Application of qPCR Technologies in Stormwater Source Tracking and Determination of Host Contributions of Fecal Indicator Bacteria

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ABSTRACT. Current methods of quantification of fecal indicator bacteria (FIB) are based on culturing techniques. These tests take a minimum of twenty-four hours to complete and offer no information as to the identity of potential host sources. This often results in human health risk advisories in recreational waters being issued after hazards have subsided. Additionally, results from these culturing techniques can be ambiguous when deployed in stormwater source tracking efforts to remediate FIB pollution.

A series of new genetic-based methods have been developed to provide more rapid and host specific techniques to measure FIB. Most promising of these are the newly approved USEPA Methods A (for determination of *Enterococci*) and B (for the determination of *Bacteroides*) in water. These techniques use TaqMan quantitative real time polymerase chain reaction (qPCR). qPCR is a molecular biology tool that amplifies the DNA of specific genes and allows for quantification of as low as one gene copy. Additionally, qPCR offers the ability to identify specific microbial hosts through the quantification of gene sequences in FIB strains unique to target species.

Coastal Carolina University's Environmental Quality Laboratory (EQL) has adopted qPCR molecular tools to identify host sources of bacterial pollution and provide quantitative estimates of host source contributions. The approach uses mammalian *Bacteroides* to infer a total FIB concentration and *Bacteroides* (a subset of *Bacteroides* unique to humans) to estimate the contribution to the FIB pool from human sources. Companion assays to quantify other known contributing hosts such as canines and Canada Geese are also being developed. Validation of these techniques in a representative group of local environments, ranging from blackwater rivers to estuarine/saltwater environments, shows the robust nature of this tool.

These newly validated qPCR source tracking tools are currently being employed in a pilot project in Withers Swash (Myrtle Beach, SC) designed to identify FIB contamination sources. This tidal creek is the major drainage for the city of Myrtle Beach. It is 303(d) listed for *Enterococcus* and fecal coliforms. The pilot project is being conducted collaboratively by the City of Myrtle Beach, CCU's EQL, and US Army Corps of Engineers using a multi-tracer, watershed investigatory approach that provides a weight-of-evidence confirmation of FIB sources. The results will be used to direct remediation efforts aimed at reducing nonpoint source FIB contamination and to provide a procedural framework that can be deployed in other similarly contaminated swashes along the Grand Strand.

INTRODUCTION

The Grand Strand coastline of northeastern South Carolina stretches for more than 60 miles in Horry and Georgetown Counties. Eighty-three (83) sites in Horry and Georgetown counties have been documented by SCDHEC as having fecal indicator bacteria (FIB) impairments. Tools are needed to identify the host sources of pathogens as represented by FIBs. To address these needs, Coastal Carolina University (CCU), in conjunction with the stormwater managers of Horry County, Georgetown County and the cities of Myrtle Beach and North Myrtle Beach, partnered with the USACOE to receive a Planning Assistance to States grant to develop local capacity to conduct multi-tracer, targeted microbial source tracking (MST) investigations. This capacity has now been created through the development of qPCR-based genotypic microbial source tracking tools for local use in collaboration with CCU's Environmental Quality Lab (EQL). Validated assays are currently being applied in Withers Swash, a tidal creek included on the federal 303(d) list of impaired water bodies due to contraventions of *Enterococcus* water quality standards (SC DHEC 2010). This pilot project is employing a multi-tracer, targeted sub-watershed investigatory approach. The latter provides geographic

and host animal source information. The resulting source assessments will advance stormwater management planning and remediation efforts in the Withers Swash basin. The genotypic tracking tools and sub-watershed investigatory protocol will be made available for application throughout Horry and Georgetown counties to address microbial source tracking needs at known sites of FIB impairments.

PROJECT DESCRIPTION

A pilot project has been designed to employ these assays during the summer of 2012 within Withers Swash Myrtle Beach, SC, a chronically impaired water body for FIB. A baseline watershed assessment has been performed to compile a suite of informational GIS-based data layers for the upper reaches of Withers Swash. Data illustrated on the watershed maps included: aerial imagery, roadways, topography, land use/zoning, Standard Industrial Classification (SIC) codes of businesses, parcel boundaries, stormwater drainage systems, sanitary sewer systems, septic systems, and underground storage tanks. These maps were used to identify eleven sub-watersheds in which targeted sampling have been designed to capture stormwater from differing land uses and densities of development.

