APPENDIX A

QUALITY ASSURANCE PROJECT PLAN

QUALITY ASSURANCE PROJECT PLAN

FOR

BRIARCLIFF ACRES WATER QUALITY STUDY HORRY COUNTY, SC

PREPARED FOR

HORRY COUNTY, SC

FINAL DRAFT
JANUARY 13, 2010

J – 22072.0000

THOMAS & HUTTON ENGINEERING CO.

SAVANNAH, GEORGIA ◆ BRUNSWICK, GEORGIA
CHARLESTON, SOUTH CAROLINA ◆ MYRTLE BEACH, SOUTH CAROLINA
WILMINGTON, NORTH CAROLINA

QUALITY ASSURANCE PROJECT PLAN

FOR

BRIARCLIFF ACRES WATER QUALITY STUDY
HORRY COUNTY, SC

Prepared by:

Thomas & Hutton Engineering Co.
935 Houston Northcutt Blvd.
Mount Pleasant, Sc 29465
Richard Karkowski, P.E., P.H.
(843) 725-5208
karkowski.r@thomas-hutton.com

Prepared for:

Horry County, South Carolina
4401 Privetts Road
P.O. Box 1236
Conway, SC 29528
Tom Garigen
(843) 915-5160
garigent@horrycounty.org

Approved by:

Thomas & Hutton Project Manager:	Richard P. Karkowski, P.E., P.H.	Date
Horry County Stormwater Manager:	Tom Garigen	Date
Coastal Carolina University Environmental Quality Lab:	Susan Libes, Ph.D.	Date
Virginia Polytechnic Institute and State University, Department of Crop and Soil Environmental Sciences:	Charles Hagedorn, Ph.D.	Date

TABLE OF CONTENTS

APPENDICES

1.0 Distribution List

Horry County, South Carolina:
Tom Garigen, Stormwater Manager
Thomas & Hutton Engineering Co.:
Richard Karkowski, P.E., P.H., Project Manager
Coastal Carolina University, Environmental Quality Lab:
Susan Libes, Ph.D., Principal Investigator
Coastal Carolina University, Environmental Quality Lab:
Joe Bennett, EQL Director
Virginia Polytechnic Institute and State University, Department of Crop and Soil Environmental Sciences:
Charles Hagedorn, Ph.D., Bacteria Source Tracking Specialist

2.0 Project Organization

Project Sponsor:
Horry County, South Carolina
Tom Garigen, Stormwater Manager
Project Coordination, Management and Report Production:
Thomas & Hutton Engineering Co.
Richard Karkowski, P.E., P.H., Project Manager
Water Quality Principal Investigator:
Coastal Carolina University, Environmental Quality Lab:
Susan Libes, Ph.D., Principal Investigator
Water Quality Sampling and Laboratory Coordination:
Coastal Carolina University, Environmental Quality Lab:
Joe Bennett, EQL Director
Bacteria Source Tracking Analysis Coordination:
Virginia Polytechnic Institute and State University, Department of Crop and Soil Environmental Sciences:
Charles Hagedorn, Ph.D., Bacteria Source Tracking Specialist

3.0 Project Definition / Background

Refer to Appendix A.

4.0 Project / Tasks Description

Refer to Appendices A and B.

5.0 Data Quality Objectives and Criteria for Measurement Data

The main objective of this monitoring program and study are:

- Document historic data and information related to elevated bacteria levels in the swash adjacent the Town of Briarcliff Acres and the adjacent Atlantic Ocean.
- Document the relative number and location of septic systems within the Town of Briarcliff Acres.
- Document the watershed of the swash adjacent the Town of Briarcliff Acres and describe potential sources and characteristics related to bacterial contamination.
- Collection of water samples and analyze for the specified parameters to determine the presence and concentration of indicator substances for bacteria and pathogens contamination.
- Determine the relative contribution of human and non-human sources of bacteria loading found in the water samples.

The ultimate goal of this monitoring program and study is to identify or disqualify septic tank systems located in the watershed as a significant source of bacteria loading in the watershed.

Once collected, the data from this study will be used to:

- Identify the relative location of bacteria loading sources in the watershed.
- Identify potential sources – human or non-human – of bacteria loading.

6.0 Special Training Requirements/Certifications

No special training requirements and/or certifications will be required for this project.

7.0 Documentation and Records

All sampling locations (see Appendix B) were located and documented during a field orientation conducted on November 5, 2009. All sites will be sampled per the Scope of Services (Appendix A). Any deviation from the locations described in the Scope of Services (Appendix A), Sample Location Map (Appendix B), or as described in the field orientation shall be identified on the field sample collection log.

All sampling activities will be documented through the use of a field sample collection log. The log will record weather, tidal flow, field measurements (e.g. pH, salinity, water temperature, DO, conductivity, etc.) and data pertinent to the collection of samples such as type of sample, time collected, samplers, and the type, number, and volume of sample containers, and preservation technique.

Analytical results, in hard copy form, will be maintained by each laboratory performing the analysis for a period of five (5) years. This period of time is in accordance with the requirements

and conditions of laboratory certification from the South Carolina Department of Health and Environmental Control (SCDHEC) and the National Environmental Laboratory Accreditation Program (NELAP).

8.0 Sampling Process Design

The sampling locations were initially located as part of preparing the Scope of Work (Appendix A) and are shown in the Sampling Location Map (Appendix B). The sites were subsequently revised following coordination with the adjacent Meher Baba Spiritual Center and during the field orientation conducted November 5, 2009. The final sites selected for sampling are described in the Scope of Work (Appendix A) and shown on the Sampling Location Map (Appendix B).

9.0 Sampling Methods Requirements

At each sampling location and for each grab sample collected, the following field sampling methods will be used:

Record the following in the field sample collection log:

- Collection Date and Time
- Tidal Stage
- Samplers
- Wind Direction
- Water Depth
- Water Flow (estimated)

Measure the following per the CCU EQL Standard Operating Procedure (SOP) No. 402 (Appendix C) and record in the field sample collection log:

- Specific Conductance
- Salinity
- Temperature
- Dissolved Oxygen
- pH

Collect laboratory samples for each of the following utilizing the appropriate SOP's listed:

- Turbidity – CCU EQL SOP No. 405 (Appendix D) or 406 (Appendix E)
- Biological Oxygen Demand - CCU EQL SOP No. 430 (Appendix F)
- Ammonia - CCU EQL SOP No. 447 (Appendix G)
- Total Suspended Solids - CCU EQL SOP No. 435 (Appendix H)
- Fecal Coliform Bacteria - CCU EQL SOP No. 502 (Appendix I)
- Enterococci Bacteria - CCU EQL SOP No. 501 (Appendix J)

- Optical Brightener Measurement by Fluorometry (Appendix K)

  Note that optical brightener measurements being conducted by CCU are to be considered provisional, since their SOP is being refined and is currently in draft form. Virginia Tech is conducting similar analyses and the CCU results shall only be used for comparison and validation.

Collect laboratory samples for bacteria source tracking analysis as described in Appendix L for the following:

- *Bacteroides* DNA primer
- esp *Enterococcus* human DNA primer
- Optical Brighteners

10.0 Sample Handling and Custody Requirements

Sample handling will be in accordance with standard, accepted field collection methods and shall meet all the requirements of the referenced SOPs. The CCU field sampling team that collects the samples will be responsible for initiating the Chain-of-Custody (COC) documentation by starting a COC form for samples to be transported to the CCU EQL and a separate COC form for samples to be shipped to the Virginia Polytechnic Institute and State University (VT) laboratory.

Each laboratory is responsible for completing the COC form as samples are received. A copy of the completed COC forms shall be provided to the Project Coordinator when analytical results are submitted by the laboratories.

11.0 Analytical Methods Requirements

Refer to the appropriate SOP (listed in Section 9.0) of the various parameters measured for the analytical methods requirements.

12.0 Quality Control Requirements

Refer to the appropriate SOP (listed in Section 9.0) for the various parameters measured for the quality control requirements.

13.0 Instrument/Equipment Testing, Inspection, Maintenance Requirements

Refer to the appropriate SOP (listed in Section 9.0) for the various parameters measured for the instrument/equipment testing, inspection, and maintenance requirements.

14.0 Instrument Calibration and Frequency Requirements

Refer to the appropriate SOP (listed in Section 9.0) for the various parameters measured for the instrument calibration and frequency requirements.

15.0 Inspection/Acquisition Requirements for Supplies and Consumables

Refer to the appropriate SOP (listed in Section 9.0) for the various parameters measured for the inspection and acquisition requirements for supplies and consumables.

16.0 Data Acquisition Requirements (Non-Direct Measurements)

Refer to the appropriate SOP (listed in Section 9.0) for the various parameters measured for the data acquisition requirements.

17.0 Data Management

A field sampling collection log will be maintained and will be used for documenting the collection of field sampling measurements and the collection of grab samples for subsequent laboratory analysis. The log will record the sampling day, time of day at each station, weather conditions, field measurements, grab samples collected, and any special notes of potential importance such as unusual conditions. No special data reduction or reporting will be needed for this project. All results from field and laboratory analysis will be provided to the Project Coordinator in hard copy form (using the laboratory's standard reporting format) and in electronic form in a computer database (i.e. MS Excel) such that data retrieval may be organized as necessary.

18.0 Assessments and Response Actions

After each sampling and analysis event, project personnel will review the information generated to assess its usability to meet project objectives. Any suggested change or refinement of a project activity to assure that project data quality and usability objectives are met will be made to the Project Coordinator. The suggested change or refinement shall be communicated promptly to the Project Coordinator so that the suggestion can be incorporated in the project, if appropriate. The Project Coordinator will assess the suggestion and will communicate the inclusion of the suggestion to the appropriate parties. Any project related documentation will be updated by the Project Coordinator and forwarded to the project team as needed.

In the event that a problem is identified during a particular activity, a corrective action will be formulated and implemented without the approval of the Project Coordinator. Proper documentation will be made of the corrective action.

19.0 Reports to Management

At the conclusion of the project, Thomas & Hutton Engineering Co. will prepare a report for distribution (with input from CCU and VT as needed). The report will present a narrative of all project activities, results of all data generating activities, and an interpretation of the results, conclusion, and other recommendations. A preliminary copy of the report will be presented to Horry County (and other stakeholders) for review prior to the publication of the final report.

20.0 Validation and Verification Methods

Refer to the appropriate SOP (listed in Section 9.0) for the various parameters measured for the validation and verification methods to be used.

21.0 Reconciliation with User Requirements

A review of each sampling events' data will be conducted when the data becomes available. The review will focus on the stated project goals (listed in Section 5.0).

Appendix A

SCOPE OF WORK

SCOPE OF SERVICES

Introduction and Background

During the past years, beach closings in Horry County have resulted due to high levels of bacterial contaminants being detected in the surf zone of the Atlantic Ocean. In 2000, Horry County commissioned a study by Davis & Floyd (D&F) to identify sources of contamination and to recommend options for improvement to water quality at storm water outfalls within the study area (*Storm Water Outfall Study – Horry County Beaches;* D&F, February 2002). In this study, it was observed:

> *"The residences throughout the Briarcliff Acres area are served by septic systems. Significant flooding was observed in this area on several occasions over the course of the study. During the study, it was common to observe homeowners in the Briarcliff Acres area pumping storm water from their homes, garages and yards into the street so that it would flow unrestricted to surface tributaries of the surf. The percentage of isolates (bacteria) from two selected samples collected from White Point Swash in the Briarcliff Acres area resulted in 50% and 57% human contribution, respectively. This was significantly higher than most of the other samples taken from the study area where the percentage of human vs. animal waste was analyzed."* (D&F 2002, pg. 3.1-3.2)

The study went on to recommend:

> *Provide Sewer to Briarcliff Acres - The Briarcliff Acres community is the only large area identified in the study that is served by septic tank and drain field systems. Flooding was observed in portions of this community during several rain events. Because of the potential for septic tank and drain field dispersion of leachate during flooding, it is recommended that Briarcliff Acres be evaluated to determine the feasibility of connecting the homes in this community to a sanitary sewer system.* (D&F 2002, pg. 3.1-3.2)

A follow-on study was conducted by D&F in 2004 (*Atlantic Intracoastal Waterway/Atlantic Ocean Surf Bacteria Investigation,* D&F, December 2004) that addressed several of the recommendations for additional study made in the first (2002) study, but did not address septic tank/sewer service at Briarcliff Acres.

In the past few years, several homes along the Atlantic Ocean where taken off septic tanks and provided with sanitary sewer service. The affect of this change on water quality, and more specifically the occurrence of beach closings have not been studied. In addition, the previous sampling implicating the existing septic tanks was limited.

Thus, this study is aimed to confirm or disprove a link between fecal coliform concentration in the waters around Briarcliff Acres and septic tanks in the area.

Our scope of services will include the following tasks:

Task 1 - Data Collection
Task 2 - Septic Tank System Survey
Task 3 - Watershed Assessment
Task 4 - Water Quality Sampling
Task 5 - Data Analysis and Report

Task 1 - Data Collection

Thomas & Hutton (T&H) will gather available data and mapping of the Briarcliff Acres area. T&H maintains an extensive in-house database of geographical data (aerials, contours, sewer and water infrastructure, roads, etc.). However, we will confirm the availability of more recent data and obtain it for use in the study if readily available.

T&H will also make contact SCDHEC Onsite Wastewater Management Division (both locally and in Columbia) and other agencies (Waccamaw COG, City of Myrtle Beach, Grand Strand Water and Sewer Authority, etc.) to collect any data relative to the situation and document information pertinent to the study (sewer system services and location of facilities, septic tank permits and/or records, etc.)

Historical water quality and other data will be collected. At this time, the known sources of this data include *Storm Water Outfall Study – Horry County Beaches;* D&F, February 2002; *Atlantic Intracoastal Waterway/Atlantic Ocean Surf Bacteria Investigation,* D&F, December 2004; data collected by the Coastal Carolina University Environmental Quality Laboratory in 2003; and 1997 to present beach monitoring data by Coastal Carolina University. Other data, such as local rainfall data will also be collected from readily available sources.

Task 2 - Septic Tank System Survey

T&H will meet with Briarcliff Acres officials and/or residents to inform them about the study, gather information concerning existing septic tanks (size, location, service interval, known problems, etc.), and gage support for potential future recommendations (connection to sewer system). T&H will prepare materials and submit to Horry County for review and approval prior to the meeting issuing.

Following the meeting, T&H will estimate the general location of the septic tanks in the study area and prepare a GIS overlay to catalogue the know/suspected septic tank locations.

Task 3 - Watershed Assessment

A limited watershed assessment of the swash draining Briarcliff Acres to the Atlantic Ocean will be conducted. A preliminary delineation of the watershed is shown in Exhibit 1. The watershed assessment will establish the watershed limits, define principal drainage features and patterns in the watershed (ponds/lakes, major culverts, wetlands, swashes, etc.), delineate major sub-basins,

identify homes served by septic tanks and by sanitary sewer collection system, and describe land uses and impervious land cover percentages.

Task 4 - Water Quality Sampling

Sampling Plan and Quality Assurance Project Plan (QAPP)

A conceptual sampling plan has been established as part of developing the scope of this study. However, the study plan has to be finalized with the study stakeholders and details must be finalized. In addition, a project specific QAPP must be prepared to ensure all sampling and laboratory testing will be defensible. The conceptual sampling plan is described in the two sections below and include dry and wet (storm event dependent) weather sampling. All sampling and standard laboratory analyses will be conducted by Coastal Carolina University – Environmental Quality Lab (DHEC Certified).

The preliminary sampling locations are illustrated in Exhibit 1. The preliminary sampling locations include:

BA-1: Outfall of the swash to draining Briarcliff Acres to the Atlantic Ocean.
BA-2: Outfall of interior Briarcliff Acres lake to the adjacent lake/wetland.
BA-3: Contributing wetland from the southwest.
BA-4: Outfall of second interior Briarcliff Acres lake.
BA-5: Interior sampling location, located on contributing drainage feature upstream of second interior Briarcliff Acres lake.
MSC-1: Interior lake sampling location at Meher Spiritual Center.

Dry Weather Sampling

Each station (excluding BA-5) will be sampled at periodic times (for a total of 4 samples each). The stations will be sampled on the same day and as close in time as possible. Samples will be collected as close to low tide as possible to minimize the effect of tidal back flow. The water at each site will be tested for a series of field parameters including:

- Water Depth
- Water Flow (estimated)
- Specific Conductance
- Salinity
- Temperature
- Dissolved Oxygen
- pH
- Turbidity

Grab samples will be taken, preserved, and shipped for laboratory analysis. The following laboratory parameters will be analyzed (by Coastal Carolina University - Environmental Quality Laboratory):

- Biological Oxygen Demand
- Ammonia
- Total Suspended Solids
- Surfactants (optical brighteners via fluorometry)
- Fecal Coliform Bacteria
- Enderococci Bacteria
- Bacteria Source Tracking Test – human vs. non-human via the human *Bacteroides* DNA marker and the *esp* human *Enterococcus* DNA marker test. *

* Note: Dr. Chuck Hagendorn at Virginia Tech University, a known expert in the field will conduct the tests. The tests will indicate the relative percentage of bacterial from human and animal sources.

Wet Weather Sampling

Three (3) wet weather sampling events will be conducted. The sampling will be conducted immediately following the start of a rainfall event and proceed during and after the event. Rainfall events with anticipated rainfall in excess of 0.75 inch will be targeted.* Three grab samples will be collected for each station after the start of the rain event.

The same field and laboratory parameters will be analyzed for each wet weather grab sample as for the dry weather samples.

Task 5 - Data Analysis and Report

The data produced from the study will be summarized and analyzed. Any conclusions that can be derived from the data as to the amount and source of fecal bacteria contamination in the watershed will be provided.

A draft report detailing the study procedures, findings and recommendations will be prepared. The report will include details of the data collection, septic tank survey, and water quality sampling. The draft report will be reviewed with Horry County, and based on the County's comments; a final report will be produced.

*Note: Subsequent to the development of the scope of work, it was decided that the project director (T&H) and field sampling manager (CCU) would work collaboratively before imminent rain events to decide to initiate a sampling event.

Appendix B

SAMPLING LOCATION MAP

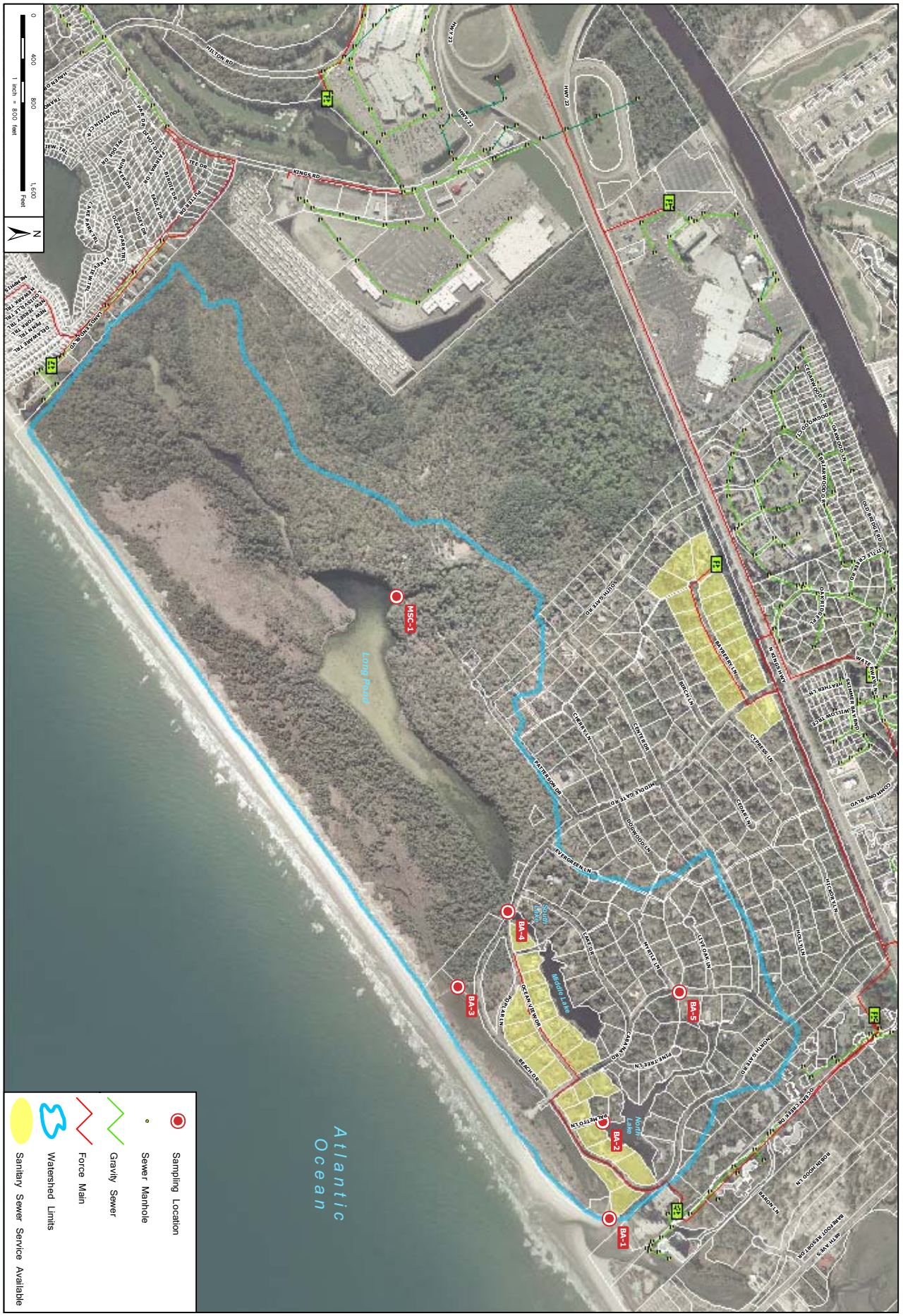

Exhibit 1

Job Number: 22072	Produced: 6/02/09	Produced By: JMN
Scale: 1 inch = 800 feet	Modified: 11/02/09	Modified By: JMN
Projection: SC State Plane	Horizontal Datum: NAD 83	Vertical Datum:
File: N:\22072\GIS\Briarcliff_SamplingLocations.mxd		

Thomas & Hutton Engineering Co. compiled the map information from the following sources:

DATA	SOURCE	DATE
Parcel Boundaries, Utilities, and Road Names	Horry County	2008
Sampling Locations & Watershed Limits	Thomas & Hutton (conceptual only)	2009
Aerial Photography	Private Source	2006

DISCLAIMER:

Where Thomas & Hutton Engineering Co. is cited as the data source, the firm has created or verified the data. For all other sources cited, Thomas & Hutton used the data "as is." has made no independent investigation of the data, and makes no representation as to the accuracy or completeness of the data. Please see each source for available documentation of its respective data sets.

Briarcliff Acres

Horry County, South Carolina

Sampling Locations

MYRTLE BEACH OFFICE
1350 FARROW PARKWAY
MYRTLE BEACH, SC 29577
(843) 839-3545

Appendix C

CCU EQL SOP NO.: 420

FIELD MEASUREMENTS WITH HACH RUGGEDIZED PROBES

Conductivity, Dissolved Oxygen, pH, Salinity, Temperature Measurements in Field with HACH Ruggedized Portable Probes

Reference Methods:
SM 2510 A. and B. (1997 online), SM 2550 B. (2000 online), SM4500-H^+ B. (2000 online), ASTM D888-05, and Hach Method 10360

Approved by: _____
Laboratory Director

Reviewed by: _____
Laboratory Technician

1.0 SCOPE/APPLICATION

HACH ruggedized probes were designed for rugged field use, easy calibrations, time-savings and results repeatability with a single HQ40d multi meter read-out. This standard operating procedure describes the procedures used by staff of the Environmental Quality Lab (EQL) to calibrate for and to measure specific conductivity, dissolved oxygen, pH, salinity, and temperature in the field with HACH ruggedized probes. Dissolved oxygen is measured using luminescent sensor technology. Specific conductance and salinity are measured by electrical conductivity. The pH is measured by the electrometric technique. Temperature is measured by thermistor.

Dissolved oxygen (DO) levels in natural and wastewaters depend on the physical, chemical, and biochemical activities in the water body. The analysis for DO is a key test in water pollution and waste treatment process control. Dissolved oxygen on the HACH ruggedized Luminescent Dissolved Oxygen (LDO) probe is determined by the luminescent measurement using luminescent sensor technology; this is not a membrane method. The EPA approved the LDO Hach method 10360 in July 2006. The HACH LDO probe is coated with luminescent material and works by transmitting blue light and red light. Blue light from an LED is transmitted to the sensor surface and excites the luminescent material. As the luminescent material relaxes, it emits a red light, and this emission time between blue and red lights is measured and used as an internal reference. Higher oxygen levels decrease the time between blue and red lights, and this correlates to oxygen concentration. The probe does not require a warm-up period, but the sensor cap must be replaced annually or when the sensor surface is < 25% black luminescent material, whichever is longer. A salinity correction must be applied when dissolved oxygen is being measured with the HACH ruggedized LDO probe in brackish or saline waters.

Conductivity is a measure of the amount of dissolved solids in a water sample. Dissolved solids are primarily ions. A conductivity meter detects the amount of electricity that is conducted by a water sample. The larger the amount of ions the greater the amount of electricity conducted and hence the greater the conductivity of the sample. Conductivity units are µmhos/cm, µS/cm, or mS/cm.

Salinity is defined as the weight in grams of the dissolved inorganic matter in 1 kg of seawater, after all bromide and iodide have been replaced by the equivalent amount of chloride, and all carbonate converted to oxide. Along with temperature, the salt content of seawater determines density. Colder water has higher density than warm and saltier water has higher density than fresh water, and density of the ocean increases with increasing depth. So, the waters of the deep sea are cold and salty. The salt content of the water is determined largely by the balance between water input and loss from the ocean. Water enters the ocean via rainfall and river runoff. It leaves via evaporation. At locations where the rate of evaporation exceeds rainfall or river runoff, the salinity of seawater is high.

The measurement of pH is one of the most important and frequently used tests in water chemistry. Many phases of water supply, wastewater treatment, and environmental water chemistry processes are pH-dependent. At a given temperature the intensity of the acidic or basic character of a solution is indicated by pH or hydrogen ion activity, where pH is defined as –log (hydrogen ion activity). In dilute solution (i.e., ionic strength <0.1) the activity of hydrogen ion in solution is approximately equal to the hydrogen ion molarity.

2.0 REFERENCES

2.1 Standard Methods 2510 A. and B. (1997 online), 2550 B. (2000 online), 4500-H^+ B. (2000 online)
2.2 Hach Method 10360
2.3 American Society for Testing and Materials (ASTM) Method D888-05
2.4 HACH "HQ Series Portable Meters" User Manual; 2006 Sept, ed. 5 (HACH catalog number: HQ40d18)
2.5 Hach.com (Hach Knowledge Base searching)

3.0 DEFINITIONS

None

4.0 SAFETY

4.1 This method is restricted to use by or under the supervision of trained analysts.
4.2 Gloves, safety glasses with side shields, and protective clothing should be worn to protect against unnecessary exposure to infectious agents (i.e., pathogens), hazardous chemicals (e.g., acids), and contaminants in potentially hazardous samples.
4.3 All activities performed while following this procedure should utilize appropriate laboratory safety systems (e.g., disinfectant, fume hoods, material safety data sheets).

5.0 METHOD

5.1 APPARATUS AND MATERIALS

5.1.1 HACH HQ40d Dual-Input, Multi-Parameter Digital Meter with 4 AA batteries
5.1.2 IntelliCAL pH Probe-Rugged (HACH product no. pH101-05, -10, -15, or -30)
5.1.3 IntelliCAL Conductivity Probe-Rugged (HACH product no. CDC401-05, -10,-15, or -30)
5.1.4 InteliCAL LDO Probe-Rugged (HACH product no. LDO101-05, -10, -15, or -30)
5.1.5 "HACH Ruggedized Field Probe CALIBRATION SHEET"
5.1.6 Deionized water
5.1.7 Delicate tissue wipes (Kimwipes or equivalent)
5.1.8 Barometer
5.1.9 Calibration flask for dissolved oxygen (250 mL glass or plastic Erlenmeyer flask with mouth large enough for probe to fit)
5.1.10 Rubber o-ring to act as a probe stopper in DO calibration
5.1.11 Parafilm

5.2 REAGENTS

5.2.1 Certified pH buffer solutions from Fisher (or equivalent): pH 4.00, 7.00, and 10.00 (Fisher catalog SB101-4, SB107-4, SB115-4; respectively)
5.2.2 Electrode Storage Solution (Fisher catalog number SE40-1 or equivalent)
5.2.3 Specific conductivity standards of various conductivities, commercially prepared (e.g., Fisher Traceable Conductivity Calibration 1000 uS/cm Standard, Cat. No. 09-328-3) and laboratory prepared (e.g., For preparation of conductivity standards see Standard Method 2510 A. (1997 online), "Conductivity Introduction"). Formulations for standards of various conductivities are provided. For example, 0.5M KCl (e.g., 37.28g KCl dissolved in deionized water and diluted to 1L) has a conductivity of 58,670 µS/cm at 25.0ºC.)
5.2.4 Conductivity control sample (e.g., 0.01 M KCl, which is 0.7456g KCl dissolved in deionized water and diluted to 1L, has a conductivity of 1,412 µS/cm at 25.0ºC)
5.2.5 Salinity Laboratory Control Samples: IAPSO P-series (35 psu) Standard Seawater (Ocean Scientific International Ltd., www.osil.co.uk), and filtered (0.45µm filter) and lab-tested seawater

5.3 DISSOLVED OXYGEN PROCEDURES

5.3.1 LDO Settings

- Push the wrench button on the HQ40d multi-meter and push the green button to 'Select' the "LDO101 Method"
- Observe the current method on the display screen. If the current method is not "INITIAL LAB" push the green button to select the currently displayed method.
- Use the up or down arrow to select "INITIAL LAB", then push the green button to select that method as the current method.
- To check or modify the current method, arrow down and then select "Modify Current Method"
- For the INITIAL LAB method the parameters and options should be as follows:
    - Measurement Options: allows you to choose resolution, lower and upper mg/L limits, **salinity correction** (enter salinity if measuring DO in brackish or seawater), pressure units (choose in mmHg), and to turn on averaging interval (choose OFF)
    - Units: allows to choose mg/L or % (choose mg/L)
    - Calibration Options: choose "User - 100%"
    - Calibration Reminder: choose "On" and set for 8 hours +30 min reminders
- To exit menus push blue button repeatedly until main menu is achieved

5.3.2 LDO Calibration Procedure

- The probe must be calibrated daily prior to use for measurements in the field or laboratory, and recalibrated after 8 hours for continuous use.

- Insert batteries in the meter if they are not present or need replacement.

- Check the condition of the probe surface. The surface should be clean and at least 25% black luminescent material. If it is necessary to clean the probe surface just rub with a wet Kimwipe and wipe dry with another Kimwipe. *The sensor cap must be replaced annually or when the sensor surface is < 25% black luminescent material, whichever is longer. A count down message appears on the screen 30 days before the sensor cap*

*expiration date. All measurements taken after the expiration date appear with the calibration **?** icon in the top left corner.*

- Place water in the calibration flask, cover the opening with parafilm and shake the flask vigorously for about 1 minute. Let the flask sit for 5-10 minutes to equilibrate and create 100% DO saturation in water saturated air.

- Remove the plastic protective bell from the LDO probe by loosening the fitting ring and sliding the bell off. Slide the rubber o-ring onto the LDO probe at a level that will allow the probe to fit loosely in the flask and the probe surface to sit directly above, but not touching, the water in the calibration flask.

- Connect LDO probe to HQ40d multi-meter (right side connection ports makes calibration activities easier but either the right or left port can be used) and turn meter on. After LDO probe has had time (5-10 minutes) to equilibrate **in the water saturated air**:
  - If the calibration screen is displayed, press cancel so you can perform a precalibration measurement.
  - Push green button to 'Read' and once the reading is displayed, fill in the pre-calibration row in the LDO probe calibration section on the "HACH Ruggedized Field Probe CALIBRATION SHEET"
  - Press blue button to 'Calibrate', and then green button to 'Read'. Once calibration reading stabilizes record calibration information on calibration sheet, push 'Done' and record that information on calibration sheet, and finally press 'Store' to save the calibration.
  - If the calibration was successful, the display screen will show OK in top left corner on the main menu. If "?" is displayed and the comment on the screen is "O_2 Sensor 0 Days Remaining" the calibration is acceptable as long as the QC checks were acceptable (see Table 1). The O_2 sensor is good as long as the sensor surface is $\geq$ 25% black luminescent material (Hach personal communication on 8-20-09). If calibration was not successful, repeat the calibration.
  - Before removing LDO probe from calibration flask, containing water saturated air, do several (at least two) post-calibration readings. Push 'Read' to perform a post-calibration reading. Wait at least 1 minute between post-calibration readings. Record these readings on the calibration sheet.

