Title: **Project to Develop and Validate a Molecular Assay for *Rhodococcus coprophilus***

Project I.D.: DNR project # 206

Investigators: Principal Investigator -Sharon C. Long, Director of Environmental Microbiology Wisconsin State Laboratory of Hygiene and Associate Professor of Soil Science
Research Intern - Jamie R. Stietz, Wisconsin State Laboratory of Hygiene

Period of Contract: *July 1, 2008 to April 30, 2009*

Background/Need: The need to protect watersheds from fecal contamination has led to an investigation of monitoring tools to supplement coliform/*E. coli* measurements. A suite or toolbox of tests developed to supplement monitoring for indicator organisms is called "Microbial Source Tracking" (MST). *Rhodococcus coprophilus* is an emerging MST tool that has been demonstrated to identify the presence of grazing animal fecal matter or manure in the presence of fecal contamination. The current method used to detect *R. coprophilus* in water samples is both labor intensive and time consuming. Three to four weeks are needed to culture and confirm the presence of this organism in water samples. It is believed that molecular techniques could give reliable results in as little as 2-4 days using polymerase chain reaction (PCR) and gel electrophoresis.

Objectives: The objective of this study was to develop and validate a molecular detection method for *R. coprophilus* in groundwater samples.

Methods: Steps in developing and validating a PCR-based method include optimizing: (1) sample concentration, (2) extraction and purification of nucleic acid, (3) PCR amplification, (4) confirmation of presence of target amplicon, and (5) quantification of nucleic acid concentration against a standard curve. Using the U.S. EPA molecular method "Rapid, PCR-Based Method for Measuring *Enterococci* and *Bacteroides* in Water Samples" as a launching point, sample concentration was accomplished using membrane filtration and nucleic acid extraction by bead beating. Various nucleic acid purification and PCR approaches were explored to assess which approach best suited detection of *R. coprophilus*. To evaluate performance of the optimized PCR-based methods using environmental samples, results were compared against standardized plating methods for the enumeration of *R. coprophilus* in MST samples collected by DNR staff and submitted to WSLH for analyses.

Results and Discussion: This research developed and validated two molecular methods for detecting *R. coprophilus* in environmental samples. These methods shortened analysis time to as little as 2 to 4 days, although samples could be frozen and batched to provide greater cost-efficiency. One method involved traditional polymerase chain reaction (PCR) and provides presence/absence detection. A second method involved quantitative real-time PCR and can yield data in calibrated cell equivalents per 100 mL of sample. Overall, the molecular methods can be used to provide the same information as the culture method in a fraction of the time.

Sample concentration was accomplished using membrane filtration and nucleic acid extraction by bead beating. Various nucleic acid purification and PCR approaches were explored to assess which approach best suited detection of *R. coprophilus*. Several commercially available clean-up kits were evaluated for their ability to eliminate/reduce environmental PCR inhibitors, and challenged with humic acid and mineral turbidity. Several volumes of PCR template and known concentrations of *R. coprophilus* were processed to achieve appropriate detection limits.

To evaluate performance of the optimized PCR-based methods using environmental samples, results were compared against standardized plating methods for the enumeration of *R. coprophilus*. Sixteen MST samples were submitted to WSLH through the duration of this project. These samples were analyzed in parallel by the culture, traditional PCR, and qPCR methods. Overall, the results for traditional PCR and the culture method compare favorably. With the exception of samples with inconclusive results, seven of eight (87.5%) of samples enumerated by traditional PCR and the culture method matched. Four of the samples contained high background bacterial levels that resulted in interference and inconclusive results for the culture method, while traditional PCR provided clear positive or negative results. For qPCR versus the culture method, six of six samples with conclusive results matched. In two of the four cases where the culture method yielded inconclusive results, qPCR yielded a result. In two of the four cases where the culture method yielded inconclusive results, qPCR also yielded inconclusive results. Comparing the two molecular methods, eight of the ten (80%) yielded consistent results and two yielded conflicting results. The qPCR method was more susceptible to sample quality (*i.e.* presence of inhibitors and background DNA) than traditional PCR.

Conclusions/ Implications/ Recommendations: The final molecular method for detection of *R. coprophilus* in groundwater samples consists of: (1) membrane filtration of samples (up to 250 mL or until membrane refusal); (2) resuspension in 300 mL AE with 150 mg of (212-300 μm, 50-70 US sieve) acid washed glass beads; (3) bead beating on "homogenize" for one minute; clean-up using the MoBio PowerClean™ kit if needed; and (4) traditional PCR of triplicate 15 μL volumes of target and/or qPCR of triplicate 5 μL volumes of target. Testing is being moved from a culture-based method to the molecular-based detection of *R. coprophilus* for MST samples analyzed by WSLH. The traditional PCR method is more robust and less subject to equivocal results; however, qPCR provides a quantitative result. With either test, results can be obtained in as little as 2 to 4 days with detection limits similar to those of the previously applied culture-based method.

Related Publications: Manuscript is currently under preparation.

Key Words: Rhodococcus coprophilus, microbial source tracking, polymerase chain reaction

Funding: Wisconsin Department of Natural Resources

Final Report: A final report containing more detailed information on this project is available for loan from Wisconsin's Water Library, University of Wisconsin - Madison, 1975 Willow Drive, Madison, Wisconsin 53706 (608) 262-3069.