbsite. Silicon dioxide, also known as Silica, is most commonly found in nature as sand or quartz. Its presence in the basin is expected due to the use of the sand filter. Rutile, titanium dioxide, is present in beach sands and finely powdered rutile is a brilliant white pigment that is used in paints for a bright white color. Bayerite and gibbsite are hydroxides of aluminum that are easily sloughed off due to their mica like structures. The large amount of aluminum stored in the basin is consistent with their measurement in the second sample where all material was vacuumed from the surface of the tops of fuel storage bundles.

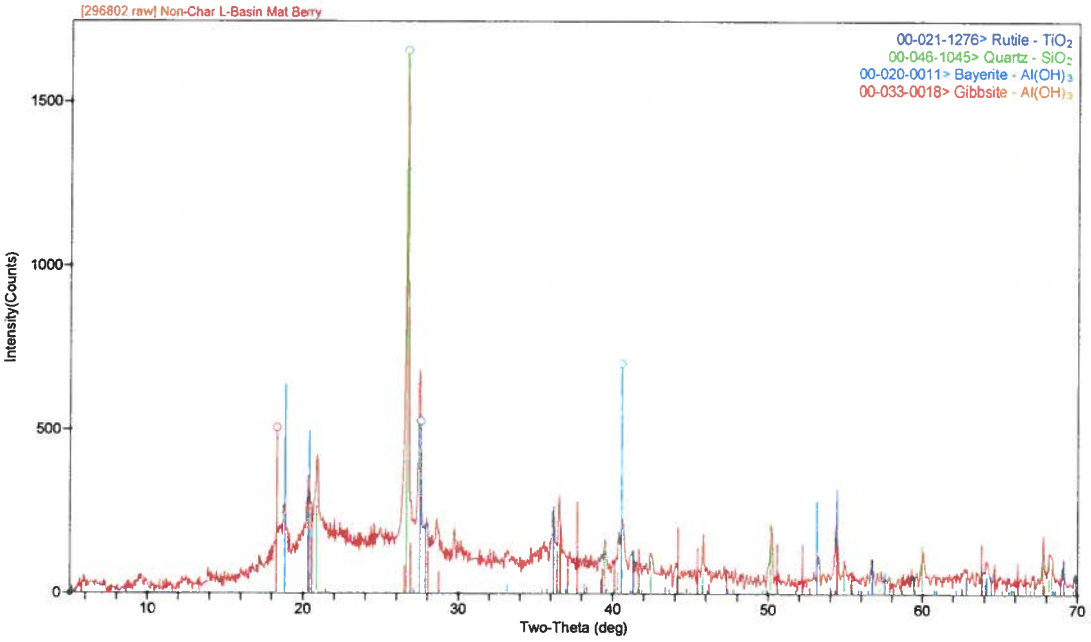


Figure 2. XRD Spectra of L-Basin Material

TOC/TIC

Filtered material was provided to Analytical Development Section (ADS) for total organic (TOC), total inorganic (TIC), and total carbon (TC) analysis. The material was provided in a moist form and was pulled directly from the sample filters. Untreated, the sample material plugged the injector of the TIC/TOC instrument. To prevent plugging, the material was sonicated and passed through a 0.45 micron filter. The results, as shown in Table 3, represent the TC in the soluble portion of the sample only, and are not representative of the entire sample. The total amount of carbon measured is approximately 0.1% of the wet weight of the original sample. It was the intent of the analyses to measure the total carbon present in the sample and combine these results with the ICPES results and obtain a mass balance on the sample, less water. However, the measurement of the soluble portion of the material, instead of all of the filtered material, precludes using the mass balance approach.

Table 3. Total Inorganic, Total Organic and Total Carbon Analytical Results

Soluble Total Inorganic Carbon (µg/g)	Soluble Total Organic Carbon (µg/g)	Soluble Total Carbon (µg/g)
131	883	1114

DNA extraction

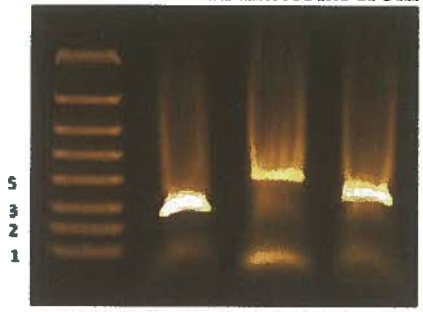
Diagnostic PCR was first conducted to determine the biological composition of the L-Basin 'cobweb' material. Specific assays were performed to discriminate the presence of bacteria, cyanobacteria, algae, and eukaryotes. This preliminary analysis clearly indicated that the sampled 'cobweb' material from L Basin was composed mostly, if not exclusively of bacteria; indicating that the material and formation was mostly likely the result of biological activities.

