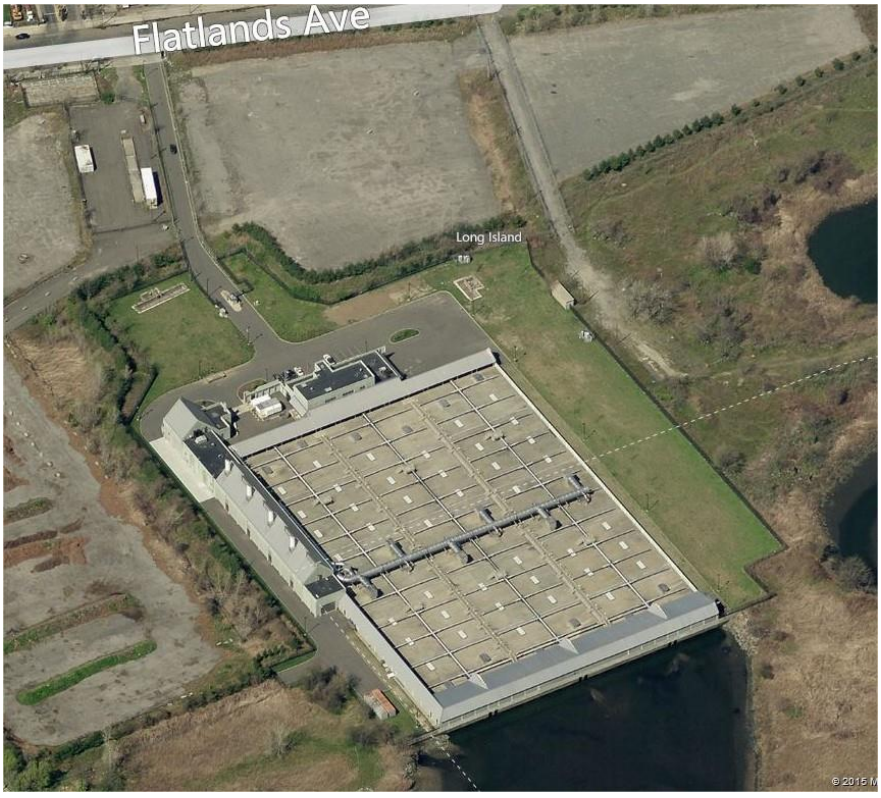
Revised Spring Creek AWWTP CSO Disinfection Demonstration Study

December 2015



The City of New York
Department of Environmental Protection
Bureau of Wastewater Treatment

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I. INTRODUCTION

This document summarizes New York City Department of Environmental Protection's (DEP) proposed combined sewer overflow (CSO) disinfection demonstration scope of work at the Spring Creek Auxiliary Wastewater Treatment Plant (AWWTP) Facility. The purpose of conducting this demonstration testing is to enable DEP to obtain operational performance data for the variable flows and loads associated with CSOs to inform the design of the disinfection facilities that may be an element of several of the waterbody specific Long Term Control Plans (LTCP).

The Spring Creek AWWTP Facility, located on Spring Creek along the Brooklyn-Queens border, is approximately one mile east of the 26th Ward Wastewater Treatment Plant (WWTP). The function of the Spring Creek AWWTP Facility is to capture CSO from tributary drainage areas.

Constructed in the early 1970s with an upgrade recently completed in 2007, the Spring Creek AWWTP Facility has an existing hypo-chlorination disinfection system that has not been used for a number of years. This disinfection system will be refurbished and upgraded under a new Job Order Contract in order to commence this demonstration study to evaluate the efficacy of CSO disinfection process, along with assessing potential chlorine toxicity issues. Details and a schedule of implementation for this contractual work are provided later in this report.

In addition to the refurbishment and upgrade of the existing hypo-chlorination disinfection system, some additional unrelated construction work is being planned at the Spring Creek AWWTP, but this is not anticipated to impact this chlorination demonstration study. The other planned construction work includes:

- Replacement of the majority of the sluice gates with tide gates. This will require that one out of the six channels be taken out of service at a time, with the remaining five channels available for the demonstration study. This work is scheduled to commence in August 2016 and be completed by January 2018.

- Upgrading of the existing instrumentation and SCADA system. Our proposed demonstration will be coordinated with this work and the new flow signals will be connected to RK-2 control panel. The RK-2 control panel is responsible for controlling the existing hypochlorination system that doses based on a level control but under this new work we'll be upgrading the RK-2 control panel and also adding additional disinfection control modes to better control dosing using the new ADS flow meters. If the new RK-2 control panel is not operational by June 2016 then an interim contingency will be reprogramming the existing panel to include a new flow paced control strategy or using the existing level-based disinfection control system with manual adjustments, if needed.

II. EXISTING CONDITIONS

Spring Creek AWWTP Facility was placed into service in the early 1970's and has a minimum storage capacity of approximately 19.3 million gallons (mg), approximately 9.9 mg in basin storage and approximately 9.4 mg in influent barrel storage. Flow is conveyed to the Facility by four overflow barrels from the Autumn Avenue regulator (26W-R3) located in the Borough of Brooklyn, and by two overflow barrels from the 157th Avenue regulator (JA-R2) located in the Borough of Queens as shown in the Process Flow Schematic (Figure 1).

The Spring Creek AWWTP Facility operates as a flow-through retention facility for tributary drainage areas in Brooklyn and Queens within the 26th Ward and Jamaica WWTP drainage areas. The retention facility is

designed to fully contain certain storms and act as a flow-through facility to maximize the reduction of CSO overflows to Spring Creek during larger storms. The total tributary area is composed of 3,256 acres, of which 1,874 acres are in Brooklyn and 1,382 acres are in Queens.

The CSO is conveyed to Spring Creek basins by four overflow barrels from the Autumn Avenue regulator (26W-R3) and two overflow barrels from the 157th Avenue regulator.

The control of influent flow to the Spring Creek AWWTP Facility is accomplished through automated control of the Autumn Avenue Regulator (26W-R3) and from overflow from the 157th Avenue Regulator (JA-R2),

The Facility has six basins with a minimum retention volume, including inline storage, of 19.3 mg. Drain-back from the Facility is by gravity to elevation -7.50 (Brooklyn Highway Datum) and by pumping below this level. Approximately 7.0 mg of CSO is stored in the basins above elevation –7.50 and approximately 8.9 mg are stored above elevation -7.5 in the influent barrels. The stored volume flows by gravity back to the collection system through 26W-R3 influent barrels. A schematic and flow diagram of the existing facility is shown on Figures 1 and Figures 2.

The original disinfection control strategy was semi-automated using the level sensors within the basins but also relied on the operators to manually sample the influent chlorine residual and then adjust the hypochlorite metering pumps accordingly. The neat hypo is pumped into a common pipe with water from the head end of the creek that is used for carrier water. The diluted hypochlorite is added via a diffuser grid into the six channels feeding the basins upstream of the CSO Tank. Some of the hypochlorite piping and diffusers grids are in need of repair but the metering pumps are in operating condition.

III. DESIGN CRITERIA

Based on work performed by DEP during a CSO disinfection pilot study for the Spring Creek AWWTP Facility in the 1990's, the chlorine dosages ranged from 9 to 20 mg/L to achieve a 3 to 4 log. However, the current goal for CSO disinfection, as outlined in certain LTCPs submitted to DEC for review, is to balance influent chlorine dosage with potential effluent toxicity due to total residual chlorine (TRC). Therefore, a 2 log targeted reduction of fecal coliform will be the goal of this demonstration test. Based on the pilot study from the 1990's, it is anticipated that the required chlorine dosage of about 10 to 15 mg/L and some subsequent bench scale testing will be done to verify the required dosing range. The projected 5 minute CSO inflows to the tank based on CY2014 InfoWorks model are provided in Figure 2.

