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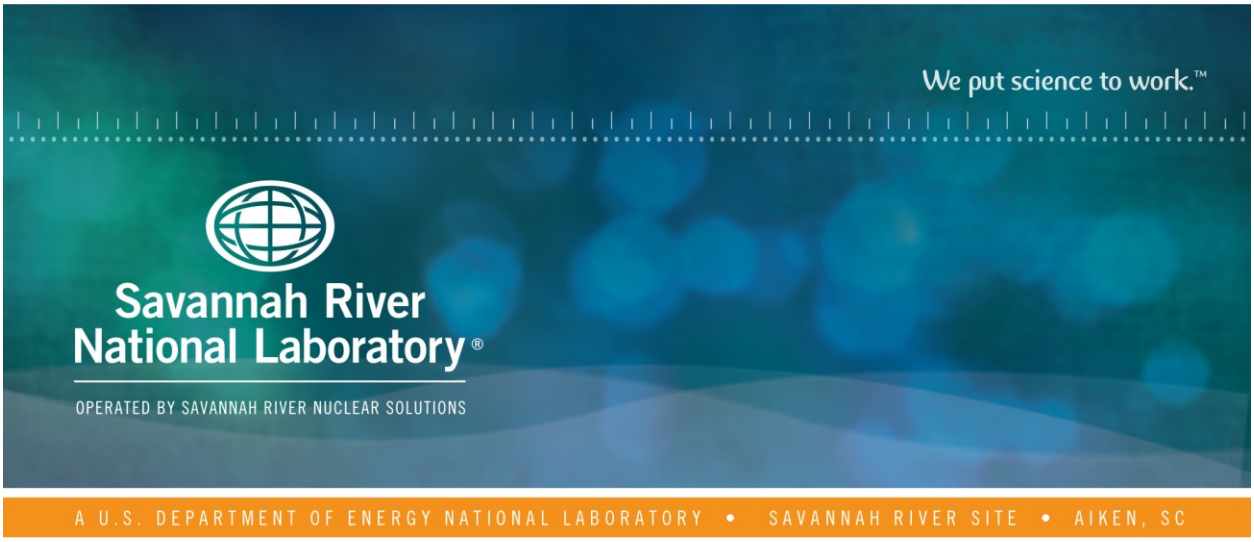

L-Basin Microbial Monitoring Program – Evaluation of 20 Years of Monitoring

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April 2021

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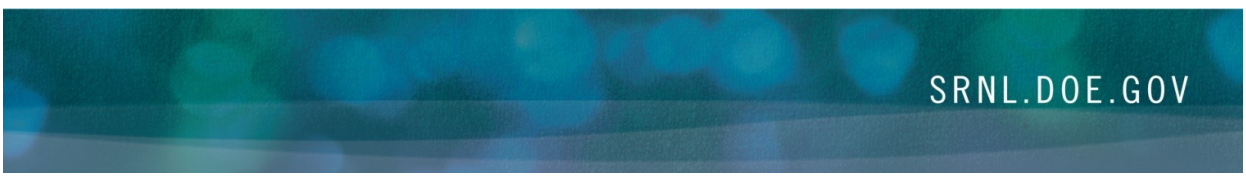

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EXECUTIVE SUMMARY

This report summarizes the results from over 20-years' of activities for microbial monitoring of the bulk water, floc analysis, and test coupon surfaces for the Savannah River Site L-Basin. The Microbial Monitoring Program provides important information for the characterization of overall water quality as it impacts the continued safe storage of spent nuclear fuel in L-Basin. The potential impacts to fuel storage are: 1) microbial-influenced corrosion of the fuel; and 2) biofouling of the fuel storage systems.

Microbial-influenced Corrosion

The L-Basin Corrosion Surveillance Program provides information on corrosion attack of aluminum fuel and storage system materials due to continuous wet storage at the prevailing water quality conditions in L-Basin. The Microbial Monitoring Program supports the Corrosion Surveillance Program with specific characterization information on microbial colonies (types and densities) and their likely contribution to corrosion of the aluminum materials. The results to date show no significant contribution of the microbial colonies to pitting corrosion attack on the aluminum materials stored in L-Basin.

This report describes the Microbial Monitoring Program including the historical use of microbial monitoring specimens for corrosion evaluation, characterization protocols, and the microbial colony characterization results. The several types of microbial colonies suggested by the literature to impact corrosion of aluminum and stainless steel, have been quantified and trended. These include Sulfate-Reducing Bacteria (SRB), an anaerobic type that generates alkaline conditions, and acid-producing (e.g. iron-oxidizing) bacteria, a general aerobic type. The data collected is separated into two phases, Phase I (2000-2012) and Phase II (2017-2020). Phase I period involved the use of the MICkit™ III measurement system. The Phase II period involved the use of the MICkit™ 5 measurement system.

In Phase I, overall bacterial density had a trend of a slight decrease over the 2000 to 2012. Metabolic diversity of microorganisms in the basin decreased dramatically. With regards to the bacterial density of specific MICkit™ III bacterial groups, the only exceptionally strong trend was that of the aerobe density decreasing, with the other groups only nominally increasing or decreasing. Aerobes were most prevalent at 3.23 average CFU/mL and acid-producing bacteria were least prevalent at 0.80 average CFU/mL.

In Phase II, using MICkit™ 5, Fe-related bacteria were most prevalent at 3.41 average CFU/mL and acid-producing bacteria were least prevalent at 0.44 average CFU/mL. All bacterial densities decreased except that of low-nutrient bacteria, which had a moderately increasing trend. In Phase II Part II, low-nutrient bacteria were most prevalent at 2.90

average CFU/mL and SRB were least prevalent at 0.19 average CFU/mL. MICkit™ 5 results for Phase II Part II data showed increasing densities for all microbe categories.

There are no distinct correlations between microbe concentrations and either water chemistry parameters or radiological conditions in L-Basin. Temperature does not appear to play a role in understanding the microbial parameters measured in the water samples. While the L-Basin is not a sterile environment, the water is filtered, artificial lighting kept low, and input from external sources to water are limited, keeping microbial activity to a minimum. It is recommended to continue characterization using the MICkit™ 5 and trend the results annually. A trend of a severalfold increase in either SRB density or acid-producing bacteria would be flagged to check for vulnerability of aluminum to MIC attack.

Biofouling – Biological Structures

The occurrence of a floc formation was first observed around 2010 on the Expanded Basin Storage racks, including on the tops of the storage bundles in those racks. The appearance of floc formation and structural consistency was that of grey biological structures. The biological structures have been readily removed by underwater vacuuming of the structures, however new formations have formed over time.

The sampling and analysis of the matter show it to consist of microorganisms, trace metals, and crystalline materials. The microorganisms are mostly, if not exclusively, bacteria with a high bacterial diversity. The highest concentration metals measured in the material include silica, aluminum, titanium, and iron. It is suggested that the bacteria produce a biofilm (bacterial precipitates, resulting in a microhabitat for further bacterial colonization) such as those of polysaccharides that can trap detritus, e.g. inorganics and organics, in the film. This microbiological matrix was dominated by many different types of heterotrophic bacteria that are dependent on external supplies of organic carbon and nutrients.

Additional investigation is required to determine specific physical/chemical processes causing the formations. Suggestions for additional investigation for formation and water treatments for mitigation are provided.

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CFU	Colony Forming Units
DAPI	4',6- diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
EDS	Energy-Dispersive X-Ray Spectroscopy
FA	Fluorescent Antibody
FITC	Fluorescein Isothiocyanate
GN	Gram Negative
GP	Gram Positive
HDPE	High-Density Polyethylene
IAEA	International Atomic Energy Agency
LCM	Laser Confocal Microscopy
MIC	Microbiologically Influenced Corrosion
RBOF	Receiving Basin for Offsite Fuel
rRNA	Ribosomal ribonucleic acid
QBSD	Quadrant Back Scattering Detector
SEM	Scanning Electron Microscopy
SRB	Sulfate-Reducing Bacteria
SRNL	Savannah River National Laboratory
SRS	Savannah River Site
SFP	Spent Fuel Project
SS	Stainless Steel
TIC	Total Inorganic Carbon
TOC	Total Organic Carbon
VTS	Vertical Tube Storage

1.0 Introduction – Microbial Monitoring of L-Basin

The SRNL L-Basin corrosion surveillance and microbial monitoring programs provide early detection and characterization of corrosion attack to the fuel and storage system materials resulting from prolonged exposure to the L-Basin water environment and of changes to and impact of the diverse microbial population, respectively [20]. The early detection of corrosion allows for adjustment of the water quality, engineering management, and fuel storage configurations to mitigate excessive corrosion attack. While microbial influenced corrosion in L-Basin has not been detected, tracking and understanding the effect of microbial populations on the stored fuel and basin water will aid in identifying remedial measures to mitigate any detrimental impact. This report reviews the microbial monitoring activities since initial characterizations in the mid-1990s

In this report, the SRNL Environmental, Materials, and Energy Sciences Directorate has evaluated biological, chemical, and radiological factors involved in the process to properly understand the problem for over 20 years. To accomplish this goal, the following was conducted and evaluated:

- An examination of the samples on receipt microscopically
- Cultured material from the radiological samples on select microbial growth media to help determine the relative densities and characterization
- Biochemical analyses of select water samples

2.0 Microbial Monitoring – Historical Overview

Microbial monitoring for the storage of spent nuclear fuel was initiated in the mid-1990s with examination of water samples from foreign fuel basins being received at the SRS Receiving Basin for Offsite Fuel (RBOF) [6]. Microorganisms that can cause microbial corrosion had been repeatedly identified in SRS basins [6, 7]. Monitoring in RBOF was initiated in 1994. The ultimate purpose was to understand if the microbiology from foreign fuel receipts would impact storage conditions in RBOF [6]. The difference between the RBOF microbiological activity and that of foreign fuel water was found to vary with the development of diverse microbial populations. The presence of these populations was not shown to have impacted corrosion [6, 7].

Atmospheric CO_2 assimilation by photosynthetic organisms is a very important event in the global carbon cycle. Cyanobacteria can use the energy contained in artificial light for photosynthesis, even at low levels as in L-Basin, which can provide the energy necessary for cyanobacteria growth. L-Basin does receive input from outside sources including airborne dust, pollen, and microorganisms that can impact microbial growth and colonization.

Microbial monitoring was extended to the other storage basins, L-Area and K-Area, during the late 1990s. In the early 2000s, pin-type coupons were added to the microbial monitoring program specifically to evaluate for the onset of microbiologically influenced

corrosion (MIC). Recent comparative analyses of 16S ribosomal ribonucleic acid (rRNA) gene amplicon libraries from L-Basin biofilm samples demonstrated microbial community and taxonomic signatures unique to the L-Basin environment [8]. While MIC microorganisms have been detected in L-Basin, actual MIC corrosion has not been identified to date. This is most likely due to the stringent water chemistry control [14].

Water samples and coupon analysis have continued with biannual water sample analyses and annual coupon analyses over 20 years. The general trends for all measured microbial parameters for density and diversity have decreased or stayed relatively low. These trends indicate that operation of the basin has not facilitated the growth of planktonic bacteria and that MIC is not a significant factor for the aluminum-clad fuel and aluminum storage systems (racks, tube bundles, and oversized storage containers) or the coupons exposed since the surveillance program began [9].

3.0 Materials and Methods

Two types of samples are monitored in the Microbial Monitoring Program, water samples and coupon samples. Water samples assess the general microbial condition of the basin. General changes in the microbial population are assessed quickly and inexpensively using water samples to monitor bacterial, fungal, and alga populations in the basin. Coupon samples are used to assess biofilm formation on metal compositions that are similar in grade to the SNF and basin hardware. The purpose of the microbiological coupons was intended to be a bounding or conservative measuring tool for observing and measuring MIC in the basin. Examination of the coupons is more labor intensive but provides the most useful information that can be related directly to MIC and continued long-term spent fuel storage in aqueous environments.