The sample was prepared for unbiased, comprehensive molecular analysis to ascertain the composition and identification of the bacteria within the 'cobweb' material. DNA was extracted by standard methods and subsequently amplified using 3 distinct primer pairs specific for bacteria. A combination of primers was used to eliminate the potential influence(s) of annealing bias imposed by any single primer pair or region of the target molecule being amplified. This approach provides 3 independent samplings and descriptions of the L Basin material.

Specific, amplification by each of the 3 primer pairs is shown in the Figure 4. Multiple amplifications were conducted per primer pair and final products purified for sequencing at The University of South Carolina's EnGenCore Lab. Sequence files were culled and analyzed using the pyrosequencing pipeline at Michigan State University (http://rdp.cme.msu.edu).

Sequencing analysis produced 22.1 Mb of genetic data from the L Basin 'cobwebs.' This quantity of data equates to over 96,000 gene sequences. The results are organized hierarchically by taxonomy, the total number of unique sequences recorded for each taxonomic category are provided in parenthesis, and then key features of each lineage are described, see Figure 5.

Figure 4. Extracted DNA material from the L-Basin



Molecular detection of bacteria from L-Basin. Marker on the side indicates length (bp).

Figure 5. Sequencing Analysis Results

norank Root (48974 sequences) [*show assignment detail*]
- domain Archaea (1)
  - phylum "Euryarchaeota" (1)
    - unclassified_"Euryarchaeota" (1)
- domain Bacteria (48712)
  - phylum "Gemmatimonadetes" (1)
    - class Gemmatimonadetes (1)
  - phylum "Thermotogae" (1)
    - class Thermotogae (1)
  - phylum "Armatimonadetes" (3)
    - class Chthonomonadetes (3)
  - phylum "Nitrospira" (3630)
    - class "Nitrospira" (3630)
  - phylum "Acidobacteria" (5620)
    - class Acidobacteria_Gp16 (11)
    - class Acidobacteria_Gp4 (10)
    - class Acidobacteria_Gp6 (31)
    - class Acidobacteria_Gp2 (143)
    - class Acidobacteria_Gp1 (112)
    - class Acidobacteria_Gp3 (5230)
    - class Holophagae (38)
    - unclassified_"Acidobacteria" (45)
  - phylum "Chloroflexi" (3)
    - class Ktedonobacteria (3)
  - phylum "Deinococcus-Thermus" (4)
    - class Deinococci (4)
  - phylum "Verrucomicrobia" (1)
    - class Spartobacteria (1)
  - phylum "Spirochaetes" (1)
    - class Spirochaetes (1)
  - phylum "Planctomycetes" (14)
    - class "Planctomycetacia" (14)
  - phylum Cyanobacteria/Chloroplast (5)
    - class Cyanobacteria (2)
    - class Chloroplast (2)
    - unclassified_Cyanobacteria/Chloroplast (1)
  - phylum "Actinobacteria" (628)
    - class Actinobacteria (628)
  - phylum Firmicutes (6)
    - class Bacilli (5)
    - class Clostridia (1)
  - phylum "Proteobacteria" (14660)
    - class Deltaproteobacteria (234)
    - class Alphaproteobacteria (4135)
    - class Betaproteobacteria (5450)
    - class Gammaproteobacteria (1758)
    - unclassified_"Proteobacteria" (3083)
  - phylum "Bacteroidetes" (438)
    - class "Bacteroidia" (1)
    - class Flavobacteria (7)
    - class "Sphingobacteria" (394)
    - unclassified_"Bacteroidetes" (36)
  - unclassified_Bacteria (23697)
- unclassified_Root (261)

