Technical Review of Report

Final Version

Tracking and Investigating Microbial Sources in Gainesville's Urban Creeks

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GENERAL COMMENTS

This document is an impartial peer review of a report prepared for Gainesville Regional Utilities by CH2MHill and Biological Consulting Services of North Florida entitled **Tracking and Investigating Microbial Sources in Gainesville's Urban Creeks.** The report carries a project number 316918 and a date of April 2007. The purpose of the microbiology study was to determine the major sources of microbial pollution (bacteria and viruses) from sewage and other fecal sources to surface waters in Gainesville Florida, which was accomplished with a suite of microbial source tracking (MST) methodologies.

The MST tests are intended to determine the source(s) of fecal pollution by detecting microbes that are specific to or highly associated with the waste of particular types of animals or humans. These tests were employed in conjunction with field reconnaissance and land use analysis to better understand the extent of the impact of human and nonhuman waste on surface water quality in several tributaries of Gainesville. These tributaries contain elevated levels of fecal indicator bacteria, which are used by the State of Florida and across the U.S. to determine the public health safety of recreational use and other uses of the water bodies. As outlined by the report, indicator bacteria levels in exceedance of regulatory standards indicates a public health concern and **possible** contamination by sewage; however the many possible sources of these bacteria greatly complicate risk assessment, and development of best management plans and remediation strategies (Scott et al 2002; Stoeckel and Harwood 2007; USEPA 2005). The determination of distinct sources of fecal pollution and other sources of indicator bacteria to water bodies is therefore necessary for improved public health protection and watershed/water quality management.

Two distinct MST approaches were used in this study: (1) library-dependent ribotyping of *Escherichia coli* (*E. coli*) and (2) library-independent tests for indicators of human fecal contamination that include the *esp* gene of *Enterococcus faecium,* human-associated *Bacteroides,* enteric viruses and human polyomaviruses. Each approach and test has advantages and disadvantages (Stoeckel and Harwood, 2007; USEPA 2005), and the use of several tests to provide a conclusion by weight of evidence is a common practice in this field.

The study was conducted in three phases. **Phase 1**, labeled "synoptic sampling, included acquisition of land use data, other knowledge about the tributaries, and sampling for three indicator bacteria groups: total coliforms, fecal coliforms and enterococci (*Enterococcus* species). **Phase 2** included the MST tests at three "high priority" sites that were selected according to elevated indicator bacteria levels. In **Phase 3** additional sites were added around "hot spots;" sites at which MST tests indicated human fecal contamination. An attempt was made to identify specific inputs of pollution (e.g. faulty central sewer infrastructure, septic tanks, stormwater runoff, pets and/or wild animals) to the tributaries.

In general, the approach used in this study is sound and reflects recent advances in methodologies for assessing the major sources of microbial contamination to water bodies. The report is well-organized and fairly comprehensive; however, certain controls could have been conducted or added to the report that would have provided better confidence in the results (see immediately below and "Specific Comments" for details).

The library-independent methods utilize polymerase chain reaction (PCR) for detection of specific genes, but no mention is made of the possibility of false-positive or false-negative results in PCR tests. The interpretation of studies using such tests should weigh the results of corroborating tests and the frequency of observation of the marker at each site; however this approach complicated in this study by analyzing only a handful of samples at each site. The table of results and interpretation of DNA marker tests (Exhibit 25) indicates that most sites were sampled between once and three times for DNA markers and/or human viruses, which does not lend itself to a weight-of-evidence approach and prevents certain analyses that would improve confidence in the study conclusions (see comments at 3-12). The enteric virus data were apparently not included in the statistical analysis and this omission is not explained. In fact, the enteric virus data are discussed very little in the report, in spite of the fact that these are true human pathogens and incontrovertible evidence of human fecal pollution.

The library-dependent MST approach of *E. coli* ribotyping, which is designed to estimate the proportion of *E. coli* contributed from various fecal sources, suffers from the difficulties of validation and interpretation which are associated with all library-dependent MST methods (Stoeckel and Harwood, 2007; USEPA 2005). These concerns are detailed below, but in general they contribute to very low confidence in the assessment of the proportion of contamination that originates from various fecal sources in the tributaries.