L-Basin water samples were pulled with 250 mL polypropylene containers that were pre-sterilized using ethanol (95 %) and then rinsed with deionized, filter-sterilized (0.2 µm pore size) water. Coupon samples were pulled by hand using aseptic techniques. Sample results and discussion are found in the results and discussion section.

Once samples were received at SRNL, they were immediately transported to the laboratory and stored at 4 °C to keep microbial parameters similar to those of the original sample until analyses were performed. Generally, analyses are performed the day samples arrived at SRNL's Sample Receiving (773-A).

Microbial water sample analyses in Phase 1 consisted of three tests to determine the density and diversities of microorganisms present in the basin. Total direct counts measured microbial densities, as determined by the total number of organisms (viable and non-viable). MICkit™ (versions III and 5) testing measured the density of specific groups of organisms (viable) associated with MIC in industrial environments. Biolog™ testing, gram negative (GN) and gram positive (GP), measured the physiological diversity of each sample using different chemical growth substrates to quantify the number of different enzymes and enzyme systems present in each sample [10].

3.1 Total Direct Counts

The total number of organisms, viable or non-viable, are determined by spotting fifty microliters (µL) of well-mixed water onto alcohol wiped microscope slides and heat fixed at 65 °C for 12 minutes. The samples are stained with fluorescein isothiocyanate (FITC), 4',6-diamidino-2-phenylindole (DAPI), or propidium iodide solution for two to five minutes, rinsed with deionized pre-filtered (0.2 µm pore size) water, and air dried at room temperature [10].

Stained microbial cells are counted using a Zeiss epifluorescent microscope and appropriate filter set. After counting the cells on each slide, the cellular density is calculated based on the sample volume, the area of each field for the microscope, and the total number of fields counted. Control wells, wells without added sample but stained, are also used in the calculation. Results are presented as counts per unit volume for water samples. The results represent all the microorganisms, both viable and nonviable, that were present in the samples.

3.2 Biolog™

While the total direct counts and MICkit™ are microbiological in nature, the Biolog™ data can be considered biochemical. Biolog™ testing, gram negative (GN) and gram positive (GP), measured the physiological diversity of each sample using different chemical growth substrates to quantify the number of Ecofunctional enzyme systems present in each sample. GN and GP Biolog™ plates (Biolog, Hayward, CA) consist of 96 small wells containing an indicator dye with 95 of the wells containing different organic substrates. The two different plate types (GN and GP) characterize most of the aerobic microbial population of microorganisms. Bacteria can be divided into two major groups based on their cell membrane structure. To prepare each test plate, 150 µL of sample water is pipetted in all the wells and allowed to incubate at 25 °C for 24-72 hours. For coupons, scrapings are taken from the surface, mixed with sterile media, and pipetted into all of the wells. Positive results are determined visually for each plate. The results of the tests profiled the metabolic diversity of the microbial community and the types of enzymes in each water sample [11, 12]. A large number of positive wells indicate increased physiological diversity and activity in the microbial population.

3.3 MICkit™ (III and 5) Testing

Viable counts of aerobic bacteria, anaerobic bacteria, sulfate-reducing bacteria (SRB), acid-producing bacteria, and iron-producing bacteria are determined using a commercially available kit, MICkit™ (Bioindustrial Technologies, Inc., Georgetown, TX). MICkit™ III was used for Phase 1 of data collection (2000-2012), and MICkit™ 5 (Figure 3-1Figure 3-1 MICkit™ 5 Test Kit for assessing specific bacteria population including aerobic, anaerobic, sulfate reducing, and acid producing) was used in Phase II of data collection (2017-2020). MICkit™ 5 does not have aerobe detection capability but does have low nutrient and iron-producing bacteria detection capabilities. Analysis of water samples and strategically placed metal coupons is conducted using MICkit™ to determine microbial population size

and Laser Confocal Microscopy (LCM) for monitoring localized corrosion, respectively. Microbial parameters were monitored with MICkit™ for assessing specific bacteria associated with corrosion including aerobic, anaerobic, sulfate reducing, and acid producing species. These species were found to be present in the samples varying from non-detectable to 10^{-4} colony forming units (CFU)/mL. A seasonal cycle was observed with the microbial populations correlating with ambient temperature. Water samples and coupon scrapings are analyzed by inoculating these kits using serial dilution and aseptic techniques. At 2, 5, and 15 days of incubation at room temperature (i.e., ~ 25 °C) the kits are visually examined for growth (i.e., turbidity), iron metabolism (i.e. rust, white, gray or green deposits), sulfate reduction (i.e., black precipitate), and acid production (i.e., change in medium color) as specified by the manufacturer. The results for each test group are reported as viable cellular concentration in water.

Figure 3-1 MICkit™ 5 Test Kit for assessing specific bacteria population including aerobic, anaerobic, sulfate reducing, and acid producing

Each bacterial group functions differently regarding MIC.

- Viable Aerobic Bacteria grow in the presence of oxygen with generally high concentrations in circulating water systems such as L-Basin. These bacteria are the predominate type of microorganisms and provide a measure of the turbidity in the system.

- Viable Anaerobic Bacteria grow in the absence of oxygen with generally low concentrations in circulating water systems. Pockets and surfaces of anaerobiosis can occur where these bacteria grow and may impact the initiation of MIC, encouraging the growth of sulfate-reducing and acid-producing bacteria [13].

- SRBs reduce sulfate to sulfide under appropriate anaerobic conditions. The sulfide may be utilized by some acid-producing bacteria in their growth processes and produce sulfuric acid as a cellular by-product. SRBs are common in MIC of carbon and stainless steel and should ideally be kept at a minimum. The SRB are strict anaerobic organisms.

- Acid-Producing Bacteria produce inorganic and organic acids that can lower the pH of the environment below a biofilm. This acidic environment can prevent repassivation of metal surfaces, precluding formation of protective films.

- Iron-related bacteria have activities ranging from a passive bioaccumulation in the slime (biofilm) or fouling growths through to active use in metabolism. These bacteria can derive respiratory or energy functions out of the reduction (ferrous) to oxidation (ferric) manipulations at metal surfaces which can lead to pitting.

3.4 Metal Coupons

The purpose of the microbiological coupons was intended to be a bounding or conservative measuring tool for observing and measuring MIC in the basin. Although the coupons were placed in the same storage environment and, in some cases, as close to the fuel as operations would allow, the coupons are not stored in the same radiation fields as the fuel. Based on the hypothesis that the radiation field reduces microbial growth and MIC processes, the coupons placement should provide a conservative or bounding indicator of microbial changes or corrosion processes in the basins. Therefore, more corrosion and microbial activity would be expected on the coupons than on the fuel.

Four types of metal coupons from L-Basin were examined for biofilm development and MIC. Coupons were only used in Phase I testing. Two of the coupons consisted of chromium-nickel stainless steels (SS 304L and SS 308) while two were aluminum-based alloys (AL 1100 and 6061). The composition of these coupons is identical to the cladding surface of most, but not all, of the types of fuels and fuel storage materials contained in the SRS storage facilities. The coupons were approximately 1.27 mm in diameter by 1.27 cm long and produced by centerless grinding (Metal Samples Co., Munford, AL).

The surfaces of these coupons were cleaned, prior to exposure, using a 70% alcohol solution and autoclaved at 121°C and 15 psi for one hour to provide an oxide layer on the aluminum and to destroy any biofilm present before introduction to the basin. Ten coupons were installed in separate grooves in one of four pre-drilled Teflon blocks and suspended into the basin. The coupons were submerged in L-Basin vertical tube storage (VTS) at approximately 4.0 meter (m), and 8.3 m from the water surface on 6/12/1997. Two sets of coupons were placed above row 15 and row 22, four sets total, just above

and next to the fuel storage racks at the levels indicated. All remaining coupons that were installed in 1997 were removed on January 14, 2004. At this time two sets of coupons were added to the basin and were placed just above the fuel (approximately 4.0 meters below the surface). All coupons were aseptically removed using sterile forceps and stored in sterile conical tubes at 4°C once received at SRNL. Storing coupons at 4°C slows the metabolic activity of the organisms on the coupons. The coupons were not returned to the basin after being analyzed.

Each coupon sampling event removed six samples for destructive and nondestructive testing. Three samples, consisting of at least one stainless steel (SS) or aluminum alloy, were evaluated using destructive techniques that removed the biofilm from the coupon surface. One coupon and autoclaved glass beads (1 mm diameter, Biospec Inc.) were vortexed with 7 mL of Fluorescent Antibody (FA) buffer solution for 2 minutes. The coupon was then rinsed with 5 mL of FA buffer twice and then used to prepare MICkit™ III test kits, Biolog™ plates and total direct counts using the same methods described above for the water samples.

The non-destructive testing was performed on the other three coupons. The testing consisted of a visual examination and a scanning electron microscopy examination of the surface of each coupon. Two coupons, one aluminum-based and one stainless steel were stained with DAPI (0.7 µg of DAPI per mL phosphate buffer, pH 7.5) for five minutes. Stained microbial cells were counted, examined, and photographed using a Zeiss epifluorescent microscope and appropriate filter set. The remaining coupon was analyzed with scanning electron microscopy. Samples were serially dehydrated for 30 minute each in 50%, 75%, and 95% tert-butanol, prior to a final 16 h dehydration step with tert-butanol 100%. Sample was placed onto copper tape, and then examined with the aid of a LEO 440 Glovebox-contained Scanning Electron Microscope, operated at 25 keV accelerating voltage. Secondary and backscatter images were captured in tiff format. Energy dispersive x-ray spectra were obtained using the Oxford Inca Energy Dispersive x-ray detector, with a thin window capable of detecting elements carbon and above.

4.0 Results and Discussion

4.1 Phase I Testing – 2000-2012 Microbial Sampling

The results for the water samples indicate that overall density was not subject to large changes or significant trends other than a slightly decreasing trendline, and the populations of viable organisms associated with MIC were low. The total density of microorganisms, as measured by FITC direct counts, in the L-Basin were slightly lower than the average value observed in the RBOF. Figure 4-1 shows the measured microbial density versus time for L-Basin and the RBOF. The trend line for the L-Area data shows a steady but slightly decreasing microbial population in the basin, with a slightly more accelerated rate of decrease toward the tail end of the sampling period.

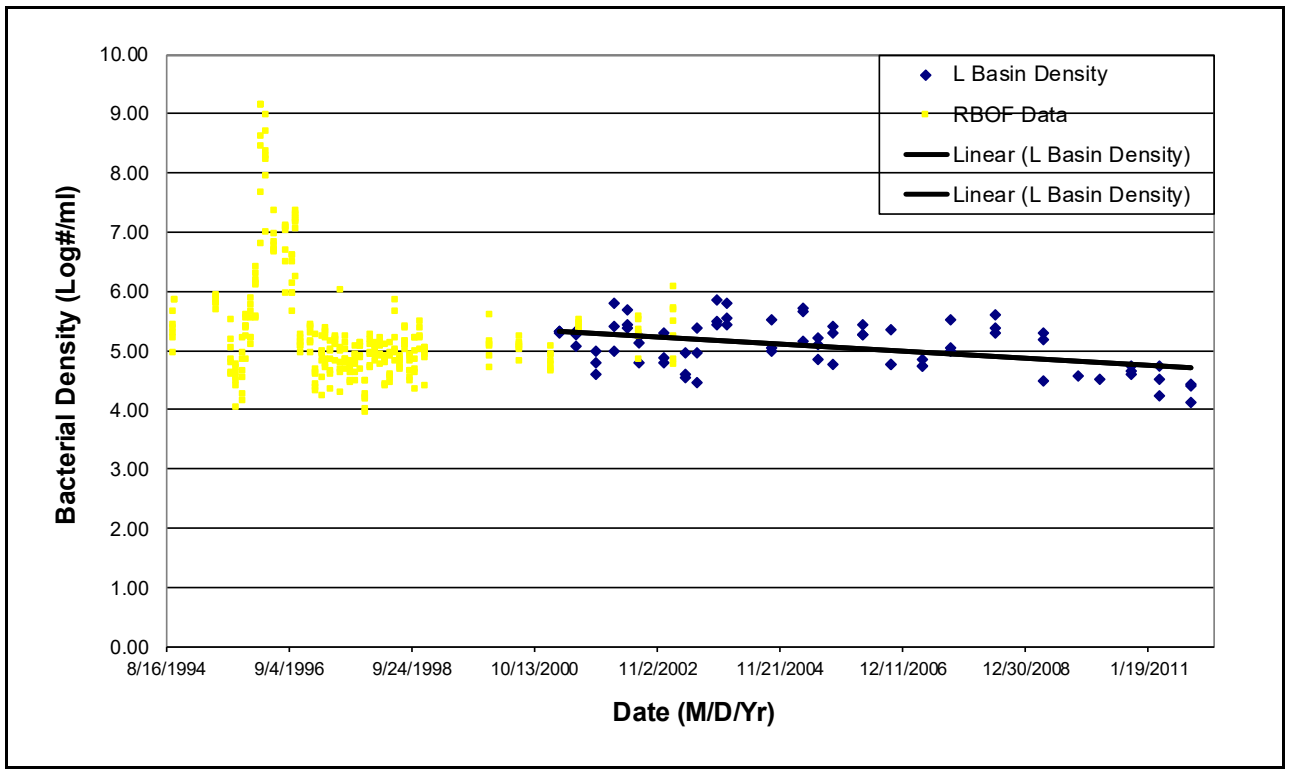


Figure 4-1 Bacterial density over time, based on FITC direct counts

With regard to the bacterial density of specific MICkit™ bacterial groups, the only exceptionally strong trend is that of the aerobe density decreasing, with the other groups only nominally increasing or decreasing (**Error! Reference source not found.**).