Based on projected InfoWorks CSO influent flow rates as shown on Figure 3 and the typical CSO TRC dosages, the following hypochlorite flow rates have been calculated assuming a 15 percent hypochlorite solution:

Table 1 – Projected Hypo Chlorite Dosages

	Percentile	5 ppm	10 ppm	15 ppm
Projected Hypo Flow Rate (gph)	90th	230	450	680
	10th	5	10	15

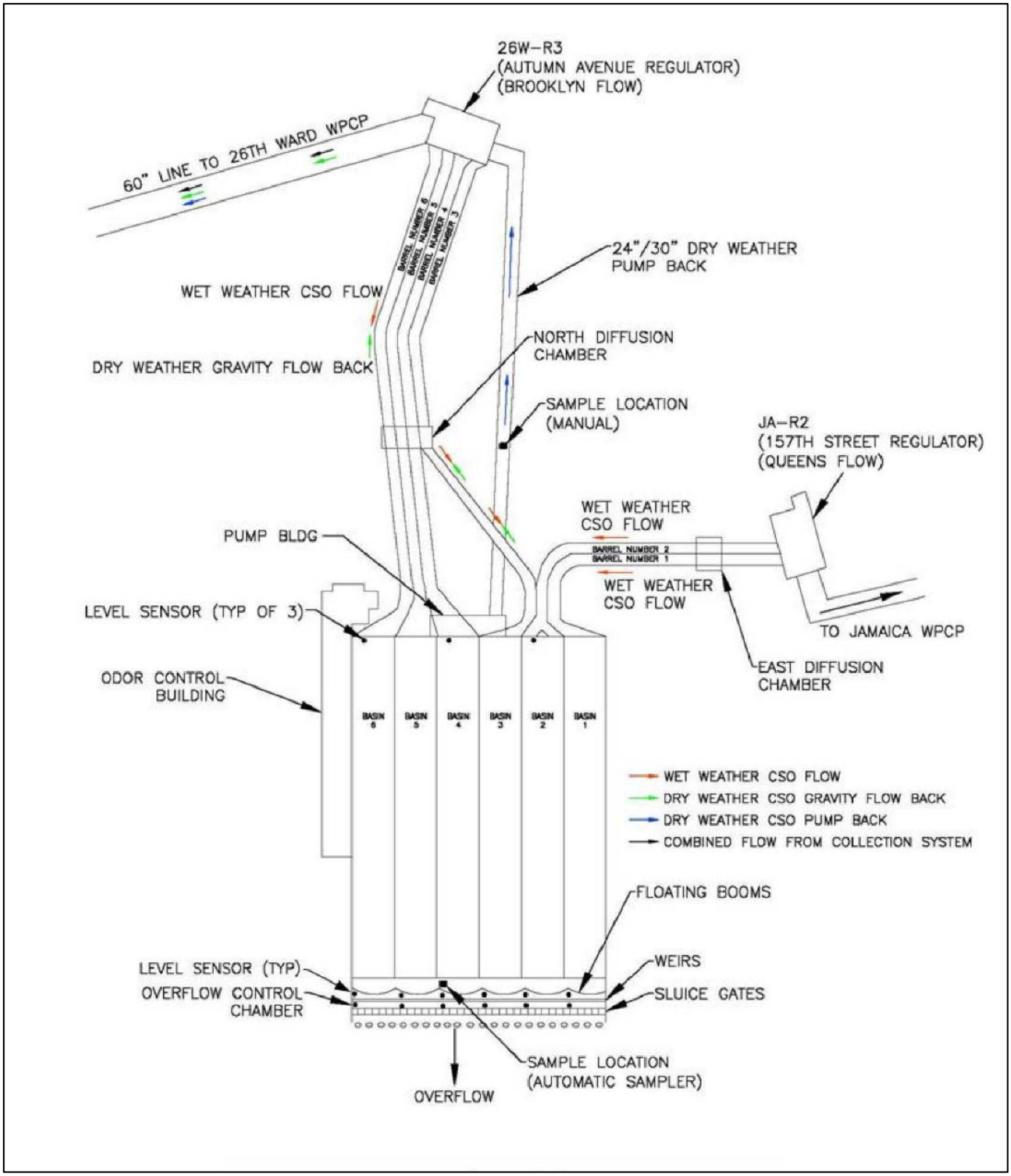


Figure 1 - Spring Creek Schematic

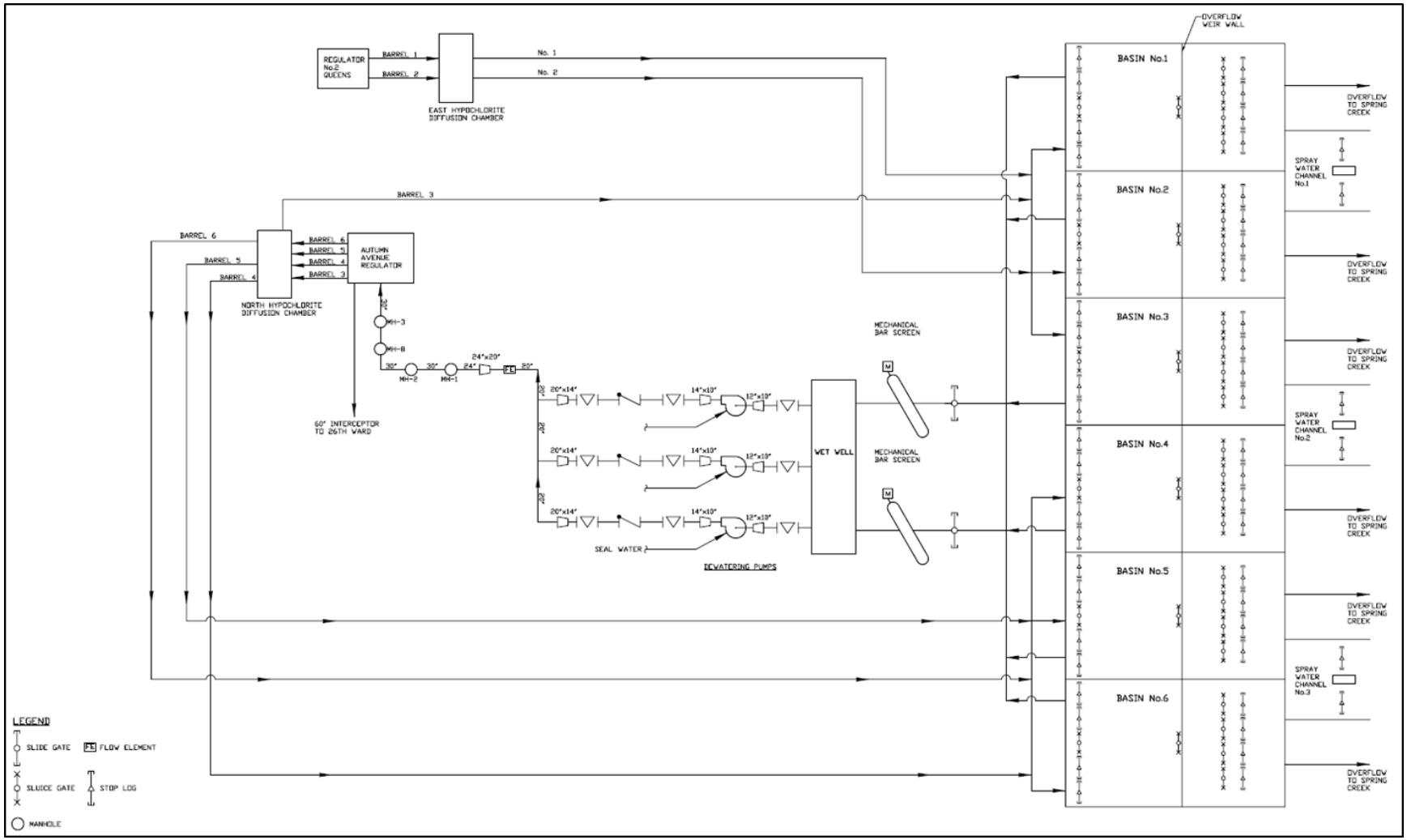


Figure 2 - Spring Creek Flow Diagram

The current disinfection system has three existing low range hypochlorite metering pumps rated for flow rates from 100 to 938 gph based on selected stroke length and have a rated turn down capacity of 10:1 for each pump based on pump speed controls. Based on the projected hypochlorite flow rates from Table 1, the low range metering pumps are properly sized to handle the majority of the CSO events. There is also sufficient hypochlorite storage capacity using the two existing 12,000 gallon sodium hypochlorite chemical storage tanks. A schematic of the existing hypochlorite system is provided in Figure 4.

