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Characterization of Microbial Activity

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University and Community College System of Nevada (UCCSN)

Scientific Investigation Plan (SIP)

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REVISION HISTORY

Revision Number	Effective Date	Description and Reason for Change
0	01/06/05	Initial issue.

1.0 Scope and Objectives

The overall goal of this study is to investigate the phenomena that affect the fate and transport of radionuclides in the environment. The objective of this task, "Characterization of Microbial Activity", is to develop a molecular biological method for the characterization of the microbial population indigenous to the Yucca Mountain Project site, with emphasis in detection and measurement of species or groups of microorganisms that could be involved in actinide and/or metal reduction, and subsurface transport. Subtasks consist of QA planning and preparation, and literature review. This task is part of a cooperative agreement between the UNLV Research Foundation and the U.S. Department of Energy (#DE-FC28-04RW12237) titled "Yucca Mountain Groundwater Characterization".

To quantify and characterize the microbial populations, including microorganisms that may be viable but are not currently physiologically active, a molecular biological approach will be utilized to amplify and detect microbial DNA present in the subsurface. This approach, termed polymerase chain reaction (PCR), results in the amplification of DNA sequences that are unique to the groups of microorganisms of interest. A quantitative PCR (QPCR) assay will be developed and used for the measurement of subsurface microbial populations. The protocol will be evaluated in laboratory tests involving representative microbial species and genera previously characterized from the subsurface. The protocols developed will also be tested by assaying available subsurface samples from the Yucca Mountain Project. It is anticipated that the results will confirm previously reported culturable bacteriological populations, but will expand the knowledge base of bacterial populations present to include those that were not detected due to the limitations of culture. This will provide a more complete determination of microbial populations that may affect the repository environment.

This work is subject to the QARD and to University and Community College System of Nevada (UCCSN) Quality Assurance (QA) Program requirements.

2.0 APPROACH

The research will be conducted in 5 subtasks. Subtask 1 will involve QA indoctrination, and equipment acquisition, installation and training. Subtask 2 will consist of an extensive review of the scientific literature. Subtask 3 will involve laboratory studies and protocol development. Subtask 4 will consist of validating the protocols developed in the laboratory. The final subtask, Subtask 5, will focus on technical and QA reviews and preparation of the final report.

Subtask 1: Planning and Preparatory Activities

The first three months of the project will involve completion of a scientific investigation plan (SIP), reviewing/updating the implementing procedures, and equipment acquisition, installation and training. The SIP will be prepared and technical and QA reviews will be obtained. Personnel new to the QA program will receive scientific notebook training and other QA-required training. In addition, the necessary equipment will be purchased, installed, and the personnel trained on the equipment use.

Subtask 2: Literature Review

A literature review and a search of the genetic sequence databases will be conducted to determine "universal" primer and probe sequences that amplify DNA from all bacterial genera. In addition, searches will be conducted to determine primers and probes that amplify specific bacterial groups of interest, such as metal reducing bacterial species.

Subtask 3: Laboratory Studies and Protocol Development

A QPCR assay protocol designed for the measurement of subsurface microbial populations will be developed. Candidate universal primer and probe sequences, identified in the literature review, that are compatible with the TaqMan® QPCR technology will be selected and tested in the laboratory. The universal primer and probes that are selected will be those that most effectively amplify DNA from different bacterial groups spanning the spectrum of diversity within the prokaryotes. Cross-reactivity with DNA from nonbacterial sources will also be tested. Similarly, group-specific primers and probes will be evaluated. PCR amplification conditions will be optimized, and quantitation standards will be prepared from various bacterial species by extracting the DNA from known concentrations of cells. A DNA extraction and concentration protocol previously developed in our laboratory will be used (Buttner *et al*., 2001).

The ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) will be used for QPCR analysis. PCR quantitation standards will be prepared from a purified suspension enumerated electronically with a Coulter Multisizer II (Beckman Coulter, Inc., Hialeah, FL) using the same DNA extraction and purification methods used to process samples. Quantitation will be achieved by amplification of standards containing DNA extracted from suspensions of known concentration of each target organism. Standards will be amplified in duplicate at the same time and under the same conditions as the replicate unknown samples. Once amplification is completed, the data will be analyzed using the software provided with the ABI Prism 7900 sequence detection system. Using the concentrations assigned to each standard, the software constructs a standard curve of *Ct* value versus concentration. *Ct* refers to the PCR cycle at which fluorescence (i.e., amplification product) is first detected; and is inversely proportional to the initial DNA template concentration. Concentration values for the unknown samples will be extrapolated from the standard curve by the software and reported as the mean of two replicates. An internal positive control (IPC) (IPC-VIC™ Probe; Applied Biosystems) will be incorporated into PCR reactions to determine whether the samples contain PCR inhibitors. The IPC kit consists of control DNA, primers and a specific probe. This fluorescent probe is labeled with a dye that is different from the target DNA probe to allow for the differentiation of fluorescent signals generated during amplification. A known amount of IPC DNA will be amplified with the sample and inhibition is observed by a change in amplification of control DNA.

Subtask 4: Protocol Validation

The QPCR protocol will be evaluated in laboratory tests involving representative microbial species and genera, with particular focus on species known to participate in metal

reduction and subsurface transport. The protocol will also be tested with available samples from the repository environment to validate the assay.

Subtask 5: Task Close-Out

The final phase of the project involves technical and QA reviews, and report writing.

Any special environmental conditions will be described in the corresponding Implementing Procedure (IP) or scientific notebook. Any processes not addressed in the IP will be documented in the scientific notebook. Special controls will be specified in the corresponding IP. The skills of the microbiologists needed to perform these measurements are outlined in their position descriptions. There are no special training requirements for this work beyond the education and experience requirements of each employee's position description.

3.0 SCHEDULE OF WORK

The project will utilize IPs previously approved by the UCCSN QA Program that have been in use at the HRC. Any additional IPs that need to be written will be completed by February 28, 2005. Reports summarizing the progress of this study will be submitted quarterly to the UNLV Research Foundation for review and signature and final submission to the DOE ORD. In accordance with QAP-3.6, "Submittal of Data", Q data will be submitted to the UCCSN Technical and Electronic Data specialist for entry into the UCCSN Technical Data Archive (TDA). Q data consists of PCR data that have been verified using the check lists included in the appropriate instrument IP and for which the associated scientific notebook has been reviewed both for QA and technical content. QA records (described in the SIP and implementing procedures) will be submitted to the UCCSN Records Office within 60 days following the completion of testing and also successful entry of data into the TDA.

4.0 INTERFACE CONTROLS

External Interfaces:
Yucca Mountain Cooperative Agreement Technical Contact:
DOE Technical Task Representative: Abe Van Luik

Internal Interfaces:

Task Lead Investigator:	Linda Stetzenbach
Co-Principal Investigators:	Mark Buttner, Patricia Cruz
Analysts:	Amy Klima-Comba, Nikki Burns-Savage, Joanne Henry
Students:	James Fischer

There are no ongoing field or laboratory investigations associated with this task. Information and/or item transfers across interfaces will be performed electronically.

5.0 STANDARDS

Standards used to quantify target DNA concentrations are not available from NIST or qualified suppliers. PCR quantitation standards will be prepared in our laboratory, as described in the corresponding IP, from a purified cell suspension enumerated electronically with a Coulter Multisizer II (Beckman Coulter, Inc., Hialeah, FL) using the same DNA extraction and purification methods used to process samples (Buttner, *et al*., 2001 and 2004; Cruz-Perez, *et al*., 2001a and 2001b). Quantitation will be achieved by amplification of standards containing DNA extracted from suspensions of known concentration of each target organism. Extraction of standards in the same manner as samples provides absolute quantitation of templates and corrects for the occurrence of external DNA present in the samples. Standards will be amplified in duplicate at the same time and under the same conditions as the replicate unknown samples.

Reference cultures of microorganisms will be obtained from the American Type Culture Collection (ATCC, Manassas, VA), the internationally recognized supplier of microbial cultures. All other standards will be obtained from NIST or qualified suppliers.