- The LDO probe saves the calibration within itself (intelliCAL), so it can be disconnected and reconnected to the same or another multi-meter and not need a recalibration.

- Proceed with sample analyses or turn power off.

5.3.3 Sample Measurement for LDO

- Insert batteries in the meter if they are not present or need replacement.

- If meter power is off, turn power on. Set date and time, if needed.

- If the salinity of the sample to be measured is greater than 1 ppt, enable a salinity correction by pushing the **wrench** button on the HQ40d multi-meter, and push the

green button to 'Select' the "LDO101 Method". Then arrow down to "Modify Current Method" and then push the green button to 'Select'. Press the green 'Select' "Measurement Options" and arrow down to "**Salinity Correction**" and push the green button to 'Select'. Input the salinity and arrow to the right until "OK" is selected. Next, push the blue button several times to 'Exit' out to main menu and salinity correction should appear on this main screen under the dissolved oxygen concentration.

- For NPDES compliance analyses, analysis must be performed within 15 minutes of sample collection.

- Place probe into the water body or collected sample to be measured and push green "Read" button. Wait for reading to stabilize (meter beeps). Record measurement value.

- Switch probe, if needed, to measure another parameter.

- After all measurements are completed, push power button to turn meter off.

- Rinse the probes with deionized water and dry with a delicate tissue wipe. Store the probes clean and dry.

5.4 CONDUCTIVITY PROCEDURES

5.4.1 **Conductivity Settings**

- Push the wrench button on the HQ40d multi-meter and push the green button to "select" the "CDC401 Method".
- Observe the current method displayed on the display screen. If the current method displayed is not "CONDUCTIVITY", push the green button to select the currently displayed method.
- On the screen that comes up, use the up or down arrow to select "CONDUCTIVITY", then push the green button to select that method as the current method.
- To check or to modify the current method, arrow down and then select "Modify Current Method"
- For the CONDUCTIVITY method the parameters and options should be as follows:
    - Parameter: Conductivity
    - Measurement Options: Units - Auto or can choose µS/cm or mS/cm if desire, Measurement Limits - 0.01 – 400,000 µS/cm, Temperature Correction – Linear, Correction Factor - 1.90%/°C, Reference Temperature - 25°C
    - Calibration Options: Custom Standard – if desire to change displayed custom standard value, input the actual standard value by selecting "Standard Value" then entering desired standard value in µS/cm. Preset standard concentrations can also be selected by selecting "Std:" then selecting the desired standard from the list.
- Push the blue button several times to 'Exit' out to the main menu.

5.4.2 **Conductivity Calibration Procedures**

- The probe must be calibrated daily prior to use for measurements in the field or laboratory, and recalibrated after 8 hours for continuous use.

- Insert batteries in the meter if they are not present or need replacement.

- **Clean** the conductivity probe with deionized water and a brush if necessary. Dry the probe with a soft cloth or paper towel.

- Power on the meter and connect the conductivity probe to the meter (right side connection ports makes calibration activities easier but either the right or left port can be used). Perform a reading in air by pressing the green "Read" button and waiting for the reading to stabilize. If the reading is ≤ 0.01 µS/cm the probe is clean and ready to calibrate.

- *Note: All conductivity probe calibrations are performed using conductivity units* µS/cm *or* mS/cm *regardless of whether conductivity, resistivity, salinity, or total dissolved solids (TDS) is measured.*

- Specify desired conductivity settings using section 5.4.1.

- If necessary, remove the plastic protective bell from the Conductivity probe by loosening the fitting ring and sliding the bell off. If the bell will remain on the probe make sure the holes in the bell line up so you can see through the bell and through the conductivity chamber. This is to ensure free flow through the chamber.

- Place probe in the conductivity calibration standard, tilt and jiggle the probe to remove air bubbles which will prevent an accurate result.

- With meter powered on and conductivity probe connected:
    - If the calibration screen is displayed, press cancel so you can perform a precalibration measurement.
    - Push green button to 'Read' and once reading is displayed fill in the pre-calibration column in the conductivity probe calibration section on the "HACH Ruggedized Field Probe CALIBRATION SHEET"
    - Press blue button to 'Calibrate', and then green button to 'Read'. Once calibration reading stabilizes record calibration information on calibration sheet, push 'Done' and record that information on calibration sheet, and finally press 'Store' to save calibration.
    - If the calibration was successful, the display screen will show OK in the top left corner of the main menu. If it was not successful, repeat the calibration.
    - Before removing conductivity probe from conductivity calibration standard, push 'Read' to perform a post-calibration reading. Record this reading on calibration sheet as well.

- Rinse the conductivity probe and dry it. Perform a post calibration conductivity control sample reading and fill in that section on the calibration sheet.

- The conductivity probe saves the calibration within itself (intelliCAL), so it can be disconnected and reconnected to the same or another multi-meter and not need a recalibration.

- Proceed with sample analyses or turn power off.

5.4.3 **Sample Measurement for Conductivity**

- Insert batteries in the meter if they are not present or need replacement.

- If meter power is off, turn power on. Set date and time, if needed.

- Make sure that the holes in the bell line up so that you can see through the bell and through the conductivity chamber. This is to make sure there is free flow of sample through the chamber

- Place probe into water body or collected sample to be measured, tilt and jiggle the probe to **remove air bubbles** which will prevent an accurate result, and push green "Read" button. Wait for reading to stabilize (meter beeps). Record measurement value.

- Switch probe, if needed, to measure another parameter.

- After all measurements are completed, push power button to turn meter off.

- Rinse the probes with deionized water and dry with a delicate tissue wipe. Store the probes clean and dry.

5.5 SALINITY PROCEDURES

5.5.1 **Salinity Settings on Conductivity Probe**

- Push the wrench button on the HQ40d multi-meter and push the green button to "select" the "CDC401 Method".
- Observe the current method displayed on the display screen. If the current method displayed is not "SALINITY", push the green button to select the currently displayed method.
- On the screen that comes up, use the up or down arrow to select "SALINITY", then push the green button to select that method as the current method.
- To check or to modify the current method, arrow down and then select "Modify Current Method"
- For the SALINITY method the parameters and options should be as follows:
    - Parameter: Salinity
    - Measurement Options: Units - $^{o}/_{oo}$, Measurement Limits - 0.0 – 40.0$^{o}/_{oo}$
    - Calibration Options: Custom Standard – usually 58,670, if desire to change displayed custom standard value, input the actual standard value by selecting "Standard Value" then entering desired standard value in µS/cm. Preset standard concentrations can also be selected by selecting "Std:" then selecting the desired standard from the list.
- Push the blue button several times to 'Exit' out to the main menu.

5.5.2 **Salinity Calibration Procedures**

- The probe must be calibrated daily prior to use for measurements in the field or laboratory, and recalibrated after 8 hours for continuous use.

- Insert batteries in the meter if they are not present.
- Turn on the meter, connect the conductivity probe (right side connection ports makes calibration activities easier but either the right or left port can be used) and check to

make sure the probe is set to read salinity (the main menu will show ‰ as the unit). If needed, follow section 5.5.1 to change the parameter to salinity.

- **Clean** the conductivity probe with deionized water and a brush if necessary. Dry the probe with a soft cloth or paper towel.

- Perform a reading in air by pressing the green "Read" button and waiting for the reading to stabilize. If the reading is ≤ 0.01 µS/cm the probe is clean and ready to calibrate.

- *Note: All conductivity probe calibrations are performed using conductivity units* µS/cm *or* mS/cm *regardless of whether conductivity, resistivity, salinity, or total dissolved solids (TDS) is measured.*

- If necessary, remove the plastic protective bell from the Conductivity probe by loosening the fitting ring and sliding the bell off. If the bell will remain on the probe make sure that the holes in the bell line up so that you can see through the bell and through the conductivity chamber. This is to make sure there is free flow through the chamber.

- Place probe in the conductivity calibration standard, tilt and jiggle the probe to remove air bubbles which will prevent an accurate result.

- With the meter powered on and the conductivity probe connected:
    - If the calibration screen is displayed, press cancel so you can perform a precalibration measurement.
    - Push the green button to 'Read' and once the reading is displayed fill in the pre-calibration row in the conductivity probe calibration section on the "HACH Ruggedized Field Probe CALIBRATION SHEET"
    - Press the blue button to 'Calibrate', and then the green button to 'Read'. Once the calibration reading stabilizes record the calibration information on the calibration sheet, push 'Done' and record that information on the calibration sheet, and finally press 'Store' to save the calibration
    - If the calibration was successful, the display screen will show OK in the top left corner of the main menu. If it was not successful, repeat the calibration.
    - Before removing the probe from the conductivity calibration standard, push 'Read' to perform a post-calibration reading. Record this reading on the calibration sheet as well.

- Rinse the conductivity probe and dry it. Perform a post calibration salinity control sample reading and fill in that section on the calibration sheet.

- Conductivity probe saves its calibration within itself (intelliCAL), so it can be disconnected and reconnected to the same or another multi-meter and not need recalibration.

- Proceed with sample analyses or turn power off.

5.5.3 **Sample Measurement for Salinity**

- Insert batteries in the meter if they are not present or need replacement.
- If meter power is off, turn power on. Set date and time, if needed.
- Make sure that the holes in the bell line up so that you can see through the bell and through the conductivity chamber. This is to make sure there is free flow of sample through the chamber
- Place the probe into water body or collected sample to be measured, tilt and jiggle the probe to **remove air bubbles** which will prevent an accurate result, and push green "Read" button. Wait for reading to stabilize (meter beeps). Record measurement value.
- Switch probe, if needed, to measure another parameter.
- After all measurements are completed, push power button to turn meter off.
- Rinse the probes with deionized water and dry with a delicate tissue wipe. Store the probes clean and dry.

5.6 pH PROCEDURES

5.6.1 **pH Settings**

- Push the wrench button on the HQ40d multi-meter and push the green button to 'Select' the "pHC101 Method"
- The current method is the default method and nothing needs changed or checked.

5.6.2 **pH Calibration Procedures**

- The probe must be calibrated daily prior to use for measurements in the field or laboratory, and recalibrated after 8 hours for continuous use.
- Insert batteries in the meter if they are not present.
- Remove the electrode storage solution cup from the probe and rinse the probe with deionized water. Pat the probe dry with a Kimwipe.
- Connect the probe to the meter (right side connection ports makes calibration activities easier but either the right or left port can be used) and power the meter on.
- If necessary, remove the plastic protective bell from the pH probe by loosening the fitting ring and sliding the bell off.
- Place the pH probe in a 7 buffer. If the calibration screen is displayed, press cancel so you can perform a precalibration measurement. Push the green button to 'Read'. Record this value in the pre-calibration section of the calibration sheet.
- Rinse, blot dry and place the pH probe immediately into a 4 buffer and push the green button to 'Read'. Record this value in the pre-calibration section of the calibration sheet.

- Push the blue 'Calibrate' button, place the pH probe in the lowest pH buffer (usually the pH 4), and press the green 'Read' button. Write the result on the calibration sheet.

- When prompted, clean and place the probe in the next higher pH buffer (usually the pH 7), and press the green 'Read' button. Write the result on the calibration sheet.

- Unless specifically required for the analyses being conducted, continue the calibration (i.e., perform a three-point calibration) and when prompted, clean and place the probe in the next high pH buffer (usually the pH 10), and press the green 'Read' button. Write the result on the calibration sheet.

- When finished reading the calibration buffers, push the up arrow under 'Done'. Record the rest of the calibration summary data on the calibration sheet.

- Press the green 'Store' button to accept the calibration.

- When calibration is successful, display will show OK in top left corner of main menu.

- Place the pH probe in a buffer closest to expected sample pHs (usually the 6 or 7) and push the green button to 'Read'. Record this value in the post calibration check standard section of the calibration sheet within the pH probe calibration box.

- Rinse the probe with deionized water and blot dry with Kimwipe.

- If storing the probe temporarily before measurements, refresh the electrode storage solution in the cup and replace it on the probe end.

- Replace the plastic protective bell to the probe.

- The pH probe saves the calibration within itself (intelliCAL), so it can be disconnected and reconnected to the same or another multi-meter and not need a recalibration for 8 hours.

- Proceed with sample analyses or turn power off.

5.6.3 **Sample Measurement for pH**

- Insert batteries in the meter if they are not present or need replacement.

- If meter power is off, turn power on. Set date and time, if needed.

- Remove the electrode storage solution cup from the probe and rinse the probe with deionized water. Pat the probe dry with a Kimwipe.

- For NPDES compliance analyses, analysis must be performed within 15 minutes of sample collection.

- Place the probe into water body or collected sample to be measured and push the green "Read" button. Wait for the reading to stabilize (it beeps). Record the measurement value.

- After measurement, rinse the probe with deionized water, and continue to next sample or store the probe clean and in the electrode storage solution.
- Switch probe, if needed, to measure another parameter.
- After all measurements are complete, push power button to turn meter off.
- Rinse the pH probe with deionized water and blot dry with a delicate tissue wipe. Store the pH probe clean and in the electrode storage solution

5.7 TEMPERATURE PROCEDURES

5.7.1 **Temperature Calibration**

- The thermistor on each probe is calibrated annually using a NIST-Traceable thermometer.
- The thermistor temperature correction factor is labeled on the probes.
- To obtain the corrected temperature apply the correction factor to the temperature read (i.e., corrected temperature = temperature read + listed temperature correction value).

5.7.2 **Sample Measurement for Temperature**

- Temperature is displayed along with the parameter result simultaneously for every probe that is connected to the meter, so temperature is determined when any parameter is being measured.
- For NPDES compliance analyses, analysis must be performed within 15 minutes of sample collection.
- Place any probe into water body or sample collected, and push the green 'Read' button. Record the temperature.
- Apply the temperature correction factor listed for the probe to the recorded temperature to determine the corrected temperature.
- Clean and store the probe as specified for the probe type being used (see sections: 5.3, 5.4, 5.5, or 5.6).

5.8 QUALITY CONTROL

5.8.1 Quality control (QC) measures for analyzing samples are summarized in Table 1 and are

as follows:

- Prior to analyzing samples, each analyst must demonstrate the ability to generate acceptable results (i.e., demonstration of capability).
- The probe must be calibrated daily prior to use for measurements in the field or laboratory, and recalibrated after every 8 hours for continuous use.
- Acceptable calibration checks (see Table 1 for calibration check acceptance criteria) must be performed immediately after calibration.
- For NPDES compliance analyses, analyses for DO, pH, and temperature must be performed within 15 minutes of sample collection.
- Any QC sample analysis (e.g., field replicates, laboratory control samples) should be subjected to exactly the same analytical procedures as those used on individual sample analyses.
- Unless otherwise specified for specific project or samples, each sampling event of up to 20 samples analyzed should include at least one field duplicate (i.e., field duplicate sample collected same location and nearly same time as initial sample) as QC sample.

Table 1. Quality Control Requirements for Measurements with Hach Ruggedized Probes

LCS = laboratory control sample QC = quality control PE = performance evaluation

QC Sample or Activity	Minimum Frequency	Acceptance Criteria	Corrective Action
Capability demonstration	Four (4) prepared samples analyzed prior to any customer sample analyses	LDO 97-104% of theoretical DO Others 75-125% R Others RPD ≤ 25%	Repeat until acceptable.
Calibration stability monitoring	Immediately before calibration measure standards	Not applicable.	Not applicable. Results are used to monitor stability of probes and evaluate need for maintenance.
Calibration	Daily prior to sample analysis and after every 8 hours	After calibration, measure calibration standards (conductivity, pH, DO % saturation of water saturated air) as sample pH ± 0.1 of expected, others 99-101% R	Investigate and fix any obvious problems. Repeat until acceptable.
Calibration check	Immediately following calibration	Measurement of calibration standards or LCS (conductivity, pH, DO % saturation of LCS or of water saturated air) Cond. 90-110% R, pH ± 0.1 of expected, DO 97-104% sat ****LDO method requires LCS to be read in duplicate with each calib. event****	Investigate and fix any obvious problems. Recalibrate and repeat until acceptable.
Field duplicate (duplicate sample collected at one of sampling sites	One (1) per sampling event	RPD ≤ 25%	Investigate problem. If system precision is in control, qualify results. If system precision is out of control, reanalyze all sampling sites if possible.
Internal PE sample	Samples and frequency determined by Lab QA Officer	75-125% R RPD ≤ 25%	Investigate all unacceptable results.
Blind PE sample	Samples and frequency determined by accrediting agencies and projects	Determined by PE provider	Investigate all unacceptable results.

MB = method blank %R = percent recovery RPD = relative percent difference

5.9 CORRECTIVE ACTIONS

5.9.1 If any of the QC requirements listed in Section 5.8 (*see Table 1*) are not satisfied, the analyst must consult with the Laboratory Director. Normally the activity must be repeated, after corrective actions are taken to correct any obvious problems, until the QC results are acceptable. If repeating the process is not possible (e.g., sample spilled), the results report will include a discussion of the problem and the client will be consulted.

5.9.2 The problem and associated corrective actions will be documented on a Nonconformance and Corrective Action Report (see EQL SOP 201).

5.10 EQUIPMENT MAINTENANCE

5.10.1 **General**

- The probes are designed to generally be maintenance-free. If the probes become dirty (see pH probe maintenance below), wipe their surface with a damp cloth or Kimwipe. Use a cotton-tipped applicator to clean or dry the connectors if they get wet.
- Wipe and clean probes and cables well after each field use.
- Store the display meter **without** batteries

5.10.2 **Luminescent Material on Dissolved Oxygen Probe**

- Check the condition of the probe surface. The surface should be clean and at least 25% black luminescent material. If it is necessary to clean the probe surface just rub with a wet Kimwipe and wipe dry with another Kimwipe. *The sensor cap must be replaced annually or when the sensor surface is < 25% black luminescent material, whichever is longer. A count down message appears on the screen 30 days before the sensor cap expiration date. All measurements taken after the expiration date appear with the calibration "**?**" icon in the top left corner.*

5.10.3 **Cleaning pH electrode**

- Cleaning the electrode may improve performance when the response and stabilization time become noticeably slower, but it should be noted that normal life expectancy for a probe is about 2 years.
    - For a mild cleaning, place the probe in a detergent solution such as Alconox for 30 minutes, and then soak in deionized water for approximately 15 minutes before use.
    - For a more vigorous cleaning, place the probe in 0.1N HCl solution for 2 minutes, rinse with deionized water, place in 0.1N NaOH solution for 2 minutes, and then rinse and place in the HCl solution again for 2 more minutes. Rinse the probe and allow it to soak in deionized water for approximately 15 minutes before use.
    - To clean organic build-up on the probe, place the probe in liquid bleach for 5 minutes, then soak in deionized water for 15 minutes before use.

5.10.4 **Cleaning conductivity probe**

- If the sample contains oils, greases, or fats, the probe may become coated. If this occurs, clean the probe with a strong detergent solution and brush. Rinse thoroughly with DI water.

6.0 WASTE DISPOSAL

Dispose of analyzed laboratory samples and waste from calibration activities down a sink sewage drain. Flush the sink with a large volume of tap water.

HACH Ruggedized Field Probe CALIBRATION SHEET (revised 10/15/09)

for field sampling date(s): | Probes used: **LONG / SHORT** | Project name(s):

pH probe calibration

date & time of cal: _____ Analyst: _____

	pH 4 Std	pH 7 Std	pH 10 Std		Post Calibration Check Std
Std LOT #, exp date				temp	°C
pre calibration				expected value	
post calibration				measured value	
calibration temperature	°C	°C	°C		
mV					

Slope: mV/pH

%:

r²:

offset: mV

LDO probe calibration

date & time of cal: _____ Analyst: _____

time	temp (°C)	D.O. (mg/L)	D.O. % saturation	pressure (mmHg)	expected D.O.
pre-calibration reading					
cal reading					
post-cal reading #1					
post-cal reading #2					
post-cal reading #3					

slope: **offset:**

Conductivity probe calibration

date & time of cal: _____ Analyst: _____

Std LOT #, exp date		*Std LOT #, exp date*	
µS/cm Cal Standard Used		**µS/cm LCS Used**	
pre calibration		**LCS value after cal.**	
temperature of cal std	°C	**temperature of LCS**	°C
cell constant	/cm		
post calibration			
temperature of cal std	°C		

Appendix D

CCU EQL SOP NO.: 405

TURBIDITY MEASUREMENT WITH HACH POCKET TURBIDMETER

Turbidity Measurement in Field or Laboratory with Hach Pocket Turbidimeter ***Reference Method: SM 2130 B. (21st ed.)***

Approved by: _____
Laboratory Director

Reviewed by: _____
Laboratory Master Technician

1.0 SCOPE/APPLICATION

Turbidity is an expression of the optical property that causes light to be scattered and absorbed rather than transmitted with no change in direction or flux level through the sample. Turbidity in water is caused by suspended and colloidal matter such as clay, silt, finely divided organic and inorganic matter, and plankton and other microscopic organisms.

In the Environmental Quality Lab (EQL) turbidity is determined by the nephelometric method. In this technique turbidimeters with scattered-light detectors located at 90° to the incident beam are used. Nephelometric measurement results are reported as the nephelometric turbidity unit (NTU).

2.0 REFERENCES

2.1 Standard Method 2130 B. (21st ed.)
2.2 Hach Pocket Turbidimeter Instrument Manual

3.0 DEFINITIONS

None

4.0 SAFETY

4.1 This method is restricted to use by or under the supervision of trained analysts.

4.2 Gloves, safety glasses with side shields, and protective clothing should be worn to protect against unnecessary exposure to corrosive infectious agents (i.e., pathogens), hazardous chemicals (e.g., standards containing formazin), and contaminants in potentially hazardous samples.

4.3 All activities performed while following this procedure should utilize appropriate laboratory safety systems (e.g., disinfectant, fume hoods, material safety data sheets).

5.0 METHOD

5.1 APPARATUS AND MATERIALS

5.1.1 Hach Pocket Turbidimeter
5.1.2 Deionized water
5.1.3 Delicate tissue wipes (Kimwipes or equivalent)
5.1.4 Hach 5 mL sample cells with caps for Pocket Turbidimeter (Catalog 52631-00)
5.1.5 Silicone oil
5.1.6 Oiling cloth

5.2 REAGENTS

5.2.1 StablCal Stabilized Formazin Standards, 1.0 NTU (Catalog 26598-42)
5.2.2 StablCal Stabilized Formazin Standards, 20 NTU (Catalog 26601-42)

5.3 PROCEDURE

5.3.1 2-Point Calibration

- When the Pocket Turbidimeter is to be used for field or laboratory measurements, a 2-point calibration is required daily within 8 hours prior to use for measurements and recalibrated for every 8 hours of continuous use.

- Thoroughly rinse the Pocket Turbidimeter sample cell with deionized water.

- Apply a small drop of silicone oil to each of the outside four vertical rectangular sides of the cell.

- Spread the oil uniformly on the optical surfaces of the cell using the oiling cloth provided. Wipe off excess oil with the oiling cloth. The cell should appear nearly dry with little or no visible oil and no dust particles.

- Pour 1-2 mL of properly mixed 1.0 NTU StablCal Standard into the clean, labeled, and oiled Pocket Turbidimeter sample cell, swirl the standard around in the cell to rinse it, then pour out the rinse.

- Pour 5 mL of properly mixed 1.0 NTU StablCal Standard into the rinsed sample cell.

- Cap the cell.

- To remove all dust particles, wipe the sample cell exterior with a delicate tissue wipe.

- Place the sample cell containing the 1.0 NTU standard into the instrument sample cell compartment.
- Cover the sample cell with the light shield and wait 30 seconds for the

standard to stabilize.

- Press and hold the **CAL** key then press the **READ** key. Release both keys. After a short delay, **dA** will flash alternating with the dark value.
- Press and hold the **READ** key until the reading is stable. Release the **READ** key to accept the new dark value.
- Press the **CAL** key. After a short delay, the display shows **C1.0** alternating with the 1.0 NTU value using the last calibration.
- Press and hold the **READ** key. When the reading is stable, release the **READ** key to save the new 1.0 NTU value.
- Pour 1-2 mL of properly mixed 20 NTU StablCal Standard into the clean, labeled, and oiled Pocket Turbidimeter sample cell, swirl the standard around in the cell to rinse it, then pour out the rinse.
- Pour 5 mL of properly mixed 20 NTU StablCal Standard into the rinsed cell.
- Cap the cell.
- To remove all dust, wipe the sample cell exterior with a delicate tissue wipe then insert it into the sample cell compartment.
- Cover the sample cell with the light shield and wait 30 seconds for instrument to stabilize.
- Press the **CAL** key. After a short delay, the display shows **C20** alternating with the 20 NTU value using the last calibration.
- Press and hold the **READ** key until the reading is stable. Release the **READ** key to save the new 20 NTU value.
- Press the **CAL** key to end the calibration. The instrument displays **CLd** to indicate a new calibration has been entered. If no data points were changed, the instrument displays **OLd** to show the previous calibration has been retained.
- Measure the turbidity of each standard following the procedure listed in Section 5.3.3.
- See Section 5.4.2 for acceptance criteria for standard measurements (i.e., within 10% of expected values). If any standard measurements do not satisfy acceptance criteria, follow corrective actions described in Section 5.4 Table 1 and Section 5.5.

5.3.2 Calibration Check Prior to Sample Analysis

- After calibration and prior to sample analysis a calibration check is required by analyzing the calibration standards.

- Thoroughly rinse the Pocket Turbidimeter sample cell with deionized water.

- Apply a small drop of silicone oil to each of the outside four vertical rectangular sides of the cell.
- Spread the oil uniformly on the optical surfaces of the cell using the oiling cloth provided. Wipe off excess oil with the oiling cloth. The cell should appear nearly dry with little or no visible oil and no dust particles.

- Pour 1-2 mL of properly mixed 1.0 NTU StablCal Standard into the clean, labeled, and oiled Pocket Turbidimeter sample cell, swirl the standard around in the cell to rinse it, then pour out the rinse.

- Pour 5 mL of properly mixed 1.0 NTU StablCal Standard into the rinsed sample cell.

- Cap the cell, then remove dust particles by wiping the cell with a delicate tissue wipe immediately before inserting it into the sample cell compartment.

- Place the sample cell containing the standard into the instrument sample cell compartment.

- Insert the sample cell into the instrument sample compartment.

- Cover the sample cell with the light shield and wait 30 seconds for instrument to stabilize.

- Press and hold the **READ** key until the reading stabilizes (approximately 5 seconds). Release the **READ** key and record the displayed reading.

- Repeat the measurement process used for the 1.0 NTU standard for the 20 NTU standard.

- See Section 5.4.2 for acceptance criteria for check standard measurement (i.e., within 10% of expected value). If check standard measurement does not satisfy acceptance criteria, follow corrective actions described in Section 5.4 Table 1 and Section 5.5.

5.3.3 Sample Analysis

- If samples are to be measured in the field, they should be analyzed on the same day collected, ideally with 15 minutes of collection. Store at 1-4°C if cannot analyze immediately, and then warm to ambient temperature before analysis.

- For measurements in the laboratory, samples must be stored in the dark

at 1-4°C and analyzed within 24 hours of collection. Samples should be warmed to room temperature prior to analysis.

- Agitate or invert the sample to be measured to uniformly distribute particles/turbidity.
- Rinse a clean sample cell 2-3 times with the mixed sample.
- Pour 5 mL of sample into the Pocket Turbidimeter rinsed sample cell. Cap the cell.
- Wipe the outside surfaces of the cell with a delicate tissue wipe to remove any liquid. Take care not to scratch the cell.
- Insert the sample cell into the instrument sample compartment.
- Cover the sample cell with the light shield and wait 30 seconds for instrument to stabilize.
- Press and hold the **READ** key until the reading stabilizes (approximately 5 seconds). Release the **READ** key and record the displayed reading. If reading exceeds the concentration of the highest calibration standard, 20 NTU, the sample must be diluted with particle free water and measured until the value is less than 20 NTU.
- Between sample measurements in the field, rinse the sample cell thoroughly with deionized water.
- After completing measurements and returning to the laboratory, clean all sample cells and caps using detergent and water. Rinse with deionized water and dry and cap the cells for storage. Do not store samples or standards in the plastic sample cells.

5.4 QUALITY CONTROL

Quality control (QC) measures for analyzing samples are summarized in Table 1 and are as follows:

- When the Pocket Turbidimeter is to be used for field or laboratory measurements, a 2-point calibration is required daily within 8 hours prior to use for measurements and recalibrated for every 8 hours of continuous use. The two standards used for calibration and for calibration checks of the turbidimeter are 1.0 NTU and 20 NTU. For an acceptable 2-point calibration, measured values of all standards must be within 10% of expected values. For calibration checks the calibration standards are again measured as samples, and their results must also be within 10% of expected values.
- Prior to analyzing samples, each analyst must demonstrate the ability to generate acceptable results (i.e., demonstration of capability).

- Samples must be analyzed within 24 hours of collection. If not analyzed immediately after collection, samples must be stored in the dark at 1-4°C, and then warmed to ambient or room temperature prior to analysis.
- Any QC sample analysis (e.g., method blank, laboratory replicate, field replicate) should be subjected to exactly the same analytical procedures as those used on individual sample analyses.

- Unless otherwise specified for specific project or samples, each batch of up to 20 samples analyzed should include at least one method blank (i.e., deionized water) and one sample analyzed in duplicate (i.e., at least 5% duplicates) as QC samples.

- Direct turbidimeter reading (i.e., not corrected for dilution) of a sample must fall within the range bracketed by the lowest and highest calibration standards (i.e., 1.0 NTU and 20 NTU). Any sample reading below the lowest calibration standard's concentration must be reported as less than 1.0 NTU, the reporting limit (see Section 6.1). For any direct sample reading above the highest calibration standard's concentration, an aliquot of the sample must be diluted with particle free water until the reading falls within the calibration range.

5.5 CORRECTIVE ACTIONS

5.5.1 If any of the QC requirements listed in Section 5.4 are not satisfied, the analyst must consult with the Laboratory Director. Normally the activity must be repeated, after corrective actions are taken to correct any obvious problems, until the QC results are acceptable. If repeating the process is not possible (e.g., sample spilled), the results report will include a discussion of the problem and the client will be consulted.

5.5.2 The problem and associated corrective actions will be documented on a Nonconformance and Corrective Action Report (see EQL SOP 201).

Table 1. Quality Control Requirements for Turbidity by Hach Pocket Turbidimeter

QC Sample or Activity	Minimum Frequency	Acceptance Criteria	Corrective Action
Capability demonstration	Four (4) prepared samples analyzed prior to any customer sample analyses	Criteria for duplicate precision	Repeat until acceptable
2-Point calibration	Daily prior to sample analysis and every 8 hours for continuing	90-110% R (measured value of all standards when analyzed as samples within 10% of expected value)	Investigate problem. Correct any obvious problems. Repeat calibration until acceptable.
Calibration check with calibration standards	Prior to sample analysis	90-110% R	Investigate problem. Correct any obvious problems including new 2-point calibration if necessary. Repeat calibration check until acceptable.
Method blank	Daily prior to sample analysis	<1.0 NTU	Clean analytical system and repeat MB analysis. Identify and eliminate source of contamination.
Sample analysis	For all sample analyses	Direct sample reading within calibration range (i.e., lowest and highest calibration standard concentrations)	If reading below range report result as < RL. If result above range dilute sample.
Sample duplicate	One (1) per preparation batch	RPD ≤ 25%	Investigate problem. If system precision is in control, qualify results. If system precision is out of control, reanalyze entire batch.
Internal PE sample	Samples and frequency determined by Lab QA Officer	75-125% R RPD ≤ 25%	Investigate all unacceptable results.
Blind PE sample	Samples and frequency determined by accrediting agencies and projects	Determined by PE provider	Investigate all unacceptable results.