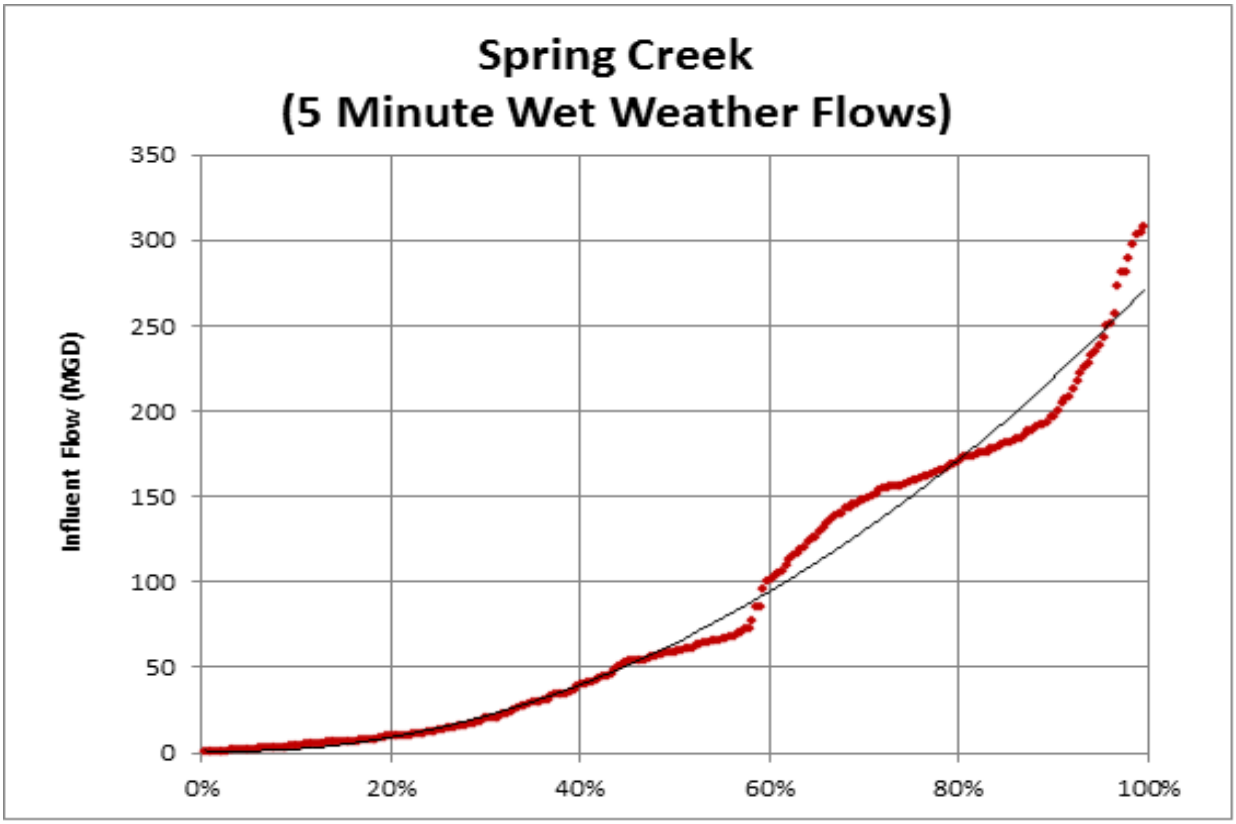


Figure 3 - InfoWorks Projected CSO Flows

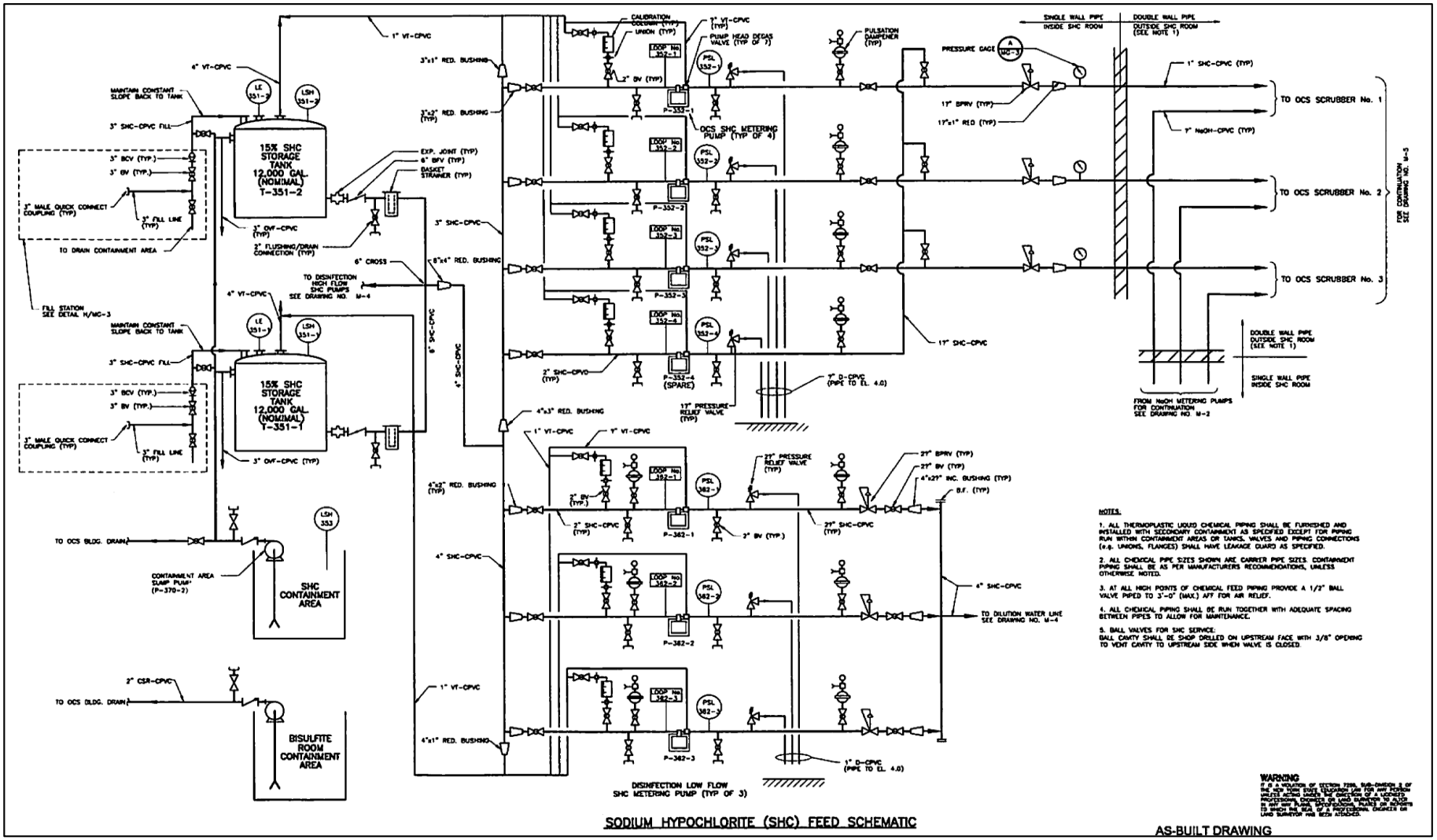


Figure 4 - Schematic of Hypochlorite System

IV. DESIGN & CONSTRUCTION SCOPE OF WORK

The proposed design and construction scope of work includes the installation of three new triton influent flow meters just upstream of the hypochlorite diffusers to monitor flow. This will require running power to the six new flow meters using the existing conduits where possible, along with some trench work and installation of new conduit and wire. In addition, a signal wire will need to be run from the flow meters to a centralized location in the hypochlorite room where the flow signals will be connected to the RK-2 control panel that is currently the control panel for existing hypochlorite system. The RK-2 control panel is also being upgraded under a separate contract and included in this scope of work will be programming some additional TRC automated control strategies using the new ADS flow meters. The hypochlorite pumps and will provide the ability to run the system either automatically or manually. DEP intends to retain the services of an outside vendor to maintain the flow meters during the startup and demonstration testing, both to calibrate and to maintain the system. Additional details on the flow metering equipment and installation are provided in Attachment 1. Provisions will also be included to tie this local control panel into the remote SCADA system in the future, so that these newly installed flow meters are used to report CSO overflow volumes and frequency in the monthly operating reports. In addition to the new flow metering and controls, some work will be necessary to refurbish some of the hypochlorite piping and hypochlorite diffuser grids prior to re-commissioning the disinfection system.