6.0 IMPLEMENTING PROCEDURES

Existing IPs that will be used are:

1. IPLV-003, “Analytical and Top-Loading Balance Use”
2. IPLV-017, “Pipettor Use and Calibration Check”
3. IPLV-8.3, “Groundwater Sample Collection and Control”

Implementing procedures expected to be written include but are not limited to:

1. DNA extraction and purification
2. DNA PCR amplification using the ABI Prism 7900 sequence detection system
3. Electronic cell enumeration

Any additional IPs that need to be written will be identified and completed. Work performed prior to development and approval of a new IP will be documented in the scientific notebook in accordance with QAP 3.0, “Scientific Investigation Control”.

7.0 SAMPLES

The protocols developed will be tested by assaying available subsurface samples from the Yucca Mountain Project. Samples will be controlled at HRC in accordance with QAP-8.0, “Identification and Control of Items and Samples”. The Chain of Custody Section of IPLV-8.3, “Groundwater Sample Collection and Control”, will be followed once custody is transferred to the HRC.

8.0 EQUIPMENT AND INSTRUMENTATION

The specific equipment used for each measurement, described in Section 2.0, will be documented in the scientific notebook or other QA record. Documentation will include the instrument manufacturer, model, and serial number as well as all applicable instrument manuals.

The calibration, accuracy, and precision requirements for all equipment are to be described in the corresponding IP. Analytical instruments will be calibrated before each use (where applicable). All Measurement and Test Equipment (MT&E) will be stored in a locked laboratory to prevent loss and tampering. Other equipment that may be used includes analytical balances and pipettors. Balances will be calibrated annually by Bechtel. The reference mass set used to check working mass sets is calibrated every two years by an organization on the Qualified Supplier List (QSL). Calibrations of pipettors are checked annually. Calibrations and calibration checks will be performed by HRC staff or by an organization on the QSL.

9.0 SOFTWARE and MODELS

The software packages used in this study include 1) The analytical instrumentation software used for data acquisition, and 2) Spreadsheet software such as Quattro Pro or Excel for data reduction. Any macros used in Excel, etc. will be qualified and documented. Use of the analytical instrumentation data acquisition and spreadsheet software will be documented or referenced, along with the specific version used, in the instrument scientific notebook or other QA record. Control of electronic data is addressed in each IP that involves electronic data management, primarily instrument system IPs.

No models will be developed for or used during this study.

10.0 PROCUREMENTS and SUBCONTRACTS

Calibration items and services are procured in accordance with QAP-7.0, “Control of Quality-Affecting Procurement and Receipt”. Balances are calibrated annually by a qualified supplier. The reference mass set used to check working mass sets is calibrated every two years by an organization on the Qualified Supplier List (QSL). Calibrations of Pipettors are checked annually. Calibrations and calibration checks will be performed by HRC staff or by an organization on the QSL.

Reference cultures of microorganisms will be obtained from the American Type Culture Collection (ATCC, Manassas, VA), the internationally recognized supplier of microbial cultures. All other standards will be obtained from NIST or qualified suppliers. The basis for acceptance of any standards that are not available from a qualified supplier will be documented in the scientific notebook.

No subcontractors will be used on this task.

11.0 HOLD POINTS

Submittals and documents must complete technical and QA reviews prior to approval. Decision points associated with the analytical measurements are addressed by use of quality controls to indicate when there is an analytical or other problem which needs action described in the IPs. It is anticipated that there will be a hold point after the completion of Subtask 3, laboratory studies and protocol development, and before the inititiation of Subtask 4, protocol validation.

12.0 QUALITY CONTROL

Objectives for precision and accuracy, for the analytical measurement and how results are evaluated are described in each corresponding IP. Precision will be determined using laboratory replicates. Accuracy of standards preparation will be determined using initial calibration checks (ICC). The calibration standard and the ICC must agree within 10% in order to proceed with sample analysis. Accuracy of the calibration curve is verified by use of the ICCs. Maintenance of that accuracy, or a lack of instrument drift, is verified by the use of continuing calibration checks. Potential sources of error and uncertainty are addressed in the associated IPs.