LCS = laboratory control sample
MB = method blank
MDL = method detection limit
MS = matrix spike
PE = performance evaluation
QC = quality control
%R = percent recovery
RL = reporting limit
RPD = relative percent difference

5.6 EQUIPMENT MAINTENANCE

5.6.1 Cleaning the Sample Cells and Caps

- To maintain clean sample cells and caps, rinse with 1:1 hydrochloric acid followed by multiple rinses with particle-free water or soak in warm water to which a mild detergent (Liqui-nox or equivalent) has been added; use a cotton swab to scrub the cells if necessary. Rinse several times with turbidity-free water.

5.6.2 Cleaning the Sample Compartment

- Clean the sample compartment and the sample cell windows with a cotton swab dampened with isopropyl alcohol or detergent and turbidity-free water. Rinse with turbidity-free water. Do not use acetone or other organic solvents to clean the sample cells; damage to the cells will result.

6.0 DATA REPORTING

6.1 REPORTING LIMIT

The reporting limit (RL) for turbidity is primarily based on the concentration of the lowest calibration standard and the overall dilution or concentration of the sample during sample analysis. Additionally, the RL cannot be lower than typical method blank results or values calculated by method detection limit determinations. The concentration of the lowest calibration standard is 1.0 NTU. Nominally, sample analysis is done without dilution. Therefore, the nominal RL is 1.0 NTU, and any measured sample concentration below 1.0 NTU must be reported as less than 1.0 NTU.

6.2 MEASUREMENT RANGE REQUIREMETS

Direct turbidimeter reading (i.e., not corrected for dilution) of a sample must fall within the range bracketed by the lowest and highest calibration standards (i.e., 1.0 NTU and 20 NTU). Any sample reading below the lowest calibration standard's concentration must be reported as less than 1.0 NTU, the reporting limit (see Section 6.1). For any direct sample reading above the highest calibration standard's concentration, an aliquot of the sample must be diluted with particle free water until the reading falls within the calibration range. If it is not possible to dilute a sample that reads greater than 20 NTU, then the result must be reported as greater than 20 NTU.

7.0 WASTE DISPOSAL

Dispose of analyzed samples and used standards down a sink sewage drain. Flush the sink with a large volume of tap water.

Appendix E

CCU EQL SOP NO.: 406

TURBIDITY MEASUREMENT WITH HACH 2100N TURBIDIMETER

Turbidity Measurement in Laboratory with Hach 2100N Turbidimeter ***Reference Method: SM 2130 B. (21st ed.)***

Approved by: _____
Laboratory Director

Reviewed by: _____
Laboratory Master Technician

1.0 SCOPE/APPLICATION

Turbidity is an expression of the optical property that causes light to be scattered and absorbed rather than transmitted with no change in direction or flux level through the sample. Turbidity in water is caused by suspended and colloidal matter such as clay, silt, finely divided organic and inorganic matter, and plankton and other microscopic organisms.

In the Environmental Quality Lab (EQL) turbidity is determined by the nephelometric method. In this technique turbidimeters with scattered-light detectors located at 90° to the incident beam are used. Nephelometric measurement results are reported as the nephelometric turbidity unit (NTU).

2.0 REFERENCES

2.1 Standard Method 2130 B. (21st ed.)
2.2 Hach 2100N Laboratory Turbidimeter Instruction Manual

3.0 DEFINITIONS

None

4.0 SAFETY

4.1 This method is restricted to use by or under the supervision of trained analysts.

4.2 Gloves, safety glasses with side shields, and protective clothing should be worn to protect against unnecessary exposure to corrosive infectious agents (i.e., pathogens), hazardous chemicals (e.g., standards containing formazin), and contaminants in potentially hazardous samples.

4.3 All activities performed while following this procedure should utilize appropriate laboratory safety systems (e.g., disinfectant, fume hoods, material safety data sheets).

5.0 METHOD

5.1 APPARATUS AND MATERIALS

5.1.1 Hach 2100N Turbidimeter
5.1.2 Deionized water
5.1.3 Delicate tissue wipes (Kimwipes or equivalent)
5.1.4 Hach 30 mL sample cells with caps for 2100N Turbidimeter
5.1.5 Volumetric Flasks, Class A, various sizes
5.1.6 Volumetric Pipets, Class A, various sizes

5.2 REAGENTS

5.2.1 Hach Formazin Turbidity Standard, 4000 NTU (Catalog 2461-42) or equivalent
5.2.2 GELEX Secondary Standards (Catalog 25890-00, including: air, stray light, 0-2, 0-20, 0-200, and 200-4000 ranges)

5.3 REAGENT PREPARATION

5.3.1 Preparation of Formazin Turbidity Standards for Instrument Calibration

- Use Class A volumetric glassware for preparing each standard.
- Use deionized water for all the following dilutions:
  - Pipet 12.5 mL of 4000 NTU standard into 50 mL volumetric flask and dilute to 50 mL (corresponds to 1000 NTU).
  - Pipet 5 mL of 4000 NTU standard into 100 mL volumetric flask and dilute to 100 mL (corresponds to 200 NTU).
  - Pipet 1 ml of 4000 NTU standard into 200 mL volumetric flask and dilute to 200 mL (corresponds to 20 NTU).
  - Pipet other volumes of standard into volumetric flasks and dilute to prepare other desired standard concentrations.
- Record preparation of standards in Standard Preparation Log, Form 205. Record following on separate line for each standard solution:
  - Type of Stock Standard to be Diluted or Dissolved
  - ID of Stock Standard
  - Conc. of Stock Standard
  - Type of Standard Prepared
  - Volume (mL) Stock Solution Dissolved
  - ID of Standard Prepared
  - Conc. of Standard Prepared
  - Volume of Standard Prepared (mL)
  - Date Prepared
  - Prepared with class A glassware by: (initials)
  - Date Discarded

5.4 PROCEDURES

5.4.1 Calibrating the Turbidimeter and Assigning Values to Gelex Standards

- Calibrating the turbidimeter and assigning new values to the GELEX secondary standards are done at least quarterly.
- Press the I/O switch on the back instrument panel to turn power on, and allow turbidimeter to warm up (minimum of 30 minutes).
- Select automatic range selection, signal average, and ratio settings (i.e., confirm the corresponding indicator lights are on or select them).
- Fill a clean sample cell to the line (approx. 30 mL) with fresh, deionized water. Wipe the cell clean. Place it into the cell holder with the triangle shape on the vial aligned with the index mark on the instrument sample compartment, and close the cell cover.
- Press the **CAL** key. The S0 annunciator lights. The NTU value of the deionized water used in the previous calibration is displayed.
- Press the **ENTER** key. The instrument display counts down from 60 to 0, and then makes a measurement. This result is stored and used to calculate a correction factor for measurement of all NTU standards.
- The instrument automatically increments to the next standard, displays the expected NTU value (e.g., 20.00 NTU), and the S1 annunciator flashes. Remove the sample cell from the holder.
- Fill a clean sample cell to the line with well-mixed 20 NTU Formazin standard. Wipe the sample cell clean. Place it into the cell holder with the triangle shape on the vial aligned with the index mark on the instrument sample compartment, and close the cell cover.
- Press the **ENTER** key. The instrument display counts down from 60 to 0, and then displays the turbidity (compensated for deionized water turbidity).
- The instrument automatically increments to the next standard, displays the expected NTU value (e.g., 200.0 NTU), and the S2 annunciator flashes. Remove the sample cell from the holder.
- Fill a clean sample cell to the line with well-mixed 200 NTU Formazin standard. Wipe the sample cell clean. Place it into the cell holder with the triangle shape on the vial aligned with the index mark on the instrument sample compartment, and close the cell cover.
- Press the **ENTER** key. The instrument display counts down from 60 to 0, and then displays the turbidity (compensated for deionized water turbidity).

- The instrument automatically increments to the next standard, displays the expected NTU value (e.g., 1000 NTU), and the S3 annunciator flashes. Remove the sample cell from the holder.

- Fill a clean sample cell to the line with well-mixed 1000 NTU Formazin standard. Wipe the sample cell clean. Place it into the cell holder with the triangle shape on the vial aligned with the index mark on the instrument sample compartment, and close the cell cover.

- Press the **ENTER** key. The instrument display counts down from 60 to 0, and then displays the turbidity (compensated for deionized water turbidity).

- The instrument automatically increments to the next standard, displays the expected NTU value (e.g., 4000 NTU), and the S4 annunciator flashes. Remove the sample cell from the holder.

- Fill a clean sample cell to the line with well-mixed 4000 NTU Formazin standard. Wipe the sample cell clean. Place it into the cell holder with the triangle shape on the vial aligned with the index mark on the instrument sample compartment, and close the cell cover.

- Press the **ENTER** key. The instrument display counts down from 60 to 0, and then displays the turbidity (compensated for deionized water turbidity).

- The display automatically increments back to the deionized water standard. The S0 annunciator lights, and the previously measured value of deionized water is displayed.

- Press the **CAL** key. The instrument stores the new calibration and returns the instrument to the measurement mode.

- To check the accuracy of the calibration, measure each standard used for the calibration as if it were a sample (i.e., Wipe the sample cell containing the standard clean. Place the standard in the sample compartment with the triangle or diamond on the vial aligned with the index mark on the instrument sample compartment. Close the sample-cell cover. Record the value displayed.)

- After the instrument is calibrated, the GELEX Secondary Turbidity Standards can be assigned a value.

- Confirm that automatic range selection, signal average, and ratio settings are on.

- Wipe the air standard sample cell clean. Place the air standard in the sample compartment with the triangle or diamond on the vial aligned with the index mark on the instrument sample compartment. Close the sample-cell cover.

- Press the **ENTER** key. Record the value displayed. Remove the air standard from the instrument, and mark this value on the vial cap.

- Repeat these steps with the stray light, 0-2, 0-20, 0-200, and 200-4000 ranges standards.

- After completing the calibration, continue with sample analyses or turn instrument power off with power switch on the back panel of instrument.

5.4.2 Sample Analysis

- Samples must be stored in the dark at 1-4°C and analyzed within 24 hours of collection. Samples should be warmed to room temperature prior to analysis.

- Turn turbidimeter power switch on, and allow meter to warm up (minimum of 30 minutes).

- Select automatic range selection, signal average, and ratio settings (i.e., confirm the corresponding indicator lights are on or select them).

- For a calibration check with the GELEX Secondary Turbidity Standards, wipe the standards clean.

- Insert the air standard into the cell holder with the triangle or diamond shape on the vial aligned with the index mark on the instrument sample compartment, and close the cell cover. Record the value. This should be within 33% of its assigned value.

- Repeat these steps with the stray light, 0-2, 0-20, and 0-200 ranges standards. The stray light standard should be within 33% of its assigned value; the other standards should be within 10% of their assigned values.

    - If the standards are not within the acceptance criteria, identify and fix the problem before conducting sample analyses. This may require a new calibration and assignment of new values to the secondary standards.
    - If the calibration check is acceptable, continue with analysis of samples.

- Fill a clean sample cell to the line (approx. 30 mL) with sample. Wipe the cell clean. Place it into the cell holder with the triangle shape on the vial aligned with the index mark on the instrument sample compartment, and close the cell cover.

- Read the digital readout for sample in NTUs and record value on the Turbidity Determination Worksheet form, Form 505. In general, take the reading when the digital readout stabilizes to a fixed value. In samples which the digital readout drifts, which sometimes occurs when sample contains numerous small swimming organisms, record the high and low measurements for the range of measurements. Direct readings less than

1.00 NTU (i.e., report limit – see Section 6.1) must be reported as less than 1.00 NTU. For direct readings above 4000 NTU (i.e., highest calibration standard), an aliquot of the sample must be diluted with particle free water and remeasured until less than 4000 NTU.

- Do not leave standards or samples in instrument after test is completed.

- Between sample measurements in the lab, rinse the sample cell thoroughly with deionized water.

- After completing sample measurements, measure the GELEX secondary standards again. If the measurements do not meet the calibration check acceptance criteria, identify the problem and fix it, if possible. Reanalyze samples if investigation data were adversely impacted.

- After completing all measurements, turn the instrument power off with the power switch on the back panel of the instrument.

- After all measurements, wash out used sample cells and caps with a detergent solution, and gently brush them with a test tube brush. Rinse thoroughly with tap water, and finally with deionized water. Turn tubes upside down and allow to drain and dry.

5.5 QUALITY CONTROL

5.5.1 Quality control (QC) measures for analyzing samples are summarized in Table 1 and are as follows:

- Prior to analyzing samples, each analyst must demonstrate the ability to generate acceptable results (i.e., demonstration of capability).

- Samples must be stored in the dark at 1-4°C and analyzed within 24 hours of collection. Samples should be warmed to room temperature prior to analysis.

- Any QC sample analysis (e.g., method blank, laboratory replicate, field replicate) should be subjected to exactly the same analytical procedures as those used on individual sample analyses.

- Unless otherwise specified for specific project or samples, each batch of up to 20 samples analyzed should include at least one method blank (i.e., deionized water) and one sample analyzed in duplicate (i.e., at least 5% duplicates) as QC samples.

- Direct turbidimeter reading (i.e., not corrected for dilution) of a sample must fall within the range bracketed by the lowest and highest calibration standards (i.e., 20.0 NTU and 4000 NTU) or the lowest and highest primary standards that were measured as samples and yielded results within 10% of expected values. A series of low turbidity standards were measured, and for this instrument the lowest formazin turbidity standard that could be measured within 10% of its expected value was 1.00 NTU. Therefore, the acceptable range for direct turbidity measurements for this

instrument is 1.00 NTU to 4000 NTU. Any sample reading below 1.00 NTU must be reported as less than 1.00 NTU. For any direct sample reading above 4000 NTU, an aliquot of the sample must be diluted with particle free water until the reading falls within the acceptable measurement range.

- Calibrating the turbidimeter and assigning new values to the GELEX secondary standards are done at least quarterly. Primary formazin turbidity standards of 20.0 NTU, 200 NTU, 1000 NTU, and 4000 NTU are used for these purposes. Step-by-step procedures are listed in Section 5.4.1.

- Each day of sample analyses, a calibration check is done before and after sample analyses with the GELEX Secondary Turbidity Standards.

5.6 CORRECTIVE ACTIONS

5.6.1 If any of the QC requirements listed in Section 5.5 are not satisfied, the analyst must consult with the Laboratory Director. Normally the activity must be repeated, after corrective actions are taken to correct any obvious problems, until the QC results are acceptable. If repeating the process is not possible (e.g., sample spilled), the results report will include a discussion of the problem and the client will be consulted.

5.6.2 The problem and associated corrective actions will be documented on a Nonconformance and Corrective Action Report (see EQL SOP 201).

5.7 EQUIPMENT MAINTENANCE

5.7.1 Cleaning the Sample Cells and Caps

- Because of the extreme sensitivity of the instrument, all sample cells must be absolutely clean inside and out. Do not allow previous test samples to dry out in cells. All residue must be removed because any matter remaining in the cell or on its outside surfaces will cause error in subsequent tests. Wash out used sample cells and caps with a detergent solution, and gently brush them with a test tube brush. Rinse thoroughly with tap water, and finally with deionized water.

- Turn tubes upside down and allow them to drain and dry.

- Any cells which are scratched or permanently stained must be discarded. To check for scratches, place the empty sample cell in the test well while the instrument is on in a dimly lighted room. With the cell cap and well cap off, rotate the tube slowly while looking down into the opening. Any scratches on the tube will be visible when the instrument's light strikes them.

Table 1. Quality Control Requirements for Turbidity by Hach 2100N Turbidimeter

QC Sample or Activity	Minimum Frequency	Acceptance Criteria	Corrective Action
Capability demonstration	Four (4) prepared samples analyzed prior to any customer sample analyses	Criteria for duplicate precision	Repeat until acceptable
4-Point calibration plus deionized water blank	At least quarterly	90-110% R (measured value of primary standards when analyzed as samples within 10% of expected values)	Investigate problem. Correct any obvious problems. Repeat calibration until acceptable.
Assign values to permanent transfer standards using formazin primary standards	At least quarterly	Measurement after acceptable 4-point calibration and values within 10% of previous established values	Investigate problem. Correct any obvious problems including replacing transfer standards if necessary. Repeat until acceptable.
Daily calibration check	Immediately prior to and after sample analysis	GELEX Secondary Turbidity Standards should read within 10% of assigned values	Investigate problem. Correct any obvious problems. If necessary reassignment of GELEX values and reanalyze samples. Repeat calibration check until acceptable.
Method blank	Daily prior to sample analysis	<1.0 NTU (i.e., < RL)	Clean analytical system and repeat MB analysis. Identify and eliminate source of contamination.
Sample analysis	For all sample analyses	Direct sample reading within acceptable measurement range (i.e., 1.00 NTU to 4000 NTU)	If reading below range report result as < RL. If result above range dilute sample.
Sample duplicate	One (1) per preparation batch	RPD ≤ 25%	Investigate problem. If system precision is in control, qualify results. If system precision is out of control, reanalyze entire batch.
Internal PE sample	Samples and frequency determined by Lab QA Officer	75-125% R RPD ≤ 25%	Investigate all unacceptable results.
Blind PE sample	Samples and frequency determined by accrediting agencies and projects	Determined by PE provider	Investigate all unacceptable results.

LCS = laboratory control sample
MB = method blank
MDL = method detection limit
MS = matrix spike
PE = performance evaluation
QC = quality control
%R = percent recovery
RL = reporting limit
RPD = relative percent difference

6.0 DATA REPORTING

6.1 REPORTING LIMIT

The reporting limit (RL) for turbidity is primarily based on the concentration of the lowest calibration standard, or the lowest primary standard measured as a sample that yielded a result within 10% of expected value, and the overall dilution or concentration of the sample during sample analysis. Additionally, the RL cannot be lower than typical method blank results or values calculated by method detection limit determinations. A series of low turbidity standards were measured, and for this instrument (Hach 2100N Laboratory Turbidimeter) the lowest formazin turbidity standard that could be measured within 10% of its expected value was 1.00 NTU. Nominally, sample analysis is done without dilution. Therefore, the nominal RL is 1.00 NTU, and any measured sample concentration below 1.00 NTU must be reported as less than 1.00 NTU.

6.2 MEASUREMENT RANGE REQUIREMETS

Direct turbidimeter reading (i.e., not corrected for dilution) of a sample must fall within the range bracketed by the RL (see Section 6.1) and highest calibration standard (i.e., acceptable measurement range of 1.00 NTU to 4000 NTU). Any sample reading below the 1.00 NTU must be reported as less than 1.00 NTU. For any direct sample reading above the highest calibration standard's concentration (i.e., 4000 NTU), an aliquot of the sample must be diluted with particle free water until the reading falls within the calibration range. If it is not possible to dilute a sample that reads greater than 4000 NTU, then the result must be reported as greater than 4000 NTU.

7.0 WASTE DISPOSAL

Dispose of analyzed samples and used standards down a sink sewage drain. Flush the sink with a large volume of tap water.

Appendix F

CCU EQL SOP NO.: 430

BIOCHEMICAL OXYGEN DEMAND (BOD)

Biochemical Oxygen Demand (BOD) ***Reference Method: SM 5210 B. (2001 online)***

Approved by: _____
Laboratory Director

Reviewed by: _____
Laboratory Technician

1.0 SCOPE/APPLICATION

General

The biochemical oxygen demand (BOD) determination is an empirical test in which standardized laboratory procedures are used to determine the relative oxygen requirements of waste-waters, effluents, and polluted waters. The test has its widest application in measuring waste loadings to treatment plants and in evaluating the BOD-removal efficiency of such treatment systems. The test measures the molecular oxygen utilized during a specified incubation period for the biochemical degradation of organic material (carbonaceous demand) and the oxygen used to oxidize inorganic material such as sulfides and ferrous iron. It also may measure the amount of oxygen used to oxidize reduced forms of nitrogen (nitrogenous demand) unless their oxidation is prevented by an inhibitor. The seeding and dilution procedures provide an estimate of the BOD at pH 6.5 to 7.5.

Measurements of oxygen consumed in a 5-day test period (5-day BOD or BOD_5), oxygen consumed after 60 to 90 days of incubation (ultimate BOD or UBOD) and continuous oxygen uptake (respirometric method,) are methods used to determine oxygen demand. Many other variations of oxygen demand measurements exist, including using shorter and longer incubation periods and tests to determine rates of oxygen uptake. Alternative seeding, dilution, and incubation conditions can be chosen to mimic receiving-water conditions, thereby providing an estimate of the environmental effects of wastewaters and effluents.

Dilution Requirements

The BOD concentration in most wastewaters and many surface waters (i.e., rivers, ponds) exceeds the concentration of dissolved oxygen (DO) available in an air-saturated sample. Therefore, it is usually necessary to dilute the sample before incubation to bring the oxygen demand and supply into appropriate balance. **Because bacterial growth requires nutrients such as nitrogen, phosphorus, and trace metals, these are added to the dilution water, which is buffered to ensure that the pH of the incubated sample remains in a range suitable for bacterial growth.** Complete stabilization of a sample may require a period of incubation too long for practical purposes; therefore 5 days has been accepted as the standard incubation period.

If the dilution water is of poor quality, the BOD of the dilution water will appear as sample BOD, and a positive bias will result. This effect will be amplified by the dilution factor. Both a dilution-water 'CHECK' and a 'BLANK' are included within the method. Seeded dilution waters are checked further for acceptable quality by measuring their consumption of oxygen from a known organic mixture; this is usually glucose and glutamic acid (contains an NH_2 component) are used, and sometimes potassium hydrogen phthalate (KHP) is used because it lacks nitrogen.

The source of dilution water is not restricted and may be distilled, tap, or receiving-stream water free of biodegradable organics and bio-inhibitory substances such as chlorine or heavy metals. Distilled water may contain ammonia or volatile organics; deionized (DI) waters often are contaminated with soluble organics leached from the resin bed. Use of copper lined stills or copper fittings attached to distilled water lines will produce water containing excessive amounts of copper.

2.0 REFERENCES

2.1 Standard Methods 5210 B. (2001 online), 5-Day BOD Test.
2.2 Laboratory Testing for BOD and CBOD, Post 1995. Brake, Perry & Raynovic, Michael. NCL of Wisconsin, Inc.
2.3 A Bug's-Eye-View of the BOD Test, January 2007. Brake, Perry.

3.0 DEFINITIONS

None

4.0 SAFETY

4.1 This method is restricted to use by or under the supervision of trained analysts.
4.2 Gloves, safety glasses with side shields, and protective clothing should be worn to protect against unnecessary exposure to infectious agents (i.e., pathogens), hazardous chemicals (e.g., acids), and contaminants in potentially hazardous samples.
4.3 All activities performed while following this procedure should utilize appropriate laboratory safety systems (e.g., disinfectant, fume hoods, material safety data sheets).

5.0 METHOD

5.1 APPARATUS AND MATERIALS

5.1.1 Glassware and containers:

- **Incubation Bottles:** 300-mL capacity. Clean bottles using a brush and warm tap water or two wash cycles in the dishwasher with no detergent, rinse with RO thoroughly, and with DI water two times. If possible, store in incubator until needed. Drain before use.

The bottles are designed to allow a water-seal as a precaution against drawing air into the dilution bottle during incubation. Obtain a satisfactory water seal by capping a full sample BOD bottle with a **ground glass stopper**, adding dilution water to the flared mouth of the BOD bottles and placing a **plastic cap** over the flared mouth of bottle to reduce evaporation of the water seal during incubation.

- **Standard Lab glassware:** Graduated cylinders, 500 mL beakers, 1000 mL beakers, Erlenmeyer flasks, and containers large enough for preparing dilution water. Clean all glassware with warm tap water or two wash cycles in the dishwasher with no detergent, rinse with RO thoroughly, and with DI water two times.

5.1.2 Air Incubator: Fisher Low Temperature BOD Incubator (model 307C is currently used in Coastal Science Bldg Room 308), thermo-statically controlled at 20°C ± 1ºC.

5.1.3: **DO meter and Probe**: YSI model 5100 Dissolved Oxygen meter (Fisher catalog #13-298-22) and YSI model 5010 BOD probe with self stirring capability (Fisher catalog # 15-176-18).

5.1.4 Membrane cap kit: YSI 5906 Membrane cap kit, 6/pk, contains membrane cap assemblies, bottle O2 probe solution, sanding disk, and instruction sheet (Fisher catalog # 14-660-225)**.**

5.1.5 Deionized water
5.1.6 Delicate tissue wipes (Kimwipes or equivalent)
5.1.7 Barometer

5.2 REAGENTS AND CHEMICALS

5.2.1 Buffer pillows -HACH BOD Nutrient Buffer Pillows

- **300 mL pillows** (HACH brand, Fisher catalog # 14160-66) to be used for 300 mL individual bottle buffering with sample volumes >150 mL)
- **6L pillows** (HACH brand, Fisher catalog #14862-66) to be used in the preparation of the bulk dilution water
- **19L/5gal pillow** (HACH brand, Fisher Catalog # 14863-98) to be used in the preparation of the bulk dilution water

5.2.2 Phosphate Buffer Solution.
Dissolve 8.5 g KH_2PO_4, 21.75 g K_2HPO_4, 33.4 g $Na_2HPO_4 \bullet 7H_2O$, and 1.7 g NH_4Cl in about 500 mL distilled water and dilute to 1L. The pH should be 7.2 without further adjustment.
Commercially prepared solution is available and acceptable.

5.2.3 Magnesium sulfate Solution:
Dissolve 22.5g $MgSO_4 \bullet 7H_20$ in distilled water and dilute to 1L.
Commercially prepared solution is available and acceptable.

5.2.4 Calcium Chloride Solution:
Dissolve 27.5g $CaCl_2$ in distilled water and dilute to 1L.
Commercially prepared solution is available and acceptable.

5.2.5 Ferric Chloride Solution:
Dissolve 0.25g $FeCl_3 \bullet 6H_2O$ in distilled water and dilute to 1L.
Commercially prepared solution is available and acceptable.

Prepare buffers immediately before use and be sure to discard if there is any sign of biological growth in the pillows.

5.2.6 Preparation of dilution water with commercial buffer pillows:

1. Place desired volume of water in a suitable clean bottle (i.e., 6L Erlenmeyer flask), aerate for no less than 1 h with a clean hollow glass rod hooked to a vacuum pump's HEPA filtered outflow, making sure to have a liquid trap flask and filter in-between the glass rod and pump exhaust.
2. After aeration of the water, place a paper towel over the bottle's opening and secure it with a rubber band. Place the bottle in the incubator at 20°C ± 1ºC for >24 h to stabilize.
3. Prepare buffered dilution water fresh daily. On day of analysis, 2-3 hours prior to sample set-up, dissolve the buffers in the desired volume of the aerated water. It is best to prepare a flask of double-strength dilution water and a flask of unbuffered water.
    - When using the **6L pillows**, add 2 pillows to 6L of aerated water, and swirl gently. This will create 6L of double-strength nutrient buffered BOD dilution water.
    - When using the **19L/5gal pillow**, pour 1 pillow into 100 mL aerated water, swirl until dissolved, then pour off until 63.2 mL remains. Finally pour this into the 6L of aerated water, and swirl gently to mix. This will create 6L of double-strength nutrient buffered BOD dilution water.
    - Other volumes of buffered double strength dilution water can be prepared by proportionately adjusting the number or volume of buffer pillows or volume of aerated water.
4. When adding the buffer to the bottles:
    - Dilution water blanks, seed controls and GGA standards are prepared with single strength buffered dilution water, (i.e., equal volumes of aerated water and double strength buffered dilution water).
    - Samples ≤150 mL will receive ≤150 mL of sample, the

selected quantity of seed, unbuffered aerated water, if necessary, to make up to 150mL total, and the remainder of the bottle volume with double-strength buffered dilution water.

- Samples >150 mL will receive > 150 mL of desired sample, one '**300 mL individual buffer pillow**', the selected quantity of seed, and remainder of the bottle volume filled with unbuffered dilution water.

5.2.7 Preparation of dilution water without commercial buffer pillows:

1. Place desired volume of water in a suitable bottle, aerate for no less than 1 h with a clean hollow glass rod hooked to a vacuum pump's HEPA filtered outflow, making sure to have a liquid trap flask and filter in-between the tube and pump discharge.

2. After aeration of the water, place a paper towel over the bottle's opening and secure it with a rubber band. Place the bottle in the incubator at 20°C ± 1ºC for >24 h to stabilize.

3. Prepare buffered dilution water daily. On day of analysis, 2-3 hours prior to sample set-up, dissolve the buffers in the desired volume of aerated dilution water. It is best to prepare a flask of double-strength dilution water and a flask of unbuffered dilution water. Add 1 mL each of the following buffers per 1 L of water for single-strength dilution water and 2 mL each per L for double-strength dilution water:

- Phosphate Buffer solution (5.2.2)
- Magnesium sulfate solution (5.2.3)
- Calcium chloride solution (5.2.4)
- Ferric chloride solution (5.2.5)

4. When adding the buffer to the bottles:

- Dilution water blanks, seed controls and GGA standards are prepared with single strength buffered dilution water, (i.e., equal volumes of aerated water and double strength buffered dilution water).
- **Samples ≤150 mL will receive ≤150 mL of sample, the selected** quantity of seed, unbuffered aerated water, if necessary, to make up to 150mL total, and the remainder of the bottle volume with double-strength buffered dilution water**.**
- **Samples >150 mL will receive > 150 mL volume of sample, one** '**300 mL individual buffer pillow**', the selected quantity of seed, and remainder of the bottle volume with unbuffered dilution water.

5.2.8 Acid and alkali solutions, 1N for neutralization of caustic or acidic waste samples.

- Acid- Dissol**ve slowly, while stirring, 28 m**L of concentrated

sulfuric acid, H2SO4, to distilled water. Dilute to 1L.

- Alkali- Dissolve 40g sodium hydroxide, NaOH, in distilled water. Dilute to 1L.

5.2.9 Sodium Sulfite Solution:
Dissolve 1.575g Na_2SO_3 in 1L distilled water. This solution is not stable; prepare daily for neutralization of chlorine.

5.2.10 Glucose-Glutamic Acid Solution (G-GA):
Dry reagent-grade glucose [Dextrose (D-Glucose) Anhydrous, CAS 50-99-7 & F.W. 180.16] and reagent-grade Glutamic Acid (L-(+)-Glutamic Acid, CAS 56-86-0 & F.W. 147.13) at 103ºC for 1 h. Cool and store in a desiccator.

For standard preparation, add 150 mg glucose and 150 mg glutamic acid to single strength buffered dilution water and dilute to 1L, or equivalent ratio. Prepare fresh immediately before use. This makes a concentration of 198 mg/L when 6 mL is added into 300 mL bottle diluted with buffered water and seeded.

5.2.11 Second-source G-GA standard: There are at least two choices for a second standard:

- NCL brand BOD Standard,
  198 ppm Glucose-Glutamic Acid Standard (NCL catalog #B-12D, call 715-449-2673 or 800-648-7836) in which 6 mL are placed into a 300mL sample bottle for 198 mg/L concentration.

- HACH BOD Standard Solution
  300 mg/L Glucose, 300 mg/L Glutamic Acid (HACH brand, Fisher catalog # 14865-10) in which 3 mL are placed into a 300 mL sample bottle for 198 mg/L concentration.

5.2.12 Potassium Hydrogen Phthalate (KHP):
Keep a clean vial with KHP in a desiccator. Add 150 mg to 500 mL DI water. This makes a concentration of 300 mg/L when 3 mL are added to a 300 mL sample bottle with dilution water, and seeded.

Discard reagents if there are any signs of biological growth in the stock bottle.

5.2.13 Seed Water:
Add one capsule Polyseed™ or NCL brand to one half liter of standard dilution water at 20ºC and stir (deep vortex in solution while stirring) for 60 minutes. Let settle for 30 minutes and decant off into another container. Stir at a gentle pace, and pipet from 1-2 inches under the surface when adding to sample with a wide-tipped pipet. Use before 6 hours after preparation.

5.3 PROCEDURE

5.3.1 Principle:

The method consists of filling with sample, to overflowing, an airtight bottle of the specified size and incubating it at 20.0°C ± 1°C for 5 days without light. An initial dissolved oxygen (DO) reading is measured and again after incubation. The BOD is computed from the difference between this initial and final DO. Because the initial DO is determined immediately after the dilution is made, all oxygen uptake, including that occurring during the first 5 min, is included in the BOD measurement.