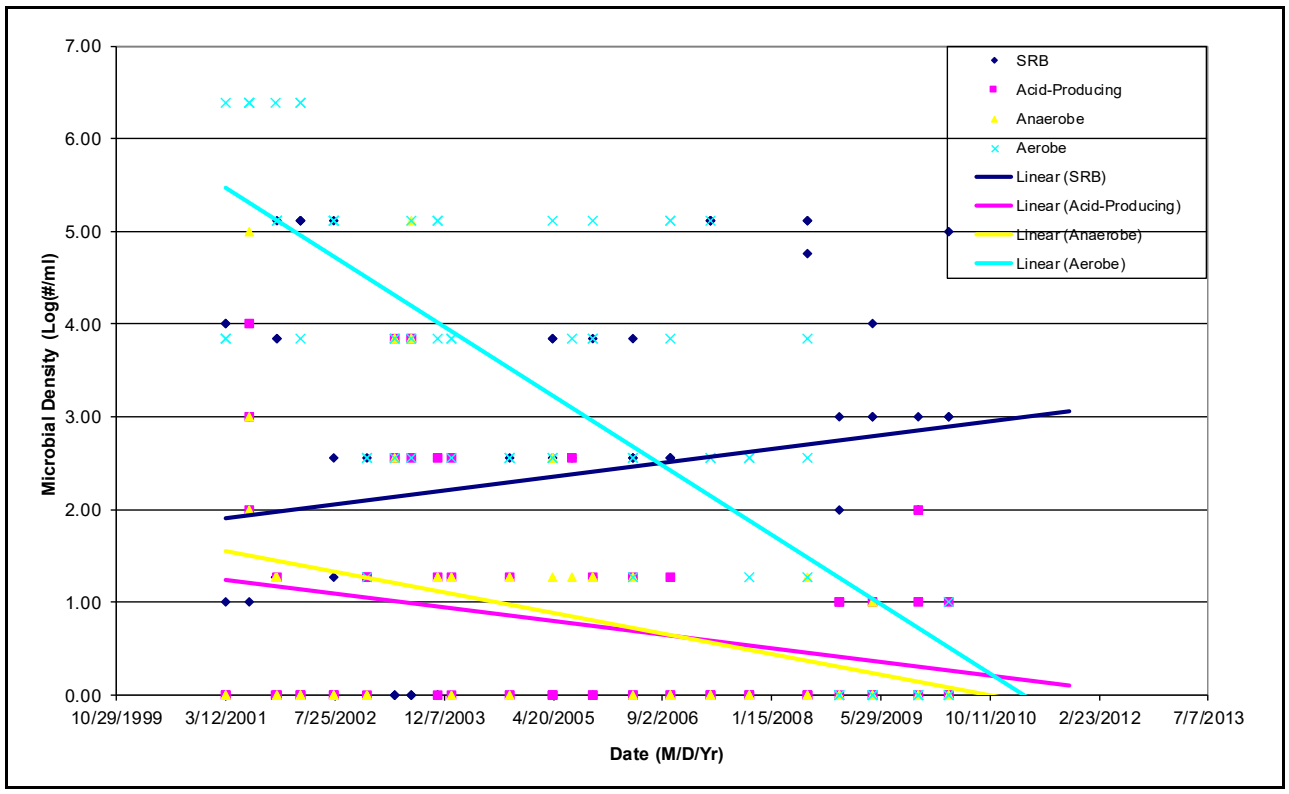


Figure 4-2 Microbial density over time from MICkit™ III results

The activity of gram-negative bacteria, as measured by Biolog™ plates, decreased in the basin (Figure 4-3) and was below average activity values in 2007-08. The activity of gram-positive bacteria decreased in the basin and was below average L-Area activity in 2006-09. Densities of Acid Producing Bacteria (APB) remained low for all samples except July 2005 and were not detected during 2007 and 2008. Sulfate Reducing Bacteria (SRB) had high variability in the last nine sample events. Anaerobic bacteria were low for all sample sets and were frequently not detected. Viable aerobic densities decreased when compared to historical densities and had large variability in the results reported. The metabolic diversity of microorganisms in the basin decreased dramatically.

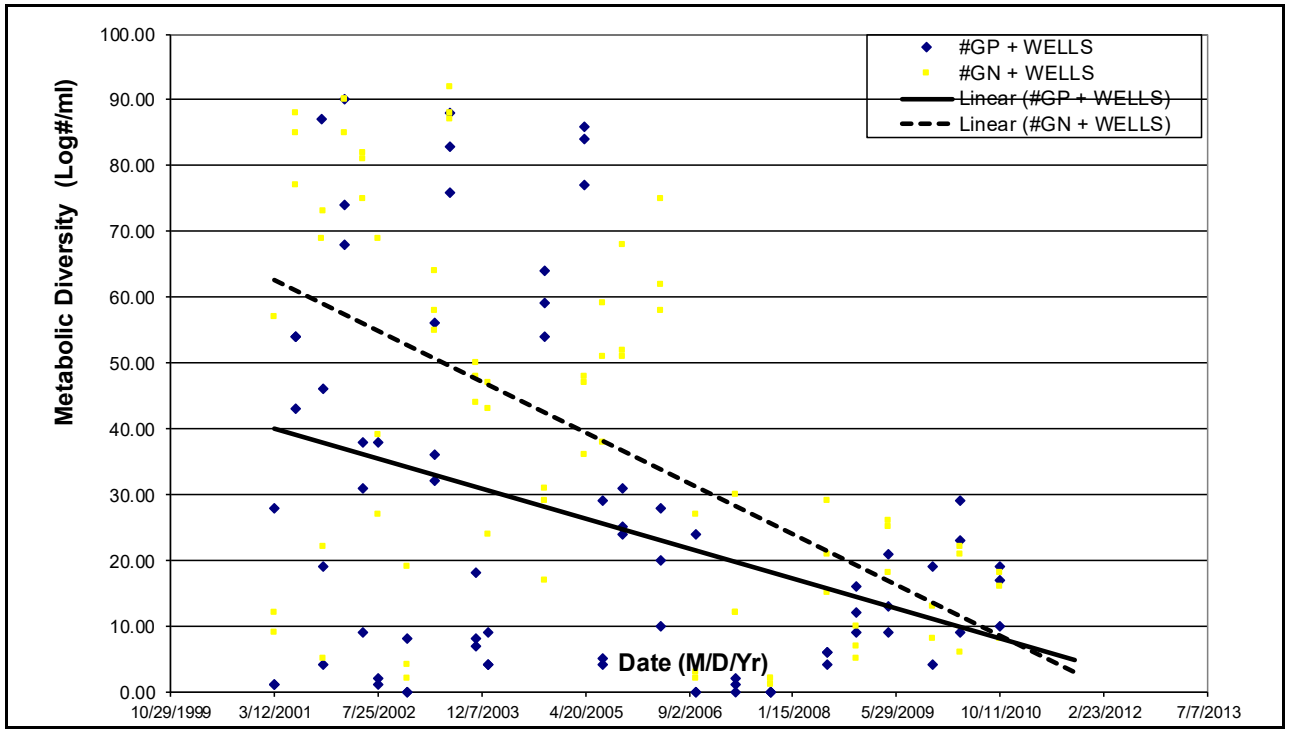


Figure 4-3 Biolog™ GN and GP Total Positive (i.e. Metabolic diversity)

Colony counts are shown in columnar form in Figure 4-4, with a dominance of aerobes and a lowest count of acid-producing bacteria.

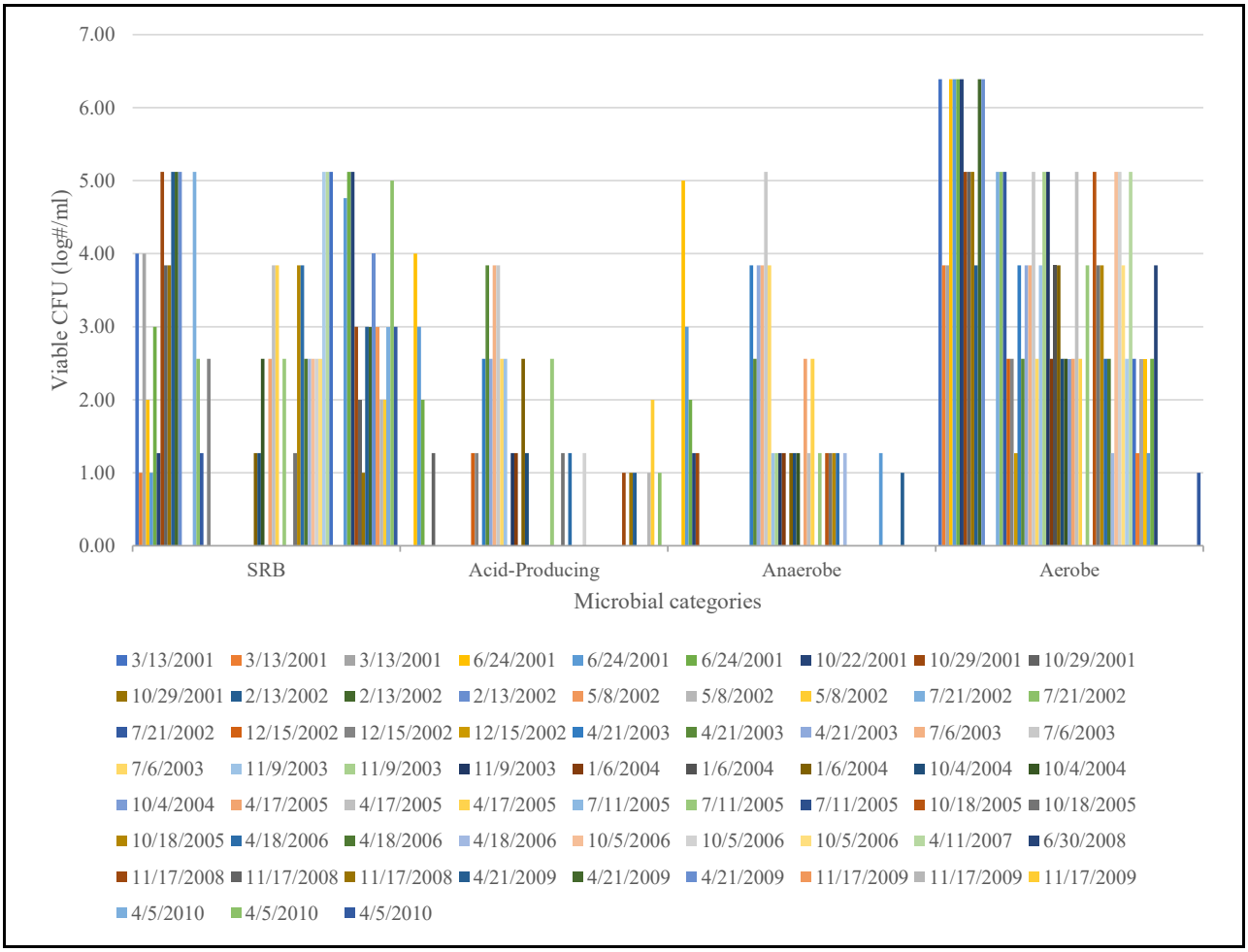


Figure 4-4 Microbiology of L-Basin: Phase I

Average viable CFU/mL counts are 2.35 for SRB, 0.80 for acid-producing, 0.89 for anaerobes, and 3.23 for aerobe bacteria.