V. PROJECT SCHEDULE

Figure 5 provides the schedule for placing the CSO demonstration facility on-line and performing the demonstration testing.

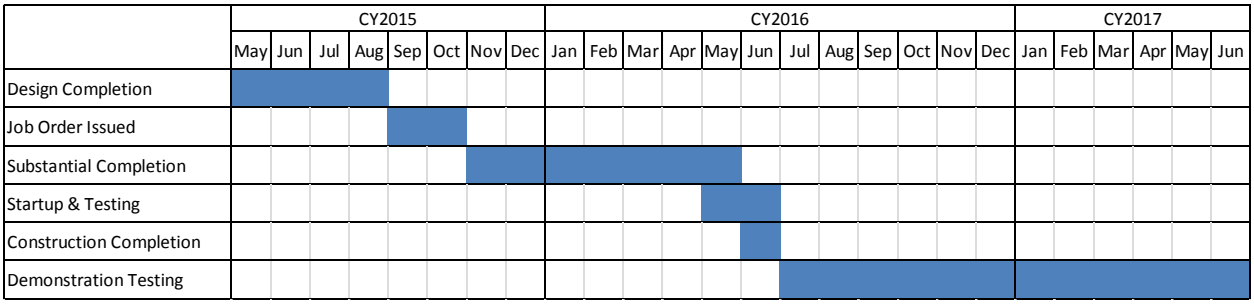


Figure 5. CSO Demonstration Facility Schedule

VI. DEMONSTRATION TESTING SCOPE OF WORK

DEP personnel will coordinate with the City College of New York (CCNY) staff about impending events to enable necessary mobilization. CCNY will go to the facility to collect and analyze samples prior to rain events that are projected to occur during the daytime hours, no manual night sampling is planned. A schematic showing the sampling locations is provided in Figure 6, with the following overview of the proposed sampling plan:

- Pre-chlorinated influent samples will be collected prior to hypo addition and the samples will be analyzed for fecal coliform and enterococcus. CCNY has obtained NELAP certification for fecal

coliform and enterococcus and will be using standard methods approved methodologies for the analysis as shown in Attachment 2.

- Chlorinated influent samples will be collected just downstream of the hypochlorite diffuser and analyzed for fecal coliform, enterococcus and TRC.

- There are limited access locations to collect samples from the middle of the tank, so a duplicate chlorinated influent sample collected will be held for fixed durations to mimic detention time in the tank, and then re-analyzed for fecal coliform, enterococcus, and TRC.

- It is not anticipated that the tank will frequently overflow; however, when it does, overflow samples will be collected and analyzed for fecal coliform, enterococcus, and TRC.

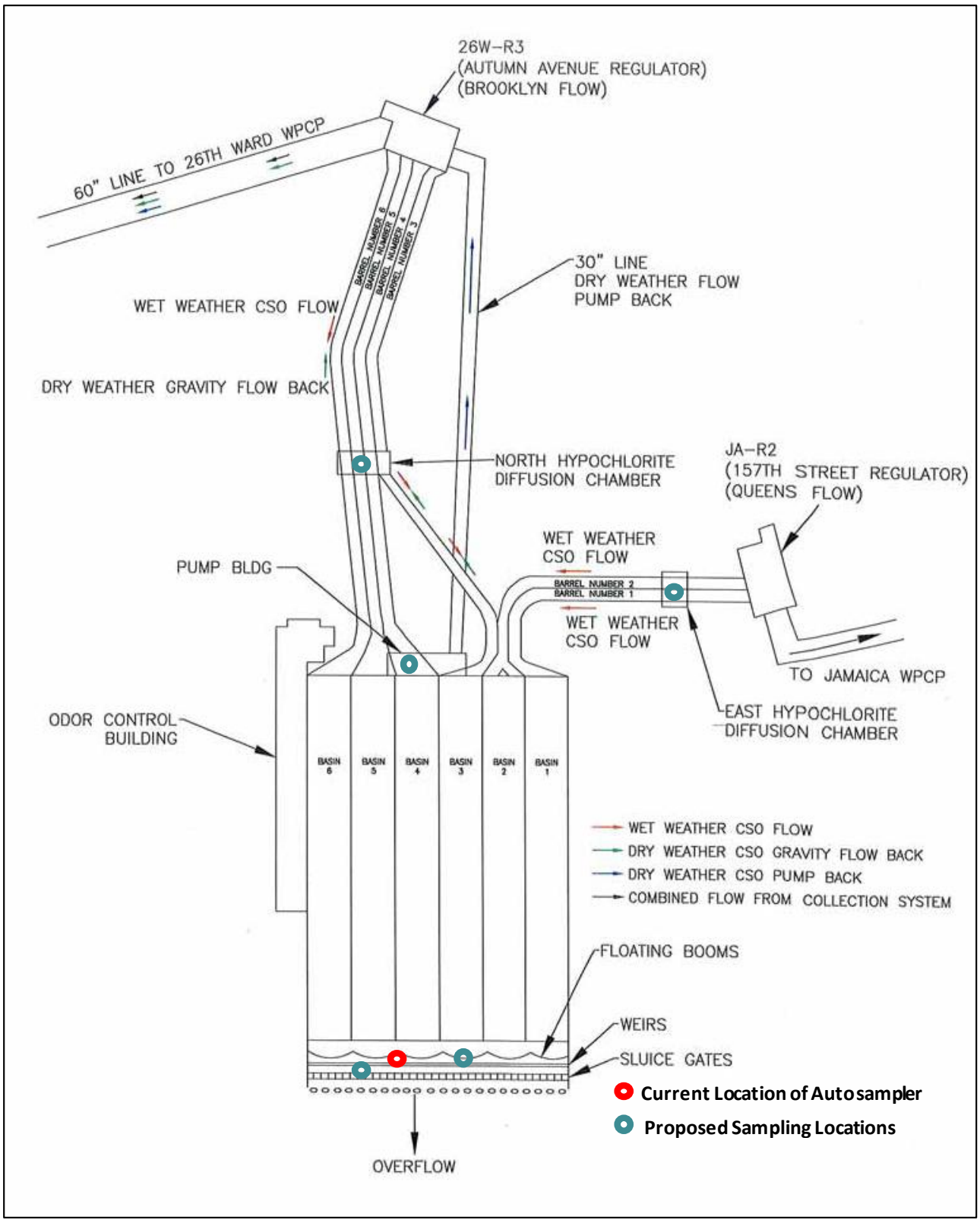
- During overflow events, manual samples will also be collected of the ambient water from the dilution water wet well from which water from the head end of Spring Creek is pumped into the hypochlorite feed lines to be used as carrier water to convey hypochlorite into the two hypochlorite diffusion chambers. A schematic of the dilution water system is provided in Attachment 3 and it is in a separate chamber from the disinfected CSO overflow and is open to the creek therefore representative of ambient conditions.