5.3.2 Sampling and storage:

Samples for BOD analysis may degrade significantly during storage between collection and analysis, which can result in low BOD values. To minimize this reduction of BOD samples should be analyzed promptly or by cooling it to ≤ 6°C during storage. However, even at low temperatures, holding times should be kept at a minimum. Samples should be warmed to 20ºC before analysis, but not in direct light. Hold time for this analysis is 48 hours, but try to begin analysis within 6 hours.

- **Grab samples**:
  If analysis is begun within 2 hours of collection, cold storage is unnecessary. If analysis is not started within 2 hours of sample collection, keep sample at ≤ 6ºC from the time of collection. Begin analysis within 6 hours of collection; when this is not possible because the sampling site is distant from the laboratory, store at ≤ 6ºC and report length and temperature of storage with the results. In no case should analysis start more than 48 hours after grab sample collection; make every effort to deliver samples for analysis within 6 hours of collection.
- **Composite samples:**
  Keep samples at ≤ 6ºC during composite collection. Limit composite collection period to 24 hours. Use the same criteria as for storage of grab samples, starting the measurement of holding time from end of compositing period. State storage time and conditions as part of the results.

5.3.3 Sample Preparation:

- **pH Neutralization:** If the sample has a pH lower than 6.0 or higher than 8.0, neutralize the sample to pH of 6.5 to 7.5 with 1N sulfuric acid (H_2SO_4) or 1N sodium hydroxide (NaOH).If the quantity of the neutralizing reagent dilutes the sample more than 0.5%, a stronger solution must be used. The sample's initial pH, final pH and mLs of H_2SO_4 or NaOH added are recorded.
- **DO super saturation:** Samples containing more than 9 mg/L oxygen at 20ºC may be encountered in cold waters or in waters

where photosynthesis occurs. To prevent loss of oxygen during incubation of such samples, reduce DO to saturation at 20ºC by bringing sample to about 20ºC in a partially filled bottle while agitating by vigorous shaking or by aerating with clean, filtered compressed air. This may also be achieved by vigorously pouring the sample back and forth from one large mouth container to another. Care must be taken to reduce loss of sample due to spills.

- **Sample temperature adjustment:** Bring samples to 20ºC before making dilutions using gradual temperature adjustments in incubators or water baths.
- **Nitrification inhibition:** Nitrification inhibition is not currently being used in this lab. If nitrification inhibition is desired, add 3 mg 2-chloro-6-(trichloro methyl) pyridine (TCMP) to each 300 mL bottle or add sufficient amounts of sample, seed and dilution water to fill bottle to make a final concentration of 10 mg/L TCMP. Pure TCMP may dissolve slowly and can float on top of the sample. Some commercial formulations dissolve more readily but are not 100% TCMP; adjust the dosage accordingly. Samples that may require nitrification inhibition include, but are not limited to biologically treated effluents, samples seeded with biologically treated effluents, and river waters. Note the use of nitrogen inhibition in reporting results.
- **Dechlorination:** If possible, avoid samples containing residual chlorine by sampling ahead of chlorination processes. As this laboratory intends to test surface waters and not sewage treatment plant discharge, chlorination is not expected to be a problem.

5.3.4 <u>Calibration of YSI model 5000 meter/YSI model 5010 probe</u>:

1. At least one hour prior to use, plug power cable into the meter, plug the DO probe cable into the meter, and press the on-off switch to turn the meter on. Rinse the DO membrane-end of probe with DI water and blot any water droplets off the membrane with a Kimwipe. Rinse the BOD bottle used for storage of the probe and fill with 1-2 inches of fresh DI water. Insert probe into the clean BOD bottle with 1-2 inches of water. This provides a 100% humidity environment. Wait at least 20 minutes for temperature to equilibrate.

2. After allowing the probe to polarize and the temperature to stabilize for at least 20 minutes, (if the calibration is performed prematurely the values will probably drift and may be out of specification) press the calibration key.

3. Make sure the display readings are stable, and there are no condensation droplets on the membrane, then press the [DO Cal] soft-key soft-key to calibrate % saturation of dissolved oxygen. Using the [UP] [DOWN] [DIGIT] soft-keys indicate 100.0% and then press enter. The message "D.O. CALIBRATION SAVED" Will be displayed for a few seconds.

4. Press [MODE] to return to the main mode. If the barometric pressure and % saturation values stabilize, the instrument is now calibrated. Otherwise repeat calibration a 2nd or 3rd time. Wait 5 minutes after calibration to start recording calibration readings in calibration log.
5. A minimum of 3 readings, at least 1 minute apart, are to be recorded in the calibration log while the probe remains in the 100% humidity oxygen saturation air. Follow the acceptance range on the calibration sheet for stability check.
6. Be sure to also record the expected DO values for the temperature and pressure. This information is provided in the calibration log insert.
7. If stability check is outside acceptable limits, recalibrate and take a minimum of 3 more readings in 100% humidity oxygen saturated air.
8. Once the 100% humidity oxygen saturated air check is acceptable, pour 300 mL of aerated DI water at 20°C into a clean BOD bottle. Place the recently calibrated DO probe into this bottle and turn on the probe stir bar. After the reading stabilizes (may take a few minutes.), record the value and check to see if it is within acceptance limits (noted on calibration sheet).

5.3.5 <u>BOD Bottle Set-up:</u>

1. Select 300 mL glass BOD bottles that have been washed in the dishwasher once on the heavy wash cycle (no soap, no drying option) and once on the normal wash cycle (no drying option) and then triple rinsed with distilled water and stored in the incubator.
2. Refer to Table #1, on page 11, for the layout of a typical BOD run utilizing two dilution blanks, one dilution blank check, two GGA standards, 5 seed dilutions, and two samples including duplicates and a spike.
3. Prepare the bench sheet with the bottle numbers, sample ID numbers, Seed and GGA lot numbers, date, times and analyst as well as the mL of seed used, mL of GGA used, and mL of sample used for each BOD bottle
4. Using the descriptions in Table #1, prepare two Dilution Blanks and one Dilution Blank Check. With the BOD meter calibrated and stabilized (Ref 5.3.4), insert the DO probe into the first bottle. The meter will beep upon stabilizing. Read and record the DO, temperature, and time for each of the two Dilution blanks. Do not insert the DO Probe into the Dilution Blank Check bottle. The final DO will be read and compared to the two Dilution Blanks initial and final readings to insure that the probe is not a contributing factor in contamination. Refill the bottles with dilution water if necessary, stopper, and cap. Place into the incubator.

5. Fill the GGA std #1 bottles half-way with single strength dilution water. Add 6 mL of GGA std #1 and 6mL seed (typically, but may vary depending on experience regarding seed strength and GGA recovery). Fill the bottle with single strength dilution water. Rinse the DO probe between each bottle to avoid cross contamination. Insert the DO probe into each bottle and allow to stabilize. Immediately read the DO, temperature and time for each bottle and record. Refill the bottles with dilution water if necessary, stopper, and cap. Place into the incubator.
6. Fill the seed bottles half-way with single strength dilution water. Add the designated amount of seed to each (Ref Table #1), and fill with single strength dilution water.
7. Immediately read the DO, temperature and time for each bottle and record. Refill the bottles with dilution water if necessary, stopper, and cap. Place into the incubator.
8. Fill the sample #1 150 mL bottle with 150mL of sample, 4 mL of seed and fill with double strength dilution water. Fill the 300 mL bottle only half-way with sample, add 4 mL seed (typically, but may vary depending on experience regarding seed strength), one 300 mL nutrient buffer pillow and fill to top with sample. Immediately read the DO, temperature and time for each bottle and record. Refill the bottles with dilution water if necessary, stopper and cap. Place into the incubator.
9. Repeat the procedure in **5.3.5.8** for all other samples. A field duplicate should be included and should be tested as an independent sample. A lab duplicate and lab spike should be run on one in every ten samples analyzed (Ref Table #1).
10. Incubate at 20ºC ± 1ºC in the dark for 5 days ± 6 hours.

Table #1 Typical BOD Preparation Guide

ID	Sample	Preparation in 300 ml BOD bottle
1	Dilution Blank	Fill with Single Strength Dilution Water
2	Dilution Blank Dup	Fill with Single Strength Dilution Water
3	Dilution Check	Fill with Single Strength Dilution Water
		Do not insert DO probe for initial DO reading
4,5	2 x GGA std #1	Single Strength Dilution Water
		6 mL prepared GGA std. (Ref 5.2.10)
		6 mL seed
6,7	2 x GGA std #2	Single Strength Dilution Water
		Correct dosage GGA std. #2 (Ref 5.2.11)
		6 mL seed
8	Seed Control 4mL	Single Strength Dilution Water
		4 mL seed
9	Seed Control 8mL	Single Strength Dilution Water
		8 mL seed
10	Seed Control 10mL	Single Strength Dilution Water
		10 mL seed
11	Seed Control 12mL	Single Strength Dilution Water
		12 mL seed
12	Seed Control 15mL	Single Strength Dilution Water
		15 mL seed
13	Sample #1 150 mL	150 mL Sample
		4 mL seed
		Double Strength Dilution Water
14	Sample #1 300 mL	300 mL Sample
		4 mL seed
		Nutrient Buffer Pillow for 300 ml bottle
15	Sample #2 150 mL	150 mL Sample
		4 mL seed
		Double Strength Dilution Water
16	Sample #2 300 mL	300 mL Sample
		4 mL seed
		Nutrient Buffer Pillow for 300 ml bottle
17	Sample #2 Dup 150 mL	150 mL Sample
		4 mL seed
		Double Strength Dilution Water
18	Sample #2 Dup 300 mL	300 mL Sample
		4 mL seed
		Nutrient Buffer Pillow for 300 ml bottle
19	Sample #2 Dup Spike	150 mL Sample
		4 mL seed
		Double Strength Dilution Water
		6 mL prepared GGA std.

5.3.6 Final DO Determination:

1. Repeat the DO Probe calibration (Ref. **5.3.4**).
2. After 5 days ± 6 hours in the incubator at 20ºC ± 1ºC, remove the caps and stoppers from the bottles.
3. Beginning with the Dilution blanks, and Dilution blank check, insert the DO probe and allow to stabilize. Read and record the final DO, temperature, and time.
4. Continue with the GGA standard bottles, the seed bottles, and the sample bottles. Be sure to rinse the DO probe between bottles to avoid cross contamination. Read and record the final DO, temperature and time.
5. Continue the procedure with the seed bottles, and all of the sample bottles. Read and record the final DO, temperature and time for each.

6.0 CALCULATIONS

6.1 When dilution water is not seeded;

$$BOD_5, \text{ mg/l.} = \frac{(D_1 - D_2) - (B_1 - B_2) \times f}{P}$$

where:

D_1 = DO of diluted sample immediately after preparation. mg/L
D_2 = DO of diluted sample after 5 days incubation at 20ºC mg/L
P = decimal volumetric fraction of sample used
f = (volume of seed in diluted sample)/ (volume of seed in seed control)
B_1 = DO of seed control before incubation mg/L
B_2 = DO of seed control after incubation mg/L

Report results as $CBOD_5$ if nitrification is inhibited.

Average results all sample bottles with a residual DO of at least 1 mg/L and a DO depletion of at least 2 mg/L.

In order to do so, at least one sample dilution must meet the criteria of a residual DO of at least 1 mg/L and a DO depletion of at least 2 mg/L, and there must be no evidence or toxicity at higher sample concentrations or the existence of an obvious anomaly.

In these calculations, do not make corrections for DO uptake by the dilution water blank during incubation. This correction is unnecessary if dilution water depletion is less than 0.2 mg/L. If the dilution water does not meet these criteria, proper corrections are difficult and results become questionable.

6.2 Control limits:

The average BOD for the bottles containing the Glucose-Glutamic acid should lie within the range of **198 ± 30.5 mg/L**. **If measured BOD for a glucose-glutamic acid check is outside the accepted control limit range, reject tests made with that seed and dilution water.**

6.3 Working range and detection limit:

The working range is equal to the difference between the maximum initial DO (7 to 9 mg/L) and minimum DO residual of 1.0 mg/L multiplied by the dilution factor. A lower detection limit of 2 mg/L is established by the requirement for a minimum DO depletion of 2 mg/L. If the sample has been diluted the minimum detection limit is 2 mg/L multiplied by the dilution factor.

7.0 QUALITY CONTROL

7.1 Quality control (QC) measures for analyzing samples are summarized in Table 2 and are as follows:

- **7.1.1** Prior to analyzing samples, each analyst must demonstrate the ability to generate acceptable results (i.e., demonstration of capability).
- **7.1.2** Any replicate sample analyses should be subjected to exactly the same analytical procedures as those used on individual sample analyses.
- **7.1.3** Unless otherwise specified for specific project or samples, each batch should include two dilution water blanks, five seed dilutions, a known standard (GGA), and at least one sample analyzed in duplicate as QC samples.
- **7.1.4** When matrix interference is suspected, a matrix spike should be added.
- **7.1.5** The minimum DO depletion of 2.0 mg/L and minimum residual DO of 1.0 mg/L is required on all measurements. The results are not considered to be valid if these criteria are not met.
- **7.1.6** Annual performance tests are to be performed using blind PE samples and results reported to accrediting agencies.

8.0 WASTE DISPOSAL

8.1 Dispose of analyzed samples and used standards down a sink sewage drain. Flush the sink with a large volume of tap water.

Table 2. Summary of QC Requirements for 5-dayBOD Analysis

QC Sample or Activity	Minimum Frequency	Acceptance Criteria	Corrective Action
Capability demonstration	Four (4) prepared samples analyzed prior to any sample analyses	Criteria for LCS recovery and duplicate precision	Repeat until acceptable
Dilution water blank (Deionized water with buffers added)	Daily prior to sample analysis	< 0.2 mg/L DO depletion	Clean analytical system and repeat MB analysis. Identify and eliminate source of contamination.
Minimum residual DO and minimum DO depletion	For all measurements	Minimum DO depletion 2.0 mg/L Residual DO in bottle ≥ 1.0 mg/L	Results will not be considered valid
Seed Control	For every preparation batch	DO uptake attributable to seed added to each bottle generally 0.6 to 1.0 mg/L but seed amount must provide acceptable GGA recovery	Investigate problem. If system accuracy is in control, qualify results. If system accuracy is out of control, reanalyze batch
Glucose, Glutamic-acid Standard	Two (2) per preparation batch of samples run	Average BOD must be 198±30.5 mg/L.	Evaluate the cause, make appropriate corrections. High values indicate too much seed; low values indicate poor quality seed or the presence of toxic material.
Glucose, Glutamic-acid Standard- Second source standard	At least one (1) per quarter (i.e., every three months)	Average BOD must be 198±30.5 mg/L.	Evaluate the cause, make appropriate corrections. Compare with, to verify the accuracy of, the Lab Prepared GGA standard.
Matrix Spike	When suspect matrix interference	BOD matrix spike Recovery of 75 – 125%	Investigate problem. If system accuracy is in control, qualify results. If system accuracy is out of control, reanalyze batch.
Sample duplicate or matrix spike duplicate	One (1) per batch	RPD ≤ 25%	Investigate problem. If system accuracy is in control, qualify results. If system accuracy is out of control, reanalyze batch.
Internal PE sample	Samples and frequency determined by Lab QA Officer	Criteria for LCS recovery and duplicate precision	Investigate all unacceptable results.
Blind PE sample	Samples and frequency determined by accrediting agencies and projects	Determined by PE provider	Investigate all unacceptable results.

LCS = laboratory control sample
MB = method blank
MDL = method detection limit
MS = matrix spike
PE = performance evaluation
QC = quality control
%R = percent recovery
RL = reporting limit
RPD = relative percent difference

Appendix G

CCU EQL SOP NO.: 447

AMMONIA (NITROGEN) BY AUTOMATED PHENATE METHOD

Determination of Ammonia (Nitrogen) by Automated Phenate Method

Reference Methods: SM 4500-NH_3⁻ G. (1997 online)

Approved by: _____
Laboratory Director

Reviewed by: _____
Laboratory Technician

1.0 SCOPE/APPLICATION

In this method, ammonia (nitrogen) is determined by the reaction of alkaline phenol and hypochlorite with ammonia to form indophenol blue that is proportional to the ammonia concentration. The blue color formed is intensified with sodium nitroprusside. Ammonia nitrogen can be determined in potable, surface and saline waters as well as domestic and industrial wastewaters over a range of 5 to 600 µg NH_3-N/L when photometric measurement is made at 630 nm in a 10 mm flow cell. Higher concentrations can be determined using dilutions.

2.0 REFERENCES

2.1 Standard Method 4500-NH_3-G. (1997 online), Automated Phenate Method.

2.2 Standard Method 4500-NH_3-A. (1997 online), Introduction, Selection of Method.

2.3 Lynn, E. (2008) Lachat Instruments Inc., QuikChem Method 31-107-06-1-B, Alkaline Phenol based method; Brackish or seawater matrix. Lachat Instruments Inc.

2.4 U. S. Environmental Protection Agency. 1983. Nitrogen, Ammonia. Method 350.1 (Colorimetric, Automated, Phenate). (Online summary) Pp.350-1.1 -- 350-1.4. In *Methods for Chemical Analysis of Water and Wastes*, EPA-600/ 4-79-020. U.S.E.P.A., Cincinnati, Ohio, USA.

3.0 DEFINITIONS

3.1 *Ammonia:* Ammonia is a binary compound made up of nitrogen and hydrogen, characterized by a pungent smell and taste. Ammonia nitrogen, by definition is only a portion of total nitrogen. Total nitrogen is comprised of ammonia nitrogen, organic nitrogen, nitrate and nitrite. Ammonia nitrogen and organic nitrogen together make TKN, Total Kjeldahl nitrogen.

4.0 INTERFERENCES

- Glassware and labware contamination can be a problem in low level nitrogen analysis. Glassware and labware should be acid washed with 25% concentrated HCl and rinsed three times with deionized water.

- Reagent grade chemicals may contain trace levels of nitrogen. Using the best quality reagents will help to avoid nitrogen contamination.

- All reagent containers should be covered to prevent contamination from airborne ammonia.

- Calcium and magnesium ions may be present in concentration sufficient to cause precipitation problems during analysis. An EDTA solution is added to the sample on-line to prevent the precipitation of calcium and magnesium ions from river water and industrial waste.

- Sample turbidity and color may interfere with this method. Turbidity must be removed by filtration prior to analysis. Sample color that absorbs in the photometric range used will also interfere.

5.0 SAFETY

- This method is restricted to use by or under the supervision of trained analysts.

- Gloves, safety glasses with side shields, and protective clothing should be worn to protect against unnecessary exposure to corrosive infectious agents (i.e., pathogens), hazardous chemicals (e.g., phenol), and contaminants in potentially hazardous samples.

- All activities performed while following this procedure should utilize appropriate laboratory safety systems (e.g., disinfectant, fume hoods, material safety data sheets).

6.0 METHOD

6.1 APPARATUS AND MATERIALS

6.1.1 Pipettes (Class A- 0.5, 1.0, 5.0 and 10.0mL)
6.1.2 Deionized water
6.1.3 Analytical balance, capable of accurately weighing to the nearest 0.0001 g.
6.1.4 50 mL Graduated cylinders
6.1.5 500 mL Graduated cylinder
6.1.6 300 mL Griffin beaker
6.1.7 250 mL Class A volumetric flasks
6.1.8 500 mL Class A volumetric flasks
6.1.9 1000 mL Class A volumetric flasks
6.1.10 PVC carrier lines
6.1.11 PVC pump tubes (Red/Red – colors are industry standard designations for pump tubing diameters)
6.1.12 PVC pump tubes (Blue/Blue)
6.1.13 PVC pump tubes (Black/Black)
6.1.14 Neoprene pump tubes (Green/Green)
6.1.15 PVC pump tubes (White/White)
6.1.16 PVC pump tubes (Orange/White)
6.1.17 Lachat QC8500 Series FIA+ Autoanalyzer with ASX-500 Series XYZ Auto sampler, RP-100 Reagent Pump, 4 channels with HP injection valve, Omnion 3.0 software, four 175CM software controlled heaters and PDS200 Dilutor
6.1.18 pH meter
6.1.19 Sonicator bath
6.1.20 Analytical Manifold for method # 31-107-06-1-B, Ammonia.
6.1.21 Glass reagent bottles for reagent storage
6.1.21.1 500-mL plastic screw-top bottle for sodium hydroxide solution

6.1.22 Glass reagent bottles for auto sampler use and storage
- **6.1.22.1** 1000-mL clear glass screw-top bottle for Carrier DIW
- **6.1.22.2** 2500-mL clear glass screw-top bottle for Auto sampler/diluter DIW rinse water
- **6.1.22.3** 500-mL plastic screw top bottle for reagent DIW rinse water
- **6.1.22.4** 500-mL clear glass screw-top bottle for disodium EDTA buffer solution
- **6.1.22.5** 500-mL dark glass stoppered bottle for sodium phenate reagent solution
- **6.1.22.6** 500-mL clear glass screw-top bottle for sodium hypochlorite solution
- **6.1.22.7** 500-mL clear glass screw-top bottle for sodium nitroprusside solution

6.2 REAGENTS

6.2.1 Ammonia-free distilled water
- **6.2.1.1** Concentrated sulfuric acid, H_2SO_4, certified ACS or equivalent

6.2.2 Phenate reagent
- **6.2.2.1** Crystalline phenol, certified ACS or equivalent

6.2.3 Sodium hydroxide, NaOH, pellets, certified ACS or equivalent

6.2.4 Sodium hypochlorite solution containing 4.00-6.00 % NaOCl.

6.2.5 EDTA buffer chelating reagent
- **6.2.5.1** Disodium ethylenediamine tetraacetate, certified ACS or equivalent
- **6.2.5.2** Sodium hydroxide pellets, certified ACS or equivalent

6.2.6 Sodium nitroprusside reagent certified ACS or equivalent

6.2.7 Stock ammonium solution

6.2.8 Anhydrous ammonium chloride, NH_4Cl, solid, certified ACS or equivalent

6.2.9 Stock ammonium solution, LCS
- **6.2.9.1** Ammonium sulfate, $(NH_4)_2SO_4$, certified ACS or equivalent

6.3 PROCEDURES

6.3.1 Sample Collection, Processing, and Preservation

- Samples for ammonia analysis must be collected in acid-cleaned, plastic bottles. If samples are analyzed within 24 hours, the samples must be stored at ≤6°C. If samples are preserved by adding H_2SO_4 to pH< 2 and refrigerated at ≤ 6°C, they can be stored for up to 28 days before analysis. If acid preservation is used, neutralize samples with NaOH or KOH immediately before making the determination. Note: Although acidification is suitable for certain types of samples, it produces interferences when exchangeable ammonium is present with unfiltered solids.

6.3.2 Preparation of Reagents

- Ammonia-free distilled water (**6.2.1**): Special precaution must be taken to ensure that distilled water is free of ammonia. All solutions must be made with the ammonia-free distilled water.

- To prepare sodium phenate solution (**6.2.2**): In a 500-mL volumetric flask, dissolve 41.5 crystalline phenol in 250 mL distilled water. In small increments and with agitation, slowly add 32g NaOH. Cool flask under running water and dilute to 500 mL. Invert to mix. Prepare fresh every 3-5 days. *Caution: Minimize exposure of personnel to this compound by wearing gloves and eye protection, and using proper ventilation. Phenol causes skin burns and is rapidly absorbed into the body through the skin.*

- To prepare sodium hypochlorite solution (**6.2.4**): Dilute 125 mL sodium hypochlorite solution containing 4.00 – 6.00% NaOCl to 250 mL distilled water. Prepare fresh daily.

- To prepare EDTA buffer chelating reagent (**6.2.5**): Dissolve 50g disodium ethylenediamine tetraacetate and 11.0 g NaOH in l-L distilled water. Stir to mix. Prepare fresh monthly.

- To prepare sodium nitroprusside solution (**6.2.6**): Dissolve 1.75 g sodium nitroprusside in 500-mL distilled water. Prepare fresh every 1-2 weeks.

6.3.3 Preparation of Calibration and Calibration Check Solutions:

- General: The weights of chemicals and volumes of solutions used in the following specific procedures for the preparation of standards can be adjusted if different volumes or different standard concentrations are needed.

- Preparation of Calibration Solutions:

  1. Prepare the ammonia **primary standard solution** by dissolving 0.1909g primary standard grade ammonia chloride (**6.2.8)** that has been dried at 105°C for one hour, in about 800 mL DIW. Dilute to 1L and invert to mix. This generates the **primary ammonia standard solution** that is 50.0 mg NH_3 N/L and should be made fresh weekly. Store solution in a glass bottle in the refrigerator at ≤ 6°C.

  2. Dilute 10 mL of the p**rimary ammonia standard solution** to 500mL with distilled water. This is the **secondary ammonia standard solution** equal to 1.0 mg NH_3–N/L and should be prepared fresh daily and stored in the refrigerator at ≤ 6°C.

  3. Dilute 60 mL of the **secondary ammonia standard solution** to 200 mL with distilled water. This is the **third ammonia standard solution** or the **working ammonia standard solution** equal to 300µg NH_3–N/L and should be prepared fresh daily and stored in the refrigerator at ≤ 6°C.

- Preparation of ammonia laboratory control sample (LCS), which is the calibration check solution:

  1. Dry ammonium sulfate, $(NH_4)_2SO_4$, (**6.2.9.1**), in an oven at 105°C for 24 h., then cool to room temperature in a desiccator.

2. Dissolve 0.1910g primary standard grade ammonium sulfate that has been dried at 105°C, in about 800 mL DIW. Dilute to 1L and invert to mix. This generates the **primary stock ammonia LCS standard solution** that is 40.49 mg NH_3 - N/L and should be made fresh weekly. Store solution in a glass bottle in the refrigerator at ≤6°C.

3. Dilute 10 mL **primary stock ammonia LCS standard solution** to 500 mL with deionized water; giving a final concentration of 0.80988 mg NH_3-N/L. This **is intermediate ammonia LCS standard solution** and should be made fresh daily. Store solution in a glass bottle in the refrigerator at ≤6°C for up to 24 hours.

4. Dilute 25mL **intermediate ammonia LCS standard solution** (0.80988 mg N/L) to 500 mL with deionized water; giving a final concentration of 40.49 µg NH_3-N/L. This is the **working ammonia LCS standard solution** and should be made fresh daily. Store solution in a glass bottle in the refrigerator at ≤6°C for up to 24 hours.

6.3.4 Calibration and Sample Analysis on Lachat QuikChem 8500 Auto analyzer

- **Turn on computer interfaced to the QuikChem and logon. All further actions on this computer are in boldface font.**
- Turn on the power strip for the auto sampler, diluter, and QuikChem.
- **Select the Omnion 3.0 icon on the start-up menu.**
- **At the upper left hand corner of the screen, click on the tab labeled OPEN.**
- **In the CCU methods folder, sort the data results files by date.**
- **Open the most recent run by clicking on the DATE tab. The run settings should be:**

Properties	**Values**
Analyte Name	NH_3
Concentration Units	µg NH_3-N/L
Calibration fit type	2nd order Polynomial
Clear calibration	"check" box
Calibration weighing	1/x
Auto dilutor trigger	"check" box
% of high standard	110
QuikChem Method	31-107-06-1-B
Chemistry	Brackish

- **A popup menu will ask if you want to change the set points of the relevant heaters. Select "Yes"**

- Check that the canopy hood is on the HIGH setting.
- Press the manual/run button on the peristaltic pump. The pump should begin pumping.
- Snap the pump tubes into place on the peristaltic pump.
- Put the line labeled "carrier" into the Carrier DWI bottle and leave pumping.
- After 5 minutes, put the line labeled "Buffer" into the EDTA NaOH Buffer Solution bottle. Leave it pumping.
- After 5 minutes, put the line labeled "Reagent" into the Sodium Phenate Solution bottle. Leave it pumping for 5 minutes.
- After 5 minutes, put the line labeled "hypochlorite" into the Sodium Hypochlorite Solution bottle. Leave it pumping for 5 minutes.
- After 5 minutes, put the line labeled "Nitro" into the Sodium Nitroprusside Solution bottle. Leave it pumping for 5 minutes.
- After all tubing is on-line; no bubbles should be visible in the tubing around the mixing loop.
- The typical working range for this analysis by EQL is 7.5 to 300µg NH_3-N/L.

  Enter the following expected concentrations of the standards shown in Table 1 into the run worksheet of the Omnion software and the listed dilution factors needed to achieve these concentrations by diluting the 300µg NH_3-N/L standard.

Table 1. Ammonia-Nitrogen Calibration Standards

Sample ID	Conc.(µgNH3-N/L)	ADF
NH_3 300	300	
NH_3 300	188	1.60
NH_3 300	150	2.00
NH_3 300	100	3.00
NH_3 300	75	4.00
NH_3 300	60	5.00
NH_3 300	30	10.00
NH_3 300	15	20.00
NH_3 300	10	30.00
NH_3 300	7.5	40.00
DIW Blank	0.000	

ADF = auto dilutor dilution factor

- Other working ranges can be utilized by adjusting the concentration of the undiluted standard and the ADFs.

- After the calibrating solutions are analyzed, the Omnion software will compute a regression equation that is used to convert sample absorbances to concentrations.

- The quality of the regression will be documented by r^2 and by calculation of percent Recovery (% R). The latter is the percent recovery which is computed by taking the determined concentration divided by the expected concentrations. The determined concentration will be reported in the results table and is computed by comparing sample absorbances to the calibration regression curve.

- Immediately after the calibrating solutions, a DIW blank is analyzed.

- Swirl the 300µg NH_3-N/L standard, uncap, and pour approximately 45 mL of the standard into a 50-mL centrifuge tube. Place the tube on the autosampler in position #1.

- Swirl the DIW blank, uncap, and pour approximately 45 mL of the DIW blank into a 50-mL centrifuge tube. Place the tube on the autosampler in position #2.

- Refer to **Table 2**, A Typical Sample Analysis Set-up for the Autoanalyzer for guidance on sample and Quality Control sequencing.

Table 2. A Typical Ammonia (Nitrogen) Sample Analysis Set-up for the Autoanalyzer

Cell ID		
1 Thru 5	Parameter Standards for Calibration curve (NH_3)	Minimum 5 standards dependent on expected sample range; Could be up to 11 standards.
6	Rinse DIW	
7	Method Blank	
8	LCS	Laboratory Control Standard
9	LCS	Laboratory Control Standard
10	Rinse DIW	
11	Rinse DIW	
12	Sample 1	
13	Sample 2	
14	Sample 3	
15	Sample 4	
16	Sample 5	
17	Sample 5 Dup.	Duplicate to be inserted with suggested one sample out of 20
18	Sample 5 Matrix Spike	Matrix Spike to be inserted with suggested one sample out of 20 with a concentration close to expected sample conc. or middle working range.
19	Sample 6	
20	Sample 7	
21	Sample 8	
22	Sample 9	
23	Sample 10	
24	Artificial Seawater with Matrix Spike	Insert to establish peak retention time windows for brackish water (i.e., brackish timing) if brackish or seawater samples are analyzed
25	CCC	Continuing Calibration Check
26	Rinse DIW	
27	Samples 11 - 20	
28	Sample Dup.	Duplicate to be inserted with suggested one sample out of 20
29	Sample Matrix Spike	Matrix Spike to be inserted with suggested one sample out of 20 with a concentration close to expected sample conc. or middle working range.
30	CCC	Continuing Calibration Check
31	Rinse DIW	

Note: The Auto dilutor is automatically triggered if any samples are above the highest standard used in the calibration. The auto dilutor is set up to automatically perform a 10 to 1 dilution at the end of the run. The additional dilution will require additional tubes in the auto-sampler. Extra tubes should be added at the beginning of each run to cover such events.

- Swirl each sample bottle, uncap, and pour approximately 8 mL of sample into an autosampler sample cup and place onto the autosampler tray. Do this for all the samples to be run. Position them in the autosampler tray in the order that they will be run. Include the QC samples specified in **Table 3** at the frequency also specified in **Table 3.**

- **On the computer, press the PREVIEW button located at the top of the screen.**

- **Enter each EQL sample ID into the run worksheet in the column labeled "Sample ID".**

- **End the preview mode by selecting the STOP tab at the top of the screen.**

- **Begin the run by pressing the START button (green arrow) located at the top of the screen. The AA will then perform the calibration followed by the analysis of samples.**

- After the run is complete, save the results in a file with the run date in the file name. **From the Run tab on the top left, select Save As, and type in the Parameter, Type of Run (MDL), and date.**

- To clean the system, first remove the nitrogen reagent lines (Buffer and Reagent) from their reagent bottles and place them in the Reagent DIW rinse bottle. Let pump for about two (2) minutes. Remove the lines from the Reagent DIW bottle.