4.2 Phase II testing – 2017-2020 Microbial Sampling: MICkit™ Results

In Phase II of testing, MICkit™ 5 was used to evaluate microbial densities as viable CFU/mL = over a span of just under 2 years (Oct. 2017 to Sept. 2019). Note that MICkit™ III was used for Phase I testing, whereas MICkit™ 5 was used for Phase II testing. MICkit™ 5 does not have aerobe detection capability but does have low nutrient and iron-producing bacteria detection. Coupons were not investigated in Phase II of data collection. Biolog™ testing was not conducted for Phase II data for determining density and diversities of microorganisms. FITC direct counts for density were not investigated in Phase II.

Results in Figure 4-5 indicate a dominance of iron-related and sulfate-reducing bacteria (SRB) that persist over time, with a minimal amount of anaerobic and acid-producing

bacteria persisting over time. Low-nutrient bacteria persisted in moderate amounts over time.

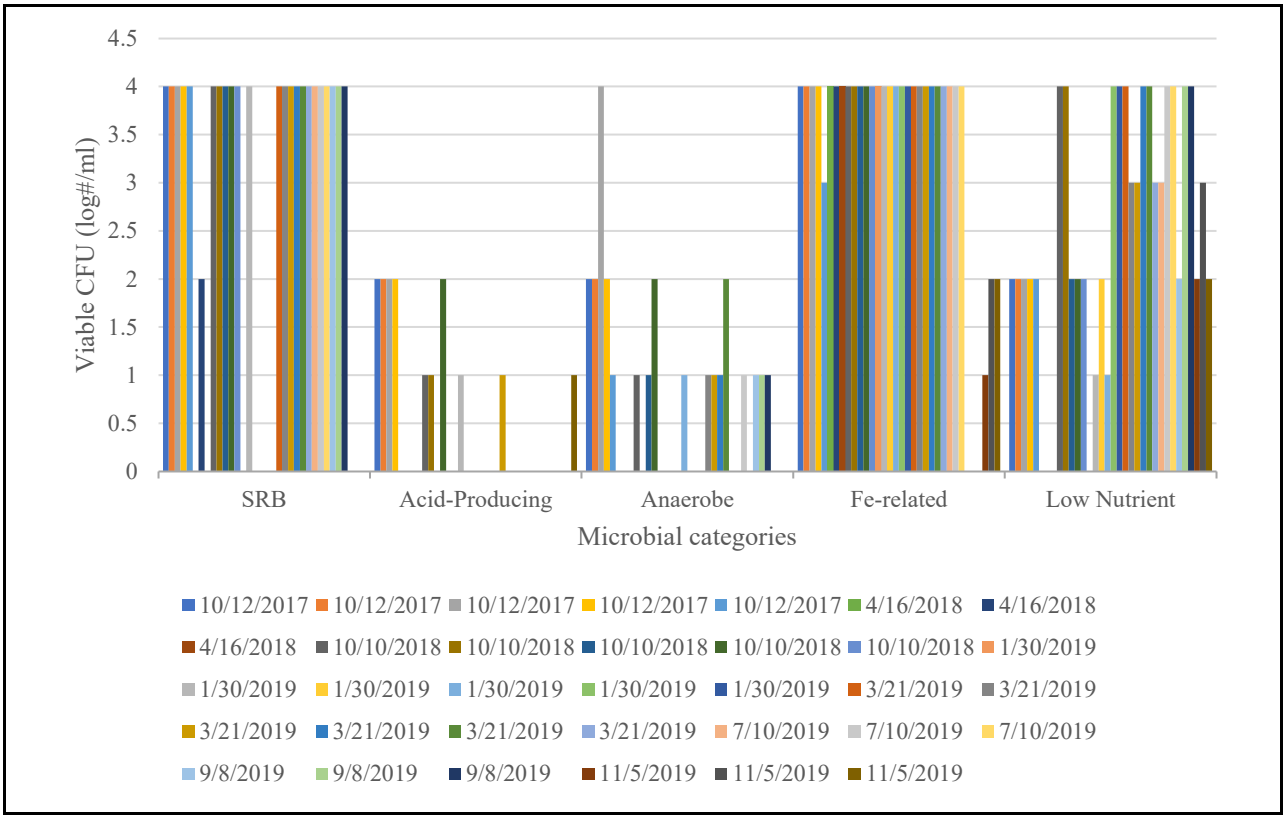


Figure 4-5 Microbiology of L-Basin: Phase II

Average viable CFU/mL counts are 2.76 for SRB, 0.44 for acid-producing, 0.74 for anaerobes, 3.41 for Fe-related, and 2.5 for low nutrient bacteria.

MICkit™ 5 results for Phase II data are shown in Figure 4-6, with all bacterial densities decreasing except that of low-nutrient bacteria, which has a strong increasing trend.

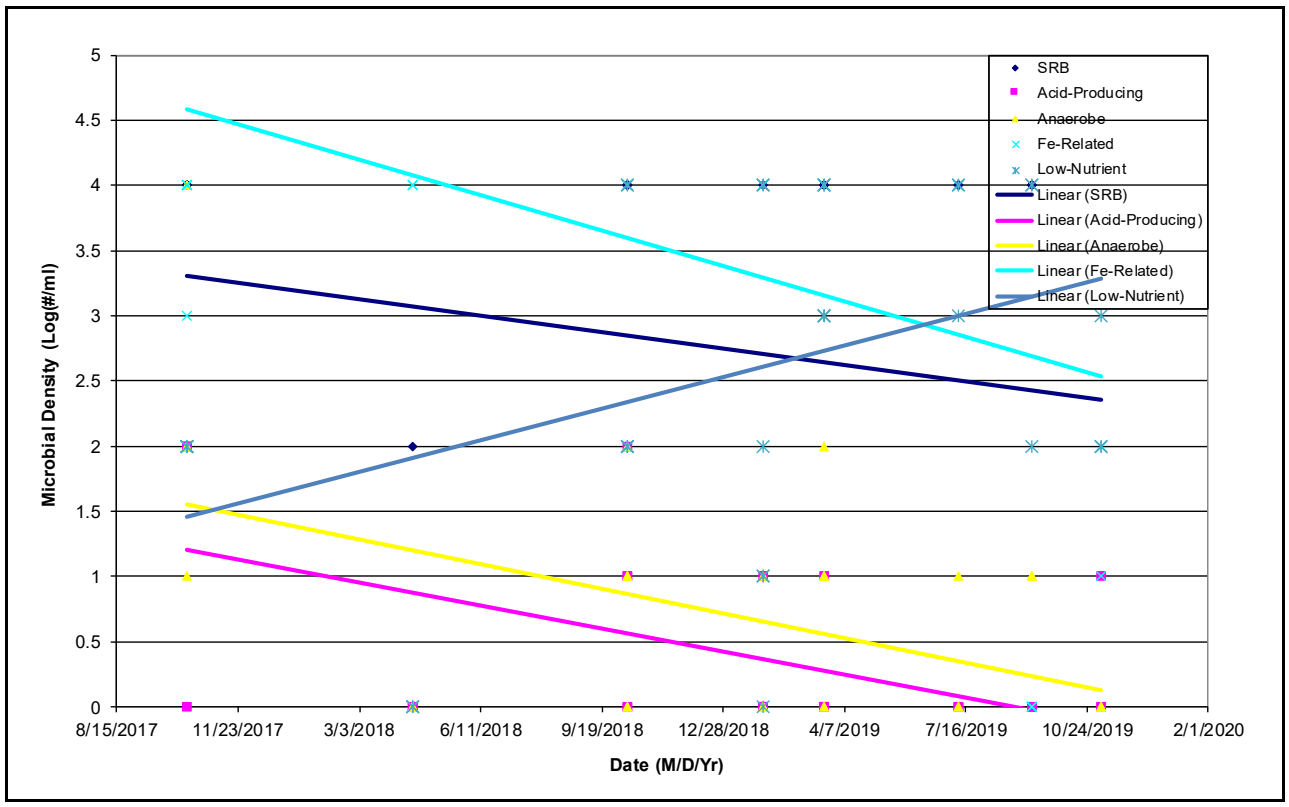


Figure 4-6 Microbial density over time from MICkit™ 5 results: Phase II

Three aqueous L-Basin samples were recently analyzed over Oct. 2020 to Nov. 2020,". Results indicated a dominance of low-nutrient and iron-related bacteria that persisted over time, with the lowest count of sulfate-reducing bacteria recorded over time. Anaerobes and acid-producing bacteria persisted in moderate counts over time (Figure 4-7). This data is separated out from the remaining Phase II data due to separate consolidated grouping and timeframe of testing.

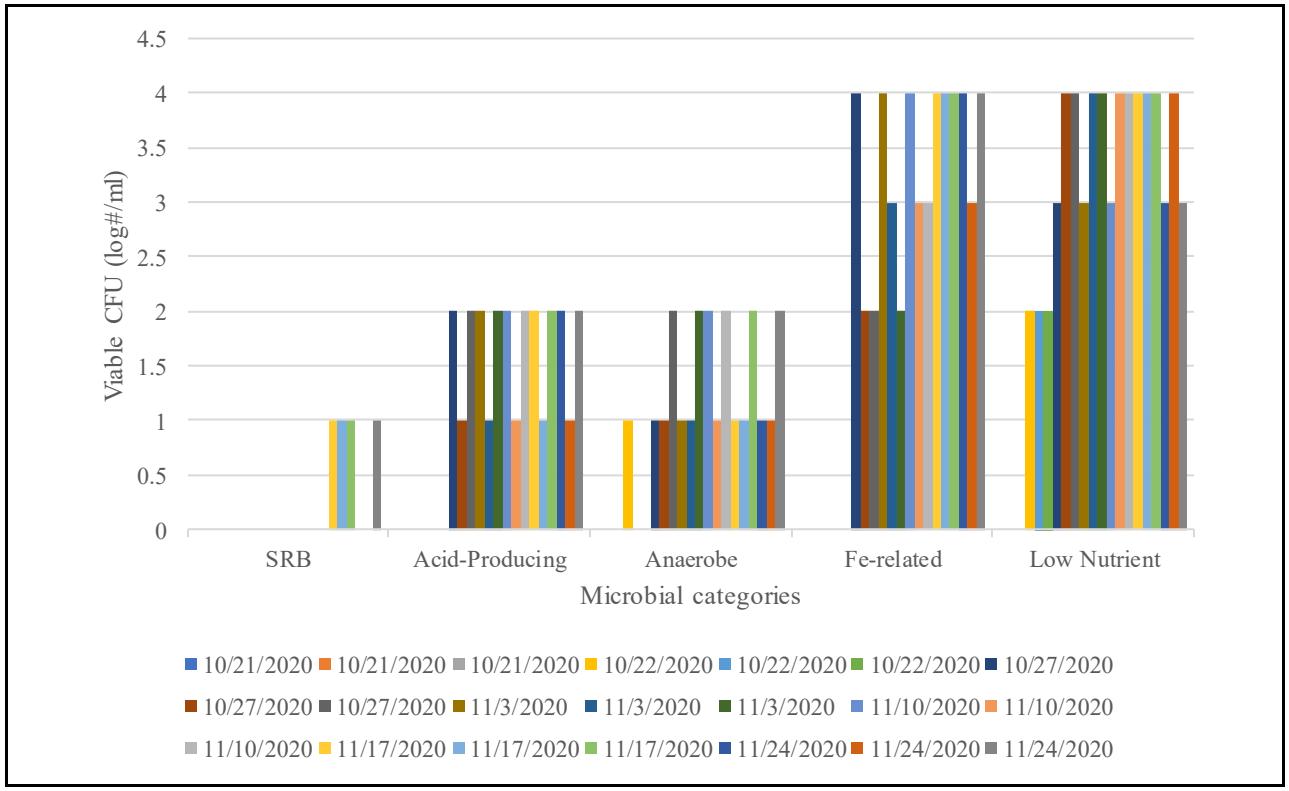


Figure 4-7 Microbiology of L-Basin: Phase II Part II

Average viable CFU/mL counts are 0.19 for SRB, 1.19 for acid-producing, 1.05 for anaerobes, 2.38 for Fe-related, and 2.90 for low nutrient bacteria.