Stormwater sampling has consisted of two base-flow and three storm-flow events. In addition to the genotypic marker profiles, the following are being measured at the eleven sites: DO/pH/SC/Sal, Turbidity, BOD, ammonia, TSS/VSS, IQ Toxicity Test, fecal coliforms, Enterococci, and optical brighteners. These indicators were selected based on recommendations from the Center for Watershed Protection as tools stormwater managers should use for tracking and distinguishing sources of various illicit discharges. They provide a weight of evidence confirmation of conclusions regarding source assessments.

Results from this field work will be used in the production of a watershed assessment report (WAR). The Withers Basin WAR will include the baseline watershed assessment, results of the sub-watershed field work, conclusion regarding the locations and host animal sources for FIB hotspots, and recommended remediation strategies that will advance stormwater management and improve water quality within the Withers Swash Drainage Basin.

BACKGROUND

Microorganisms of fecal origin are an important type of water pollution. These pollutogens appear to be increasingly prevalent and of growing ecological (Lafferty and Kuris 2005) and economic impact (Santo Domingo and Ashbolt 2010). Epidemiological studies have shown various groups of these fecal bacteria to be correlated to the occurrence of human illnesses. To prevent these human health risks, the Beaches Environmental Assessment and Coastal Health Act (BEACH Act) of 2000 amended the Clean Water Act requiring states to adopt water quality standards and implement monitoring programs for recreational waters using FIB. The available approved conventional methods inherently have a number of limitations in their ability to accurately predict human health risks. The most notable of these limitations is that they culture and measure viable indicator bacteria as a proxy for the agents that actually cause the human disease. Thus, any number of variables can influence the balance of pathogens and indicators which are the bases for the regulations.

FIBs are not host species specific. Epidemiological studies have shown that the resulting human health risk varies greatly at the same concentration of FIB depending on the source organism. Additionally, this lack of specificity can produce ambiguous outcomes when employed in MST efforts. Thus, the interpretation of results to direct remediation efforts can only identity the locations of problematic source areas and not the specific host animal sources of the FIBs.

Recent and future advances in genetic technology promise solutions to the problems associated with FIB culturing methods. A number of different methods and assays have been advanced, each requiring unique molecular reagents to target nucleic acid sequences that are presumably host-associated and environmentally refractory. Currently, quantitative polymerase chain reaction (qPCR) methods are considered the most promising of these methods (Roslev and Bukh 2011). qPCR is a widely used molecular biology tool which amplifies DNA of specific genes and allows for simultaneous quantification of as low as one gene copy. This is accomplished by quantifying a fluorescent tracer liberated during amplification.

A number of PCR-based methods have been developed to detect and quantify the presence of fecal-sourced bacteria in environmental waters (Hagedorn et al. 2011). One rapidly emerging technique utilizing qPCR technology examines 16S ribosomal RNA (16S rRNA) markers. The 16S rRNA gene codes for a subunit of the prokaryotic ribosome and is found in nearly all bacteria and archaea, making this gene useful for constructing bacterial phylogenies and species identification. Based on these principles, the US EPA has developed two methods for rapid detection and enumeration of FIB. The first, Method A targets *Enterococcus*, a FIB commonly used for recreational water quality standards in marine waters (US EPA 2010a). The second, Method B, targets *Bacteroidal*es, an

order of bacteria commonly found in the feces of humans and other warm-blooded animals (US EPA 2010b).

The use of these genetic methods also allows for the identification of subgroups of bacteria based on small changes in their genetic composition. This is important because of the linkage between gut microbes and their hosts. Many animal hosts have developed unique communities of gut bacteria over time due to mutations of the bacterial DNA. Through this process, unique sequences in the 16S rRNA gene have emerged, allowing for targeted assays to be developed for the identification of the host animal source of FIBs.

While current genetic techniques are not robust enough to serve as standalone methods for all MST applications, they offer a significant step forward, particularly as part of a weight of evidence investigative approach. As the use of qPCR technology in the regulatory and MST fields continues to rapidly expand, the number of assays to identify source organisms will also increase. One of qPCR's strengths is the opportunity to concurrently run multiple assays at detection levels as low as one gene. These attributes should allow for the development of targeted assays to detect the bacterial and viral pathogens causing illness rather than the proxy FIBs.