SPECIFIC COMMENTS

EXECUTIVE SUMMARY

The indicator bacteria regulatory criteria (pg ES-2, 2nd para) should be more clearly stated, although in the authors' defense they are confusing. The U.S. Environmental Protection Agency (U.S. EPA) no longer recognizes total coliforms or fecal coliforms as appropriate indicators for recreational water use. However, the U.S. EPA does recognize the use *Escherichia coli* and the enterococci as appropriate indicators (http://www.epa.gov/fedrgstr/EPA-WATER/2004/November/Day-16/w25303.pdf; U.S. EPA 2004). The fecal coliform standard is based on criteria set by the Florida Department of Environmental Protection (FDEP). Although the FDEP's criteria do include the 800 CFU/100 ml one-time maximum, in practice the 400 CFU/100 ml standard, which is not to be exceeded by more than 10% of samples, is employed for beach monitoring (http://esetappsdoh.doh.state.fl.us/irm00beachwater/default.aspx). FDEP has recently (2006) recommended discontinuing the use of total coliforms to monitor surface water quality and regulate its uses.

INTRODUCTION

In the future inclusion of water body ID (WBID) numbers used by the FDEP to refer to specific sub-basins within a watershed would be useful (I-2 2nd para).

METHODS

A control site (no markers or fecal contribution from certain sources expected) would be very useful for interpretation of MST data. The control site(s) can be used to validate the predictions made by MST, i.e. rural site with no upstream contribution from human sources could be used as a control for detection of human sources of fecal pollution.

Pg 2-4 The amount of rainfall that constitutes a rain event is not specified, but should be. Many investigators (including myself) have found that cumulative rainfall data collected over a period of 3, 5 or 7 days prior to sampling correlates with other variables such as microbial counts, while 24 hour prior rainfall does not. I would suggest further analysis of the rainfall data in future studies.

Pg 2-5 The methods for enumerating indicator bacteria (total coliforms, fecal coliforms and enterococci) should be specified, but are not.

Pg 2-5 paragraph 3: What was basis for selecting 5 isolates per sample for ribotyping analysis? Because of the high concentration of *E. coli* in the water, this sample size cannot be representative of population. The undersampling problem is a common deficiency in library-dependent MST methods; while the ribotyping method may provide some qualitative information on the source of fecal contamination, it is obvious that it does not provide a "snapshot" of the composition of the *E. coli* population in the sample if sample size is far too small.

Pg 2-8 PCR Detection of Human Enteric Viruses. The sample volume is not given for human polyomaviruses or enteric viruses. It is difficult to interpret the results for enteric viruses (most tests are NEGATIVE) without knowing sample volumes or the types of enteric viruses that were tested for.

P2-8 Ribotype Profile Database: No mention was made of the composition of the library (e.g. how many source categories, the number of isolates per source category, the accuracy of library or how was it was validated - see for example USEPA 2005; Stoeckel and Harwood 2007). Without this analysis, the confidence in the accuracy of source assignment cannot be assessed. Unfortunately, the presentation of the library as a "black box" is an all-too-common practice in reports and sometimes even publications on MST. Managers should know that there are many ways to evaluate the usefulness of MST methods, and should require them to be specified in proposals and reports.

P2-9 Statistical Analysis of Ribotype Profiles... This section is somewhat confusing. If I read it correctly, the same ribotype database was used for the initial analysis (all sources) and the second analysis – but I am not sure. The number of source categories specified for the initial analysis should be given. It would be very useful to know the how close to isolates must be to be called a "match." If a given isolate's pattern has no match what is the call (what is its designation)?

RESULTS

The site maps that include the test results are very helpful for easy reference.

3-1 Exhibit 4 - Land Use Assessment. It would be much more useful to separate pets and wildlife as potential sources, since one (pets) can be considered an anthropogenic (human-associated) factor and can be relatively readily controlled while the other (wildlife) is natural and is much more difficult to control.

3-3 and throughout: Geomean values are frequently used to analyze microbial data because the reduce the effect of a few very large observations (data points). Recommend using these alone or in conjunction with arithmetic means in future studies.