- Remove the reagent and carrier lines from their DIW rinse bottles. Leave pumping air about two (2) minutes.

- Wrap all line ends loosely in aluminum foil.

- Unsnap the pump tubes from the peristaltic pump to relax the tension on the tubes.

- Cap all reagent bottles and place in refrigerator.

- Print the report:

    - Click on the Tools Bar
    - Click on Custom Report
    - Click on Format
    - Click on Charts
    - Click on Calibration
    - Under File, Click on Print
    - Choose printer based on Room number.

- **Close the Omnion program.**

- **Turn off the computer.**

- Log the run into the Autoanalyzer Run Log book. Note any problems or maintenance that was performed.

6.4 QUALITY CONTROL

6.4.1 Quality control (QC) measures for analyzing samples are summarized in **Table 3** and are as follows:

- Prior to analyzing samples, each analyst must demonstrate the ability to generate acceptable results (i.e., demonstration of capability).

- Any replicate sample analyses should be subjected to exactly the same analytical procedures as those used on individual sample analyses.

- Unless otherwise specified for specific project or samples, each batch of up to 20 samples analyzed should include a DIW blank, a method blank (i.e., deionized water or synthetic seawater in a sample bottle that accompanied samples and received the same processing), a laboratory control sample (i.e., sample of known concentration of ammonia), and at least one sample analyzed in duplicate (i.e., at least 5% duplicates) as QC samples.

- An artificial seawater sample should be run to establish brackish timing if brackish or seawater samples are being analyzed.

Table 3. Summary of QC requirements for Determination of Ammonia Using the Automated Phenate Method

QC Sample or Activity	Minimum Frequency	Acceptance Criteria	Corrective Action
Capability demonstration	Four (4) prepared samples analyzed prior to any sample analyses	Criteria for LCS recovery and duplicate precision	Repeat until acceptable
Initial NH_3 calibration with standards (at least 5-point calibration plus blank)	Daily prior to sample analysis	Calibration standards approximately, evenly spaced over calibration range. Standards processed like samples with 90-110% R (measured value of each standard within 10% of expected value)	Investigate problem. Correct any obvious problems. Repeat calibration until acceptable.
Method blank	Daily with sample batch	<7.50 µg/L	Clean analytical system and repeat MB analysis. Identify and eliminate source of contamination. If significant adverse impact on results, reanalyze batch.
Laboratory control sample	At least one (1) per preparation batch	80-120% R	Investigate and identify the problem. If system accuracy is in control, no corrective action needed. If system is out of control, reanalyze batch.
Matrix Spike	When suspect matrix interference	Ammonia-(Nitrogen) 75-125%	Investigate problem. If system accuracy is in control, qualify results. If system accuracy is out of control, reanalyze batch.
Sample analysis	For all sample analyses	Direct sample reading within calibration range (i.e., above lowest standard and below highest standard)	If reading below range report result as < RL. If result above range, dilute sample.
Sample duplicate or matrix spike duplicate	At least one (1) per preparation batch	RPD ≤ 25%	Investigate problem. If system precision is in control, qualify results. If system precision is out of control, reanalyze batch.
Artificial Seawater sample with matrix spike	When brackish or sea water samples analyzed	To establish peak retention time windows for brackish water (i.e., brackish timing) and seawater	If analyte peaks not within integration windows, manually adjust peak integration windows to windows established for seawater.
Internal PE sample	Samples and frequency determined by Lab QA Officer	Criteria for LCS recovery and duplicate precision	Investigate all unacceptable results.
Blind PE sample	Samples and frequency determined by accrediting agencies and projects	Determined by PE provider	Investigate all unacceptable results.

LCS = laboratory control sample
MB = method blank
MDL = method detection limit
MS = matrix spike
PE = performance evaluation
QC = quality control
%R = percent recovery
RL = reporting limit (i.e., conc. of lowest cal. std adjusted for dilutions)
RPD = relative percent difference

6.5 CORRECTIVE ACTIONS

6.5.1 If any of the QC requirements listed in Section 5.4 are not satisfied, the analyst must consult with the Laboratory Director. Normally the activity must be repeated, after corrective actions are taken to correct any obvious problems, until the QC results are acceptable. If repeating the process is not possible (e.g., sample spilled), the results report will include a discussion of the problem and the client will be consulted.

6.5.2 The problem and associated corrective actions will be documented on a Nonconformance and Corrective Action Report (see EQL SOP 201).

7.0 CALCULATIONS

7.1 The autoanalyzer computes sample results in concentration units of mg NH_3-N/L by converting sample absorbances to concentrations using a regression curve determined from the calibration solutions.

7.2 The auto dilutor is automatically triggered if any samples are above the highest concentration standard used in the calibration. The autodiluter is set up to automatically perform a 10 to 1 dilution. This is done at the end of the run. In the results file, the dilution factor (10) is reported in the column labeled "ADF". The results need to be corrected for the dilution as follows:

Undiluted sample conc. = [Diluted sample conc.] x [Dilution factor]

8.0 WASTE DISPOSAL

Phenol, a reagent used in this analysis, is hazardous. Any analysis waste solutions containing phenol, or any other hazardous substances, must be segregated and stored in the hazardous waste accumulation area in room 303. Waste is periodically disposed of by shipment to a hazardous waste disposal contractor by Coastal Carolina University's Science Lab Manager.

Dispose of analyzed samples, used standards, and used reagents not containing phenol or any other hazardous substances down a sink sewage drain. Flush the sink with a large volume of tap water.

9.0 EQUIPMENT MAINTENANCE

9.1 Check pump tubes for wear and replace as needed.
9.2 Replace o-rings every 6 months.
9.3 Replace flow cell flares and O-rings once a year.
9.4 Clean surfaces of the manifolds, dilutor, and autosampler every day they are used.
9.5 Check for leaks during every run.
9.6 Replace all manifold tubing every year.
9.7 Clean pump tube adapters, ports and valve connectors monthly.
9.8 Check the cadmium column to see that air has not gotten into the column. If some air does get into the column, run the nitrogen reagents and carrier DIW through the column for about 15 minutes.

Appendix H

CCU EQL SOP NO.: 435

TOTAL SUSPENDED SOLIDS

Total Suspended Solids Dried at 103-105ºC ***Reference Method: SM 2540 D. (1997 online)***

Approved by: _____
Laboratory Director

Reviewed by: _____
Laboratory Technician

1.0 SCOPE/APPLICATION

This standard operating procedure describes the procedures used by staff of the Environmental Quality Lab (EQL) to determine total suspended solids (TSS) in natural waters. The primary goal of TSS measurement is to provide an estimate of the suspended particles present in a water sample.

TSS is measured gravimetrically as follows: a well-mixed sample is filtered through a weighed standard glass-fiber filter. The residue retained on the filter is dried to a constant weight at 103 to 105°C. The increase in weight of the filter represents the mass of suspended solids. To obtain a concentration in mg per liter of sample, the mass is divided by the volume of filtered sample.

Large floating particles or submerged agglomerates of nonhomogeneous materials should be excluded from the sample if it is determined that their inclusion is not desired in the final result. Because excessive residue on the filter may form a water-entrapping crust, limit the sample size to that yielding no more than 200 mg residue. For samples high in dissolved solids, thoroughly wash the filter to ensure removal of dissolved material. Prolonged filtration times resulting from filter clogging may produce high results owing to increased colloidal materials captured on the clogged filter.

2.0 REFERENCES

2.1 Standard Method 2540 D. (1997 online) Total Suspended Solids Dried at 103-105°C

3.0 DEFINITIONS

Total Suspended Solids: Total suspended solids (TSS) in this standard operating procedure document is referring to the total mass of solids retained by a glass-fiber filter which remains after rinsing with deionized water to remove dissolved solids and then drying at 103-105°C to remove water. The latter is meant to evaporate water and not lead to the volatilization of residues. Likewise, the deionized water rinses are not meant to solubilize particles previously retained by the filter.

4.0 SAFETY

4.1 This method is restricted to use by or under the supervision of trained analysts.
4.2 Safety glasses and protective clothing should be worn to protect against unnecessary exposure to water samples which are potentially contaminated with infectious agents (i.e., pathogens) and hazardous chemicals.
4.3 All activities performed in the laboratory while following this procedure should utilize appropriate laboratory safety systems (e.g., disinfectant, fume hoods, material safety data sheets).

5.0 SAMPLE COLLECTION, HANDLING, AND PRESERVATION

5.1 Samples should be refrigerated at ≤ 6°C immediately upon collection and in the laboratory until analysis.

5.2 Filtration must take place within 7 days of sample collection.

6.0 METHOD

6.1 APPARATUS AND MATERIALS

6.1.1 Drying oven, for operation at 103 to 105 ºC

6.1.2 Analytical balance capable of weighing to 0.1 mg.

6.1.3 Desiccator: provide with a desiccant containing a color indicator of moisture content.

6.1.4 Glass filter funnel and filter stand with fritted disk for filter support.

6.1.5 1- liter Erlenmeyer filter flask for the filter stand and funnel.

6.1.6 500-ml Erlenmeyer filter flask to act as a trap for the mechanical pump.

6.1.7 Rubber vacuum hoses, three-way stopcock, and quick disconnect adapters.

6.1.8 Glass fiber filter disks: Whatman grade 934 AH 47 mm diameter or equivalent

Commercially prepared and preweighed filters for TSS analysis are available and acceptable.

6.1.9 Aluminum weighing dishes (3" diameter) with an Awl ID# (engraved with an awl).

To clean, wipe with a kimwipe dampened with deionized water.

6.1.10 Flat-blade forceps. Clean by rinsing with deionized water and wiping with a kimwipe.

6.1.11 Mechanical vacuum pump.

6.1.12 Certified balance weights (200 mg to 100 g).

6.2 REAGENTS

6.2.1 Deionized water

6.3 PROCEDURES

6.3.1 Filter Preparation

(1) Using the forceps, place a rinsed (three times with 20 mL deionized water), dried (103 - 105ºC), ignited (15 minutes at 550ºC), and weighed 47-mm Whatman GFF 934-AH filter disk with wrinkled side up (grid side down) into filtration apparatus.

(2) Apply vacuum (150 mm Hg) and wash the filter with three successive 20 mL volumes of deionized water. Continue suction to remove all traces of water.

Discard washings.

(3) Using the forceps, remove the filter from the filtration apparatus and transfer to a clean labeled aluminum weighing dish being careful not to tear the filter.

(4) Dry in an oven at 103 to 105ºC for at least 2 hours.

(5) If volatile solids are also to be measured, ignite at 550 ºC for 15 minutes in a muffle furnace. Cool in desiccator 2 hours or more to room/balance temperature and weigh filter only to 0.000X g.

(6) Repeat the cycle of drying, cooling, igniting, desiccating and weighing until a constant weight is obtained or until the weight change is less than 0.5 mg. If filter weights are not within 0.5 mg, the repeat the cycle of drying, cooling, igniting, desiccating and weighing until the last two weighings agree within 0.5 mg.

(7) Record the weights on the **Data Recording Worksheet for Filter Preparation for TSS/VSS**. Also record the analyst name, Awl ID, date and time of each weighing, the filter type, lot, and filter batch. Compute the weight change in mg between repetitive weighings.

(8) Enter all data into the **Total Suspended Solids and Volatile Suspended Solids Worksheet: Filter Prep** to obtain a computation of weight change, % weight change, and determination that a constant weight has been met. Follow the instructions in the template for saving and file naming.

(9) Store the prepared filters in a desiccator until needed.

6.3.2 Selection of sample volume

Select a sample volume that will yield about 2.5 to 100 mg of dried solids and not to exceed 200 mg. For environmental samples, this usually ranges in volume from 200 to 1000 mL. This is sufficient to provide a dark visible residue on the filter. It is important to filter smaller volumes (20mL) if the sample has a high sediment load as a large amount (100mL) poured in all at once may clog the filter before it can be pulled by the vacuum through the filter and make it difficult to determine the actual volume of analyte that has passed through the filter paper.

6.3.3 Sample analysis

(1) Place the filtration stand on the filter flask. Wet the frit with a small amount of deionized water. Use forceps to place a prepared filter wrinkled side up onto the filter stand. Place the filter cup onto the filter stand with filter in place and clamp together.

(2) Thoroughly shake the sample bottle. Then pour an aliquot of the desired volume of the homogenized sample into a 100 mL or smaller graduated cylinder. It is also permissible place a magnetic sir bar in the sample bottle and place bottle on magnetic stirrer to stir the sample with a magnetic stirrer and while stirring, pipet a measured volume onto the seated glass fiber filter.

(3) Pour the sample into the filtration cup. Try to pour directly onto the filter to reduce the retention of solids on the walls of the filtration cup.

(4) Apply vacuum (150 mm Hg).

(5) Rinse the graduated cylinder with deionized water (about 10 mL).

(6) After the filter cup has emptied, pour the rinse water from the graduated cylinder into the filter cup taking care to wash any residue from the side of the filter funnel. It is also useful to use a squirt with DI water to wet down the inside of the filtering funnel to make sure all the solids reach the filter.

(6) Wash with two additional 10 mL volumes of reagent grade water (**NOTE:** Samples with dissolved solids (e.g., seawater) may require additional washings.). Allow complete drainage between washings and continue suction for about 3 minutes after filtration is complete. This serves to quantitatively transfer any sediments retained in the 100mL graduated cylinder to the filtering funnel.

(7) Using the forceps, carefully remove the filter from filtration apparatus and transfer it back into its labeled aluminum-weighing dish. If analyzing multiple samples, wash the forceps, 100mL graduated cylinder and filter cup with DI water between each of the samples to avoid contamination. Also, when filtering multiple samples, pay attention to the filtrate level in the filtering flask to avoid drawing water into the vacuum trap between the filtering flask and the pump. Empty the filtrate as needed.

(8) Dry the sediment laden filter for at least 1 h at 103 to 105^{o}C in an oven, and then cool in desiccator to room/balance temperature.

(9) Perform a balance check with a 200 mg certified weight. Record this in the **TSS Data Recording Worksheet**.

(10) Weigh the dried and cooled filter to 0.000X g.

(11) Repeat the cycle of drying, cooling, desiccating, and weighing until a constant weight is obtained or until the weight change is less than 4% of the previous weight or 0.5 mg, whichever is less. If filter weights are not within 0.5 mg, then repeat the cycle of drying, cooling, igniting, desiccating and weighing until the last two weighings agree within 0.5 mg.

(12) In the **TSS Data Recording Worksheet, r**ecord the sample ID, Dish ID (if commercial preweighed filter used), Awl ID, filter weight, the volume of sample filtered, and the repetitive weights of the filter + residue. Also record the analyst name, analysis batch, the date and time of each weighing. Compute the weight change in mg between repetitive weighings.

(13) Enter all data into the **Total Suspended Solids and Volatile Suspended Solids Worksheet: TSS** to obtain a computation of weight change, % weight change and determination that a constant weight has been met. The weight of the residue will also be computed along with the TSS concentration (mg/L). Follow the instructions in the template for saving and file naming.

(14) If volatile solids are to be determined, use the forceps to return the filter + residue to the aluminum dish and treat according to the SOP for Volatile Suspended solids (VSS).

6.4 CALCULATIONS

$$\text{mg total suspend solids/L} = \frac{(A - B) \times 1000}{C}$$

Where:

A = weight of filter + dried residue, mg
B = weight of filter, mg
C = sample volume, mL

6.5 QUALITY CONTROL

6.5.1 Quality control (QC) measures for analyzing samples are summarized in Table 1 and are as follows:

- Prior to analyzing samples, each analyst must demonstrate the ability to generate acceptable results (i.e., demonstration of capability).

- Any replicate sample analyses should be subjected to exactly the same analytical procedures as those used on individual sample analyses.

- Unless otherwise specified for specific project or samples, each batch of up to10 samples analyzed should include a method blank (i.e., deionized water in a sample bottle that accompanied samples and received same processing),,and at least one sample analyzed in duplicate (i.e., at least 10% duplicates) as QC samples.

TABLE 1. SUMMARY OF QC REQUIREMENTS FOR TSS

QC Sample or Activity	Minimum Frequency	Acceptance Criteria	Corrective Action
Capability demonstration	Four (4) prepared samples analyzed prior to any customer sample analyses	90 – 110% R < 10% RSD	Repeat until acceptable
Balance Calibration Check	Prior to weighing any sample filters	Weight of certified 200 mg weight: 0.1998 – 0.2002 g	Investigate problem including cleaning weight and balance. If balance is out of calibration attempt recalibration or use another balance until obtain acceptable calibration check.
Method Blank	At least one (1) per analysis batch of up to 10 samples	For 1.0 L blank filtered: < 1.0 mg/L	Investigate, identify, and correct the problem. If system accuracy is in control, qualify results. If system accuracy is out of control, correct problem before analyzing samples
Sample analysis	For all sample analyses	Total residue on filter: ≥2.5 mg to ≤ 200 mg	If total residue on filter < 2.5 mg report result as < RL If total residue on filter > 200 mg filter a smaller volume of sample.
Laboratory Control Sample	At least one (1) per year	90 – 110% R	Investigate, identify, and correct problem. If system accuracy is in control, qualify results. If system accuracy is out of control, correct problem before analyzing samples.
Sample duplicate	One (1) per preparation batch of up to 10 samples	RPD ≤ 5%	Investigate problem. If system precision is in control, qualify results. If system precision is out of control, reanalyze entire batch.
Internal PE sample	Samples and frequency determined by Lab QA Officer	Criteria for LCS recovery and duplicate precision	Investigate all unacceptable results.
Blind PE sample	Samples and frequency determined by accrediting agencies and projects	Determined by PE provider	Investigate all unacceptable results.

LCS = laboratory control sample
MB = method blank
MDL = method detection limit
MS = matrix spike
PE = performance evaluation
QC = quality control
%R = percent recovery
RL = reporting limit where RL = (2.5 mg /mL filtered) x 1000 mL
RPD = relative percent difference
RSD = relative standard deviation

6.6 CORRECTIVE ACTIONS

6.6.1 If any of the QC requirements listed in Section 6.5 are not satisfied, the analyst must consult with the Laboratory Director. Normally the activity must be repeated, after corrective actions are taken to correct any obvious problems, until the QC results are acceptable. If repeating the process is not possible, the results report will include a discussion of the problem and the client will be consulted.

6.6.2 The problem and associated corrective actions will be documented on a Nonconformance and Corrective Action Report (see EQL SOP 201).

6.7 EQUIPMENT MAINTENANCE

6.7.1 Balance Calibration Checks

- Balances are certified annually by a commercial provider
- Monthly calibration checks are performed by EQL personnel using EQL SOP 203.

7.0 WASTE DISPOSAL

7.1 Dispose of analyzed samples and used standards by discharging into a laboratory sink drain. Flush the sink with a large volume of tap water.

7.2 Discard filters in regular trash.

Appendix I

CCU EQL SOP NO.: 502

FECAL COLIFORM MEASUREMENT BY

DIRECT TEST (A-1 MEDIUM)

Fecal Coliform Measurement by Direct Test (A-1 Medium)

Reference Methods: SM 9221 C. (1999 online) SM 9221 E.2. (1999 online)

Approved by: _____
Laboratory Director

Reviewed by: _____
Laboratory Master Technician

1.0 SCOPE/APPLICATION

This procedure describes an elevated-temperature test for distinguishing bacteria of the total coliform group that also belong to the fecal coliform group. The test uses A-1 medium and is a single-step method. Using A-1 broth the test can directly isolate fecal coliforms from water without prior enrichment in a presumptive medium.

The test using A-1 medium is applicable to source water, seawater, and treated wastewater.

2.0 REFERENCES

2.1 Standard Method 9221 C. (1999 online)
2.2 Standard Method 9221 E.2. (1999 online)

3.0 DEFINITIONS

3.1 *Bacterial Indicators:* Bacterial measures commonly used to estimate the amount of sewage in contaminated water. Although these bacteria themselves are not pathogenic, they are used to "indicate" the presence of human pathogens. Current federal and state recreational water quality standards include or recommend at least one of the following bacterial indicators: total coliforms, fecal coliforms, enterococci, and *E. coli*.

3.2 *Colony Forming Unit (CFU):* A visible mass of organisms developing from one or a group of bacteria. The mass results from the bacteria reproducing themselves.

3.3 *E. coli:* A species of bacteria that occurs in the intestine of warm-blooded animals. It is part of the fecal coliform group.

3.4 *Fecal Coliforms:* A group of bacteria found in the feces of various warm-blooded animals. It is a subgroup of the total coliform group and has been used as a more definitive indicator for fecal pollution.

3.5 *Most Probable Number:* When multiple tubes are used in the fermentation technique, results of the examination of replicate tubes and dilutions are reported in terms of the Most Probable Number (MPN) of organisms present. This number, based on certain probability formulas, is an estimate of the mean density of coliforms in the sample.

3.6 *Pathogens:* Organisms that cause disease.

3.7 *Total Coliforms:* A large group of bacteria that can originate from soil, plants, human waste, and animal waste. Commonly used as a bacterial indicator.

4.0 SAFETY

4.1 This method is restricted to use by or under the supervision of trained analysts.

4.2 When using the autoclave or Bunsen burner, be careful to avoid touching hot surfaces and to not expose yourself to steam or flames.

4.3 Gloves, safety glasses with side shields, and protective clothing should be worn to protect against unnecessary exposure to infectious agents (i.e., pathogens), hazardous chemicals, and contaminants in potentially hazardous samples.

4.4 All activities performed while following this procedure should utilize appropriate laboratory safety systems (e.g., disinfectant, fume hoods, material safety data sheets).

5.0 METHOD

5.1 APPARATUS AND MATERIALS

5.1.1 Incubator at 35.0 ± 0.5°C, Fisher Isotemp 600 Series Standard Incubator or equivalent
5.1.2 Refrigerator at ≤ 6°C
5.1.3 Water bath at 44.5 ± 0.2°C with water circulation pump
5.1.4 Autoclave
5.1.5 Bunsen burner
5.1.6 Inoculating loop, nickel-chromium alloy
5.1.7 Sterile plastic, serological pipets, 10 mL size graduated in 1/10 mL (Fisher Cat. No. 13-678-11 E or equivalent)
5.1.8 Sterile plastic, serological pipets, 1.0 mL size graduated in 1/100 mL (Fisher Cat No. 13-678-11 B or equivalent)
5.1.9 Glass pipets, volumetric, assorted sizes
5.1.10 Pipet bulbs
5.1.11 pH electrode, Orion or equivalent
5.1.12 pH meter, Fisher Accumet AR25 or equivalent
5.1.13 Glass bottles for dilution water, 300 mL
5.1.14 Glass culture tubes, 10x75 mm, for inversion (Fisher Cat. No. 14-961-25 or equivalent)
5.1.15 Glass culture tubes, 25 mL, with rubber lined, screw close caps (Fisher Cat. No. 14-959-25 E or equivalent)
5.1.16 Thermometers for controlled temperature equipment

5.2 REAGENTS

5.2.1 A-1 dehydrated medium (BD Difco ™ or equivalent)

5.2.2 Tryptic soy broth dehydrated medium (BD Bacto ™ or equivalent)
5.2.3 Buffered dilution water
5.2.4 Bacteria control cultures (MicroBiologics® or equivalent)
Escherchi coli, ATCC 25922
Enterobacter aerogenes, ATCC 13048
Other acceptable bacteria depending on availability those currently used
5.2.5 pH buffer solutions, 4.00, 7.00, 10.00
5.2.6 Electrode storage solution
5.2.7 Electrode filling solution
5.2.8 Sodium hydroxide
5.2.9 Hydrochloric acid

5.3 REAGENT PREPARATION

5.3.1 A-1 Broth

- This medium may be used for the direct isolation of fecal coliforms from water. Prior enrichment in a presumptive medium is not required.

- Prepare from dehydrated media according instructions on bottle. Heat if necessary to dissolve media.

- Make A-1 broth of such strength that adding the sample aliquot will not significantly reduce the medium concentration below that of the original medium. For 10 mL sample aliquots prepare double-strength medium.

- Suspend 31.5 grams in 1 liter of deionized water and boil, if necessary, to dissolve completely. For preparing double strength medium suspend 63.0 grams in 1 liter. Other volumes of medium can be prepared as long as these proportions of medium and water are used.

- Measure pH and adjust pH, using dilute NaOH solution if necessary, so that the final pH after sterilization will be 6.9 ± 0.1 at 25ºC. Experience in preparing each media will determine the need to adjust pH, especially for compensating for pH changes during sterilization. pH usually drops slightly during sterilization (up to ~0.1), so adjust pH to close to 7.0 before sterilization.

- Dispense 10 mL into 20-30 mL screw culture tubes then insert inverted fermentation vials. Do not insert inverted vials into two of the culture tubes with medium (These tubes will be used for final pH measurements).

- Sterilize the media in the autoclave. The total time of the sterilization cycle cannot exceed 40 minutes and must include exposure at 121-124ºC for 10 minutes. Promptly remove sterilized medium from the autoclave when sterilization is complete.

- Measure final pH in the tubes without inverted vials. Final pH must be 6.9 ± 0.1 at 25ºC. Discard entire batch of medium if pH is not within limits. Record pH in Media Preparation Log, Form 907.

- Store medium in refrigerator at ≤ 6°C in tightly closed screw-cap containers for no more than 7 days. Ignore formation of precipitate.

5.3.2 Tryptic Soy Broth Medium

- This medium is used as a general growth medium for bacteria.
- Prepare from dehydrated media according instructions on bottle. Heat if necessary to dissolve media.
- Make tryptic soy broth (TSB) of such strength that adding the sample aliquot will not significantly reduce the medium concentration below that of the original medium. For 10-50 mL sample aliquots prepare double-strength medium.
- Suspend 30.0 grams in 1 liter of deionized water and warm slightly, if necessary, to dissolve completely. For preparing double strength medium suspend 60.0 grams in 1 liter. Other volumes of medium can be prepared as long as these proportions of medium and water are used.
- Measure pH and adjust pH, using dilute NaOH or HCl if necessary, so that the final pH after sterilization will be 7.3 ± 0.2 at 25ºC. Experience in preparing TSB medium will determine the need to adjust pH, especially for compensating for pH changes during sterilization. pH usually drops slightly during sterilization (up to ~0.1), so adjust pH to close to 7.4 before sterilization.
- Dispense 10 mL into 20-30 mL culture tubes with screw close caps and 25-50 mL in 100-300 mL glass bottles with screw close caps.
- Sterilize the media in the autoclave. The sterilization cycle must include exposure at 121ºC for 15 minutes. Promptly remove sterilized medium from the autoclave when sterilization cycle is complete.
- Measure final pH using two of the sterilized tubes of TSB. Final pH must be 7.3 ± 0.2 at 25ºC. Discard entire batch of medium if pH is not within limits. Record pH in Media Preparation Log, Form 907.
- Store sterile TSB in refrigerator at ≤ 6°C in tightly closed screw-cap containers for up to 3 months.

5.3.3 Buffered Dilution Water

- Dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 mL of deionized water, adjust to pH 7.2 ± 0.25 with 1 N sodium hydroxide (NaOH) and dilute to 1 liter with deionized water.
- Dissolve 81.2 g of magnesium chloride ($MgCl_2 \bullet 6H_2O$) to 1 liter with deionized water.

- To prepare buffered dilution water: dilute 1.25 mL stock phosphate buffer solution and 5.0 mL magnesium chloride solution in 1 liter deionized water.

- Dispense in amount that will provide 99 ± 2.0 mL in bottles or 9 ± 0.2 mL in tubes after autoclaving for 15 minutes.

- Alternatively purchase sterile buffered dilution water (e.g., Fisher or IDEXX).

- Store in dark at room temperature.

5.4 PROCEDURE

5.4.1 Locate samples from cooler according to the chain of custody and analysis request forms.

5.4.2 Arrange fermentation tubes in three rows of five each for each sample tested. The first row should contain double strength A-1 broth and the next two rows should contain regular strength A-1 broth. At least one tube of each set of tubes for a sample should be labeled by sample number and dilution on the sample tube with a permanent marker (usually double strength tube for each sample).

5.4.3 Sample analysis should begin immediately, preferably within 2 hours of collection. The maximum transport time to the laboratory is within 6 hours of sample collection. Samples should be processed within 2 hours of receipt at the laboratory. Immediately prior to sample analysis shake sample vigorously 25 times before inoculating tubes.

5.4.4 Dispense with sterile 10 ml pipet, 10 ml of sample in each tube of the first row (double strength A-1).

5.4.5 Dispense with sterile 1.0 ml pipet, 1.0 ml of sample in each tube of the second row.

5.4.6 Dispense with sterile 1.0 ml pipet, 0.1 ml of sample per tube in the third row.

5.4.7 If additional dilutions are required due to anticipated large bacterial concentration, additional rows containing 0.01 sample per tube and 0.001 ml sample per tube can also be inoculated. The following guidelines are for using volumes less than 0.1 mL of sample to inoculate tubes:

- For a desired sample volume of 0.01 or 0.001 mL add 1.0 mL of sample to 99 mL of sterile, buffered dilution water.

- Shake diluted sample vigorously 25 times.

- If 0.01 mL sample is desired, transfer 1 mL of diluted sample.

- If 0.001 mL of sample is desired, transfer 0.1 mL of diluted sample.

5.4.8 Mix the contents of the tubes with gentle agitation.

5.4.9 Incubate inoculated tubes for 3 hours at 35.0 ± 0.5°C in incubator. Transfer tubes to a water bath at 44.5 ± 0.2°C for an additional 21 ± 2 hours.

5.4.10 Remove tubes from the water bath.

5.4.11 Gas production (i.e., gas accumulation in top of inverted vial in tube) in any A-1 broth culture tube within 24 hours or less is a positive reaction indicating the presence of fecal coliforms. Record analysis date and analysis data in Fecal (A-1) Run Log, Form 909.

5.5 QUALITY CONTROL

5.5.1 Quality control (QC) measures for analyzing samples are as follows:

- Prior to analyzing samples, each analyst must demonstrate the ability to generate acceptable results (i.e., demonstration of capability).
- Sample analysis should begin immediately, preferably within 2 hours of collection. The maximum transport time to the laboratory is within 6 hours of sample collection. Samples should be processed within 2 hours of receipt at the laboratory.
- Any replicate sample analyses should be subjected to exactly the same analytical procedures as those used on individual sample analyses.
- Unless otherwise specified for specific project or samples, each batch of up to 20 samples analyzed should include at least one sample analyzed in duplicate (i.e., at least 5% duplicates) as QC samples.

5.5.2 Sterility of sample bottles must be assured prior their use. Bottles are cleaned and tested for sterility as described in QAM Section 3.4.2 and EQL SOP 301. Bottles must pass the sterility test, and test results are recorded on the Sterility Check Log, Form 910.

5.5.3 Sterility of blank dilution water in glass bottles must be assured prior their use. Bottles are cleaned following the same procedures as for sample bottles (see EQL SOP 301 and Microbiology QAM Section 3.4.2). Bottles containing blank dilution water are prepared and tested for sterility as described in this SOPs Section 5.3.2 and Microbiology QAM Section 3.4.4. Bottles must pass the sterility test, and test results are recorded on the Sterility Check Log, Form 910.

5.5.4 Effectiveness of the autoclave for sterilization is tested monthly as described in EQL SOP 205. The autoclave must pass the sterility test, and the test results are recorded on Autoclave Sterility Check Log, Form 905.

5.5.5 All glassware that comes in contact with the sample must be sterile, and a sterilization record must be kept on the Sterility Check Log, Form 910, unless the items are purchased presterilized.

5.5.6 QC requirements for controlled temperature equipment are provided in EQL SOP 204, "Controlled Temperature Equipment". In summary:

- Incubator temperatures must be checked to the nearest 0.1 ºC and documented at least twice daily when in use, with at least a four-hour separation between measurements.
- Refrigerator/freezer temperatures must be checked to the nearest 1ºC and documented at least twice daily when in use. Readings should be at least 4 hours apart.
- Thermometers used to determine temperatures must be checked against an NIST or NIST-traceable thermometer at least annually, and for the temperature range used the thermometers must differ from the expected temperature by <1.0 °C and must be tagged with the temperature correction factor.
- Temperatures will be recorded on the Temperature Monitoring Log, Form 901, attached to each device.