- In addition to these ambient grab samples, DEP will be conducting a controlled, bench-top chlorine degradation analysis using different predetermined doses of chlorine, adding it to a raw CSO sample, simulating detention time of the CSO tank, and then dosing it to a fixed volume of Jamaica Bay water and measuring the observed TRC residuals A detailed description of the bench scale ambient TRC decay studies in included in Attachment 3 along with some additional details on ambient sampling location.

Below is a summary of the proposed analytical methods to be used. Samples will be analyzed on site and at the City College Laboratory for analysis. A schematic with the sampling locations is provided on Figure 6.

Methods for Analysis		
Analyte	CCNY method	Note
Coliform, Fecal	HACH Method 8074	EPA 9222 D
Enterococci	Method 1600	EPA 821-R-09-016
TRC	D1253-08	

Figure 6. Spring Creek AWWTP Sampling Locations



Attachment 1
ADS Flow Meters

HARDWARE

ADS TRITON+™

The new ***ADS TRITON+***™ is a "Fit-for-Purpose" open channel flow monitor for use in sanitary, combined, and storm sewers. It is designed to be the most versatile flow monitoring system available for wastewater collection applications. It supports single pipe or dual pipe flow measurement installations and is certified to the highest level of Intrinsic Safety.

ADS TRITON+

This multiple technology flow monitor will power almost every available sensor technology that is used in wastewater applications today. It is the most versatile and cost-effective, multiple-technology flow monitor on the market. The ***TRITON+*** includes three multiple technology sensor options: a Peak Combo Sensor, a Surface Combo Sensor, and an Ultrasonic Level Sensor (see inside for technology and specifications). This array of monitoring technologies provides for unmatched flexibility in a fully integrated, fit-for-purpose monitoring platform.

The ***TRITON+*** platform adapts to a wide range of customer applications and budgets. It can be configured as an economical single sensor monitor or dual sensor monitor. It offers a longer battery life and fewer parts for a more reliable system. This provides a lower purchase price and a lower ownership cost over the life of the monitor. The ***TRITON+*** has the lowest operational cost per data sample of any Intrinsically Safe flow monitor available.

About ADS

A leading technology and service provider, ADS Environmental Services® has established the industry standard for open channel flow monitoring and has the only ETV-verified flow monitoring technology for wastewater collection systems. These battery-powered monitors are specially designed to operate with reliability, durability, and accuracy in sewer environments.

TRITON+ Features

- Versatile performance that is easy to install and operate
- Two sensor ports supporting 3 interchangeable sensors providing up to 6 sensor readings at a time
- Single or dual pipe/monitoring point measurement capabilities
- Multi-carrier cellular or serial communication to help optimize coverage and cost
- Industry-leading battery life with a 3G/4G UMTS/HSPA+ wireless connection providing up to 15 months at the standard 15-minute sample rate (*varies with sensor configuration*)
- External power and Modbus network connectivity option available with an ADS External Power and Communications Unit (ExPAC) and a 9-36 VDC power supply
- Analog and digital I/O expansion (4-20 mA and dry contacts) available with an ADS External I/O unit (XIO)
- Modbus protocols enabling RTUs to help simplify SCADA system integration
- Supports the delivery of CSV files to an FTP site at user-defined intervals
- Supports actuation of a water quality sampler for flow proportional or level-based operation
- Monitor-Level Intelligence (MLI®) enables the ***TRITON+*** to effectively operate over a wide range of hydraulic conditions
- Superior noise reduction design for maximizing acoustic signal detection from depth and velocity sensors
- Five software packages for accessing flow information: Q*start*™ (configuration and activation); Profile® (data collection, analysis, and reporting); IntelliServe® (web-based alarming); Sliicer.com® (I/I analysis); and FlowView Portal® (online data presentation and reporting)
- Intrinsically-Safe (IS) certification by IECEx for use in Zone 0/Class I, Division 1, Groups C & D, ATEX Zone 0, and CSA Class I, Zone 0, IIB
- Thick, seamless, high-impact, ABS plastic canister with aluminum end cap (meets IP68 standard)
- Innovative circuit board dome-enclosure protects and limits exposure of electronics when opening the canister to change the battery

To Learn more, visit www.adsenv.com/TRITON+

ADS ENVIRONMENTAL SERVICES®
A Division of ADS LLC

Multiple Technology Sensors

The ***TRITON+*** features three depths and two velocities with three sensor options. Each sensor provides multiple technologies for continuous running of comparisons.

Peak Combo Sensor

Dimensions: 6.76 inches (172 mm) long x 1.23 inches (31 mm) wide x 0.83 inches (21 mm) high

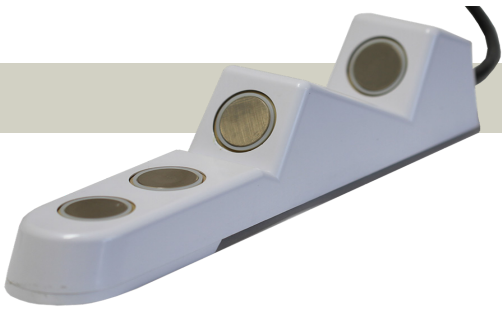
This versatile and economical sensor includes three measurement technologies in a single housing: ADS-patented continuous wave peak velocity, uplooking ultrasonic depth, and pressure depth.

Continuous Wave Velocity
Range: -30 feet per second (-9.1 m/s) to +30 ft/sec (9.1 m/s)
Resolution: 0.01 feet per second (0.003 m/s)
Accuracy: +/- 0.2 feet per second (0.06 m/s) or 4% of actual peak velocity (whichever is greater) in flow velocities between -5 and 20 ft/sec (-1.52 and 6.10 m/s)

Uplooking Ultrasonic Depth
Performs with rotation of up to 15 degrees from the center of the invert; up to 30 degrees rotation with Silt Mount Adapter
Operating Range: 1.0 inch (25 mm) to 5 feet (152 cm)
Resolution: 0.01 inches (0.254 mm)
Accuracy: 0.5% of reading or 0.125 inches (3.2 mm), whichever is greater

Pressure Depth
Range: 0-5 PSI up to 11.5 feet (3.5 m); 0-15 PSI up to 34.5 feet (10.5 m); or 0-30 PSI up to 69 feet (21.0 m)
Accuracy: +/-1.0% of full scale
Resolution: 0.01 inches (0.25 mm)

Surface Combo Sensor



Dimensions: 10.61 inches (269 mm) long x 2.03 inches (52 mm) wide x 2.45 inches (62 mm) high

This revolutionary new sensor features four technologies including surface velocity, ultrasonic depth, surcharge continuous wave velocity, and pressure depth.

Surface Velocity *
Minimum air range: 3 inches (76 mm) from the bottom of the rear, descended portion of the sensor
Maximum air range: 42 inches (107 cm)
Range: 1.00 to 15 feet per second (0.30 to 4.57 m/s)
Resolution: 0.01 feet per second (0.003 m/s)
Accuracy: +/-0.25 feet per second (0.08 m/s) or 5% of actual reading (whichever is greater) in flow velocities between 1.00 and 15 ft/sec (0.30 and 4.57 m/s)

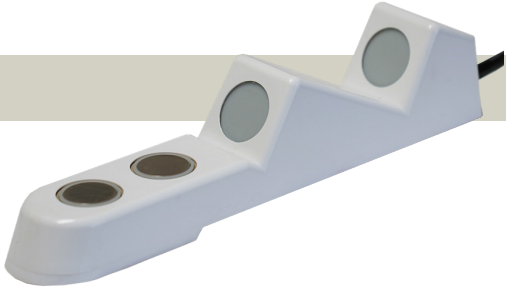
** The flow conditions existing in some applications may prevent the surface velocity technology from being used.*

Ultrasonic Depth
(*Does not require electronic offsets*)
Minimum dead band: 1.0 inches (25.4 mm) from the face of the sensor or 5% of the maximum range, whichever is greater
Maximum operating air range: 10 feet (3.05 m)
Resolution: 0.01 inches (0.25 mm)
Accuracy: +/- 0.125 inches (3.2 mm) with 0.0 inches (0 mm) drift, compensating for variations in air temperature

Surcharge Continuous Wave Velocity (*Under submerged conditions, this technology provides the same accuracy and range as* **Continuous Wave Velocity** *for Peak Combo Sensors*)

Surcharge Pressure Depth (*Under submerged conditions, this technology provides the same accuracy and range as* **Pressure Depth** *for Peak Combo Sensors*)

Ultrasonic Level Sensor



Dimensions: 10.61 inches (269 mm) long x 2.03 inches (52 mm) wide x 2.45 inches (62 mm) high

This non-intrusive, zero-drift sensing method results in a stable, accurate, and reliable flow depth calculation. Two independent ultrasonic transducers allow for independent cross-checking.