METHODS

The strategy developed for this project uses two qPCR procedures related to EPA Method B. The first qPCR assay utilizes a quantitative primer set for detection of order *Bacteroidales* bacteria (GenBac 3F), a common gut bacteria found in warm-blooded animals. A second qPCR assay targets *Bacteroide*s spp. (BacHum-160f), a subgroup within the *Bacteroidales* community (order) that is strongly human-associated. By adopting this strategy, two important criteria are satisfied; quantitative detection of *Bacteroidales* fecal bacteria derived from mammals (Bernhard and Field 2000, Siefring et al. 2008, USEPA 2010b) and *Bacteroides* spp. derived primarily from humans (Seurinck et al. 2005, Kildare et al. 2007). The general *Bacteroidales* assay is used to estimate total mammalian fecal pollution in a water sample while the human-associated *Bacteroides* assay provides an estimate of the contribution of human-sourced fecal pollution.

To guarantee the successful deployment of these assays within the study area, the validation process considered the following factors: (1) matrix interference in local samples that might require optimization of published assays, (2) false negative results that could arise from geographic genotypic variability in local bacteria and their host animals and (3) false positive results caused by cross reactivity. To resolve these issues and ensure that the genotypic marker tools could detect the sources in the diverse marine and freshwater aquatic systems found within Horry and Georgetown Counties, validation work was conducted in known FIB hot spots and pristine locations. The primary validation test sites were: Crabtree Canal/Swamp (Horry County), Murrells Inlet (Georgetown County), White Point Swash (North Myrtle Beach), and Withers Swash (Myrtle Beach). Each of these sites is on the SCDHEC's 303(d) list of impaired waterbodies and lies within areas regulated under the National Pollutant Discharge Elimination System (NPDES) Phase II Stormwater Program. In the case of Murrells Inlet, a fecal coliform TMDL was approved in 2005. Sampling was also conducted at secondary validation sites where the EQL routinely collects FIB data, at a farm pond, and in mixed liquor from a local wastewater treatment plant (WWTP) to provide samples with a variety of bacterial compositions and abundances.

Matrix interferences were examined by spiking fecal samples into deionized water and pristine ambient waters. False negatives and positives were tested for by spiking ambient waters with human and animal fecal matter. Results from this validation work showed only a low level cross reactivity from house cat fecal matter on the BacHum assay, suggesting some cross reactivity that might be attributable to co-habitation with humans. Spiking experiments conducted with human feces did generate positive detects as did the mixed liquor from the WWTP sample. All of these spiked samples generated GenBac 3F detects. These results also were internally consistent (all GenBac results were higher than BacHum), showed good agreement between duplicate and replicate samples, and blanks were generally below detection limits for both assays.

Additionally, assay robustness across varied matrixes was tested on sixty environmental water samples from the validation sites using the two qPCR assays. All samples tested positive for the presence of total fecal *Bacteroidales* (60 of 60). The highest responses were locations known to have FIB impairments, such as the tidal creeks listed on the 303(d) list and Murrells Inlet. The lowest concentrations (about three orders of magnitude lower) were located in the main body of the Waccamaw River, an area known to be pristine. The assays detecting the presence of human-associated *Bacteroides* was both less frequent (28 of 60) and of much lower mean intensity than the total *Bacteroidales* pool. These results were much less consistent within sites suggesting that human influences are more episodic in nature. Additionally a single sample of settled mixed liquor collected from a local WWTP showed that human-associated *Bacteroides* was ~20% of the magnitude of the total *Bacteroidales* concentration.

Conventional culture methods for four FIB (*Enterococci*, total coliforms, fecal coliforms, and *E.*

coli) were concurrently conducted on the environmental samples to establish relationships with the qPCR results. Most pairwise comparisons exhibited significant relationships ($p \leq 0.00$; 10 of 15) and strong correlations ($0.64 < r < 0.95$). Only the BacHum qPCR assay did not show significant correlations to the other indicators. The BacHum assay exhibited a weak correlation with Gen Bac and no correlation with the conventional FIBs. This was likely driven by the low rate of detection for this assay in the environmental samples.

CONCLUSIONS

Quantitative assays for total *Bacteroidales* and a human-associated subset of *Bacteroides* have been developed and validated. They are now in routine use in CCU's EQL. This represents a great increase in local capacity to conduct MST investigations and to enhance regulatory FIB monitoring programs. During the summer of 2012, these methods are being employed as part of a multi-tracer pilot storm water study in Withers Swash, Myrtle Beach, SC. This study will provide information on the relative contributions of human-sourced fecal pollution within eleven subwatersheds of Withers Swash and serve as a model for other regional investigations.

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