domain Bacteria (47290 sequences) [*show assignment detail*]
- phylum "Nitrospira" (27)
  - class "Nitrospira" (27)
- phylum Cyanobacteria/Chloroplast (2)
  - class Chloroplast (2)
- phylum "Spirochaetes" (3)
  - class Spirochaetes (3)
- phylum "Chlamydiae" (12)
  - class Chlamydiae (12)
- phylum "Acidobacteria" (689)
  - class Acidobacteria_Gp4 (7)
  - class Acidobacteria_Gp16 (1)
  - class Acidobacteria_Gp1 (54)
  - class Acidobacteria_Gp2 (85)
  - class Holophagae (18)
  - class Acidobacteria_Gp3 (232)
  - class Acidobacteria_Gp6 (255)
  - unclassified_"Acidobacteria" (37)
- phylum "Deinococcus-Thermus" (127)
  - class Deinococci (127)
- phylum "Actinobacteria" (20)
  - class Actinobacteria (20)
- phylum "Planctomycetes" (161)
  - class "Planctomycetacia" (161)
- phylum "Proteobacteria" (9839)
  - class Betaproteobacteria (130)
  - class Alphaproteobacteria (386)
  - class Deltaproteobacteria (13)
  - class Gammaproteobacteria (8613)
  - unclassified_"Proteobacteria" (697)
- phylum "Bacteroidetes" (21)
  - class "Bacteroidia" (6)
  - class "Sphingobacteria" (15)
- phylum Firmicutes (3)
  - class Clostridia (2)
    - unclassified_Firmicutes (1)
- unclassified_Bacteria (36386)

Generalized Properties & Capabilities of Detected Bacterial Lineages

Gemmatimonas. Little know group. Characterized representatives were obtained from wastewater treatment systems. Bacteria are capable of phosphorous removal and accumulation.

Kosmotoga, Chthonomonas, Thermosporothrix. Bacteria that thrive at high temperatures. Generally tolerant of high concentrations of soluble metals.

Nitrospira. Bacteria that gain energy by oxidizing nitrite.

Acidobacteria. Poorly characterized group of bacteria. Ubiquitous in the environment, best studied for role in soils. Metabolically diverse.

Halophagae. Tolerant of high levels of salts.

Chloroflexi. Metabolically diverse group of bacteria. A few divisions can grow by photosynthesis, and many others are capable of organic toxin or contaminant metabolism and degradation.

Deinococcus / Thermus. Bacteria capable of tolerating high degrees of stress; e.g., temperature, radiation, toxins, soluble metals and radionuclides. The deinococci are particularly well studied for metal/radionuclide reduction and organic toxin oxidation. The bacteria are also capable of catalyzing the dissolution of minerals and have been shown to form biofilms on metal alloys and to promote corrosion.

Verrucomicrobia. Poorly studied group. Largely uncharacterized but most known to inhabit soils. Functions unknown.

Spirochaetes. Diverse group of bacteria, best studies for causative agents of disease, though other representatives are capable of degrading a variety of organic contaminants.

Planctomycetes. Typical aquatic bacteria.

Actinobacteria. Highly diverse group of bacteria. Abundant in the all environments, having tremendous metabolic capabilities. Many divisions are especially well known for high levels of tolerance to environmental stress, so much as even thriving in toxic or contaminated environments (organic, inorganic, radioactive). Many representatives excrete organic acids for metal-mineral dissolution, local environmental acidification.

Firmicutes / Bacillus. Common environmental bacteria. High metabolic capability. Representatives known for inhabiting toxic environments, metabolizing metals, biofilm formation, as well as promoting corrosion of metal alloys.

δ-Proteobacteria. Many different divisions of organic contaminate degrading bacteria / metal (iron, uranium) respiring bacteria.

α-Proteobacteria. Divisions detected include taxa, a group of organisms judged to be a unit, known for reducing gaseous N to ammonium, lineages known for organic contaminant degradation (e.g., *Sphingomonas*). These bacteria possess high metabolic capability. They are often among the dominant bacteria present in biofilms of man-made aquatic environments (*Pedomicrobium*) and also include taxa known for metal metabolism and accumulation. *Hyphomicrobium* are quite capable of adapting to stress or nutrient starvation, and they participate in metal oxide formations.

β-Proteobacteria. Bacteria maintain lots of metabolic capability, including model systems for organic contaminant degradation and tolerance of heavy metals (*Oxalobacteria, Comamonadaceae, Cupriavidus*). Inhabitants of aquatic environments, including industrial wastewater systems. *Cupriavidus* are capable of tolerating high levels of dissolved metals. *Oxalobacter* sp. excrete high concentrations of organic acid (oxalic acid) for metal-mineral dissolution; potential for promoting corrosion of metal alloy surfaces.