Ultrasonic Depth (*See* **Ultrasonic Depth** *Specifications Above*)

TRITON+ Specifications

Connectors
U.S. Military specification MIL-C 26482 series 1, for environmental sealing, with gold-plated contacts

Communications
- Hepta band UMTS/HSPA+ cellular wireless modem
- Direct connection to PC using an ADS USB serial cable

Monitor Interfaces
- Supports simultaneous interfaces with up to two combo sensors
- Supports optional Analog and Digital I/O with ADS XIO: two 4-20 mA inputs and outputs, two switch inputs and two relay outputs

Power
Internal - Battery life with a cellular modem:
- Over 15 months at a 15-minute sample rate*
- Over 6 months at a 5-minute sample rate*

External - Optional external power available with ADS External Power and Communications Unit (ExPAC) with an ADS- or customer-supplied 9-36 Volt DC power supply

** Rate based on collecting data once a day and varies according to sensor configuration and operating temperature*

Operating and Storage Temperature
-4 degrees to 140 degrees F (-20 degrees to 60 degrees C)

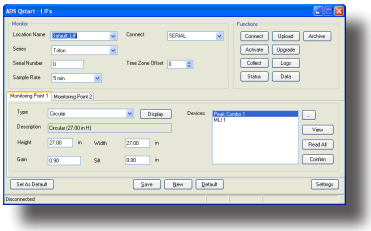
Connectivity
- Modbus ASCII: Wireless; Wired using ExPac
- Modbus RTU: Wireless; Wired using ExPac
- Modbus TCP: Wireless only

Intrinsic Safety Certification
- Certified under the ATEX European Intrinsic Safety standards for Zone 0 rated hazardous areas
- Certified under IECEx (International Electro technical Commission Explosion Proof) Intrinsic Safety standards for use in Zone 0/Class I, Division 1, Groups C&D rated hazardous areas
- CSA Certified to CLASS 2258 03 - Process Control Equipment, Intrinsically Safe and Non-Incendive Systems - For Hazardous Locations, Ex ia IIB T3 (152 degrees C)

Other Certifications/Compliances
- FCC Part 15 and Part 68 compliant
- ROHS (lead-free) compliant
- Carries the EU CE mark
- Canada IC CS-03 compliant

ISO 9001 sira CERTIFICATION UKAS MANAGEMENT SYSTEMS 011
Certificate No: 940056

ADS Flow Monitoring Software

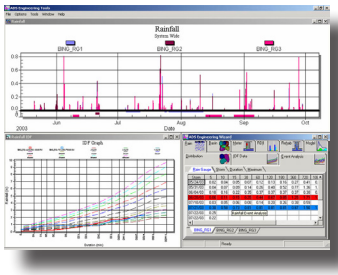


Qstart is desktop software providing field crews with a simple, easy-to-use tool for quickly activating and configuring ADS flow monitors. *Qstart* enables the user to collect and review the monitor's depth and velocity data in hydrograph and tabular views simultaneously.

FlowView Portal is web-hosted software providing robust report delivery, enabling the user to manage data, customize reports, and select viewing parameters. FlowView Portal has a virtually unlimited database for storing and accessing historical data, using data for comparison and trend analysis purposes, and sharing information electronically.

IntelliServe is web-hosted software providing real-time operational intelligence on the status of flow activity throughout the wastewater collection system. IntelliServe utilizes dynamic (or smart) alarming to inform clients about the occurrence of rain events, flow performance abnormalities, and data anomalies at the flow monitoring locations.

Sliicer.com is web-hosted software providing a powerful set of engineering tools designed for both the consulting and municipal engineer. Sliicer.com's inflow and infiltration tools examine wastewater collection system dry and wet weather flow data and provide rigorous performance measurements in one-tenth the time of other analysis tools.



Profile is desktop software providing the industry's best data analysis tools, from basic flow monitoring data to complex hydraulic analysis. Profile is intuitive software that saves time and improves data quality by compiling project data into one location for analysis and reporting.

FLOW MONITORING APPLICATIONS

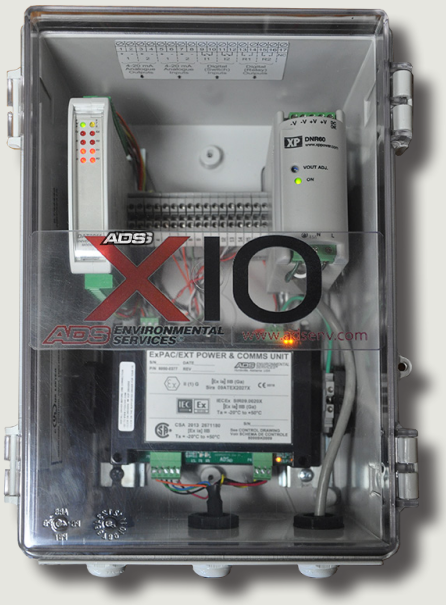
- Billing
- Inflow/Infiltration
- Model Calibration
- Combined Sewer Overflows (CSOs)
- Stormwater Monitoring
- Capacity Analysis
- Spill Notification

ADS' Self-Contained Solution for Power, Communication, Analog and Digital I/O

The new ADS External Input and Output device (**ADS XIO™**) is Intrinsically Safe and expands the monitoring and controlling capabilities of the ***TRITON+*** flow monitor. The XIO converts MODBUS RTU communications from the ***TRITON+*** to analog and digital inputs and outputs; the XIO also supplies external power to the ***TRITON+*** to allow continuous power operation in order to achieve near real-time data acquisition.

XIO Features

- Process variables measured by the ***TRITON+*** can be converted to a 4-20 mA loop signal for SCADA systems or local display and control
- Logging capabilities of the ***TRITON+*** can be used for 4-20 mA input process variables measured by other instrumentation
- Alarms produced by the ***TRITON+*** Monitor-Level-Intelligence (MLI) device can be output on the XIO relay contacts for process actuation
- Digital inputs such as switch or relay contacts can be sampled and logged
- Supports easy plug and play configuration and start-up
- Design facilitates easy field wiring
- Certified as Intrinsically Safe for Zone 0 / Class I, Division 1, Groups C & D
- Rugged indoor/outdoor NEMA 4x Case with hinged clear cover



XIO Specifications

Power Input: 85-264 VAC, 120-375 VDC; 47-63 Hz; 1.10 A @ 110/0.59 A @ 250 VAC

Power Output (to monitor): 8-11.5 VDC, 500mA, Intrinsically Safe

Analog Inputs: Two (2) 4-20 mA inputs; Isolation: 1500 VAC. Accuracy 0.05% F.S; Linearity 0.1% F.S; Thermal Drift 100ppm/C

Analog Outputs: Two (2) 4-20 mA outputs; 500 ohm. Isolation: 1500 VAC. Accuracy 0.1% F.S; Linearity 0.05% F.S; Thermal Drift 100ppm/C

Digital Inputs: Two (2) Switch or dry contacts; Input impedance 4.7 Kilo-ohms.