5.5.7 QC requirements for TSB medium are as follows:

- TSB medium (purchased from MicroBiologics® or equivalent) must be stored in the dark at 4-30°C.

- Sterility check:
  - Each batch of TSB medium prepared must be checked and be acceptable for sterility before use.
  - Place one tube from the batch into the 35°C incubator and incubate for 24-48 hours.
  - Acceptable result is no growth (i.e., medium clear after incubation).
  - **Discard the entire medium batch if growth is detected.**
  - Record the results of the sterility test in the Sterility Check Log, Form 910.

- Positive control checks:
  - Each batch of TSB medium prepared must be checked before use for the ability to promote growth of bacteria for the test(s) to be performed. Appropriate bacteria used are (appropriate others can also be used if those currently used are not available):

Escherchi coli	ATCC # 25922
Enterobacter aerogenes	ATCC # 13048

  - Bacteria cultures are prepared by swabbing MicroBiologics® or equivalent in 5-10 mL TSB and then incubating for 24 hours at 35ºC. Clouding or precipitation of the medium after incubation indicates growth of the bacteria.
  - Acceptable results are growth of all bacteria cultures.
  - **Discard the entire medium batch if growth is not detected.**
  - Record results on Positive/Negative Culture Medium Checks, Form 908.

5.5.8 QC requirements for A-1 medium are as follows:

- A-1 medium (purchased from (BD Difco™ or equivalent) must be stored in the dark at 4-30°C.
- Sterility check:
  - Each batch of TSB medium prepared must be checked and be acceptable for sterility before use.
  - Place one tube from the batch into the 35°C incubator and incubate for 24-48 hours.
  - Acceptable result is no growth (i.e., medium clear after incubation).
  - **Discard the entire medium batch if growth is detected.**
  - Record the results of the sterility test in the Sterility Check Log, Form 910.

- Positive and negative control checks:
  - Each batch of A-1 medium prepared must be checked before use with a positive and negative bacteria culture. Preferred QC and currently available bacteria are (appropriate others can also be used if those currently used are not available):

Positive:	*Escherchi coli*	ATCC # 25922
Negative:	*Enterobacter aerogenes*	ATCC # 13048

  - Bacteria cultures are prepared by by swabbing MicroBiologics® or equivalent in 5-10 ml trypticase soy broth and then incubating for 24 hours at 35ºC. If the broth had been stored refrigerated, allow it to incubate overnight at room temperature, then only use it if no growth is observed (i.e., it is clear after overnight in the incubator).
  - Clouding or precipitation of the medium with culture pill after incubation indicates growth of the bacteria.
  - Separate culture tubes of A-1 medium are inoculated with a culture of each bacteria using a 3mm inoculating loop. The loop must be flame sterilized until it glows with a Bunsen burner, then air cooled prior to submersing in the culture-containing broth.
  - Submerge the loop into the tube with A-1 medium to inoculate it, then resterilize the inoculating loop.
  - Process the inoculated A-1 medium like a normal sample (i.e., SOP steps 5.4.8 - 5.4.11).
  - For A-1 medium gas formation in tubes indicates growth. Presence of growth for the positive control culture is satisfactory as a QC check. Absence of growth is a satisfactory check for the negative control culture. In summary:

Culture	Growth Seen	QC Passes?
Positive	Yes	Yes
Negative	No	Yes
Positive	No	No
Negative	Yes	No

For A-1 medium record the positive and negative control tests in the Fecal Coliform (A-1) Run Log, Form 909. Also record test results in Media Preparation Log, Form 907. In lieu of the sample number write "E. coli" for the positive and "Enterobacter aerogenes or equivalent" for the negative control check. The lot number of the media is recorded in the run log form as well. This identifies the control culture check for that particular batch of media.

5.5.9 Storage of Dehydrated Culture Media

Store dehydrated media in tightly closed bottle at less than 30ºC. Do not use them if they are discolored or become caked and lose the character of a free flowing powder. Purchase media in as small as quantities as possible so that they will be used up preferably within 6 months. Dehydrated media may be used until the manufacturer's expiration date. If possible the dehydrated media should be stored in a desiccator.

5.6 CORRECTIVE ACTIONS

5.6.1 If any of the QC requirements listed in Section 5.5 are not satisfied, the analyst must consult with the Laboratory Director. Normally the activity must be repeated, after corrective actions are taken to correct any obvious problems, until the QC results are acceptable. If repeating the process is not possible (e.g., sample spilled), the results report will include a discussion of the problem and the client will be consulted.

5.6.2 The problem and associated corrective actions will be documented on a Nonconformance and Corrective Action Report (see EQL SOP 201).

6.0 CALCULATIONS

To calculate coliform density, compute in terms of the Most Probable Number (MPN). The MPN values for a variety of dilution series and results are given in Tables 1, 2, and 3 of this section. Included in these tables are the 95% confidence limits for each MPN value determined. If the sample used are those found in the table, report the value corresponding to the number of positive and negative results in the dilution series as the MPN/ 100 mL or report as fecal coliform presence or absence.

The sample volumes indicated in Tables 1 and 2 relate to finished waters. Table 3 illustrates value for combination of positive and negative results when five 10 mL, five 1.0 mL and five 0.1 mL volumes of sample are tested. When the series of decimal dilution is different from that in the table (i.e., series differ by factor of 10 but largest volume is not 10 mL), select the MPN value from Table 3 and for the combination of positive tubes and then calculate according to the following formula:

$$\text{Actual MPN/100 ml} = \text{MPN value (from table)} \times \frac{10}{\text{largest volume tested in dilution series used for MPN determination}}$$

For example if sample volumes of five 1.0 mL, five 0.1 mL and five 0.01 mL the actual MPN would be calculated by multiplying the MPN value from Table 3 by 10.

When more than three dilutions are used in a decimal series of dilution use the results from only three of them in computing the MPN. To select the three dilutions to be used in determining the MPN index, choose the highest dilution that gives positive results in all five portions tested (no lower dilution giving any negative results) and the two next succeeding higher dilutions. For example, if all five tubes with 10 mL sample were positive, three tubes with 1 mL sample were positive and 0 tubes with 1 mL sample were positive the MPN would be calculated to be 79 CFU/100mL using Table 3.

In the case such that a positive occurs in a dilution higher than the three chosen according to the rule, incorporate it in the result for the highest chosen dilution. For example if dilutions of 10, 1.0, 0.1 and 0.01 are used and five positives are found in the 10ml tubes, 3 positives are found in the 1.0 mL tubes and 1 positive is found in the 0.1 and 0.01 mL tubes each. The MPN is calculated as if 5 positives were found in the 10 mL tubes, 3 positives in the 1.0 mL tubes and 2 positives in the 0.1 ml tubes and none in the 0.01 ml tubes. The MPN calculation would be 140 CFU/100mL.

When it is desired to summarize with a single MPN value the results from a series of samples, use the geometric mean or the median.

The MPN for a combination not appearing in the table or for other combination of tubes or dilutions, may be estimated using Thomas' simple formula:

$$\text{MPN/100 mL} = \frac{\text{Number of positive tubes} \times 100}{\sqrt{(\text{mL sample in negative tubes}) \times (\text{mL sample in all tubes})}}$$

Table 1

MPN Index and 95% Confidence limits for various combinations of positive and negative results when five 20 mL portions are used.

Number of Tubes Giving Positive Reaction Out of 5 of 20 mL Each	MPN Index/ 100 mL	95 % Confidence Limits	
		Lower	Upper
0	<1.1	0	3.5
1	1.1	0.051	5.4
2	2.6	0.40	8.4
3	4.6	1.0	13
4	8.0	2.1	23
5	>8.0	3.4	Infinite

Table 2

MPN Index and 95% Confidence limits for various combinations of positive and negative results when ten 10 mL portions are used.

Number of Tubes Giving Positive Reaction Out of 10 of 10 mL Each	MPN Index/ 100 mL	95 % Confidence Limits	
		Lower	Upper
0	<1.1	0	3.4
1	1.1	0.051	5.9
2	2.2	0.37	8.2
3	3.6	0.91	9.7
4	5.1	1.6	13
5	6.9	2.5	15
6	9.2	3.3	19
7	12	4.8	24
8	16	5.8	34
9	23	8.1	53
10	>23	13	Infinite

Table 3

MPN Index and 95% Confidence limits for various combinations of positive and negative results when five tubes are used per dilution (10mL, 1.0mL and 0. 1mL).

Combination of Positives	MPN Index/ 100mL	95% Confidence Limits		Combination of Positives	MPN Index/ 100mL	95% Confidence Limits	
		Lower	Upper			Lower	Upper
0-0-0	<1.8	—	6.8	4-0-3	25	9.8	70
0-0-1	1.8	0.090	6.8	4-1-0	17	6.0	40
0-1-0	1.8	0.090	6.9	4-1-1	21	6.8	42
0-1-1	3.6	0.70	10	4-1-2	26	9.8	70
0-2-0	3.7	0.70	10	4-1-3	31	10	70
0-2-1	5.5	1.8	15	4-2-0	22	6.8	50
0-3-0	5.6	1.8	15	4-2-1	26	9.8	70
1-0-0	2.0	0.10	10	4-2-2	32	10	70
1-0-1	4.0	0.70	10	4-2-3	38	14	100
1-0-2	6.0	1.8	15	4-3-0	27	9.9	70
1-1-0	4.0	0.71	12	4-3-1	33	10	70
1-1-1	6.1	1.8	15	4-3-2	39	14	100
1-1-2	8.1	3.4	22	4-4-0	34	14	100
1-2-0	6.1	1.8	15	4-4-1	40	14	100
1-2-1	8.2	3.4	22	4-4-2	47	15	120
1-3-0	8.3	3.4	22	4-5-0	41	14	100
1-3-1	10	3.5	22	4-5-1	48	15	120
1-4-0	10	3.5	22	5-0-0	23	6.8	70
2-0-0	4.5	0.79	15	5-0-1	31	10	70
2-0-1	6.8	1.8	15	5-0-2	43	14	100
2-0-2	9.1	3.4	22	5-0-3	58	22	150
2-1-0	6.8	1.8	17	5-1-0	33	10	100
2-1-1	9.2	3.4	22	5-1-1	46	14	120
2-1-2	12	4.1	26	5-1-2	63	22	150
2-2-0	9.3	3.4	22	5-1-3	84	34	220
2-2-1	12	4.1	26	5-2-0	49	15	150
2-2-2	14	5.9	36	5-2-1	70	22	170
2-3-0	12	4.1	26	5-2-2	94	34	230
2-3-1	14	5.9	36	5-2-3	120	36	250
2-4-0	15	5.9	36	5-2-4	150	58	400
3-0-0	7.8	2.1	22	5-3-0	79	22	220
3-0-1	11	3.5	23	5-3-1	110	34	250
3-0-2	13	5.6	35	5-3-2	140	52	400
3-1-0	11	3.5	26	5-3-3	170	70	400
3-1-1	14	5.6	36	5-3-4	210	70	400
3-1-2	17	6.0	36	5-4-0	130	36	400
3-2-0	14	5.7	36	5-4-1	170	58	400
3-2-1	17	6.8	40	5-4-2	220	70	440
3-2-2	20	6.8	40	5-4-3	280	100	710
3-3-0	17	6.8	40	5-4-4	350	100	710
3-3-1	21	6.8	40	5-4-5	430	150	1100

3-3-2	24	9.8	70	5-5-0	240	70	710
3-4-0	21	6.8	40	5-5-1	350	100	1100
3-4-1	24	9.8	70	5-5-2	540	150	1700
3-5-0	25	9.8	70	5-5-3	920	220	2600
4-0-0	13	4.1	35	5-5-4	1600	400	4600
4-0-1	17	5.9	36	5-5-5	>1600	700	—
4-0-2	21	6.8	40				

7.0 WASTE DISPOSAL

All incubated samples, used media, and control cultures must be sterilized by autoclaving for 30 minutes at 121°C. Place test racks with used media tubes into polypropylene tubs and autoclave for 30 minutes at 121°C. Remove tubs from autoclave. Dispose of autoclaved media down the sink's sewage drain. Rinse tubes and all other containers in the sink with tap water. Flush sink with lots of water. Discard broken glass in a glass waste container.

Appendix J

CCU EQL SOP NO.: 501

ENTEROCOCCI MEASUREMENT BY

IDEXX ENTEROLERT™-QUANTI-TRAY™ METHOD

Enterococci Measurement by IDEXX Enterolert™-Quanti-Tray™ Method ***Reference Method: Enterolert***

Approved by: _____
Laboratory Director

Reviewed by: _____
Laboratory Master Technician

1.0 SCOPE/APPLICATION

This procedure is for the detection and quantification of enterococci bacteria such as *E. faecium and E. faecalis* in fresh and marine water.

This test method is based on IDEXX Defined Substrate Technology® (DST™) and utilizes a nutrient indicator that fluoresces when metabolized by enterococci. When the reagent is added to the sample and incubated, bacteria down to one CFU in a 100 mL sample can be detected within 24 hours.

2.0 REFERENCES

2.1 IDEXX Enterolert™ Test Kit instructions (IDEXX 06-02150-07)

3.0 DEFINITIONS

3.1 *Bacterial Indicators:* Bacterial measures commonly used to estimate the amount of sewage in contaminated water. Although these bacteria themselves are not pathogenic, they are used to "indicate" the presence of human pathogens. Current federal and state recreational water quality standards include or recommend at least one of the following bacterial indicators: total coliforms, fecal coliforms, enterococci, and *E. coli*.

3.2 *Colony Forming Unit (CFU):* A visible mass of organisms developing from one or a group of bacteria. The mass results from the bacteria reproducing themselves.

3.3 *E. coli:* A species of bacteria that occurs in the intestine of warm-blooded animals. It is part of the fecal coliform group.

3.4 *Enterococci:* A group of bacteria in the genus *Streptococcus* that occurs in the intestine of warm-blooded animals. Commonly used as a bacterial indicator.

3.5 *Fecal Coliforms:* A group of bacteria found in the feces of various warm-blooded animals. It is a subgroup of the total coliform group and has been used as a more definitive indicator for fecal pollution.

3.6 *Most Probable Number:* When multiple well trays are used in the technique, the number of positive wells on a tray is reported in terms of the Most Probable

Number (MPN) of organisms present. This number, based on certain probability formulas, is an estimate of the mean density of *Enterococci* in the sample.

3.7 *Pathogens:* Organisms that cause disease.

3.8 *Total Coliforms:* A large group of bacteria that can originate from soil, plants, human waste, and animal waste. Commonly used as a bacterial indicator.

4.0 SAFETY

4.1 This method is restricted to use by or under the supervision of trained analysts.

4.2 When using the autoclave or Bunsen burner, be careful to avoid touching hot surfaces and to not expose yourself to steam or flames.

4.3 Gloves, safety glasses with side shields, and protective clothing should be worn to protect against unnecessary exposure to infectious agents (i.e., pathogens), hazardous chemicals, and contaminants in potentially hazardous samples.

4.4 When using the UV lamp be sure the light is facing away from your eyes.

4.5 All activities performed while following this procedure should utilize appropriate laboratory safety systems (e.g., disinfectant, fume hoods, material safety data sheets).

5.0 METHOD

5.1 APPARATUS AND MATERIALS

5.1.1 Incubator at 35.0 ± 0.5°C, Fisher Isotemp 600 Series Standard Incubator
5.1.2 Incubator at 41.0 ± 0.5°C, Fisher Isotemp 600 Series Standard Incubator
5.1.3 Refrigerator at 1 – 4°C
5.1.4 IDEXX Quanti-Tray™ Sealers
5.1.4.1 Original Model (back-up instrument)
5.1.4.2 Model 2X
5.1.5 IDEXX 51-well Quanti-Trays (catalog # WQT100)
5.1.6 IDEXX 97-well Quanti-Trays (catalog # WQT2K)
5.1.7 Autoclave
5.1.8 Bunsen Burner
5.1.9 Inoculating loop, nickel-chromium alloy
5.1.10 Sterile plastic, serological pipets, 10 mL size graduated in 1/10 mL
5.1.11 Glass pipets, volumetric, assorted sizes
5.1.12 Pipet bulbs
5.1.13 UV lamp, 6 watt, 365 nm wavelength
5.1.14 pH meter, Accumet AR25
5.1.15 Glass bottles for dilution water, 300 mL
5.1.16 Glass culture tubes, 25 mL, with rubber lined, screw close caps
5.1.17 Thermometers for controlled temperature equipment

5.2 REAGENTS

5.2.1 Enterolert for 100 mL water samples (IDEXX catalog # WENT200)
5.2.2 Tryptic soy broth dehydrated medium
5.2.3 Sterile dilution water
5.2.4 Bacteria control cultures (MicroBiologics® or equivalent)
Enterococcus faecalis, ATCC 29212
Escherichia coli (gram -), ATCC 25922
Seratia marcescens (gram -), ATCC 14756
Aerococcus viridans (gram +), ATCC 700406
Staphylococcus aureus (gram +), ATCC 43300
Other acceptable bacteria depending on availability those currently used
5.2.5 pH buffer solutions, 4.00, 7.00, 10.00
5.2.6 Electrode storage solution
5.2.7 Sodium hydroxide
5.2.8 Hydrochloric acid
5.2.9 Food color

5.3 REAGENT PREPARATION

5.3.1 Tryptic soy broth

This medium is used as a general growth medium for bacteria.

- Prepare from dehydrated media according instructions on bottle. Heat if necessary to dissolve media.

- Make tryptic soy broth (TSB) of such strength that adding the sample aliquot will not significantly reduce the medium concentration below that of the original medium. For 10-50 mL sample aliquots prepare double-strength medium.

- Suspend 30.0 grams in 1 liter of deionized water and warm slightly, if necessary, to dissolve completely. For preparing double strength medium suspend 60.0 grams in 1 liter. Other volumes of medium can be prepared as long as these proportions of medium and water are used.

- Measure pH and adjust pH, if necessary, so that the final pH after sterilization will be 7.3 ± 0.2 at 25ºC. Experience in preparing TSB medium will determine the need to adjust pH, especially for compensating for pH changes during sterilization. pH usually drops slightly during sterilization (up to ~0.1), so adjust pH to close to 7.4 before sterilization.

- Dispense 10 mL into 20-30 mL culture tubes with screw close caps and 25-50 mL in 100-300 mL glass bottles with screw close caps.

- Sterilize the media in the autoclave. The sterilization cycle must include exposure at 121ºC for 15 minutes. Promptly remove sterilized medium from the autoclave when sterilization cycle is complete.

- Measure final pH using two of the sterilized tubes of TSB. Final pH must be 7.3 ± 0.2 at 25ºC. Discard entire batch of medium if pH is not within limits. Record pH in Media Preparation Log, Form 907.

- Store sterile TSB at 4°C in tightly closed screw-cap containers for up to 3 months.

5.3.2 Blank dilution water

- Prepare blank dilution by placing 90 mL of fresh deionized water in a clean, glass dilution bottle.

- Loosely screw on the cap to the bottle.

- Record the dilution bottle preparation in the Dilution Bottle Preparation Log, Form 919.

- Sterilize the blank dilution water in bottles in the autoclave for at least 15 minutes at 121°C.

- Allow the bottles to cool to room temperature.

- Each batch of blank dilution water should be checked for sterility by adding to 50 mL of the dilution water, 50 mL of sterile, double strength, non-selective broth (e.g., trypticase soy broth). If the broth had been stored refrigerated, allow it to incubate overnight at room temperature, then only use it if no growth is observed (i.e., it is clear after overnight at room temperature).

- Incubate at 35.0 ± 0.5°C for 24 hours and check for growth. **Discard all dilution water in batch if growth is detected.**

- Record sterility check of dilution water on Sterility Check Log, From 910.

5.4 PROCEDURE

5.4.1 Turn on the Quanti-Tray Sealer; it takes approximately 5 minutes to warm-up at which time the green ready light will come on.

5.4.2 Locate samples from cooler according to the chain of custody and analysis request forms.

5.4.3 Based on observation of the samples, site histories, and analysis experience, segregate the samples into those expected to have low and high enterococci concentrations. Process the samples expected to have low concentrations first to minimize possible carryover contamination between samples.

5.4.4 Sample analysis should begin immediately, preferably within 2 hours of collection. The maximum transport time to the laboratory is within 6

hours of sample collection. Samples should be processed within 2 hours of receipt at the laboratory. Immediately prior to sample analysis shake sample bottle vigorously at least 25 times.

5.4.5 Carefully open a dilution bottle and sample bottle without contaminating either bottle.

5.4.6 A sample dilution of at least 10-fold (one volume of sample plus nine volumes of sterile dilution water) is used for analysis of marine water samples. For samples suspected to have very high bacteria concentrations, higher dilutions may be used (e.g., 5 mL sample added to 95 mL of dilution water for 20-fold dilution). For fresh water samples dilution of the sample is not mandatory.

5.4.7 Using an individually wrapped sterile 10 ml pipet transfer 10 ml of sample to a dilution bottle containing 90 ± 2ml of sterile water or other sample and dilution water combinations to give a final volume of 100 mL. However, no more than 10 mL of marine water can be used.

5.4.8 Carefully separate an Enterolert snap pack from its strip, taking care not to accidentally open the next pack.

5.4.9 Tap the reagent snap pack to ensure that all of the Enterolert powder is in the bottom of the pack.

5.4.10 Open the pack by snapping back the top at the scoreline, being careful not to touch the opening of the pack.

5.4.11 Add the Enterolert reagent to the 100 mL of sample plus dilution water. **Because the Enterolert contains buffer, do not use dilution water that contains buffer.**

5.4.12 Aseptically cap and seal the dilution water bottle.

5.4.13 Shake to completely dissolve reagent.

5.4.14 Select either a 51-well (for suspected low enterococci concentration) or 97-well (for suspected high enterococci concentration) Quanti-Tray™.

5.4.15 Pour the sample and reagent mixture into a Quanti-Tray™ as follows:

- Use one hand to hold a Quanti-Tray upright with the well side facing the palm.
- Squeeze the upper part of Quanti-Tray so that the Quanti-Tray bends towards the palm.
- Open the Quanti-Tray pulling the foil tab away from the well side. Avoid touching the inside of the foil or tray.

- Pour the reagent and sample mixture directly into the Quanti-Tray avoiding contact with the foil tab.
- Allow foam to settle.
- Place the sample-filled Quanti-Tray onto the rubber tray carrier of the Quanti-Tray Sealer with the well side (plastic) of the Quanti-Tray facing down to fit into the carrier.

5.4.16 If the Quanti-Tray Sealer green ready light is on, slide the carrier with the Quanti-Tray on top into the chute on the side of the sealer until they engage the roller and are pulled through the sealer. The carrier and sealed Quanti-Tray are ejected out of the opposite side of the sealer.

5.4.17 Place the sealed Quanti-Tray in an incubator at 41.0 ± 0.5ºC.

5.4.18 Incubate the sealed Quanti-Tray for 24–28 hours at 41.0 ± 0.5ºC.

5.4.19 Record the following information on the Enterococcus Analysis Log, Form 252:

- Sequential laboratory ID assigned by the lab
- Analysis start date
- Analysis start time (time sample was placed in the incubator)
- mL of sample added to the dilution blank
- mL of dilution water used for sample analysis
- Lot identification for sample bottles, sterile pipets, dilution water bottles, Enterolert media, and Quanti-Trays
- Initials of setup analyst

5.4.20 After 24-28 hours (as close to 24 hours as possible) remove the tray from the incubator and read the results by placing a 6 watt 365 nm wavelength UV light within five inches of the tray in a dark environment. Be sure the light is facing away from your eyes and towards the vessel. Any amount of blue fluorescence indicates the presence of enterococci and is recorded as a positive well.

5.4.21 Record the following information on the Enterococcus Analysis Log, Form 252:

- Date and time the sample was read
- Number of positive wells
- Enterococci colonies/100 mL for diluted sample
- Enterococci colonies/100 mL of undiluted sample
- Initials of read analyst

5.5 QUALITY CONTROL

5.5.1 Quality control (QC) measures and acceptance criteria for analyzing samples are summarized in Table 1 and are as follows:

- Prior to analyzing samples, each analyst must demonstrate the ability to generate acceptable results (i.e., demonstration of capability).
- Sample analysis should begin immediately, preferably within 2 hours of collection. The maximum transport time to the laboratory is

within 6 hours of sample collection. Samples should be processed within 2 hours of receipt at the laboratory.

- Any QC sample analysis (e.g., method blank, laboratory replicate, field replicate) should be subjected to exactly the same analytical procedures as those used on individual sample analyses.
- At least weekly prior to analysis of samples, a method blank (i.e., sterile dilution water) should be analyzed.
- Unless otherwise specified for specific project or samples, at least weekly one sample should be analyzed in duplicate and also each large batch of approximately 20 samples analyzed should include at least one sample analyzed in duplicate (i.e., at least 5% duplicates).

5.5.2 Sterility of sample bottles must be assured prior their use. Bottles are cleaned and tested for sterility as described in QAM Section 3.4.4 and EQL SOP 301. Bottles must pass the sterility test, and test results are recorded on the Sterility Check Log, Form 910.

5.5.3 Sterility of blank dilution water in glass bottles must be assured prior their use. Bottles are cleaned following the same procedures as for sample bottles (see EQL SOP 301 and Microbiology QAM Section 3.4.2). Bottles containing blank dilution water are prepared and tested for sterility as described in this SOPs Section 5.3.2 and Microbiology QAM Section 3.4.4. Bottles must pass the sterility test, and test results are recorded on the Sterility Check Log, Form 910.

5.5.4 Effectiveness of autoclave for sterilization is tested monthly as described in EQL SOP 205. The autoclave must pass the sterility test, and test results are recorded on Autoclave Sterility Check Log, Form 905.

5.5.5 All glassware that comes in contact with the sample must be sterile, and a sterilization record must be kept on the Sterility Check Log, Form 910, unless the items are purchased presterilized.

5.5.6 QC requirements for controlled temperature equipment are provided in EQL SOP 204, “Controlled Temperature Equipment”. In summary:

- Incubator temperatures must be checked to the nearest 0.1 ºC and documented at least twice daily when in use. Readings should be at least 4 hours apart.
- Refrigerator/freezer temperatures must be checked to the nearest 1ºC and documented at least twice daily when in use. Readings should be at least 4 hours apart.
- Thermometers used to determine temperatures must be checked against an NIST or NIST-traceable thermometer at least annually, and for the temperature range used the thermometers must differ from the expected temperature by <1.0 °C and must be tagged with the temperature correction factor.
- Temperatures must be recorded on the Temperature Monitoring Log, Form 901, attached to each device.

Table 1. Summary of QC Requirements for enterococci analysis by Enterolert.

QC Sample or Activity	Minimum Frequency	Acceptance Criteria	Corrective Action
Capability demonstration	Four (4) prepared samples analyzed prior to any customer sample analyses	Criteria for LCS recovery and duplicate precision	Repeat until acceptable
Media sterility check	Prior to use of new lot of Enterolert and weekly	No fluorescence	Investigate problem. Eliminate contaminations. Obtain new lot of Enterolert if necessary. Repeat until successful before using Enterolert lot.
Media positive check with control culture	Prior to use of new lot of Enterolert and weekly	Fluorescence	Investigate problem. Obtain new lot of Enterolert if necessary. Repeat until successful before using Enterolert lot.
Media negative checks with control cultures (gram+ and gram-)	Prior to use of new lot of Enterolert	No fluorescence	Investigate problem. Eliminate contaminations. Obtain new lot of Enterolert if necessary. Repeat until successful before using Enterolert lot.
Method blank	At least weekly, prior to sample analysis	$\leq$ 20 CFU/100 mL	Clean analytical system and repeat MB analysis. Identify and eliminate source of contamination.
Sample duplicate or matrix spike duplicate	At least one (1) weekly, and one with all large sample batches (~20 samples)	RPD $\leq$ 200% for <150 CFU/100 mL RPD $\leq$ 100% for $\geq$ 150 CFU/100 mL	Investigate problem. If system precision is in control, qualify results. If system precision is out of control, reanalyze entire batch.
Internal PE sample	Samples and frequency determined by Lab QA Officer	Criteria for LCS recovery and duplicate precision	Investigate all unacceptable results.
Blind PE sample	Samples and frequency determined by accrediting agencies and projects	Determined by PE provider	Investigate all unacceptable results.

LCS = laboratory control sample
MB = method blank
MDL = method detection limit
PE = performance evaluation
QC = quality control
%R = percent recovery
RL = reporting limit
RPD = relative percent difference

5.5.7 Effectiveness of the Quanti-Tray Sealer operation must be checked monthly as follows:

- Add a dye (e.g., food color or Enterolert reagent) to 100 mL of water.

- Seal the colored water in a Quanti-Tray following the normal sealing procedure (see SOP Steps 5.4.14 - 5.4.16).

- If the colored water is observed outside the wells of the sealed Quanti-Tray, the sealer must be repaired or the back-up sealer used.
- The results of this test must be recorded in the Quanti-Tray Sealer Check Log, Form 912.

5.5.8 QC requirements for Enterolert media are as follows:

- Enterolert medium (purchased from IDEXX) must be stored in the dark at 4-30°C.

- Initial sterility check:
  - Before use each lot of Enterolert medium must be checked and be acceptable for sterility.
  - Analyze 100 mL of sterile, deionized water like a normal sample, except a presence/absence test is sufficient (i.e., incubate the water plus reagent in the dilution bottle not in a Quanti-Tray).
  - Acceptable result is no fluorescence.
  - Record the results of the QC sterility test in the Enterolert QC Log, Form 913.

- Initial positive and negative control checks:
  - Each lot of Enterolert medium must be checked before use with a positive control culture and negative control cultures of both gram negative and gram positive bacteria. Preferred QC bacteria currently available are (appropriate others can also be used if those currently used are not available):

Positive:	*Enterococcus faecalis*	ATCC # 29212
Negative (gram-):	*Escherichia coli*	ATCC # 25922
Negative (gram+):	*Aerococcus viridans*	ATCC #700406

  - Bacteria cultures are prepared by swabbing MicroBiologics® or equivalent in 5-10 mL trypticase soy broth and then incubating for 24 hours at 35ºC. If the broth had been stored refrigerated, allow it to incubate overnight at room temperature, then only use it if no growth is observed (i.e., it is clear after overnight at room temperature). Clouding or precipitation of the inoculated medium after incubation indicates growth of the bacteria.

- Separate 100 mL bottles of sterile dilution water are inoculated with a culture of each bacteria using a 1 µL inoculating loop. The loop must be flame sterilized until it glows in the flame of a Bunsen burner, then air cooled prior to submersing in the culture-containing broth.
- Submerge the loop into the sterile dilution water to inoculate it, and then resterilize the inoculating loop.
- Process the inoculated dilution water like a normal sample, except a presence/absence test is sufficient (i.e., samples are incubated in the dilution bottle).
- For Enterolert medium, blue fluorescence under UV light indicates growth in medium. Presence of growth for the positive control culture is satisfactory as a QC check. Absence of growth (i.e., no fluorescence) is a satisfactory check for the negative control cultures. In summary:

Culture	Growth (Fluorescence) Seen ?	QC Passes?
Positive	Yes	Yes
Negative	No	Yes
Positive	No	No
Negative	Yes	No

- For Enterolert medium record the results of the positive and negative control tests in the Enterolert QC Log, Form 914.
- If QC data are acceptable, the medium can be used.
- If QC data are not acceptable, the medium cannot be used and the problem must be investigated.

- Weekly Enterolert sterility and positive control checks:
  - Once per week a known positive control (enterococci pure culture) and sterile, deionized water blank must be analyzed using the Enterolert medium. A presence/absence test is sufficient. These QC measurements are to be performed following the procedures used for the initial Enterolert Positive Control Check and the Initial Enterolert Sterility Check.
  - Record the results of the weekly QC tests in the Enterolert QC Log, Form 914.
  - Acceptable results are growth (i.e., fluorescence) for the positive control and no growth (i.e., no fluorescence) for the sterility test.
  - If weekly QC data are acceptable, the medium may be used for the next week or until the manufacturer's expiration date, whichever is sooner.
  - If QC data are not acceptable, the medium cannot be used and the problem must be investigated.