γ-Proteobacteria. Contains many divisions of bacteria with tremendous metabolic capability; including organic contaminant degradation, resistance to high concentrations of soluble metals, and ability to affect metal and radionuclide redox chemistry.

Discussion and Summary

Biological and chemical characterization of the 'cobweb' material was sought to establish a reliable picture of the current state, stability, or potential evolution of spent fuel materials presently stored in L Basin. What do we know from combined chemical and biological sampling of L-Basin material?

The material contains precipitated silica, aluminium (oxy-) hydroxides, titanium, iron, and an exceptionally diverse bacterial community. It remains unclear whether biological properties contributed to 'cobweb' formation. XRD and ICP-ES did not detect polymeric substances (proteinaceous polysaccharides) or elemental signatures expected from bacterial biofilm formation. SEM examination may provide additional structural information.

These results are suggestive of a high energy system capable of supporting unexpected levels of bacterial diversity (and metabolic requirements) that does not appear to rely solely on photosynthetically derived carbon inputs (i.e., algae). Our analysis did detect a few corresponding sequences for photosynthetic cyanobacteria and eukaryotes (algae) however these organisms are certainly not abundant or pervasive within the 'cobweb' material. Organic carbon levels measured from the sampled material were not insignificant; they are comparable to surface waters of lakes and rivers. This matrix is dominated by many different kinds and types of heterotrophic bacteria, meaning that they are dependent upon external supplies of organic carbon and nutrients. Without these supplies the bacteria cannot sustain themselves and the levels of diversity measured imply that these requirements are certainly being met.

Gene sequences detected correspond to or are closely aligned to those from authentic bacteria previously characterized for accelerating the corrosion of metal alloys (including aluminum alloys) and the ability to tolerate high concentrations of soluble toxic metals.

- Many different bacteria capable of forming biofilms on metal alloy surfaces.
- Many bacteria capable of tolerating high concentrations of toxic soluble metals; others that immobilize or accumulate phosphate to mitigate Al toxicity.
- Presence of specific bacteria known to catalyze or promote corrosion of metal alloy surfaces.

Visual examination of the underlying metal structures did not show any bulk corrosion, discoloration, or any other indications that the material has impacted basin metals. This examination was cursory and not investigative but large scale corrosion events, linked to the chemistry in the storage basin, and tests designed to increase corrosion rates have shown observable corrosion. The cobweb-like material also seems to be localized on the surfaces, measurement of bacterial densities in the water have not shown an increase. Such an increase could impact basin operations through plugging.

Mitigation/Path forward

There are a series of possible path forwards and potential mitigations that could be employed to fully understand the presence of this microbial based material, to determine if the material is actively impacting metals in the basin, or to remove the material from the basin. These activities are bulleted below.

- Continue to monitor material – use existing materials, coupons, and monitoring techniques
- Location and growth rate determination – perform specific monitoring in harvested areas, examination of historical underwater films, and examination of other basin areas.
- Water carbon analyses – in-depth analyses to determine the source of material providing growth to the organisms. This analysis would include low level carbon analyses of the water to determine carbon sources that exist in the basin.
- Evaluate existing coupons - examine and compare the biological material on existing coupons to the material we pulled from the basin to determine if the existing coupons can be used for monitoring of the material and its activity.
- Add new coupons – add new coupon or seed coupons with basin material and place the coupons in areas with similar conditions in the basin.
- Examine Fuel – Using 10X visual tools to examine the casks and storage racks, use the upcoming surveillance activities to examine the lower portion of the fuels, and pull storage rack materials for full material testing.
- Microcosm testing - Use basin water and sampled materials with non-basin materials in a test environment with radiation to study the interaction of the biological material and metals. Light, radiation, and nutrient levels could be varied. Materials analyses with a corrosion focus and microbiological monitoring would be done.
- Material removal – the current options include vacuuming the material and using chemical treatment. pH adjustment is probably not an option due to aluminum solubility

issues but the use of hydrogen peroxide has been demonstrated(Hofstetter and Ausmus, 1989). Both methods may need to be repeated periodically and pretesting may be required for peroxide dosing.

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