13.0 DATA RECORDING, REDUCTION, AND REPORTING

Data packages consisting of the hard copies of raw data generated from each instrument will be referenced by the analysis date and will be attachments to the scientific notebook. Data recording requirements for each scientific notebook are described in the corresponding IP. A summary of the data generated from each instrument (described in section 2.0) is exported to a spreadsheet (Microsoft Excel) where final data reduction is performed. Reduction of data involves calculating the concentration of the DNA templates in each sample based on the slope and intercept of an instrument response curve and also calculating averages when multiple amplifications are performed. A hard copy of the spreadsheet containing the reduced data will be included in the data package. The concentration of the DNA template per reaction is calculated using the analytical instrumentation software. The calculation of averages is performed using the spreadsheet software. Manual and electronic data transfers will be verified by use of file utilities which have built-in cyclic redundancy checks (CRCs) or by visual inspection. All verifications will be documented in the scientific notebook. Data will be submitted to the TDA following verification in accordance with QAP-3.6. Raw and reduced data will be controlled in accordance with QAP-3.1, “Control of Electronic Data”, by being stored on password protected computers in locked rooms. The data will be backed up to the HRC server on a regular basis. The final verified spreadsheet of reduced data for submittal to the TDA will also be controlled in accordance with QAP-3.1. It is anticipated that no data will be obtained from sources external to the task.

14.0 FIELD SURVEYING

Not applicable for this task.

15.0 REVIEWS AND VERIFICATIONS

Internal verification of all data will be performed by someone other than the originator to check compliance to the procedures and to verify the accuracy of data reduction. Internal technical reviews will be performed and documented on the data, scientific notebooks, all reports, and all journal articles (non-deliverables) generated in this task. Three copies of reports, reprints, conference papers, etc. will be submitted to UNLVRF for submittal to DOE as soon as possible after the event occurs (10 CFR 605.19(a) (3)). Any report of data generated without full internal verification will be labeled as “preliminary” data. Data review and verification will include the following:

1) Check for compliance with the QA/QC criteria described in each IP using the associated checklist. The completed checklists will be included with the data packages.
2) Compare DNA template concentrations printed in the data packages to the reduced data.
3) Final data submitted for entry into the TDA will be visually compared to the reduced data spreadsheets contained within the data packages to ensure that the reported concentration data are accurate.

Data will be acceptable when the data review and verification steps 1–3 above are successfully completed. Scientific notebooks as well as data packages and attachments will be reviewed in accordance with QAP-3.0, "Scientific Investigation Control", when complete or prior to submittal of data to the TDA. Technical reports will be reviewed in accordance with QAP-3.4, "Technical Reports". Internal technical reviews will be conducted during subtask 5, task close-out.

16.0 RECORDS AND SUBMITTALS

QA records are handled in accordance with QAP 17.0, "Quality Assurance Records", and submitted to the Document Control Coordinator (DCC). Records designated as QA records in the UCCSN QAPs and IPs listed include but are not limited to:

1) Hard copies and/or electronic media containing raw and reduced data including calibration and QC results.
2) Scientific Notebooks including attachments.
3) Data review check sheets.
4) Chain of custody forms.
5) Copies of quality affecting submittals.

Submittals will be delivered to the UNLV Research Foundation for review, signature and final submission to DOE in accordance with the cooperative agreement and include but are not limited to:

1) Quarterly progress reports.
2) The final technical report will be submitted to the UNLV Research Foundation for review, signature and final submission to the DCC.

Records will be stored for protection in a locked fire-proof cabinet until they are completed.

17.0 REFERENCES

Buttner, M.P., P. Cruz, L.D. Stetzenbach, A.K. Klima-Comba, V.L. Stevens, and T.D. Cronin. 2004. Determination of the Efficacy of Two Building Decontamination Strategies by Surface Sampling with Culture and Quantitative PCR Analysis. Appl. Environ. Microbiol. **70:**4740-4747.

Buttner, M.P., P. Cruz-Perez, and L.D. Stetzenbach. 2001. Enhanced Detection of Surface-associated Bacteria in Indoor Environments using Quantitative PCR. Appl. Environ. Microbiol.**67**:2564-2570.

Cruz-Perez, P., M.P. Buttner, and L.D. Stetzenbach. 2001. Specific Detection and quantitation of *Aspergillus fumigatus* in Pure Culture using Quantitative Polymerase Chain Reaction. Mol. Cellular Probes. **15**:81-88.

Cruz-Perez, P., M.P. Buttner, and L.D. Stetzenbach. 2001. Specific Detection of *Stachybotrys chartarum* in Pure Culture using Quantitative Polymerase Chain Reaction. Mol. Cellular Probes. 15: 129-138.