5.6 CORRECTIVE ACTIONS

5.6.1 If any of the QC requirements listed in Section 5.5 are not satisfied, the analyst must consult with the Laboratory Director. Normally the activity must be repeated, after corrective actions are taken to correct any obvious problems, until the QC results are acceptable. If repeating the process is not possible (e.g., sample spilled), the results report will include a discussion of the problem and the client will be consulted.

5.6.2 The problem and associated corrective actions must be documented on a Nonconformance and Corrective Action Report (see EQL SOP 201).

6.0 CALCULATIONS AND DATA REPORTING

6.1 From the number of fluorescing wells on the Quanti-Tray, use appropriate Quanti-Tray most probable number (MPN) chart (Tables 2 and 3) to determine bacteria colony density for the diluted sample.

6.2 The density of the undiluted sample is determined by multiplying the density of the diluted sample by the dilution factor (i.e., 10 for 10-fold dilution, which is 100 divided by the volume of sample pipeted into the dilution bottle). The resulting value is the enterococci density per 100 mL of sample.

For example, if 3 wells were positive (i.e., fluorescing) and the sample had been prepared by adding 10 mL of sample to 90 mL of sterile dilution water (total volume of 100 mL), the result from the table would be 3.1 while the reported value would be 31 CFU/100 mL.

6.3 Report trays without positive wells as <10 when 10 mL of sample was used (i.e., dilution factor of 10).

7.0 WASTE DISPOSAL

All incubated samples, used media, and control cultures must be sterilized by autoclaving for 30 minutes at 121°C. Place Trays in three to five gallon metal pots. Autoclave pot for 30 minutes at 121 °C. Remove pot from autoclave. The autoclaving procedure will burst most of the Quanti-Trays. Remove trays while allowing autoclaved media to drain in a sink. Cut the paper backing of the unburst trays with a metal rod (i.e., screwdriver, dull knife), so the trays can drain completely. Rinse the trays with tap water. The used trays are sharp and tend to tear plastic bags. Thus, draining of trays prevents spillage. Place used trays in a plastic garbage bag and discard. Dispose of autoclaved media down the sink's sewage drain. Rinse all other containers in the sink with tap water. Flush sink with lots of water. Discard glass in a glass waste container.

Table 2. 51-Well Quanti-Tray MPN Table

Number of wells giving a positive reaction per 100 ml of sample.	Most Probable Number (MPN/100 ml)	95 % Confidence Limits	
		Lower	Upper
0	<1	0.0	3.7
1	1.0	0.3	5.6
2	2.0	0.6	7.3
3	3.1	1.1	9.0
4	4.2	1.7	10.7
5	5.3	2.3	12.3
6	6.4	3.0	13.9
7	7.5	3.7	15.5
8	8.7	4.5	17.1
9	9.9	5.3	18.8
10	11.1	6.1	20.5
11	12.4	7.0	22.1
12	13.7	7.9	23.9
13	15.0	8.8	25.7
14	16.4	9.8	27.5
15	17.8	10.8	29.4
16	19.2	11.9	31.3
17	20.7	13.0	33.3
18	22.2	14.1	35.2
19	23.8	15.3	37.3
20	25.4	16.5	39.4
21	27.1	17.7	41.6
22	28.8	19.0	43.9
23	30.6	20.4	46.3
24	32.4	21.8	48.7
25	34.4	23.3	51.2
26	36.4	24.7	53.9
27	38.4	26.4	56.6
28	40.6	28.0	59.5
29	42.9	29.7	62.5
30	45.3	31.5	65.6
31	47.8	33.4	69.0
32	50.4	35.4	72.5
33	53.1	37.5	76.2
34	56.0	39.7	80.1
35	59.1	42.0	84.4
36	62.4	44.6	88.8
37	65.9	47.2	93.7
38	69.7	50.0	99.0
39	73.8	53.1	104.8
40	78.2	56.4	111.2
41	83.1	59.9	118.3
42	88.5	63.9	126.2
43	94.5	68.2	135.4
44	101.3	73.1	146.0
45	109.1	78.6	158.7
46	118.4	85.0	174.5
47	129.8	92.7	195.0
48	144.5	102.3	224.1
49	165.2	115.2	272.2
50	200.5	135.8	387.6
51	>200.5	146.1	infinite

Table 3. 97-Well Quanti-Tray MPN Table

Table 3. 97-Well Quanti-Tray MPN Table

Table 3. 97-Well Quanti-Tray MPN Table

Table 3. 97-Well Quanti-Tray MPN Table

Table 3. 97-Well Quanti-Tray MPN Table

Table 3. 97-Well Quanti-Tray MPN Table

Table 3. 97-Well Quanti-Tray MPN Table

Table 3. 97-Well Quanti-Tray MPN Table

Table 3. 97-Well Quanti-Tray MPN Table

Table 3. 97-Well Quanti-Tray MPN Table

Appendix K

CCU EQL SOP NO.: 602

OPTICAL BRIGHTENER MEASUREMENT

BY FLUOROMETRY

Optical Brightener Measurement by Fluorometry ***Reference Method:* Standard Method***

Approved by: _____
Laboratory Director

Reviewed by: _____
Laboratory Master Technician

1.0 SCOPE/APPLICATION

Optical brighteners in laundry and dishwashing detergents fluoresce when exposed to certain ultraviolet wavelengths, so water samples that fluoresce under those same wavelengths are contaminated by residues from laundry and dishwashing detergents (human sources). There are at least two major potential human sources of contamination that could contain optical brighteners, and these include leachates from improperly functioning on-site wastewater systems (OWS) and leaking pipes from community wastewater treatment systems. In rural areas where the majority of homes are served by on-site systems, optical brighteners in water samples indicate failing conditions within OWS in close proximity to the sampled bodies of water.

*The method used in this SOP is based on:

Cao Y, Griffith JF, Weisberg SB (2009) Evaluation of optical brightener photodecay characteristics for detection of human fecal contamination. Water Res 43:2273–2279,

Dickerson, J.W.J., Hagedorn, C., Hassall, A., 2007. Detection and remediation of human-origin pollution at two public beaches in Virginia using multiple source tracking methods. Water Research 41, 3758–3770.

Hartel, P.G., Hagedorn, C., McDonald, J.L., Fisher, J.A., Suluta, M.A., Dickerson, J.R., Gentit, L.C., Smith, S.L., Mantripragada, N.S., Ritter, K.J., Belcher, C.N., 2007a. Exposing water samples to ultraviolet light improves fluorometry for detecting human fecal contamination. Water Research 41, 3629–3642.

Hartel, P.G., McDonald, J.L., Gentit, L.C., Hemmings, S.N.J., Rodgers, K., Smith, K.A., Belcher, C.N., Kuntz, R.L., Rivera-Torres, Y., Otero, E., Schroder, E.C., 2007b. Improving fluorometry as a source tracking method to detect human fecal contamination. Estuaries and Coasts 30, 551–561.

2.0 REFERENCES

2.1 Turner TD-700 Laboratory Fluorometer Operating Manual

3.0 DEFINITIONS

None

4.0 SAFETY

4.1 This method is restricted to use by or under the supervision of trained analysts.

4.2 Gloves, safety glasses with side shields, and protective clothing should be worn to protect against unnecessary exposure to infectious agents (i.e., pathogens), hazardous chemicals (e.g., acids, organic solvents), and contaminants in potentially hazardous samples.

4.3 All activities performed while following this procedure should utilize appropriate laboratory safety systems (e.g., disinfectant, fume hoods, material safety data sheets).

5.0 METHOD

5.1 APPARATUS AND MATERIALS

5.1.1 100mL Griffin beakers (acid leached and rinsed x 10 with Type I water); One beaker for each field sample.
5.1.2 LDPE squirt bottle
5.1.3 Aluminum foil
5.1.4 Stopwatch or suitable electronic timer
5.1.5 Refrigerator, or other means to store sample at 1-4° C in the dark
5.1.6 Aluminum foil
5.1.7 Type I water, UV irradiated
5.1.8 Refrigerator, or other means to store sample at 1-4° C in the dark
5.1.9 Polymethacrylate cuvettes (10mm x 10mm x 45mm, Turner Designs, Sunnyvale, CA)
5.1.10 Delicate tissue wipes (Kimwipes or equivalent)

5.2 REAGENTS

5.2.1 Fluorescent Brightener 28 (Cat No. 158067, MP Biomedicals, Solon, OH
5.2.2 Liquid Tide detergent

5.3 PROCEDURE

5.3.1 Sample Preparation

5.3.1.1 Store samples in amber glass bottles at 1-4° C for up to 8 days prior to analysis.

5.3.1.2 Under dark conditions, decant approximately 100mL of sample from amber bottles to 100mL beakers. Completely cover beakers with aluminum foil to prevent any light exposure during sample warming.

5.3.1.3 Transport foil-covered sample beakers to 20°C incubator and allow samples to warm to 20°C. This process takes approximately 1 hour.

5.3.2 Pre-irradiation Sample Analysis

5.3.2.1 To ensure the Optical Brightener module is installed in the Turner Trilogy fluorometer. If it is not installed, make sure the Trilogy is turner off before removing the existing unit by pulling straight up

on the small handle located on top of the installed unit. Insert the Optical Brightener module by orienting the notches on the module with the appropriate notches on the Trilogy. The module only fits in one orientation, so do not apply unnecessary force if resistance is encountered. Firmly press down until the module is seated in the Trilogy.

5.3.2.2 Work in subdued light (i.e., shades drawn, overhead lights off, only fume hood lights on).

5.3.2.3 Immediately prior to analysis, ensure the UV light source in the Trilogy is warm by selecting **<tools>, <continuous sampling>, <continuous sampling ON>, <total number of measurements 40>.** It is not necessary for an actual sample to be in the Trilogy for this process, which takes approximately 4 minutes. The fluorometer is ready for use after this warm-up step, and will remain ready for 2 hours. If for any reason the fluorometer is left unused for longer than 2 hours, repeat the warm-up procedure prior to analysis.

5.3.2.4 Reprogram the Trilogy measurement mode from continuous sampling to single sampling by selecting **<tools>, <continuous sampling>, <continuous sampling OFF>**.

5.3.2.5 Using PMMA cuvettes that have met the criteria for background fluorescence (**5.3.5**), select one cuvette for each sample.

5.3.2.6 To ensure that each cuvette is clean, fill each with Type I water and obtain a blank value. If the blank is greater than **1400 RFU's**, rinse x10 with Type I water and reanalyze. Repeat this process for each cuvette being used for the sample analysis. Do not use any cuvette that has a blank value **>1400 RFU's**.

5.3.2.7 For each sample, rinse each cuvette x3 with Type I water using the squirt bottle and then x3 with sample. Thoroughly clean all sides of the cuvette with a clean Kimwipe prior to presentation to the fluorometer.

5.3.2.8 Insert cuvette into fluorometer with the marked, optimized cuvette face facing the front of the instrument **(5.3.5)**

5.3.2.9 Press **<Measure Raw Fluorescence>** on the Trilogy screen and record the raw fluorescence units (RFU) number after measurement is completed. (**Important: Each sample can only be measured ONCE in the fluorometer since the actual analysis exposes the sample to UV light degrading the analyte. Pressing <Measure> a second time on the same sample already analyzed will result in a lower reading from this degradation and is not a valid measurement**).

5.3.2.10 Remove the cuvette and discard its sample. Repeat **5.3.2.7**, but **DO NOT** put the sample in the fluorometer. Place the cuvette in the light-proof sample box making sure that it is labeled in a way that will allow you to identify the sample from other cuvettes. Each

cuvette is engraved with a unique identification number that should be recorded in the lab book along with the sample analysis data.

5.3.2.11 Analyze the remaining samples using steps **5.3.2.7 – 5.3.2.10** until all samples have been run and are stored in the light-proof box.

5.3.2.12 Proceed to section **5.3.3**

5.3.3 Sample UV Irradiation

5.3.3.1 30 minutes prior to irradiating the samples, turn on the 2 UV lights in the 20°C incubator to allow them to warm up. Make sure to wear appropriate eye protection. Ensure that the lid is closed on the light-proof sample box before transporting the samples.

5.3.3.2 Under dark room conditions (with the exception of the UV lights in the incubator), transfer each of the sample cuvettes from the light-proof box to the incubator. Orient the cuvettes so that each one is aligned directly in front of the horizontal UV light and directly under the hanging UV light.

5.3.3.3 Set a timer to 5.0 minutes and close the incubator door. It is important that the samples are irradiated exactly 5.0 minutes.

5.3.3.4 After 5.0 minutes of irradiation remove each sample cuvette (under subdued light conditions), and place into the light-proof box. Close the lid and transport the samples back to the fluorometer.

5.3.4 Post UV-Irradiation Sample Analysis

5.3.4.1 Work in subdued light (i.e., shades drawn, overhead lights off, only fume hood lights on).

5.3.4.2 Remove the first irradiated cuvette from the light-proof box and thoroughly wipe all sides with a Kimwipe. Present the cuvette to the fluorometer with the marked, optimized cuvette face facing the front of the instrument (**5.3.5**)

5.3.4.3 Press **<Measure Raw Fluorescence>** on the Trilogy screen and record the raw fluorescence units (RFU) number after measurement is completed.

5.3.4.4 Discard the analyte and rinse the cuvette x3 with the squirt bottle.

5.3.4.5 Repeat steps **5.3.4.1** through **5.3.4.4** for all remaining samples in the light-proof box.

5.3.4.6 Proceed to section **5.3.6** to use the data to determine if optical brighteners are present in the samples. Both qualitative (**5.3.6.1**) and quantitative (**5.3.6.2**) determinations are possible.

5.3.5 PMMA Cuvette Optimization

5.3.5.1 Each polymethacrylate cuvette will have a different background level, even when running the same sample. It is important to determine this level and ensure it is low enough to be used for actual sample analysis.

5.3.5.2 Fill a 2.5L glass jug with water before starting this analysis. The water dispensed by the Milli-Q system can have varying levels of background fluorescence at different times (depending on how much it is being dispensed by other users), so it is critical to use the **same** water for this analysis. **Do not** assume that water dispensed at different times will have the same background fluorescence.

5.3.5.3 Allow the jug to come into thermal equilibrium with the room prior to use. It is common for the water to gradually grow warmer as it is dispensed from the Milli-Q (due to the reverse osmosis system supplying the Miili-Q), so it is a good practice to draw water the day prior to analysis and allow it to sit overnight in a tightly closed 2.5L glass jug.

5.3.5.4 Immediately prior to analysis, ensure the UV light source in the Trilogy is warm by selecting **<tools>, <continuous sampling>, <continuous sampling ON>, <total number of measurements 40>.** It is not necessary for an actual sample to be in the Trilogy for this process, which takes approximately 4 minutes. The fluorometer is ready for use after this warm-up step, and will remain ready for 2 hours. If for any reason the fluorometer is left unused for longer than 2 hours, repeat the warm-up procedure prior to analysis.

5.3.5.5 Work in subdued light (i.e., shades drawn, overhead lights off, only fume hood lights on).

5.3.5.6 Fill a cuvette with water from **5.3.5.2** and dry all sides with a clean Kimwipe.

5.3.5.7 Present the cuvette to the fluorometer, close the lid and press **<Measure Raw Fluorescence>**. After analysis, write this value down. Open the fluorometer and rotate the cuvette 90° in the holder, close the lid and re-measure. Write down the value, and repeat so that the fluorescence values from all 4 cuvettes sides has been measured.

5.3.5.8 **The cuvette must have background fluorescence lower than 1400 RFU's on at least one side to be acceptable for sample analysis**. If this criterion is met, engrave the cuvette ID number into the top section of the cuvette (consult the lab book for the next available ID number). Engrave a small circle in the top section of the cuvette that identifies the optimum side determined by the previous analysis.

5.3.5.9 Repeat **5.3.5.10 - 5.3.5.12** to optimize the remaining cuvettes.

5.3.6 Data Analysis

5.3.6.1 Qualitative Data Analysis

5.3.6.1.1 The presence of optical brighteners in a sample can be inferred by the decrease of a sample's fluorescence after exposure to UV light. Optical brighteners "burn out" very quickly, and a fluorescence decrease of 15% or greater after a sample irradiation is indicative of their presence.

5.3.6.1.2 Use the following formula to calculate the % decrease in fluorescence:

$$\textbf{\% Decrease} = [(RFU_{post\text{-}irradiation}) / (RFU_{pre\text{-}irradiation})] \times 100$$

% Decrease = Percent fluorescence lost after irradiation
$RFU_{pre\text{-}irradiation}$ = Fluorescence signal before UV exposure
$RFU_{post\text{-}irradiation}$ = Fluorescence signal after UV exposure

5.3.6.2 Quantitative Data Analysis

5.3.6.2.1 Measured fluorescence can be quantitatively analyzed by use of an 8 point calibration curve constructed using FB-28 standard diluted to the appropriate concentrations detailed below.

5.3.6.2.2 Make a primary standard by measuring 5.0 mg of FB-28 standard and bringing it to volume with Type I water in a 100mL volumetric flask (acid leached and rinsed x10 with Type I water) for a final concentration of 50 ppm. Store standard in an amber glass bottle at 1-4 °C for up to 14 days.

5.3.6.2.3 Make a working standard on a daily basis by bringing a 10mL aliquot of 50 ppm standard to volume in a 1000mL volumetric flask for a final concentration of 500 ppb.

5.3.6.2.4 Make a 250 ppb standard by bringing 50mL aliquot of 500 ppb standard to volume in a 100 mL volumetric flask.

5.3.6.2.5 Make a 100 ppb standard by bringing 20mL aliquot of 500ppb standard to volume in a 100mL volumetric flask.

5.3.6.2.6 Make a 50ppb standard by bringing a 10mL aliquot of 500ppb standard to volume in a 100mL volumetric flask.

5.3.6.2.7 Make a 25ppb standard by bringing a 5mL aliquot of 500ppb standard to volume in a 100mL volumetric flask.

5.3.6.2.8 Make a 10ppb standard by bringing a 10mL aliquot of 500ppb standard to volume in a 500mL volumetric flask.

5.3.6.2.9 Make a 5ppb standard by bringing a 5mL aliquot of 500ppb standard to volume in a 500mL volumetric flask.

5.3.6.2.10 Starting with a Type I water blank, run all of the concentrations (up to 500ppb) using the same cuvette, ensuring that the cuvette is rinsed x3 with Type I water and x3 with sample between each analysis.

5.3.6.2.11 Obtain the regression line equation by entering the RFU's for each standard into a spreadsheet program (Excel or equivalent).

5.3.6.2.12 To determine the concentration of optical brighteners in each of the previously analyzed samples, subtract the post-irradiation RFU's from the Pre-irradiation RFU's and use regression line analysis to determine the concentration of optical brighteners in each sample.

5.3.6.2.13 Conversely, since many current publications report analysis of field samples in units of specific detergents, it may also be desirable to report the optical brightener concentration in "Tide units". This can be accomplished by subtracting the post-irradiation RFU's from the pre-irradiation RFU's and setting it up in a ratio where 100ppb liquid Tide detergent = 151,752 RFU's.

5.4 QUALITY CONTROL

5.4.1 Quality control (QC) measures for analyzing samples are summarized in Table 1 and are as follows:

- Prior to analyzing samples, each analyst must demonstrate the ability to generate acceptable results (i.e., demonstration of capability).
- Store samples in dark at 1-4° C prior to filtering. Analyze no longer than 8 days after collection.
- Any QC sample analysis (eg., method blank, laboratory replicate, field replicate) should be subjected to exactly the same analytical procedures as those used on individual sample analyses.
- Unless otherwise specified for specific project or samples, each batch of up to 20 samples analyzed should include at least one method blank (i.e., filtered and extracted deionized water, which preferably had been placed in an empty sample bottle and stored and transported with samples) and one sample analyzed in duplicate (i.e., at least 5% duplicates) as QC samples.
- Direct fluorometer reading (i.e., not corrected for dilution) of a sample must fall within the range bracketed by the lowest and highest calibration standards. Any sample reading below the lowest calibration standard's concentration must be reported as less than the reporting limit. For any direct sample reading above the highest calibration standard's concentration, an aliquot of the sample must be diluted until the reading

falls within the calibration range.

Table 1. Determination of Optical Brighteners by Turner Fluorometer

QC Sample or Activity	Minimum Frequency	Acceptance Criteria	Corrective Action
Capability demonstration	Four (4) prepared samples analyzed prior to any customer sample analyses	Criteria for duplicate precision	Repeat until acceptable
5-Point calibration	Quarterly	90-110% R (measured value of all standards when analyzed as samples within 10% of expected value)	Investigate problem. Correct any obvious problems. Repeat calibration until acceptable.
Solid secondary standard value establishment	Quarterly	Measurement after acceptable 5-point calibration and value within 10% of previous established value	Investigate problem. Correct any obvious problems including obtain new solid secondary standards if necessary
Calibration check with solid secondary standards	Daily prior to sample analysis	90-110% R	Investigate problem. Correct any obvious problems including new 5-point calibration if necessary. Repeat calibration check until acceptable.
Method blank	Daily prior to sample analysis	< RL	Clean analytical system and repeat MB analysis. Identify and eliminate source of contamination.
Sample analysis	For all sample analyses	Direct sample reading within calibration range (i.e., lowest and highest calibration standard concentrations)	If reading below range report result as < RL. If result above range dilute sample.
Sample duplicate	One (1) per preparation batch	RPD ≤ 25%	Investigate problem. If system precision is in control, qualify results. If system precision is out of control, reanalyze entire batch.
Internal PE sample	Samples and frequency determined by Lab QA Officer	75-125% R RPD ≤ 25%	Investigate all unacceptable results.
Blind PE sample	Samples and frequency determined by accrediting agencies and projects	Determined by PE provider	Investigate all unacceptable results.

LCS = laboratory control sample
MB = method blank
MDL = method detection limit
MS = matrix spike
PE = performance evaluation
QC = quality control
%R = percent recovery
RL = reporting limit
RPD = relative percent difference

5.5 CORRECTIVE ACTIONS

5.5.1 If any of the QC requirements listed in Table 1 are not satisfied, the analyst must consult with the Laboratory Director. Normally the activity must be repeated, after corrective actions are taken to correct any obvious problems, until the QC results are acceptable. If repeating the process is not possible (e.g., sample spilled), the results report will include a discussion of the problem and the client will be consulted.

5.5.2 The problem and associated corrective actions will be documented on a Nonconformance and Corrective Action Report (see EQL SOP 201).

6.0 DATA REPORTING

6.1 REPORTING LIMITS

Reporting criteria will be determined either qualitatively by a decrease in sample fluorescence after UV irradiation (>15% being indicative of optical brightener presence) or quantitatively (with >50 ppb indicative of optical brightener presence) dependent on the needs of the client.

7.0 WASTE DISPOSAL

Dispose of samples in a designated and labeled beaker and place in fume hood. Allow liquid extracted sample to evaporate and discard glass fiber filter remnants into waste receptacle.

Appendix L

VIRGINIA TECH

IDENTIFYING SOURCES OF FECAL POLLUTION
IN IMPAIRED WATERS

Identifying Sources of Fecal Pollution in Impaired Waters

Principal Investigator
Dr. Charles Hagedorn, Professor
Department of Crop and Soil Environmental Sciences
330 Smyth Hall
Virginia Polytechnic Institute and State University
Blacksburg, VA 24061-0404

Voice: 540-231-4895
Fax: 540-231-3431
E-mail: chagedor@vt.edu

1. Critical Statewide Water Problem

Nearly 65% of the impaired stream segments in Virginia are contaminated by fecal pollution. Fecal bacteria are the major cause of impairments in Virginia's waterways, according to the Virginia Department of Environmental Quality (DEQ, 1998), with agriculture and urban runoff listed as the primary sources of contamination (http://www.deq.state.va.us/water/98-305b.html). This fecal contamination results in increased health risks to persons exposed to the water, degradation of recreational and drinking water quality, and shellfish bed closures. In Virginia, DEQ and the Department of Conservation and Recreation (DCR) face a mandate from USEPA to complete numerous TMDLs within a short timeframe (over 600 within each 10-year period beginning in 2000). Virginia contains approximately 49,000 miles of streams and waterways and less than one-third of these waterways have been adequately monitored to date by the DEQ (FORVA, 2001). Some 2,166 miles are currently listed as impaired, including large sections of the Chesapeake Bay and adjacent shellfish-bearing waters. Based on results from those waters that have been adequately monitored, it is believed that thousands of additional miles of impairments will be added as monitoring is expanded in future years. Estimates vary widely regarding the time needed to fully comply with the TMDL requirements for surface waters, but most estimates are in the 30 to 50 year range (FORVA 2001). Regulatory agencies are slowly coming to the realization that microbial source tracking (MST) methodology will have to be used in virtually every fecal impairment in order to accurately determine the source(s) of that pollution, and that watershed modeling approaches alone will not be sufficient (USEPA, 1999a). Until recently the source(s) of fecal pollution in water could not be readily determined, but source tracking technology has now made such determinations possible, reasonable, and accurate. Knowledge of the type (or types) of pollution sources will aid in the restoration of water quality, reduce the danger of infectious disease from exposure to recreational waters, and

with best management practices (BMP) implementation, reduce the amounts of nutrients that are removed from land and transported into surface waters.

2. Project Benefits

While many of Virginia's waters test positive for fecal coliforms, no useful watershed restoration plans or accurate TMDLs for bacteria can be developed until the source(s) of the fecal contamination can be identified with confidence. If our procedures can reliably and accurately separate different fecal sources (e.g. human, cattle, pets, birds, wildlife), they can provide an essential tool to those who are responsible for public health and environmental quality and are charged with reducing water pollution, protecting public health, and improving water quality. By using appropriate sampling frequency protocols it should be possible to develop stream-monitoring plans utilizing source tracking that will adequately characterize fecal bacteria pollution sources for any given watershed. MST projects can provide a mechanism for non-point source (NPS) problem identification in fecal contaminated waters and provide the necessary information for determining TMDLs for fecal bacteria based on specific source(s) of the bacteria. The possibility of establishing TMDLs for fecal bacteria by specific source(s) is both novel and unique. Target audiences for BST results include the many small communities and the agricultural industry located in watersheds, local and state officials, and regulatory agencies. The MST community has the potential to provide agencies responsible for water quality and public health with a mechanism to determine sources of fecal contamination and, until such sources of pollution are identified, the risk to communities cannot be accurately assessed and water quality improvements will remain a hit-or-miss affair.

While the main "driver" for water quality improvement in rural areas is TMDLs, it is stormwater for urban areas. Regarding stormwater, runoff from storms has been identified as a potential threat to human and ecosystem health due to the high levels of chemical and biological contaminants it contains that have been directly linked to disease outbreaks (Curriero et al., 2001; Gaffield et al., 2003), toxic effects in aquatic life (Bay et al., 2003; Heaney et al., 1999), and dramatic negative impacts on water quality (Ahn et al., 2005; Makepeace et al., 1995). As precipitation washes over land, it picks up and transports a variety of chemicals, pesticides, metals, petroleum products, sediment, and human and animal fecal wastes. Knowledge of the composition of the resultant runoff, as well as its delivery pathways and distribution in the environment, is crucial in managing the overall risk associated with stormwater runoff.

Discharge of stormwater runoff onto recreational beaches in the US is particularly problematic in terms of public health, as it is the largest known cause of beach closures and advisories in the US (Dorfman, 2006). Many of these advisories (75% in 2005) are in response to elevated fecal indicator bacteria (FIB) levels that exceed USEPA recommended beach water quality standards (Dorfman, 2006; USEPA, 1986). Furthermore, US recreational waters serve as a known route of exposure to human pathogens, with 95 documented recreational water-associated outbreaks occurring from 1996-2000 (Arnone and Walling, 2007). Evidence from epidemiological studies of recreational water-associated health effects suggests a causal dose-related relationship between gastrointestinal symptoms and FIB counts and strong relationships between urban runoff and illness (Pruss, 1998). Therefore, mitigating stormwater runoff to decrease loading rates of FIB and viral, bacterial, and protozoan pathogens to recreational beaches is a direct way to improve beach water quality and protect public health.

3. Sources of Fecal Pollution

There are several different potential sources of fecal contamination in Virginia's waters. Ground and surface waters (and potentially well water) can become contaminated with septage from leaking or faulty septic drainfields or tanks. Surface waters can become contaminated from a variety of sources, including surface run-off of livestock and poultry manure and litter that has been applied as fertilizer, or through direct stream contamination by livestock (Alderisio and DeLuca, 1999). Untreated poultry waste can also enter surface waters, either from surface run-off of improperly treated manure used as fertilizer, or from improperly impounded manure piles. Fecal material from wild animals may also enter surface waters by run-off or direct deposit. In more urban landscapes, storm runoff from impervious surfaces carries waste and fecal bacteria from sources such as dogs and birds into surface waters, and dog wastes can be present in substantial quantity along parks and walkways adjacent to streams. Each of these sources of pollution are of concern, because the contamination of natural waters with untreated or partially treated fecal material results in an increased risk of transmission of diseases to the humans who use those waters. Many pathogenic bacteria, viruses, and protozoa are passed from one host to the next by the "fecal-oral route" of transmission, and water usually serves as the carrier for these organisms. Of particular concern are bacterial pathogens such as *Salmonella*, *Shigella*, diarrheagenic *Escherichia coli* (including *E. coli* 0157:H7), *Campylobacter* and *Vibrio*. Many viruses can be transmitted by water, including poliovirus, rotaviruses, Norwalk viruses, and hepatitis A and E viruses. Protozoan pathogens that can be transmitted by water include *Giardia* and *Cryptosporidium*.

Different pathogens are often transmitted by different sources. For example, *Giardia* and *Cryptosporidium* outbreaks are often associated with cattle, and *E. coli* 0157:H7 outbreaks are usually tied to beef products. *Giardia* is also spread by deer (backpacker's disease), and Canada geese feces have been found to contain *Cryptosporidium*, *Giardia,* and *Campylobacter*. Salmonellosis is commonly associated with hogs (as well as *Vibrio*), poultry, and waterfowl, and *Campylobacter* is widespread in poultry (Holmberg et al., 1984). Most viral gastroenteritis is caused by contact with human feces. All of these organisms pose a substantial risk to human public health. Thus, knowledge of the type of pollution is an important factor in determining the degree of risk. It would be desirable to be able to determine the sources of the fecal material, both to determine the risk to the people who are exposed to the waters, and source identification will allow development of strategies to reduce pollution levels.

Livestock and poultry producers often carry the largest share of the burden of correcting fecal contamination problems. Livestock and poultry producers in particular, and the public, in general, will benefit greatly from improved microbial source tracking (MST) methodologies. More cost-effective techniques will improve modeling efforts that are used to determine how fecal contamination problems are to be corrected. In addition, these techniques will support stream water quality monitoring efforts, presenting a more complete picture of stream water quality.

MST should be used in every TMDL project that contains impairments due to fecal bacteria. When this is not done, decisions about sources can be made based on other factors. For example, federal and state officials performed a TMDL project on the Cottonwood Creek watershed in Idaho, without including a source-tracking component (US-EPA 1999b). At public meetings regulatory officials reported that, based on professional judgment, livestock was a major contributor to fecal pollution in the watershed. After ranchers raised serious objections to this conclusion, the conclusion was changed to indicate that wildlife (probably elk) were the major fecal contributors to the impaired stream. It did not seem to matter that elk were well

away from the creek while in their summer range. This is an example of what can happen when MST is not used, and actual results from employing MST should be preferred in place of opinion whenever possible.

4. Project Scope, Monitoring, and Source Tracking

The scope of this MST program is to use a variety of source tracking methodologies to determine source(s) of fecal pollution in natural waters. Numerous source-tracking methods are under development for use in determining sources of fecal pollution in watersheds. Both molecular methods such as ribotyping, pulse-field gel electrophoresis, and randomly amplified polymorphic DNA, and non-molecular methods such as ARA, nutritional patterning, cell wall fatty acid analysis, and strain specific coliphages have been used (and published) in source tracking projects (for a complete list see http://filebox.vt.edu/users/chagedor/biol_4684/BST/BST.html).