MICkit™ 5 results for Phase II Part II data are shown in Figure 4-8, with all bacterial densities increasing.

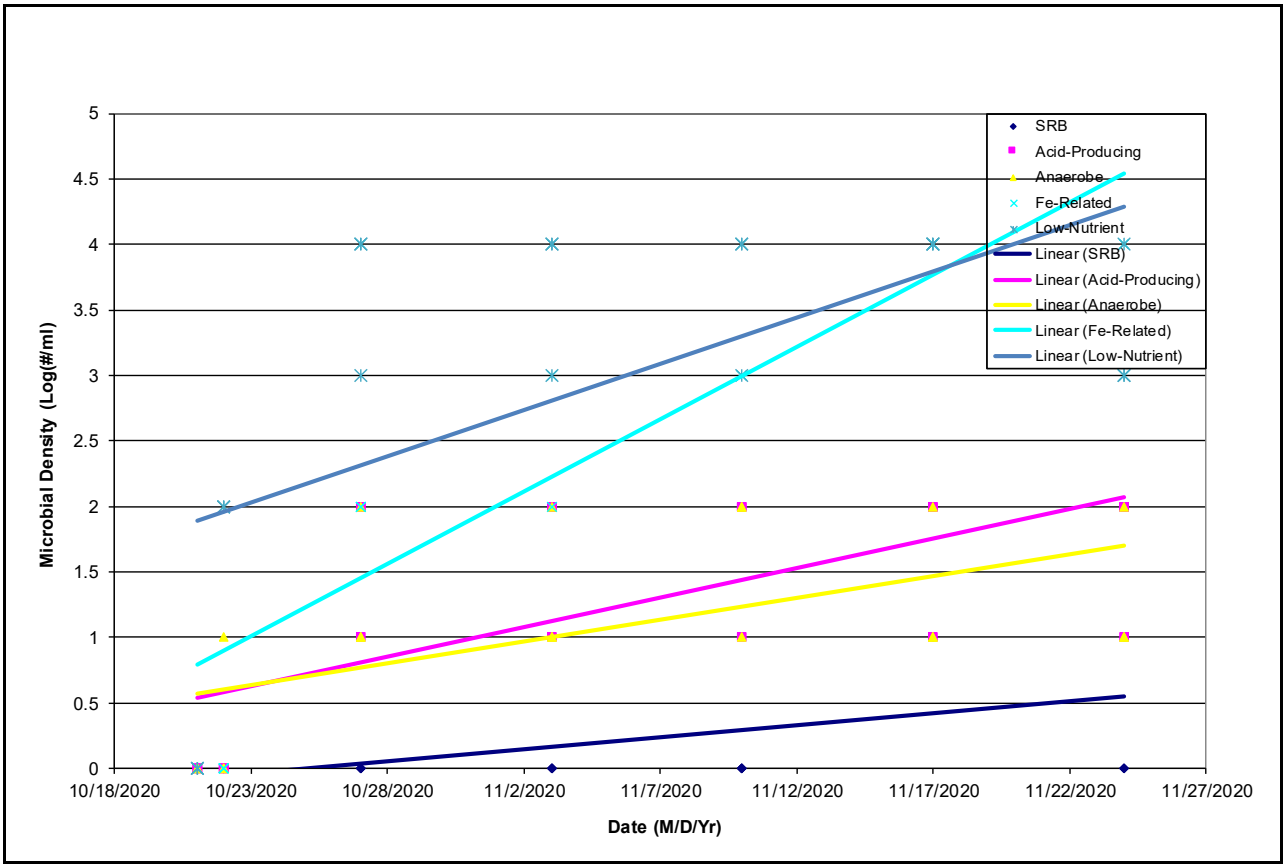


Figure 4-8 Microbial density over time from MICkit™ 5 results: Phase II Part II

All average bacterial CFU/mL counts are summarized in Table 4-1 below.

Table 4-1 Average CFU/mL by data set

Data Set	SRB (CFU/mL)	Acid-Producing (CFU/mL)	Anaerobe (CFU/mL)	Aerobe (CFU/mL)	Fe-related (CFU/mL)	Low-nutrient (CFU/mL)
Phase I	2.35	0.80	0.89	3.23	N/A	N/A
Phase II	2.76	0.44	0.74	N/A	3.41	2.50
Phase II Part II	0.19	1.19	1.05	N/A	2.38	2.90

4.3 Biofouling Results

Microbial growth has led to biofilm formation that has caused limited biofouling in the basin (Figure 4-9) [15]. Severity scales are based on standardized scales from the Institute of Validation Technology [25]. There has been growing concern over the reoccurrence of biological structures in the basin. Two sampling events were completed to obtain biological structures from L-Basin to identify the material and determine potential impacts the material may have on the basin. These biological structures and associated

biofouling build-up could interfere with operations, causing problems identifying numerical tags on containers stored in 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. The bacteria produce a biofilm including polysaccharides that can trap detritus, e.g. inorganics and organics, in the matrix. 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 [8]. Without these external inputs the bacteria cannot sustain themselves.

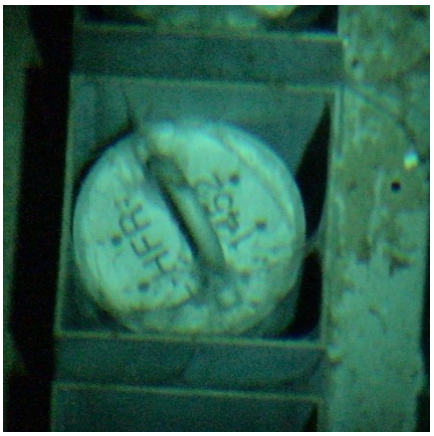
Water quality in L-Basin is strictly maintained by continuous filtration and deionization to ensure the long-term structural integrity of the aluminum alloy cladded fuels and assemblies stored in the basin. Additionally, water samples are analyzed biannually for specific microbiological pathways known to contribute to the pitting and corrosion of Al clad nuclear fuels in wet storage. While straightforward to conduct, there are a number of technical issues with these assays (how they are conducted, what they measure, and how the results are interpreted), and separately, they have not been useful in predicting or indicating the occurrence of biological structures in the basin. Biological structures began to appear in L-Basin on and around spent nuclear fuel in 2011 [15]. Since, the accepted strategy has been to remove the biological structures by vacuum filtration, which generates a secondary waste stream and added cost, but this temporary solution does not address the actual problem as the biological structures are a persistent and reoccurring condition in L-Basin. The specific conditions contributing to the formation and reoccurrence of the biological structures have not been resolved. The higher occurrence of low-nutrient bacteria and decline of some of the other bacteria might be a factor in biological structure formation, but this is speculative. Biocides cannot be used in the basins due to corrosion issues. The chemical and/or biological factors involved must first be identified to properly understand the problem, and only then can we offer a reliable and lasting solution.

An initial sampling of the unknown material 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 biological structures were observed in 7% of the basin and on 40% of the fuel. Figure 4-9 shows five levels of severity used during the basin inventory in 2011 [15] The levels were assigned to basin storage locations based on a visual comparison to these pictures. 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.

In L-Basin, suspected microbial colonies or biological structures often appear as amorphous white flocculent material on metal surfaces. This white material was sampled

from locations within the storage basin and precipitated for chemical and spectroscopic characterization, and microbial composition through deoxyribonucleic acid (DNA) analysis [8]. The spectroscopic analyses demonstrated the precipitant to be primarily amorphous to crystalline aluminum (oxy) hydroxides with associated Fe, Si, Ti, and U. High levels of organic carbon were found in the precipitants as well. It was concluded that episodic changes in the physical and chemical properties of the basin contribute to the polymerization of aluminum (oxy) hydroxides, which accumulates nutrients, organic carbon, and bacteria from the surrounding basin water. These precipitants then establish conditions favorable for bacterial colonization and growth. Comparative analyses of 16S rRNA genes were performed and microbial community and taxonomic signatures unique to the L-Basin environment were revealed. The biological component of the biological structures was determined to be almost exclusively heterotrophic bacteria [15]. Spectroscopy did verify the chemical composition of the non-biological component as Fe, Si, Ti, and Al to be 2.5% of the total mass [16].

Severity Level 1



Severity Level 2

Severity Level 3

Severity Level 4

Figure 4-9 Severity Classifications of the unknown material on a 0 to 5 Scale [15]

A diverse microbiological community was evident by high throughput DNA sequencing analysis performed directly on collected biological structures. The majority of these microbes were heterotrophic, though few autotrophic signatures were detected,

suggesting that outside sources of organic carbon (not algae growing in the basin) are more likely responsible for maintaining high bacterial densities and diversity. This result suggested a potential mitigation strategy whereby point sources of organic carbon could be controlled as a means of restricting microbiological growth and activity in the system.

Scanning electron microscopy (SEM) was used to examine these biological structures (see Figure 4-10 and Figure 4-11), as reported in SRNL-L4330-2012-00070 [17]. The sample was filtered and vacuum dried prior to processing and analysis. An SEM examination was done on the biological structures and the filter together. These SEM images, along with the original samples, are representative of the conditions and biological structures but do not necessarily represent the exact biological structures from Figure 4-9. There were no clear structures observed during energy dispersive X-ray spectroscopy (EDS) analysis compared to the background image of the filter. Instead, there were isolated spots and particles evident against the filter background. The location was examined using EDS with a Quadrant Back Scattering Detector (QBSD). Figure 4-12 shows representative EDS results at individual spots. Overall, the background EDS results of the filter from QBSD analysis indicated the presence of iron and stainless steel, some aluminum, chloride, manganese, tantalum, magnesium, zinc, silicon, and oxygen. One spot contained plutonium, but the SEM operators surmised, based on their experience, that this was likely contamination from the SEM enclosure. One spot contained gold, but this was also attributed to SEM contamination. The lack of structure observed during analyses may be attributed to long hold times of the sample prior to analysis and SEM preparation procedures. Hold time refers to the time between sampling and analyzing the material (i.e., the time when the samples were not held on ice or refrigerated).

Conducted in parallel, various chemical and spectroscopic analyses failed to reveal strong evidence that the biological structure was principally of biological origin or composition. The chemical species detected were inorganic crystalline precipitates, containing high levels of Al and U in what appeared to be mostly (oxy) hydroxides. The elemental composition of the biological structure was generally confirmed by SEM and EDS; however, the structural integrity of the biological structure matrix was not preserved during the dehydration steps in sample preparation for viewing. This outcome is not uncommon for these types of biological materials (i.e., biofilms); preparative osmodehydration of the sample has resulted in disruptive shrinkage and loss of surface features, or in this case the make-up of the matrix itself. More advanced techniques in nondestructive staining and imaging by confocal microscopy or environmental electron microscopy; SRNL does not possess these capabilities for analysis of radiological samples. Furthermore, SRNL lacks sufficient detailed information to elucidate the aqueous phase chemical reactions that could potentially occur under the basin conditions to result in the formation and precipitation of these materials. The rough surfaces observed here would be conducive to microbial attachment, colonization, and biofilm formation.