Digital Outputs: Two (2) SPST Relays; Max load 2 A @250 VAC, 2A @ 30 VDC; Min load 5 VDC, 20 mA

Dimensions: 11.024" (280 mm) high x 7.485 (190 mm) wide x 5.031(127.8 mm) deep

Enclosure: Indoor/Outdoor NEMA 4X (IP 66), PBT and Polycarbonate plastic with hinged clear cover

Operating and Storage Temperature: 14 degrees to 122 degrees F (-10 degrees to 50 degrees C)

Certifications: Intrinsically-Safe (IS) certification by IECEx for use in Zone 0/Class I, Division 1, Groups C & D; ATEX Zone 0 ; and CSA Class I, Zone 0, IIB

ADS ENVIRONMENTAL SERVICES®

ADS. An IDEX Water Services & Technology Business.

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Specifications subject to change without notice.

Photo 1 – North Meter Chamber and access point for temporary and permanent meters

Photo 4 – East Meter Chamber and access point for temporary meters

Attachment 2
Analytical Procedures

Coliforms—Fecal

DOC316.53.001209

USEPA Membrane Filtration Method **Method 8074[1]**

m-FC and m-FC/RA

Scope and Application: For potable water, nonpotable water, recreation water and wastewater.

[1] USEPA approved 9222 D.

Introduction

The Membrane Filtration (MF) method is a fast way to estimate bacterial populations in water. The MF method is especially useful when evaluating large sample volumes or performing many coliform tests daily.

Method

In the initial step, an appropriate sample volume passes through a membrane filter with a pore size small enough (0.45 micron) to retain the bacteria present. The filter is placed on an agar plate prepared with a culture medium that is selective for coliform growth. The petri dish is incubated, upside down, for 24 hours at the appropriate temperature. After incubation, the colonies that have grown are identified and counted using a low-power microscope.

PourRite™ Ampules contain prepared selective media. This eliminates the measuring, mixing, and autoclaving needed when preparing dehydrated media. The ampules are designed with a large, unrestrictive opening that allows media to pour out easily. Each ampule contains enough medium for one test.

Test preparation

Before starting the test:

When the sample is less than 20 mL (diluted or undiluted), add 10 mL of sterile dilution water to the filter funnel before applying the vacuum. This aids in distributing the bacteria evenly across the entire filter surface.
The volume of sample to be filtered will vary with the sample type. Select a maximum sample size to give 20 to 200 colony-forming units (CFU) per filter. The ideal sample volume of nonpotable water or wastewater for coliform testing yields 20–80 coliform colonies per filter. Generally, for finished, potable water, the volume to be filtered will be 100 mL.
If using PourRite™ ampules, allow the media to warm to room temperature before opening.
Disinfect the work bench with a germicidal cloth, dilute bleach solution, bactericidal spray or dilute iodine solution. Wash hands thoroughly with soap and water.

Nonpotable waters procedures

Wastewater, river, bathing, and other nonpotable waters usually are tested for fecal coliforms. In testing for fecal coliforms, a special medium and an elevated incubation temperature inhibit growth of nonfecal coliforms. Fecal coliforms growing on the membrane form an acid that reacts with an aniline dye in the medium, producing a blue color.

Use m-FC Broth with Rosolic Acid to increase specificity when high levels of non-coliform bacteria may be present, unless all the organisms in the sample are stressed or injured.

Confirmation of fecal coliforms (m-FC or m-FC/RA), method 8074

1. Place a sterile absorbent pad in a sterile petri dish using sterilized forceps. Replace the petri dish lid.

Do not touch the pad or the inside of the petri dish.

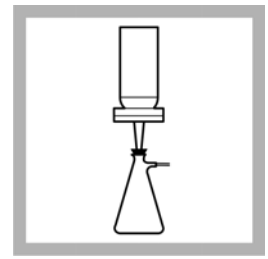
To sterilize forceps, dip forceps in alcohol and flame in an alcohol or Bunsen burner. Let forceps cool before use.

Petri dishes with pads are available.

2. Invert an m-FC Broth PourRite Ampule 2 to 3 times to mix the broth. Use the ampule breaker to open an ampule. Carefully pour the contents evenly onto the absorbent pad. Replace the petri dish lid.

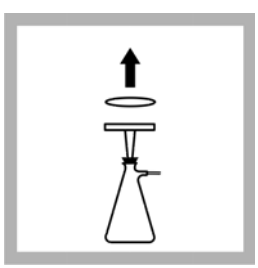
Use m-FC Broth with Rosolic Acid to increase specificity when high levels of non-coliform bacteria may be present, unless the organisms are stressed or injured.

3. Set up the Membrane Filter Assembly. Use sterilized forceps to place a membrane filter, grid side up, into the assembly.

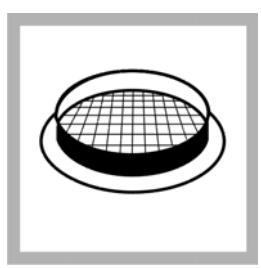


4. Prepare the necessary dilutions to obtain the proper sample size. Invert the sample for 30 seconds to mix. Pour sample into the funnel. Apply vacuum and filter the sample. Rinse the funnel walls with 20 to 30 mL of sterile buffered dilution water. Apply vacuum. Repeat rinsing step, two more times.

Release the vacuum when the filter is dry to prevent damage to the filter.



5. Turn off the vacuum and lift off the funnel top. Use sterile forceps to transfer the membrane filter to the previously prepared petri dish.



6. With a slight rolling motion, center the filter, grid side up, on the absorbent pad. Check for air trapped under the filter and make sure the entire filter touches the pad. Replace the petri dish lid.

7. Invert the petri dish and incubate at 44.5 ± 0.2 °C for 24 ± 2 hours.

To eliminate environmental Klebsiella from the fecal coliform population elevate the temperature to 45.0 ± 0.2 °C.

Alternatively, a water bath with rack may be used for incubation by placing the petri dishes into a sealed bag.

8. After incubating, count the blue colonies using a 10 to 15X microscope.

Confirmation of fecal coliforms (m-FC or m-FC/RA), method 8074 (continued)

9. Record the results of the test. See *Interpreting and reporting results*.

To verify results, follow *Verifying fecal coliforms, method 8074*

Confirmation of total coliforms (Lauryl Tryptose and Brilliant Green Bile)

For potable water samples, confirm typical colonies to ensure they are coliforms. (Confirm sheen colonies, up to a maximum of five.) Inoculate parallel tubes of Lauryl Tryptose (LT) Single Strength (SS) Broth and Brilliant Green Bile (BGB) Broth by transferring growth from each colony. Growth and gas production in both tubes verifies that the suspect organisms are coliforms. Most Probable Number (MPN) coliform tubes are ideal for this purpose.

Use the swabbing technique for fecal coliforms or *E. coli*:

- When determining only the presence or absence of total coliforms
- When inoculating EC or EC/MUG media

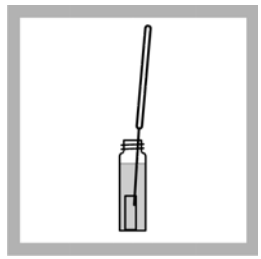
Inoculate in this order:

1. EC or EC/MUG
2. LT SS Broth
3. BGB

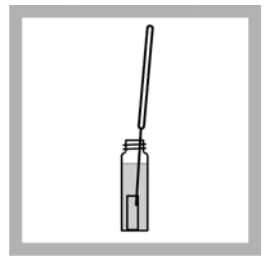
Confirmation of total coliforms (LT and BGB), method 8074

1. Sterilize an inoculating needle, or use a sterile, disposable inoculating needle.

To sterilize an inoculating needle, heat to red hot in an alcohol or Bunsen burner. Let the needle cool before use.

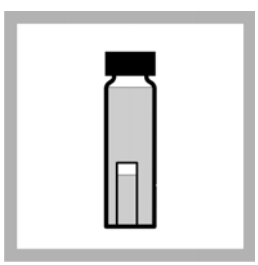


2. Touch the needle to the coliform (sheen) colony grown on m-Endo Broth. Transfer to a single-strength Lauryl Tryptose (LT) Broth tube.