4.A. Monitoring by Membrane Filtration

Bacterial monitoring using *Escherichia coli* (*E. coli*) populations from all freshwater samples is performed with EPA Method 1603: *Escherichia coli (E.coli)* in Water by Membrane Filtration Using Modified membrane-Thermotolerant *Escherichia coli* Agar (Modified mTEC) (September 2002) (PDF, 13 pp., 129 KB) - This method (EPA 821-R-02-023) combines information from a 1985 publication (Test methods for *Escherichia coli* and Enterococci in Water by the Membrane Filter Procedure, EPA 600-4-85-076) and a subsequent March 2000 manual (Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and *Escherichia coli*, EPA/821/R-97/004). Method 1603 is a revised membrane filter (MF) procedure, a single-step method that uses one medium, modified mTEC Agar, and does not require the transfer of the membrane filter to another medium or other substrate. For marine water samples, *Enterococcus* (EPA Method 1600) is used. EPA Method 1600 provides a direct count of enterococci in water based on the development of colonies on the surface of a membrane filter. A water sample is filtered through the membrane that retains the bacteria. Following filtration, the membrane containing the bacterial cells is placed on a selective medium, mEI agar, and incubated for 24 hours at 41°C. All colonies (regardless of color) with a blue halo are recorded as enterococci colonies. Magnification and a small fluorescent lamp are used for counting to give maximum visibility of colonies.

4.B. Monitoring by Most-Probable Number

Colilert-18® is used for enumeration of total coliforms and *E. coli* and Enterolert™ is used for *Enterococcus* (IDEXX Laboratories, Westbrook, ME). Duplicate samples are typically diluted to 10 ml, 1 ml, or 0.1 ml per 100 ml with deionized water. The reagents are added to each sample, dissolved by shaking 10 times, poured into a Quanti®-Tray/2000 tray, and incubated overnight at 35°C for Colilert-18® and 41°C for Enterolert™ as required by manufacturer's instructions. Trays are counted according to manufacturer's instructions and most probable number (MPN) per 100 ml of sample is determined using the manufacturer's MPN tables. For each set of duplicate MPN values (per dilution), average MPN values are calculated using the dilution set that yielded usable counts, with preference to the least diluted sample set if more than one set was usable.

5. Source Tracking Protocols

Our lab at Virginia Tech uses five different MST methods, depending on the project and the goals and specific objectives of project sponsors.

5.A. Gene Expression Systems in the Virginia Tech Bacterial Source Tracking Program

i. Antibiotic Resistance Analysis (ARA)

ARA has been performed on the enterococci, fecal coliforms, and *E. coli* (Harwood et al, 2000; Hagedorn et al., 1999; Wiggins, 1996; Wiggins et al., 1999). This method relies on different antibiotic resistance patterns in fecal bacteria that can be related to specific sources of fecal pollution, and is predicated on the rationale that antibiotics exert selective pressure on the fecal flora of the animals that ingest or are treated with the antibiotic(s), and that different types of animals receive differential exposure to antibiotics. Resistance patterns are highest in humans, moderate in livestock, pets, and poultry, and low in birds and wildlife. Benefits of ARA include use of simple laboratory techniques, requiring only basic equipment, and can be performed at a relatively low cost compared to most other methods.

Twenty-eight concentrations of seven antibiotics are used to determine antibiotic resistance patterns in *E. coli*. Each of the twenty-eight antibiotic/concentrations is added separately to flasks of autoclaved and cooled Trypticase Soy Agar (TSA, BBL) from stock antibiotic solutions to achieve the desired concentration, and then poured into sterile 15x100mm petri dishes. Control plates (no antibiotics) are included with each set. Isolates are transferred from the microwell plate using a stainless steel 48-prong replica plater (Sigma). The replicator is flame-sterilized (95% ethanol) after inoculation of each TSA plate. The inoculant is allowed to soak into the agar and the plates are then incubated for 48 hours at 37^{o}C. Resistance to an antibiotic is determined by comparing each isolate to the growth of that isolate on the control plate. A one (1) is recorded if that isolate grew (a round colony, mostly filled) and a zero (0) is recorded for no growth.

To date, ARA has been used in more source tracking projects around the US than any other method. In addition, high levels of separation between known source bacterial isolates have been found comparable to those reported for molecular methods (Parveen et al., 1997; Bernhard and Field, 2000; Dombek et al., 2000).

ii. Nutrient Utilization Patterns (NUP)

The NUP system is based on nutrient utilization profiles (or fingerprints). It is a nearly foolproof system because it uses an electronic plate reader that removes judgment decisions by laboratory personnel when evaluating plates (Hagedorn et al., 2003). In the NUP system, each well in a 96-well microplate contains a single nutrient source (one well is a water blank) and a metabolic dye (tetrazolium violet). Each isolate of *E. coli* or *Enterococcus* is grown for 12 to 24 hrs at 37^{o}C on commercial Blood Agar (BBL) and then diluted to a standardized concentration in a liquid medium that contains all nutrients for growth except a carbon source. The isolate from the Blood Agar plate is transferred to a liquid medium with a sterile cotton swab, and enough is transferred to reach an optical density of 0.23 to 0.25 absorbance on a spectrophotometer. Then, 150 μl of the liquid medium is added to each of the 96 wells in a plate with an automated 8-row pipettor. After incubation for 12 to 24 hrs at 37^{o}C, a color forms (from the metabolic dye) in any well where the isolate was able to use the carbon compound in that well and grow. The pattern of positive wells, out of a total of 95, is used as a metabolic profile. Positive wells are recorded as growth (1) and clear wells as no growth (0). The results are determined and recorded with an electronic plate reader connected to a computer

5.B. Gene Fingerprint Systems

i. Pulsed-Field Gel Electrophoresis (PFGE)

The PFGE procedure is the same as that reported by Simmons (Simmons and Herbein, 1998; Simmons et al., 1995). Pulsed field gel electrophoresis differentiates closely related isolates of the same species by detection of variations in the position of chromosomal restriction sites. In this technique chromosomal DNA is carefully extracted and cleavage of the DNA is carried out using a "rare cutting" restriction enzyme such as *Not*I. The discrete fragments of DNA are separated using pulsed-field gel electrophoresis, which resolves the fragments into distinct bands. The gel is nonspecifically stained with a dye such as ethidium bromide, allowing comparison of the banding pattern of various isolates. The molecular weight of each DNA band is then determined by comparison with a standard DNA ladder. The banding pattern of a particular isolate is the set of variables that is analyzed by statistical analysis. Dr. George Simmons closed his research laboratory in the Biology Department at Virginia Tech in July 2000. His PFGE library, *E. coli* culture collection (some 2,800 isolates from known sources) and BioRad PFGE equipment is now located in Dr. Hagedorn's laboratory. The culture collection contains a large selection of isolates from wildlife sources plus some from humans, pets, and livestock. This collection served as the basis for a much larger *E. coli* PFGE library and a new Enterococcus library to be developed in Hagedorn's laboratory. PFGE has now been used as a cross-validation tool in numerous source tracking projects around the US.

For source tracking purposes, the gene expression and PFGE profiles are analyzed by discriminant analysis (DA) using JMP-In™ statistical software (version 7.0, SAS Inc). First, known source isolates are analyzed and placed in various categories such as human, livestock, wildlife, or cow, deer, horse, waterfowl, etc., depending on the level of classification desired when developing the known source library. Discriminant analysis (DA) assigns a predicted source to each isolate: human and animal in a 2-way split, and human, livestock, urban (dogs and cats), or wildlife in a 4-way split, or a larger split based on specific sources (deer, cow, goose, etc.) as a result of the fingerprints of the isolates in the library. Unknown source isolates (isolates from water samples) are then compared against the library to classify them by source, with the match probability set at 80% or greater correct rate.

ii. *Bacteroides* Human-Specific (HS) DNA-Marker

This method is one of the newer approaches and does not require either a host-origin library or cultivation of the target microbe as DNA is extracted directly from water samples. We use the recently developed PCR method for *Bacteroides thetaiotamicron*, which is an exciting candidate for an alternative indicator of fecal contamination. *Bacteroides* sp. make up approximately one-third of the human fecal microflora, considerably outnumbering *Enterococcus* and *E. coli*. The *Bacteroidales* group belongs to a group of nonspore forming, gram negative, obligate anaerobes, so there is little concern over regrowth in the environment. More importantly, a range of human and animal specific *Bacteroides* sp. markers have been developed, increasing the value of this potential indicator (Field et al. 2003). The species *B. thetaiotamicron* is highly abundant in human fecal waste, has been demonstrated to be tightly related to the presence of human fecal contamination, and is typically found in very low numbers or not at all in animal feces. Finally, bacterial markers such as *Bacteroides* sp. have been shown to be potentially useful source tracking tools. In Griffith et al. (2003) the *Bacteroides sp.* markers correctly identified human sources of fecal pollution when present in mixed water samples delivered blind to the laboratory. This method does not require a library for comparison and does not require cultivation of microbes as the DNA is extracted directly form water samples and then processed with PCR and the correct human-origin primers to determine if complimentary DNA sequences are present in the DNA extracted from the water.

5.C. Chemical Detection Systems

i. Fluorometry

Our program was the first to evaluate the use of a fluorometer in estuarine and coastal zone environments to determine if the equipment could detect a human waste signature. The fluorometer detects compounds that fluoresce under ultraviolet light such as fecal sterols, detergent surfactants and optical brighteners. Optical brighteners in laundry and dishwashing detergents fluoresce when exposed to certain ultraviolet wavelengths, so water samples that fluoresce under those same wavelengths are contaminated by residues from laundry and dishwashing detergents (human sources). There are at least two major potential human sources of contamination that could contain optical brighteners, and these include leachates from improperly functioning on-site wastewater systems (OWS) and leaking pipes from community wastewater treatment systems. In rural areas where the majority of homes are served by on-site systems, optical brighteners in water samples indicate failing conditions within OWS in close proximity to the sampled bodies of water.

Detectors from different manufacturers were evaluated, and the portable fluorometer from Turner Designs, Inc., performed the best in both laboratory and field tests. The detector located fluorescent plumes in water samples taken in coastal rivers where a human signature was known to exist based on microbial source-tracking results. Additionally, the detector correctly identified samples in controlled laboratory tests that had been spiked with detergents and/or septage. Samples without septage or detergents (or containing detergents without optical brighteners) all failed to fluoresce. The instrument was then used successfully on a variety of waterways (both salt and fresh) where human sources of pollution were suspected or could be confirmed with microbial source tracking technology. In larger bodies of water, fluorescent plumes could be identified and mapped with the fluorometer, and then traced back to the shore and directly to locations that appeared to be the source of the pollution. The fluorescent signals appeared to be stable over seasons, storage in refrigeration for at least four months, and over different water conditions (Hagedorn and Weisberg, 2009). Whenever fluorescent plumes were found, microbial source tracking tests demonstrated a human signature in every case where source tracking was performed.

6. Molecular Analyses of Water Samples

For molecular analyses, duplicate 100 ml samples are vacuum filtered through 0.4µm polycarbonate (PC) filters (GE Osmonics, Minnetonka, MN). The filters are placed into sterile, DNase/RNase-free microcentrifuge tubes and stored at -80°C. DNA extractions are performed on the filters using the UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA) following the protocol for maximum yield, with extracts stored at -20°C. Quantitative PCR (QPCR) is performed on extracted DNA using the fecal *Bacteroides* spp. primer-probe set (Converse et al., 2009). The DNA template in each reaction rangee from 1-50 ng, determined fluorometrically with PicoGreen (Invitrogen Carlsbad, CA) using a Turner TBS-380 Fluorometer. QPCR is conducted on a SmartCycler® II (Cepheid, Sunnyvale, CA) using TaKaRa Ex Taq version 2.1 (Mirus Bio Madison, WI), with each 25 µl reaction containing the following: 300 µmol l-1 dNTPs, 4 mmol l-1 MgCl2, 0.05 U Taq polymerase, 1X Taq buffer, and 5 µl DNA template. Reaction conditions are as follows: 95°C for 2 min, followed by 45 cycles of 95°C for 15 s and 60°C for 45 s. Quantification was conducted using *Bacteroides thetaiotaomicron* cells (Converse et al., 2009) enumerated via epifluorescence microscopy (Noble and Fuhrman, 1998). Quantified cells are used to establish a 4-log standard curve, with

reactions run in duplicate. QPCR amplification efficiencies of >90% and R2 values of >0.95 were documented for all standard curves.

Conventional PCR is performed on the DNA extracts using the human-specific *Bacteroides/Prevotella* marker, referred to in this manuscript as "HS" (Bernhard and Field, 2000) using primers targeting a segment of the 16S rRNA gene from the human feces-specific group (HS183F and BAC708R). PCR master mix is composed of 1.25 U Hot Master polymerase (Eppendorf, Westbury, NY), 1X Taq Polymerase self-adjusting magnesium buffer (Eppendorf, Westbury, NY), 1 μ mol l-1 each primer, 200 μmol l-1 dNTPs, and 640 ng μl-1 191 bovine serum albumin. PCR is performed on a Genius thermal cycler (Techne, Burlington, NJ) using the following cycling parameters: 2 min at 94°C, then 30 cycles of 94°C for 30 s, 58°C for 30 s, and 68°C for 30 s, followed by 5 min at 68°C. PCR products are visualized in a 1.2% agarose gel stained with 1 μg ml-1 ethidium bromide and compared to a 100-bp DNA ladder (Promega, Madison, WI).

7. Statistical Analyses

Normality tests on non-transformed data from previous projects indicate that the FIB data are not normally distributed. Therefore, FIB measurements are log10 transformed prior to all statistical analyses. Normality tests are conducted for the datasets to select the appropriate statistical analyses. Independent sample t-tests examine significant differences (alpha (α) = 0.05, two-tailed) between FIB concentrations for storm and baseflow samples and for seasonal comparisons. Levene's test for equality of variances is used to determine whether equal variances were or were not assumed (α = 0.05, two-tailed). Seasonal differences for FIB concentrations are determined using the one-way ANOVA with post-hoc comparison (e.g. Abdi, 2007). A significant relationship is determined with respect to an alpha of 0.05 (two-tailed). The percent of samples positive for the molecular markers is calculated by dividing the number of samples with positive detection of target by the total number of samples and multiplying by 100. Previous and ongoing research is being conducted on fecal *Bacteroides* spp. QPCR data to establish relationships among fecal contamination types. Preliminary work (Coulliette and Noble, 2008) suggests an "action threshold" of 5,000 cells per 100 ml (as determined by QPCR), i.e. water samples exhibiting concentrations of fecal *Bacteroides* spp. Results above this concentration should be further examined for the potential presence of human fecal contamination using other "toolbox" approaches such as those presented by (Noble et al., 2006 and Hagedorn et al., 2007). Furthermore, ratios of *Enterococcus* spp. and *E. coli* and the fecal *Bacteroides* spp. numbers as determined by QPCR have been suggested to potentially be predictive of source (Converse et al., 2009).

8. Quality Assurance (QA) / Quality Control (QC)

Our MST laboratory typically will implement a formal quality control program to substantiate the validity of the collected analytical data. Components usually include:

i. Field Duplicates

Ten percent of all field-collected samples will be duplicates. Each field duplicate will be collected at the same time as the regular sample from that location, placed into a common container, and then the field sample and field duplicate will be made from that single sample. If situations should arise where the results for a given duplicate are noticeably different from its companion sample (greater than 10% after log normalization of the plate counts), then the statistical procedure described in Section 1010 B. Statistics, Part 1-3, (APHA, 1998) can be used

to determine if the "outlier" result should be rejected. This procedure provides a basis for deciding to keep or reject outliers, as no data should ever be arbitrarily rejected. A "T" statistic is calculated for the set of data from a particular site, resulting in an average and standard deviation for every sampling site. Depending upon the desired level of accuracy, data outliers that fall outside either a 10%, 5%, or 1% level of significance (outside of the standard deviation) can be either accepted or rejected. It will be necessary to evaluate the results for each sampling site over a period of time before sufficient data will be available to obtain a reliable average and standard deviation. It may be necessary to have 2 or 3 years of results so that a reasonable average and standard deviation for each individual site can be determined. Any data that appears to be a possible outlier can then be compared against a meaningful average and a decision made at that time regarding including or rejecting the apparent outlier. The same procedure is used for both duplicate field samples and laboratory replicates. Outliers are identified only after a "track record" for each sampling site has been obtained so that a reliable site average and standard deviation can be developed.

ii. Laboratory Replicates, Standards, and Precision

Ten percent of all positive sample results will be retested. The average and range of the cumulative retested samples will be performed so that, as each additional set is retested, it can be used as a precision check. Any results that are outside of the cumulative range indicates loss of precision and that a reevaluation of the retesting procedure is needed (U.S. EPA, 2005).

In microbiological analyses, reference and performance standards are usually not incorporated into QA/QC plans since microbes are living organisms and any standards will change as cells either die or reproduce, depending upon the conditions under which the standards are stored or maintained. Standard Methods for the Examination of Water and Wastewater (20^{th} Edition, referenced under Section 4.4.1) does not recommend reference or performance standards for microbiological analyses. The EPA Manual (1978) indicates that reference and performance standards should be run once a year only when available. While there is no reference standard available for *Enterococcus*, the positive control with a reference strain of *Enterococcus* (described under Section 4.4.4), diluted to a specific number of cells before filtration, will serve as a performance standard for each set of twice yearly samples.

For measurement of analyst precision, duplicate analyses are performed on the first 15 typical samples with positive results. Each set of duplicates is performed by the same analyst. The counts are log normalized and the range and the mean of the range is then calculated for each pair of transformed duplicates. After this initial test, 10% percent of all positive sample results are retested and the range and mean of the range is calculated for each pair of transformed duplicates. If the range of the subsequent pairs is greater than 3.27 times the range mean, then analyst precision is out of control and all analytical results since the last precision check must be discarded. The analytical problem must be identified and resolved before doing further analyses. This approach is specified in:

For each set of samples, field duplicates, lab replicates, and positive controls, after membrane filtration and incubation all plates will be counted by two analysts, consisting of the laboratory supervisor and one assistant. Comparison of the counts obtained by the two analysts will provide cross-validation of the counts. Ten percent of the plates will be recounted by each analyst, and the second counts should differ by no more than 5% for the same analyst and no more than 10% between the two analysts.

iii. Field Blanks

In accordance with U.S. EPA guidance (U.S. EPA, 2005), 5 percent of all field-collected samples will be field blanks. Field blanks will consist of sterile water placed into a laboratory sample bottle and placed into the decontaminated plunge pole or other sampling device that mimics the collection process. Field blanks will be submitted to the laboratory as regular numbered samples.

iv. Method Controls and Validations (with *Enterococcus* as an example)

In order to verify that no contamination is introduced during the process of sample collection and analysis, both positive and negative controls will be included with each sample batch. Positive controls are performed with the reference strain *Enterococcus faecalis* (Andrewes and Horder, PCI 1305, American Type Culture Collection No., 10741, ATCC, Manassas, Va). A pure-culture suspension of the reference strain is prepared in dilution buffer equivalent to approximately 10 viable cells/mL and is then filtered with each batch of samples. As ten mL of this reference sample is filtered, approximately 100 *Enterococcus* colonies should appear on the mEI agar plates after incubation. A suspension of the reference strain is also streaked with an inoculating loop onto a plate of mEI agar whenever a new batch of media is either prepared in the lab or purchased from a commercial supplier. Negative samples include the same dilution buffer, sterilized, but no bacteria have been added to these sterile blanks so there should be no colonies on the mEI plates after incubation. Positive and negative controls will be analyzed with each set of samples collected twice annually. Negative controls will serve as an indicator of contamination and will be included at the start of each set of samples and following every tenth sample.

The U.S. EPA definition of fecal enterococci is based on hydrolysis of esculin, growth on brain heart infusion agar in the presence of 6.5% NaCl, and a negative reaction to the catalase test. Five percent of colonies on mEI plates from each sample will be verified for quality assurance and for source tracking purposes. Obtaining the number of colonies from the mEI plates needed for source tracking usually results in more that 5% of the colonies being verified as *Enterococcus*. Colonies on mEI plates are confirmed as *Enterococcus* prior to performing microbial source tracking by placing the colonies into wells in a microtiter plate containing enterococcosel agar. If the suspected colony is an *Enterococcus*, it will hydrolyze esculin in the enterococcoseal broth and produce a black color in the wells after incubation. As mEI is a highly selective agar medium for the enterococci, it is very rare to select a colony that will not be confirmed as *Enterococcus*. All esculin-positive cultures are transferred to brain heart infusion agar containing 6.5% NaCl. After incubation, those colonies that are esculin positive and grew in the presence of 6.5% NaCl are tested for catalase by adding a drop of 8.82M H_2O_2 to each colony on the brain heart infusion agar plates. No reaction is a negative test and is the final step in verification that a selected isolate is an *Enterococcus*.

All membrane filters will be obtained from a manufacturer who certifies that the filters exhibit full retention and recovery of the organisms to be cultivated, stability in use, and are free of any chemical extractables that may inhibit bacterial growth and development.

9. References

Abdi, H. 2007 "Bonferroni and Sidak corrections for multiple comparisons", in N.J. Salkind (ed.): *Encyclopedia of Measurement and Statistics*. Thousand Oaks, CA: Sage Publishing.

Ahn, J.H., Grant, S.B., Surbeck, C.Q., DiGiacomo, P.M., Nezlin, N.P. and Jiang, S., 2005. Coastal water quality impact of stormwater runoff from an urban watershed in southern California. Environ. Sci. Tech. 39 (16), 5940-5953.

Alderisio, K.A. and N. DeLuca. 1999. Seasonal Enumeration of Fecal Coliform Bacteria from the Feces of Ring-Billed Gulls (*Larus delawarensis*) and Canada Geese (*Branta canadensis*). Appl. Environ. Microbiol. Vol. 65, no. 12: 5628-5630.

American Public Health Association, American Water Works Association, Water Pollution Control Federation. 1998. Standard Methods for the Examination of Water and Wastewater. 20th ed. American Public Health Association, Washington D.C.

Arnone, R.D. and Walling, J.P., 2007. Waterborne pathogens in urban watersheds. Journal of Water and Health. 5(1):149-162.

Bay, S., Jones, B.H., Schiff, K. and Washburn, L., 2003. Water quality impacts of stormwater discharges to Santa Monica Bay. Mar. Environ. Res. 56 (1-2), 205-223.

Bernhard, A.E. and Field, K.G., 2000. A PCR assay To discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA. Appl. Environ. Microbiol. 66 (10), 4571-4574.

Bernhard, A. E., and K. G. Field. 2000. Identification of Nonpoint Sources of Fecal Pollution in Coastal Waters by Using Host-Specific 16S Ribosomal DNA Genetic Markers from Fecal Anaerobes. Appl. Environ. Microbiology 66:1587-1594.

Brownell, M.J., Harwood, V.J., Kurz, R.C., McQuaig, S.M., Lukasik, J. and Scott, T.M., 2007. Confirmation of putative stormwater impact on water quality at a Florida beach by microbial source tracking methods and structure of indicator organism populations. Water Research. 41(16):3747-3757.

Converse, R. R., Griffith, J. F. and R. T. Noble. 2009. Rapid QPCR-Based Assays for Fecal *Bacteroides* and Enterococcus Speciation as Tools for Assessing Fecal Contamination in Recreational Waters. J. Water Research. doi:10.1016/j.watres.2009.06.036

Coulliette, A.D. and Noble, R.T. 2008. Impacts of rainfall on the water quality of the Newport River Estuary (Eastern North Carolina, USA). J Water Health 6 (4), 473-482.

Curriero, F.C., Patz, J.A., Rose, J.B. and Lele, S., 2001. The association between extreme precipitation and waterborne disease outbreaks in the United States, 1948-1994. American J. Public Health 91 (8), 1194-1199.

Dickerson, J.W. Jr., C. Hagedorn, and A. Hassall. 2007. Detection and remediation of human origin pollution at two public beaches in Virginia using multiple source tracking methods. Water Research 41:3758-3770.

Dombek, Priscella E., LeeAnn K. Johnson, Sara T. Zimmerley, and Michael J. Sadowsky. 2000. Use of Repetitive DNA Sequences and the PCR To Differentiate *Escherichia coli* Isolates from Human and Animal Sources. Appl. Environ. Microbiol. Vol. 66, no. 6: 2572-2577.

Dorfman, M., 2006. Testing the waters: a guide to water quality at vacation beaches. National Resource Defense Council.

Field, K.G., E.C. Chern, L.K. Dick, J. Fuhrman, J. Griffith, P.A. Holden, M.G. LaMontagne, J. Le, B. Olson, and M.T. Simonich. 2003. A comparative study of culture-independent genotypic methods of fecal source tracking. J. Water Health 1: 181-194.

Friends of the Rivers of Virginia (FORVA). 2001. State of Our Rivers Report for the Commonwealth of Virginia. Roanoke, Va: FORVA.

Gaffield, S.J., Goo, R.L., Richards, L.A. and Jackson, R.J., 2003. Public health effects of inadequately managed stormwater runoff. Amer. J. Public health 93 (9), 1527-1533.

Griffith, J. F., S.B. Weisberg, and C.D. McGee. 2003. Evaluation of microbial source tracking methods using mixed fecal sources in aqueous test samples. J. Water Health 1:141-151.

Hagedorn, C., S. L. Robinson, J. R. Filtz, S. M. Grubbs, T. A. Angier, and R. B. Reneau, Jr. 1999. Using Antibiotic Resistance Patterns in the Fecal Streptococci to Determine Sources of Fecal Pollution in a Rural Virginia Watershed. Appl. Environ. Microbiol. 65:5522-5531.

Hagedorn, C., J. B. Crozier, K. A. Mentz, A. M. Booth, A. K. Graves, N. J. Nelson, and R. B. Reneau, Jr. 2003. Carbon Source Utilization Profiles as a Method to Identify Sources of Fecal Pollution in Water. . J. Appl. Microbiol. 94:1-8.

Hagedorn, C., and S.B. Weisberg. 2009. Development of chemical-based fecal source tracking methods. Rev. Environ. Sci. Bio/Technol. In Press.

Haile, R.W., Witte, J.S., Gold, M., Cressey, R., McGee, C., Millikan, R.C., Glasser, A., Harawa, N., Ervin, C., Harmon, P., Harper, J., Dermand, J., Alamillo, J., Barrett, K., Nides, M. and Wang, G., 1999. The health effects of swimming in ocean water contaminated by storm drain runoff. Epidemiology (Cambridge, Mass.) 10 (4), 355-503.

Harwood, V. J., J. Whitlock, and V. H. Withington. 2000. Classification of the Antibiotic Resistance Patterns of Indicator Bacteria by Discriminant Analysis: Use in Predicting the Source of Fecal Contamination in Subtropical Florida Waters. Appl. Environ. Microbiol. 66:3698-3704.

Heaney, J.P., Pitt, R. a 504 nd Field, R., 1999. Innovative urban wet-weather flow management systems. EPA/600/R-99/029.

Holmberg, S. D., M. T. Osterholm, K. A. Senger, and M. L. Cohen. 1984. Drug-resistant *Salmonella* from Animals Fed Antimicrobials. N. Engl. J. Med. 311:617-622.

Jeong, Y., Grant, S.B., Ritter, S., Pednekar, A., Candelaria, L. and Winant, C., 2005. Identifying pollutant sources in tidally mixed systems: case study of fecal indicator bacteria from marinas in Newport Bay, southern California. Environmental science & technology 39 (23), 9083-9093.

Krometis, L.A., Characklis, G.W., Simmons, O.D., III, Dilts, M.J., Likirdopulos, C.A. and Sobsey, M.D., 2007. Intra-storm variability in microbial partitioning and microbial loading rates. Water Research 41 (2), 506-516.

Makepeace, D.K., Smith, D.W. and Stanley, S.J., 1995. Urban stormwater quality - summary of contaminant data. Critical Reviews in Environmental Science and Technology 25 (2), 93-139.

Noble, R.T. and Fuhrman, J.A., 1998. Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. Aquat. Microb. Ecol. 14, 113-118.

Noble, R.T., Griffith, J.F., Blackwood, A.D., Fuhrman, J.A., Gregory, J.B., Hernandez, X., Liang, X., Bera, A.A. and Schiff, K., 2006. Multitiered approach using quantitative PCR to track sources of fecal pollution affecting Santa Monica Bay, California. Appl Environ Microbiol 72 (2), 1604-1612.

Parveen, S., R. L. Murphree, L. Edmiston, C. W. Kaspar, K. M. Portier, and M. L. Tamplin. 1997. Association of Multiple-Antibiotic-Resistance Profiles with Point and Nonpoint Sources of *Escherchia coli* in Appalachicola Bay. Appl. Environ. Microbiol. 63:2607-2612.

Pruss, A., 1998. Review of epidemiological studies on health effects from exposure to recreational water. International journal of epidemiology 27 (1), 1-9.

Simmons, G. M., Jr., Herbein, S.A. and James, C.M. 1995. Managing Nonpoint Fecal Coliform Sources to Tidal Inlets. Water Resource Update 100: 64-74.

Simmons, G. M., Jr. and Herbein, S.A. 1998. Potential Sources of *Escherichia coli* to a Children's Pool in La Jolla, CA. Final Report for the City of San Diego and the County of San Diego Department of Environmental Health, San Diego, CA.

U.S. Environmental Protection Agency. Microbiological Methods for Monitoring the Environment: Water and Wastes. (1978) Bordner, R., J. Winter and P. Scarpino (eds.). EPA No. 600/8-78-017.

USEPA, 1986. Ambient water quality criteria for bacteria - 1986. (EPA440/5-84-002), 18 p.

U.S. Environmental Protection Agency. 1999a. Total Maximum Daily Load (TMDL) Program. [Online] http://www.epa.gov/owowwtr1/tmdl/tmdlfs.html (verified 28 April 2002).

U.S. Environmental Protection Agency. 1999b. National Watershed Database 305b. [Online] http://www.epa.gov/surf3/states/VA (verified 28 April 2002).

Virginia Department of Environmental Quality. 1998. 1998 305(b) Water Quality Assessment Report. [Online.] http://www.deq.state.va.us/water/98-305b.html. [19 September 2000, last date accessed.]

Wiggins, B. A., R. W. Andrews, R. A. Conway, C. L. Corr, E. J. Dobratz, D. P. Dougherty, J. R. Eppard, S. R. Knupp, M. C. Limjoco, J. M. Mettenburg, J. M. Rinehardt, J. Sonsino, R. L. Torrijos, and M. E. Zimmerman. 1999. Use of Antibiotic Resistance Analysis To Identify Nonpoint Sources of Fecal Pollution. Appl. Environ. Microbiol. Vol. 65, no. 8: 3483-3486.

Wiggins, B. A. 1996. Use of discriminant analysis of antibiotic resistance of fecal streptococci to differentiate between sources of fecal pollution in natural waters. Appl. Environ. Microbiol. 62:3997-4002.

9. Principal Investigator

Dr. Charles Hagedorn (http://fbox.vt.edu/cals/cses/chagedor/CH.html) is Professor of Soil Microbiology in the Department of Crop and Soil Environmental Sciences at VT. He has received awards for outstanding service from the USDA, the American Society for Microbiology, and the USEPA. His research and scientific expertise has been recognized by awards of 67 state, private, and federal competitive research grants; publication of 126 refereed journal articles; 17 invited review articles; 8 invited book chapters; co-editor of one book; 55 invited presentations at international, national, and state conferences; 21 invited memberships on proposal review panels; 12 refereed bulletins; and 135 abstracts and presentation papers. Fourteen Ph.D. and twenty-one M.S. students have completed degrees under his direction and he has generated in excess of $4,645,000 in external grants and contracts to support his environmental microbiology program, including public health, microbial pathogens in the environment, waste management, the impact of releasing genetically modified organisms into the environment, and determining sources of fecal pollution in water.

Over the past fifteen years, he has been involved in the development of microbial source tracking methods, and has deployed these methods to determine sources of fecal pollution in 40+ projects in Virginia and 14 in other states. His research program on source tracking has been supported by competitive awards from NSF, USDA-NRI, EPA, NOAA, and USGS. His previous source tracking research projects included such diverse locations as Clarke County and Northumberland County, Va., Washington, D.C., Myrtle Beach SC, Oyster Bay, NY, Huntington Beach, CA, Nashville, TN, and Tampa Bay, FL. He participated in all three national method comparison studies that have been performed to compare and contrast the different methodologies being developed for source tracking. Most recently he has been involved in development of a microbial source tracking research program on China's Yangtze River, with the Institute of Hydroecology, Chinese Academy of Sciences, and Zhejiang University, P.R. China.