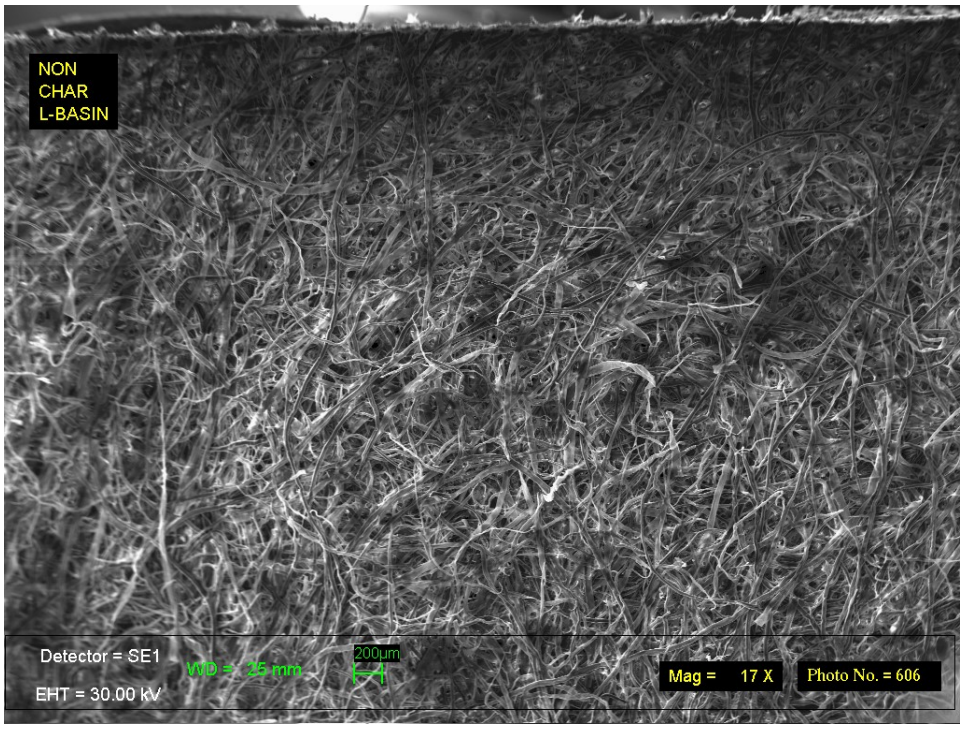


Figure 4-10 QBSD Image of Filter 17X [17]

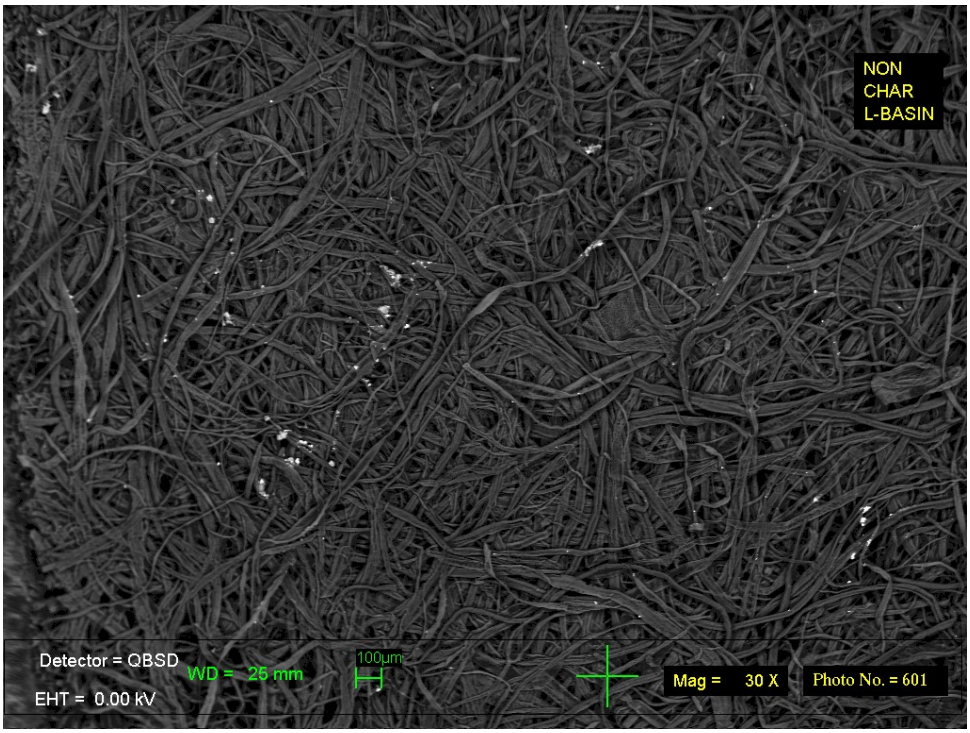


Figure 4-11 QBSD Image of Filter 30X [17]

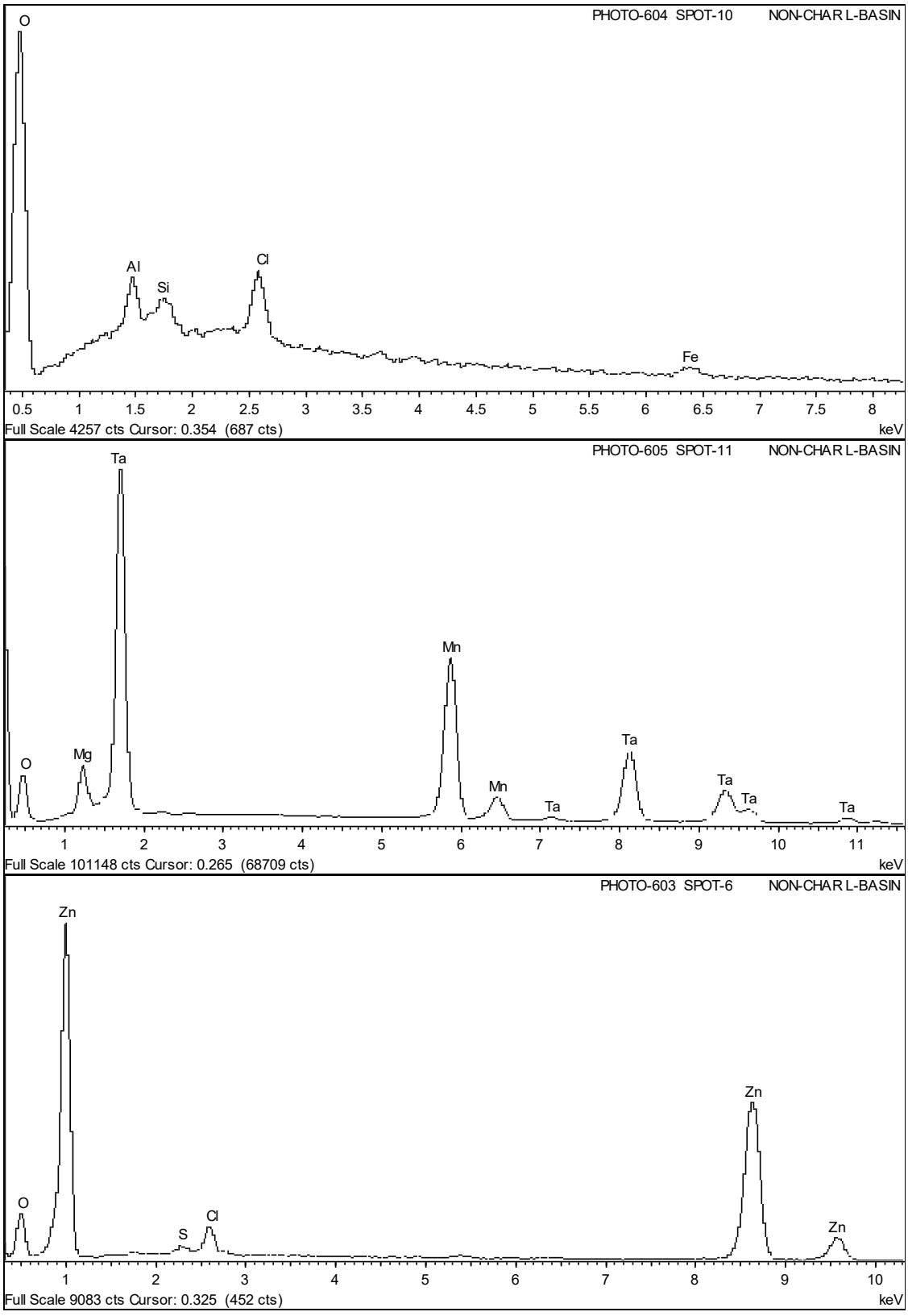


Figure 4-12 EDS results for individual spots [17]

The biological structure matrix of polymeric material combined with trace elements could function as a 'raft' for microbial colonization and organic carbon in the system; resulting in a unique 'hot spot' for higher than typical rates of microbial growth and activity, thus serving as a source for the proliferation of microbial colonies. This conclusion is speculative but is not disproven by previous measurements showing high TIC/TOC values for the biological structure.

Having only periodic data and observations to work from, SRNL cannot confidently deduce the events which led to the formation of these biological structures; though because bacterial diversity was unexpectedly high, SRNL concluded that biological processes could have a role to play in the formation and spread of these structures in the basin. Low-level basins are frequently conductive to microbial growth, and bacterial precipitates establish favorable microhabitats for bacterial colonization [8]. The contributing condition(s) of physical / chemical processes is likely; though explanation and mitigation would involve much more work and effort. Control of biological processes by minimizing microbial growth, accumulation, and attachment are more tenable alternatives in the long-term.

4.4 Radiological and Chemical Results

Of interest in Section 4.3 are any correlations between microbe concentrations and water chemistry parameters in L-Basin, as well as insights into radiological conditions at the sampling points.

In regard to radiological data, the information is not particularly insightful as the both dose above the water and tritium air sampling are non-detectable during this time period (2/2/19-1/1/20). Alpha particle results (Figure 4-13) were below the 1 dpm/mL instrument detection limit.

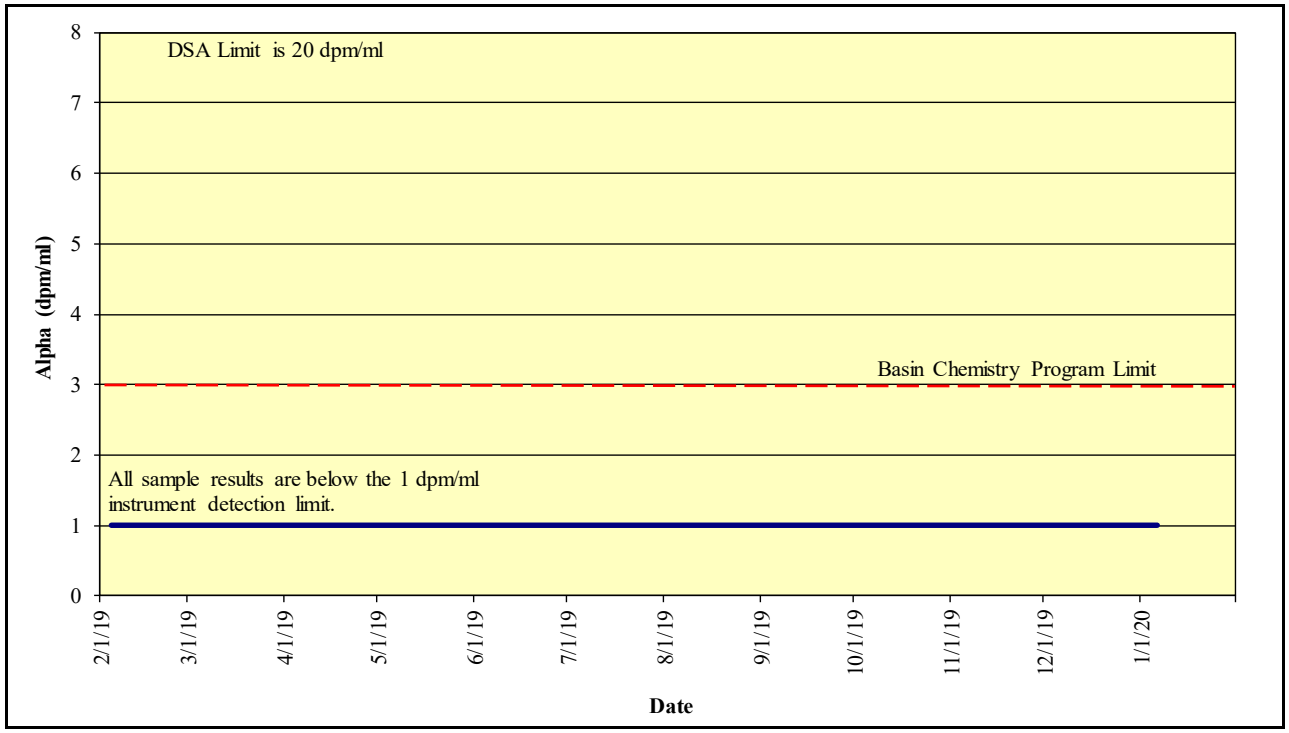


Figure 4-13 Alpha particle activity [18]

Figure 4-14 shows tritium activity in L-Basin.