3-5 (1st paragraph) and throughout: More consistency should be used in citing Florida regulatory criteria. Florida Administrative Code 62-302.530, "Criteria for Surface Water Quality Classifications" states that for Class III waters (so designated for recreational use and for maintaining a healthy ecosystem) fecal coliform concentrations are not to exceed 800 CFU/100 ml for any given observation, and are not to exceed 400 CFU/100 ml in 10% or more of samples (see Table below). The CH2MHill report cites the one-time maximum standard (800 CFU/100 ml) as the regulatory limit for fecal coliforms, although in practice the Florida Department of Health uses the **400 CFU/100 ml** standard for posting beach advisories (http://esetappsdoh.doh.state.fl.us/irm00beachwater/terms.htm). In current practice, regulatory agencies base more decisions on the 400 CFU/100 ml level than the higher one.

The recreational water standard that is based on enterococci concentrations is recommended by the U.S. Environmental Protection Agency for freshwater and marine beaches, and has been adopted by Florida for beach monitoring and advisory purposes. In the most recent federal rule (**Federal Register** / Vol. 69, No. 131 / Friday, July 9, 2004 / Proposed Rules) the enterococci single sample maximum for designated bathing beaches is specified only for marine waters, and it is **104 CFU/100 ml** (see table below). This is also the standard used for issuing advisories at Florida beaches (http://esetappsdoh.doh.state.fl.us/irm00beachwater/terms.htm). This value (104 CFU/100 ml) is therefore the most comparable to the 800 (or 400) CFU/100 ml fecal coliform standard; not the 33 CFU/100 ml or 61 CFU/100 ml (Exhibit 9) values.

TABLE 2.—PROPOSED AMBIENT FRESH WATER QUALITY CRITERIA FOR BACTERIA

A indicator	B geometric mean	C single sample maximum (per 100 ml)			
		C1 designated bathing beach (75% confidence level)	C2 moderate use coastal recreation waters (82% confidence level)	C3 light use coastal recreation waters (90% confidence level)	C4 infrequent use coastal recreation waters (95% confidence level)
E. coli	126/100 ml [a]	235 [b]	298 [b]	409 [b]	575 [b]

Footnotes to table in paragraph (c)(1):
[a] This value is for use with analytical methods 1106.1 or 1600 or any equivalent viable method.
[b] Calculated using the following: single sample maximum = geometric mean * 10^(confidence level factor * log standard deviation), where the confidence level factor is: 75%: 0.68; 82%: 0.94; 90%: 1.28; 95%: 1.65. The log standard deviation from EPA's epidemiological studies is 0.4.

TABLE 3.—PROPOSED AMBIENT MARINE WATER QUALITY CRITERIA FOR BACTERIA

A indicator	B geometric mean	C single sample maximum (per 100 ml)			
		C1 designated bathing beach (75% confidence level)	C2 moderate use coastal recreation waters (82% confidence level)	C3 light use coastal recreation waters (90% confidence level)	C4 infrequent use coastal recreation waters (95% confidence level)
Enterococci	35/100 ml [a]	104 [b]	158 [b]	276 [b]	501 [b]

Footnotes to table in paragraph (c)(2):
[a] This value is for use with analytical methods 1103.1, 1603, or 1604 or any equivalent viable method.
[b] Calculated using the following: single sample maximum = geometric mean * 10^(confidence level factor * log standard deviation), where the confidence level factor is: 75%: 0.68; 82%: 0.94; 90%: 1.28; 95%: 1.65. The log standard deviation from EPA's epidemiological studies is 0.7.

3-12 The truncation of the fecal coliform concentrations is due to the analysis of too few dilutions of the sample. This practice results in loss of information (as noted in the report) and should be modified for future studies.

3-12 – Exhibit 11 This table is mis-identified in the text as Exhibit 10. It presents the numbers of samples in which human-specific markers were co-detected (0, 1, 2, or 3 markers detected), and presents some frequencies of detection. However, there is no statistical analysis of the frequency of co-detection at each site. In other words, when results for polyoma DNA are positive at Site A, how frequently are results positive for *Enterococcus* DNA at site A? Is there a statistical association between positive results for these markers? Unfortunately, most sites have been tested for DNA markers only 1-3 times so it is difficult to do this analysis, but some sites in close proximity could be combined into a larger data set to do the analysis.

Another statistical analysis that would help interpretation is to determine which site(s) more frequently score positive for the human markers. These data and analyses would greatly help to determine if there is confidence in the assessment of human contamination at each site. Again, data from sites that are close to one another would probably have to be combined to do the analysis.