Figure 4-14 Tritium activity in L-Basin [18]

Figure 4-15 shows Cs-137 activity.

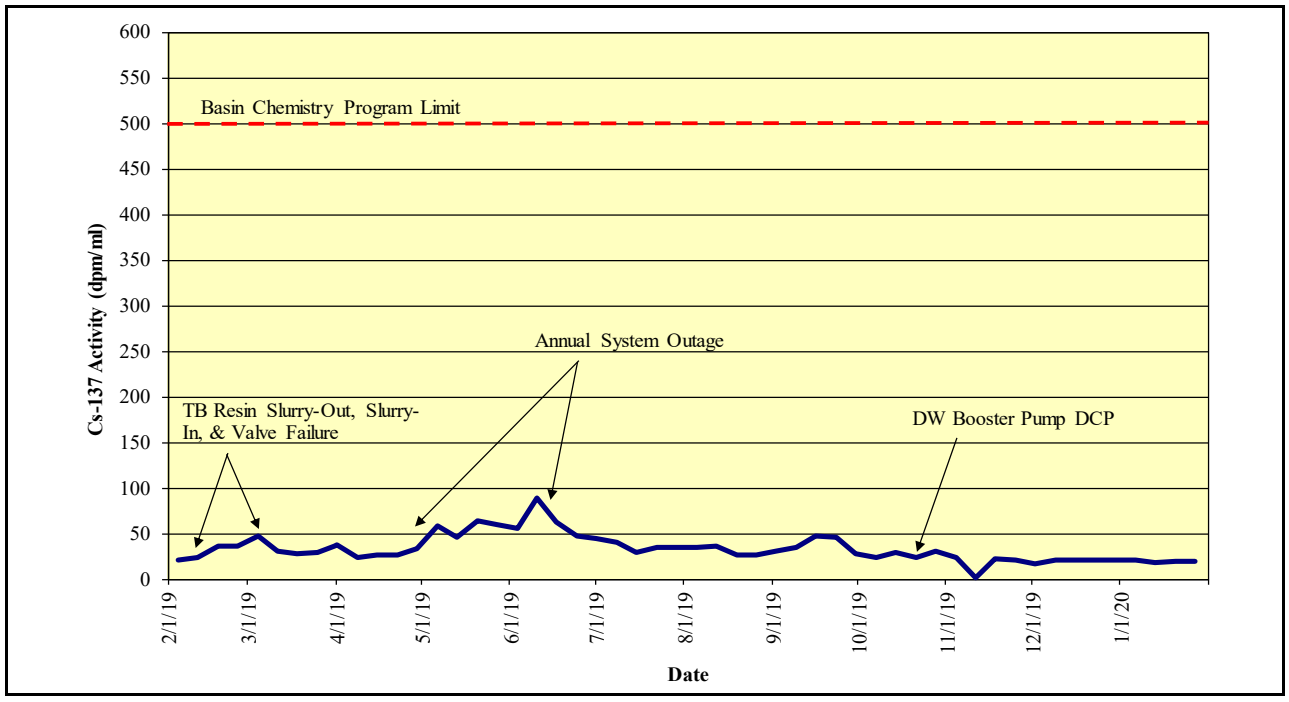


Figure 4-15 Cs-137 activity in L-Basin [18]

L-Basin was tested twice for dissolved oxygen content at different depths and locations. All the results show that the basin water is saturated with oxygen at all depths and locations due to the circulation system. The atmospheric bubbler sample results are always less than detectable, and the dissolved oxygen does not vary with depth. Essentially, the basic chemistry does not have appreciable variation with depth. Graphs of conductivity (Figure 4-16) and pH (Figure 4-17) show stable parameters.

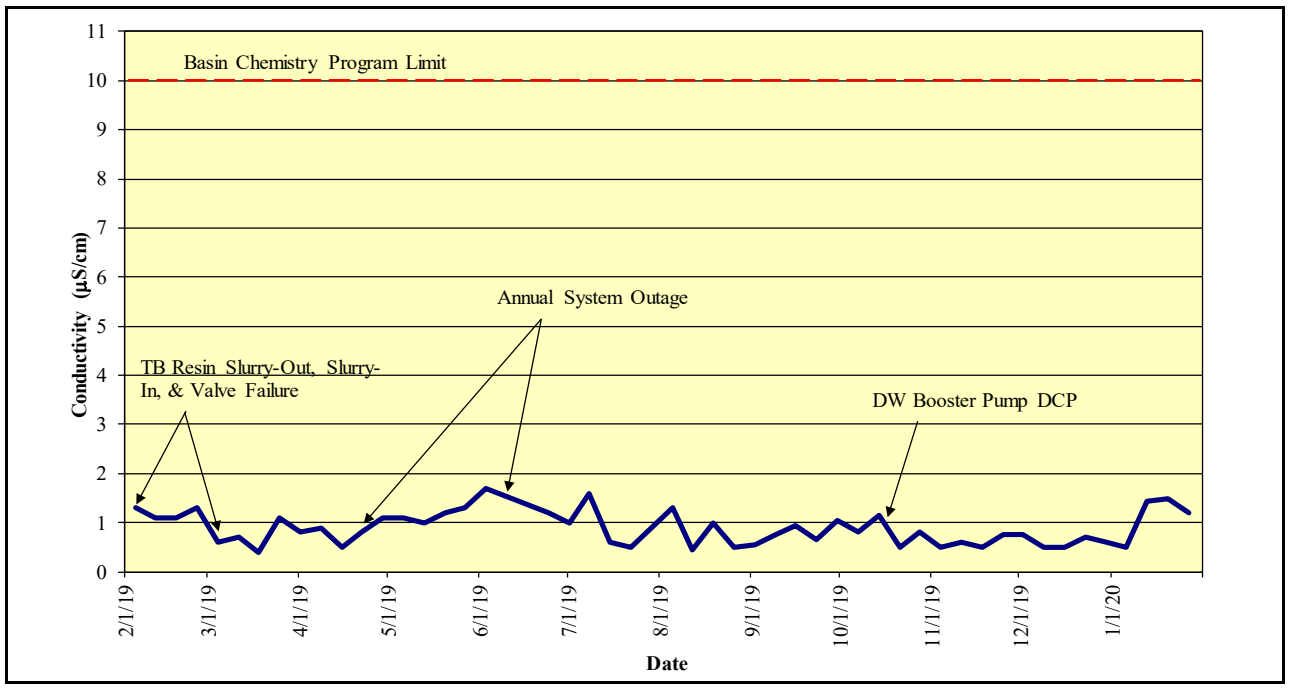


Figure 4-16 Conductivity in L-Basin [18]

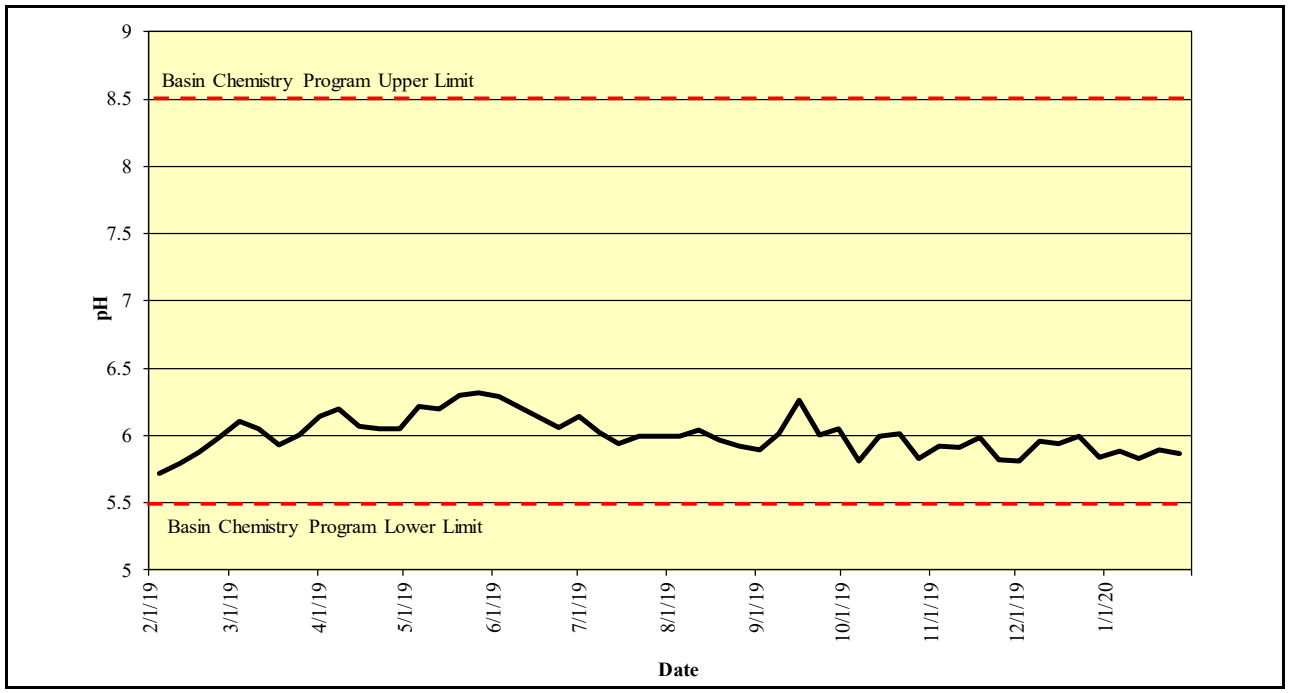


Figure 4-17 pH in L-Basin [18]

Lastly, the temperature does not vary with depth across the time span (Figure 4-18).