3-13 How were dry and wet conditions determined? This is not specified. Furthermore, there does not appear to be a significant difference in bacterial concentrations observed under dry vs. wet conditions, although the text suggests a difference. Statistical analysis is not specified, but should be – for a report that uses some very sophisticated statistical methods, there are also glaring omissions of statistical analysis such as this one.

3-13 The conclusions of the last paragraph on 3-13 about bacterial sources are unwarranted both in terms of logic applied and in terms of the weak (non-statistical) comparison of the data. Many factors besides microbial source could contribute to the relative bacterial concentrations under wet and dry conditions; and these interpretations are not supported by statistical analysis.

3-14 Again, statistical comparison is needed in comparing data collected at sites upstream vs. downstream of homeless camps.

3-15 Sec 3.3.3 A summary table of the regression results would be very useful here as this section is confusing. The use of PCA to construct new variables is supported in the literature but is not commonly used in microbiology studies, and should be referenced and supported in the report.

DISCUSSION

Pg 4-2 Section 4-3 **Interpretive Value.** First bullet There is no explanation for the term "residual fecal contamination." No rationale is given for interpretation of marker patterns in Exhibits 23 – 25, but this would be very helpful for understanding the interpretation.

Pg 4-3 Exhibit 23 I am not aware of documentation of re-growth in the environment of the specific *Enterococcus* strain(s) detected by the "Human Enterococcus" test. It is clear that certain members of the genus can proliferate outside of their host, but the test is highly specific for *Enterococcus faecium* that carries a specific gene – therefore this comment may not apply to the bacteria detected by the test.

Pg 4-6 second to last paragraph Be careful about the use of the term "significant!" This term should be applied to statistical analysis only to avoid confusion.

Pg 4-10 Section 4.4 This section is confusing. Does the report conclude that traditional bacterial indicators are useful or not? For example, "....traditional bacterial indicators showed elevated levels of coliforms and enterococci in nearly every sample... ...indicators not indicating much valuable information." But t at the end of page 4-10 the comment appears: ..."show evidence of the ability of these traditional tests to detect human sources..." Furthermore, the meaning of this sentence is unclear: "Comparison of the positive human hit through ribotyping..... Considering the importance of this section to interpretation of the report, it should be written more carefully. Apparently the authors conclude that the DNA tests are needed to interpret the significance of the high bacterial numbers by ascertaining the extent of human fecal contamination in these waters. But I think that most of those who work in the area of water quality know by now that there are many possible sources of fecal indicator bacteria other than sewage.

SUMMARY (5)

Bullet 3 In reference to the statement beginning: "Ribotyping results...." It is not accepted by the scientific community that library-based analysis can yield this level of accuracy in predicting the relative contribution of sources. Furthermore, too few isolates per sample were analyzed to make any quantitative assessment of the results.

The authors should mention that human feces carry high risk level compared to others, therefore greater efforts to remove its sources are warranted. This is found at end of recommendations, but should be more prominent.

RECOMMENDATIONS (5.1)

Is Ring Park included in the list of stream reaches that show evidence of human fecal contamination?

REVIEWER'S RECOMMENDATIONS

COMMENT: One of the major drawbacks of this study is the design of the sampling plan, which was so widespread and sporadic (sites sampled only 1-4 times for DNA markers) that it is difficult to interpret the MST results. RECOMMENDATION: Choose one or two highly polluted sub-watersheds and determine the major pollution sources there, then move on to others. Follow the implementation of remediation recommendations (e.g. replacement of sewer pipes) to determine whether water quality improves.

COMMENT: The lack of validation of the MST methods requires managers and reviewers to accept the accuracy of the tests "on faith." RECOMMENDATION: Watershed managers should become familiar with the basic criteria for MST validation so that they can require presentation and application of these criteria in proposals, in studies and in reports.

COMMENT: The MST markers used were all directed at human sources, which is not a criticism of the study since that was what was available when the study was conducted. RECOMMENDATION: Some new markers for species such as dogs and birds are becoming available that may prove useful in future studies if their use can be validated in Florida waters.

COMMENT: In a similar vein, The MST markers gave presence/absence (+/-) results, which is not a criticism of the study since that was what was available when the study was conducted. RECOMMENDATION: New methodologies for quantitative PCR of the MST markers will allow better assessment of the relative contribution of various fecal sources to the pollution in these tributaries.

REFERENCES

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