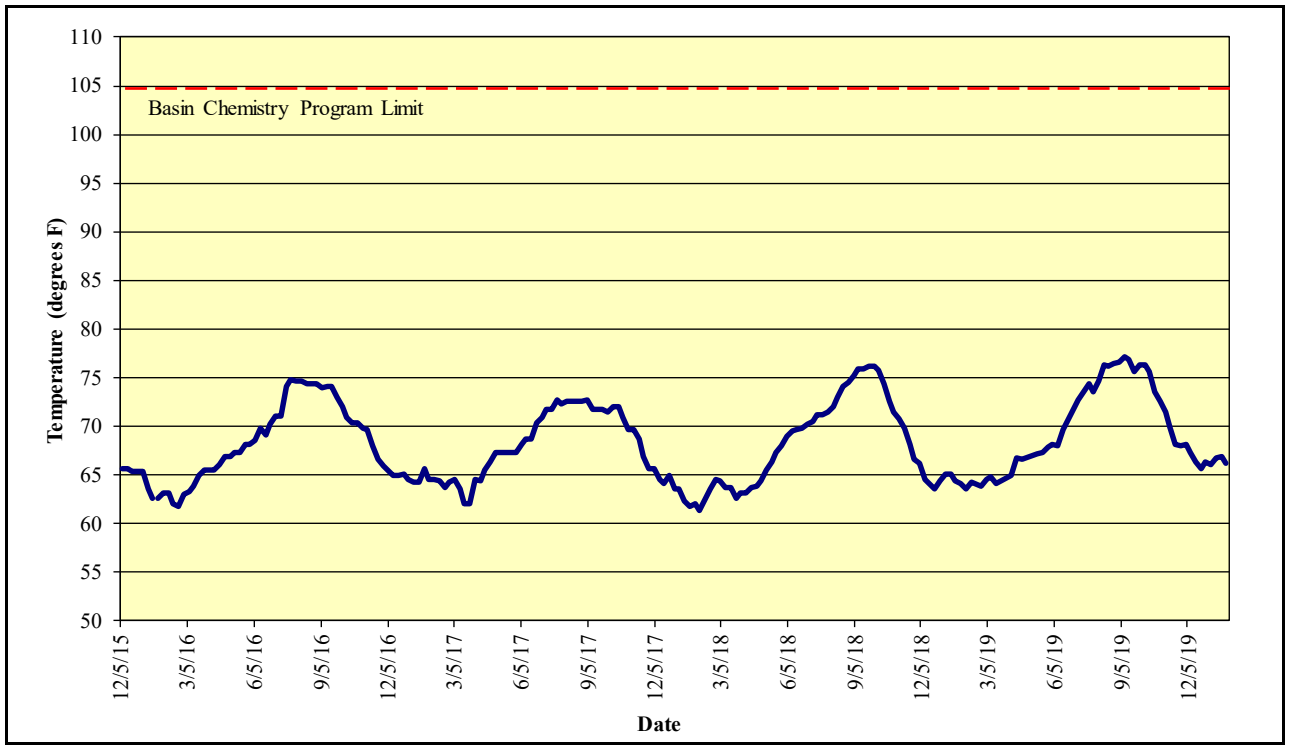


Figure 4-18 Temperature in L-Basin [18]

There are no distinct correlations between microbe concentrations and either water chemistry parameters or radiological conditions in L-Basin. Temperature does not appear to play a role in understanding the microbial parameters measured in the water samples.

4.5 L Fuel Bundles Water Sample Results (Stable Isotope Probing Test)

This section is taken from N-ESR-L-00021 [21].

> *Water samples were taken from inside ten L Bundles stored in VTS racks in L-Basin for water chemistry and microbe analyses. L Bundles store fuel assemblies in tubes with holes in the top and bottom that allow water transfer with the bulk basin. The samples were gathered for information purposes. The results provide an L Bundle water chemistry baseline that can be utilized in future fuel examinations. The bundles sampled contain the fuel assemblies chosen for the visual inspection.*
>
> *The water samples inside the L Bundles were taken on December 14, 2012 with a syringe sampler. The centrifuge tubes were transported to the Biotechnology Section of SRNL for microbe cell counts. The 250 mL High-Density Polyethylene (HDPE) sample bottles were transported to F Lab for conductivity, pH, total alpha activity, Cs-137 gamma activity, chloride, mercury, iron, copper, and aluminum*

analyses. While at the F Lab, about 25 mL of sample from each bundle sample bottle was transferred to a 30 mL glass bottle. The glass bottles were transported to the Analytical Development Section of SRNL for Total Inorganic (TIC) and Total Organic Carbon (TOC) analyses.

The bundle water sample results and comparative basin water sample results are shown in Table 4-2 , *Table 4-3* , and *Table 4-4* . *All the routine basin water analyses were performed on the bundle water samples, except for the tritium analysis. There is no tritium source in the basin, and the water tritium concentration has no connection to the corrosion of materials in the basin.*

The basin water sample results are typical or average values obtained by the routine sample pump. The conductivity, pH, chloride, iron, copper, and mercury results for the bundle water and typical or average basin water values are shown in Table 4-2. These parameters impact the corrosion rate of aluminum bundles and fuel assembly metals. Except for pH, to reduce the corrosion rate of metals the values of these parameters should be minimized. For pH, to minimize corrosion of aluminum the pH should be maintained around the neutral 7.0. Except for the chloride results, all these results are within the L-Basin Water Chemistry Control Program [14] limits. The bundle chloride sample results were accidentally diluted at the laboratory, so the detection limits were higher than the typical basin sample detection limits. The fuels have no chloride source, so the chloride concentration is not expected to be concentrated in a fuel bundle.

Table 4-2 Sample Results for Corrosion Rate Parameters [21]

Sample Location	Cond. (µS/cm)	pH	Cl (ppm)	Cu (ppm)	Fe (ppm)	Hg (ppm)
L-HFR-1435	3.95	6.61	<1.0	<0.05	<0.05	<0.002
L-RA3-0787	6.25	6.75	<1.0	<0.05	<0.05	<0.002
L-RA3-0794	7.95	6.75	<1.0	<0.05	<0.05	<0.002
L-RA3-0817	6.25	6.71	<1.0	<0.05	<0.05	<0.002
L-RA3-0822	7.3	6.73	<1.0	<0.05	<0.05	<0.002
L-IEA-R1-0625	7.65	6.75	<1.0	<0.05	<0.05	<0.002
L-ENEA-0024	7.9	6.78	<1.0	<0.05	<0.05	<0.002
L-ENEA-0352	7.4	6.78	<1.0	<0.05	<0.05	<0.002
L-ENEA-0356	5.5	6.59	<1.0	<0.05	<0.05	<0.002
L-HIFAR-1918	3	6.33	<1.0	<0.05	<0.05	<0.002
Basin	1.5	6.19	<0.1	<0.05	<0.05	<0.002

The Cs-137 activity, alpha activity, and aluminum concentration results for the bundle water and typical or average basin water in 2012 are shown in Table 4-3 [20]. These parameters in water indicate corrosion of fuel assemblies, so low values are preferable. Nine of the ten bundles contain fuel that was received pitted, so some Cs-137 and alpha activity is expected in a bundle. The Cs-137 and aluminum results are within the program limits. The alpha results for the

water in seven of the bundles were higher than the 3 dpm/mL program limit for the entire basin. The program limits protect the Safety Basis assumptions for activity in the entire basin, so it is acceptable to have a higher activity in the small volume of a fuel bundle. The activity in a bundle has no impact on the fuel corrosion rate or microbial activity.

Table 4-3 Sample Results for Corrosion Indicating Parameters [21]

Sample Location	Cs-137 (dpm/mL)	Alpha (dpm/mL)	Al (ppm)
L-HFR-1435	33.5	<1	<0.05
L-RA3-0787	38.3	<1	<0.05
L-RA3-0794	47.6	<1	<0.05
L-RA3-0817	44.2	17	<0.05
L-RA3-0822	39.42	17	<0.05
L-IEA-R1-0625	84.58	20	<0.05
L-ENEA-0024	57.5	18	<0.05
L-ENEA-0352	48.5	18	<0.05
L-ENEA-0356	31.7	18	<0.05
L-HIFAR-1918	29.1	19	<0.05
Basin	38.62	<1	<0.05

The TIC, TOC and microbe concentrations for the bundle water and basin water are shown in Table 4-4. Certain microbes can increase the corrosion rate of fuel and bundle materials. Carbon is an energy source for microbes. The bundle water TIC and TOC sample results were just slightly higher than the basin result. The microbe concentrations in the fuel bundles water are much less than the average microbe concentration in the basin water.

Table 4-4 Sample Results for Microbe Related Parameters [21]

Sample Location	TIC (ppm)	TOC (ppm)	Microbes (cells/mL)
L-HFR-1435	1.4	0.672	8.52E+03
L-RA3-0787	1.73	0.956	2.76E+03
L-RA3-0794	1.78	0.928	2.43E+03
L-RA3-0817	1.82	0.82	5.04E+03
L-RA3-0822	1.64	0.896	2.55E+03
L-IEA-R1-0625	1.81	0.884	2.67E+03
L-ENEA-0024	1.67	0.872	3.33E+03
L-ENEA-0352	1.73	1.06	6.30E+02
L-ENEA-0356	1.57	0.844	7.77E+03
L-HIFAR-1918	1.37	0.4	3.81E+03
Basin	0.804	0.4	1.60E+05

5.0 Conclusions

A summary of microbial monitoring activities for L-Basin of over 20 years was compiled, resulting in the microbial colony characterization contained within this report. The data was separated into two phases, Phase I (2000-2012) and Phase II (2017-2020).

In Phase I, overall bacterial density was not subject to larger changes or significant trends other than a slightly decreasing trendline, with a slightly more accelerated rate of decrease in density toward the tail end of the sampling period. Metabolic diversity of microorganisms in the basin decreased dramatically. With regard to the bacterial density of specific MICkit™ III bacterial groups, the only exceptionally strong trend is that of the aerobe density decreasing, with the other groups only nominally increasing or decreasing. Aerobes were most prevalent at 3.23 average CFU/mL and acid-producing bacteria were least prevalent at 0.80 average CFU/mL.

In Phase II, using MICkit™ 5, Fe-related bacteria were most prevalent at 3.41 average CFU/mL and acid-producing bacteria were least prevalent at 0.44 average CFU/mL. All bacterial densities decreased except that of low-nutrient bacteria, which had a moderately increasing trend. In Phase II Part II, low-nutrient bacteria were most prevalent at 2.90 average CFU/mL and SRB were least prevalent at 0.19 average CFU/mL. MICkit™ 5 results for Phase II Part II data showed increasing densities for all microbe categories.

Biofouling results from microbial growth leading to biofilm formation through biological structures are included, along with L fuel bundles water sample results. SRNL cannot confidently deduce the events which led to the formation of these biological structures; though because bacterial diversity was unexpectedly high, SRNL concluded that biological processes could have a role to play in the formation and spread of these structures in the basin.

There are no distinct correlations between microbe concentrations and either water chemistry parameters or radiological conditions in L-Basin. Temperature does not appear to play a role in understanding the microbial parameters measured in the water samples.

6.0 Recommendations/Improvements/Mitigation Strategies and Path Forward

The Microbiological Monitoring program should include the following items:

- Perform microbiological monitoring of the L-Basin bulk water twice per year, once during the spring and fall.
- Remove one set of coupons from L-Basin for examination each calendar year.
- Continue to reduce the amount of artificial lighting from the basin in order to reduce the organic carbon in the system and reduce alga growth.

- Remove as much organic material as possible from the basin through filtration. This will keep the bacteriological growth low since the limiting nutrient is organic carbon. Periodic evaluation of the particulate and dissolved organic carbon in the basin will serve as a QA/QC for L-Area operations.

- Microbial densities in the basins should be carefully scrutinized. A trend of increased microbial populations may directly increase MIC in the future or increase carbon loading in the basin causing an indirect potential stimulation of unwanted microbial activity. Deterioration of aging infrastructure (e.g. concrete, piping, etc.) could enhance microbial growth opportunities [22].

- Minimize the sample hold times prior to analysis to clarify the observable structures during SEM and EDS analyses.

This program should continue sampling/monitoring of the L-Basin water and coupons. Technical support provided by this program will continue to provide help with issues such as microbial floc formations, alga growth, mosquitoes, addition of chemicals into the basin, unusual colored growth associated with the spent fuel, and cloudy basin water. Additionally, there are a series of possible paths forward and potential mitigation strategies that could be employed to fully understand the presence of the microbial-based biological structures, 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, internal and external, that enter the basin.

- Evaluate existing coupons – examine and compare the biological material on existing coupons to the material 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.

- 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. These microcosms

could be tested “in situ” with designed retrievable/reusable cells. Materials analyses with a corrosion focus and microbiological monitoring would be done.

- Material removal – the current options include vacuuming the material with ultrafiltration 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 [19]. Both methods may need to be repeated periodically and pretesting may be required for peroxide dosing.

7.0 References

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