3. Again touch the same coliform colony with the needle. Transfer to a Brilliant Green Bile (BGB) Broth tube.

4. Invert both tubes to eliminate any air bubbles trapped in the inner vials. Incubate the tubes at 35 ± 0.5 °C. After one hour, invert the tubes to remove trapped air in the inner vial, then continue incubation.



5. After 24 ± 2 hours, check the inner vials for growth and gas bubbles. Growth (turbidity) and gas bubbles in both the LT and BGB Broth tubes verify that the colonies are coliforms. If one or both tubes do not show gas, continue incubating both tubes for an additional 24 hours

6. If no gas is present in the LT Broth tube after 48 hours, the colony is not a coliform and additional testing is unnecessary.

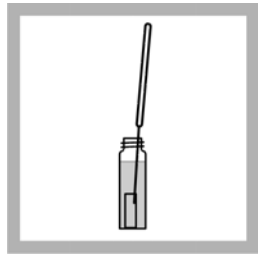
Record the results of the test. See *Interpreting and reporting results*

Confirm positive results. If growth and gas are produced in the LT Broth tube but not in the BGB Broth tube, inoculate another BGB tube from the gas-positive LT Broth tube. Incubate this BGB Broth tube and check for growth and gas after 24 hours and/or after 48 hours. If growth and gas are produced within 48 ± 3 hours, the colony is confirmed as coliform.

Verifying fecal coliforms, method 8074

1. Sterilize an inoculating needle, or use a sterile, disposable inoculating needle.

To sterilize an inoculating needle, heat to red hot in an alcohol or Bunsen burner flame. Let the needle cool before use.



2. Touch the needle to a typical blue colony and transfer to a Lauryl Tryptose (LT) Broth tube. Repeat steps 1 and 3 for each test being verified.

Steps 3 and 4 can be performed simultaneously if multiple incubators are available.

3. Invert the tubes to eliminate air trapped inside the inner vials. Incubate the tubes at 35 ± 0.5 °C. After one hour, invert the tubes to remove trapped air in the inner vial and continue incubation. Check tubes for growth and gas production at 24 hours. If no change has occurred, continue incubation for another 24 hours.

If growth and gas are not produced in 48 ± 3 hours, the colony was not coliform. If growth and gas are produced in 48 ± 3 hours, use a sterile loop to inoculate one EC Medium Broth tube from each gas-positive LT Broth tube.

4. Invert the tubes to eliminate air trapped inside the inner vials. Incubate the EC Medium tubes at 44.5 ± 0.2 °C for 24 ±2 hours. After one hour, invert the tubes to remove trapped air in the inner vial.

5. Growth and gas production at 44.5 °C within 24 ± 2 hours confirms the presence of fecal coliforms.

Record the results of the test. See *Interpreting and reporting results*.

Interpreting and reporting results

Report coliform density as the number of colonies per 100 mL of sample. For total coliforms, use samples that produce 20 to 80 coliform colonies, and not more than 200 colonies of all types, per membrane to compute coliform density. For fecal coliform testing, samples should produce 20 to 60 fecal coliform colonies.

Use ***Equation A*** to calculate coliform density. Note that “mL sample” refers to actual sample volume, and not volume of the dilution.

Equation A—Coliform density on a single membrane filter

$$\text{Coliform colonies per 100 mL} = \frac{\text{Coliform colonies counted}}{\text{mL of sample filtered}} \times 100$$

- If growth covers the entire filtration area of the membrane, or a portion of it, and colonies are not discrete, report results as “Confluent Growth With or Without Coliforms.”
- If the total number of colonies (coliforms plus non-coliforms) exceeds 200 per membrane or the colonies are too indistinct for accurate counting, report the results as “Too Numerous To Count” (TNTC).

In either case, run a new sample using a dilution that will give about 50 coliform colonies and not more than 200 colonies of all types.

When testing nonpotable water, if no filter meets the desired minimum colony count, calculate the average coliform density with Equation B.

Equation B—Average coliform density for 1) duplicates, 2) multiple dilutions, or 3) more than one filter/sample

$$\text{Coliform colonies per 100 mL} = \frac{\text{Sum of colonies in all samples}}{\text{Sum of volumes (in mL) of all samples}} \times 100$$

Controls:
Positive and negative controls are important. *Pseudomonas aeruginosa* is recommended as a negative control and *Escherichia coli* as a positive control. Use the AQUA QC-STIK™ Device for quality control procedures. Instructions for use come with each AQUA QC-STIK Device.

Potable water samples from municipal treatment facilities should be negative for total coliforms and fecal coliforms.

Consumables and replacement items

Confirmation of fecal coliforms (m-FC or m-FC/RA)

Required media and reagents

Description	Unit	Catalog number
m-FC prepared agar plates	15/pkg	2811515
m-FC Broth Ampules, plastic	50/pkg	2373250
m-FC w/Rosolic Acid Broth Ampules, plastic	50/pkg	2428550
m-FC Broth PourRite™ Ampules (for fecal coliform presumptive)	20/pkg	2373220
m-FC with Rosolic Acid Broth PourRite™ Ampules (fecal coliform presumptive)	20/pkg	2428520

Required apparatus

Description	Unit	Catalog number
Ampule Breaker, PourRite™	each	2484600
Counter, hand tally	1	1469600
Dish, Petri, with pad, 47-mm, sterile, disposable, Gelman	100/pkg	1471799
Dish, Petri, with pad, 47-mm, sterile, disposable, Millipore	150/pkg	2936300
Filter Holder, magnetic coupling (use with 24861-00)	1	1352900
Filter Funnel Manifold, aluminum, 3-place (use with 13529-00)	1	2486100
Filters, Membrane, 47-mm, 0.45-µm, gridded, sterile, Gelman	200/pkg	1353001
Filters, Membrane, 47-mm, 0.45-µm, gridded, sterile, Millipore	150/pkg	2936100
Filtering Flask, 1000-mL	1	54653
Forceps, stainless steel	1	2141100
Incubator, Culture, low profile, 110 VAC, 50/60 Hz	each	2619200
Incubator, Culture, low profile, 220 VAC, 50/60 Hz	each	2619202
Inoculating Needle, disposable	25/pkg	2748925
Loop, inoculating, disposable	25/pkg	2749125
Microscope, compound	each	2942500

Optional media and reagents

Description	Unit	Catalog number
Bags, Whirl-Pak®, without dechlorinating agent, 207 mL	100/pkg	2233199
Incubator, Water Bath, 110 VAC, 50/60 Hz	each	2616300
Incubator, Water Bath, 220 VAC, 50/60 Hz	each	2616302

Confirmation of total coliforms (brilliant green bile broth and lauryl tryptose broth)

Required media and reagents

Description	Unit	Catalog number
Brilliant Green Bile Broth Tubes (for total coliform confirmation)	15/pkg	32215
Lauryl Tryptose Broth Ampules, sterile (for enrichment technique)	20/pkg	1472520
Lauryl Tryptose Broth Tubes, single-strength (for total coliform confirmation)	15/pkg	2162315

Required apparatus

Description	Unit	Catalog number
Alcohol Burner	1	2087742
Ampule Breaker, PourRite™	each	2484600
Burner, Bunsen	each	2162700
Incubator, Culture, low profile, 110 VAC, 50/60 Hz	each	2619200
Incubator, Culture, low profile, 220 VAC, 50/60 Hz	each	2619202
Isopropyl alcohol	500 mL	1445949
Loop, inoculating, disposable	25/pkg	2749125
Pad, absorbent, with dispenser	1000/pkg	1491800

Optional media, reagents and apparatus

Description	Unit	Catalog number
Adapter for rechargeable battery pack, 230 VAC (for 2580300)	each	2595902
Alcohol Burner	1	2087742
Autoclave, 120 VAC, 50/60 Hz	each	2898600
Bag, for contaminated items	200/pkg	2463300
Bags, Whirl-Pak®, without dechlorinating agent, 207 mL	100/pkg	2233199
Bags, Whirl-Pak®, without dechlorinating agent, 720 mL	10/pkg	1437297
Bags, Whirl-Pak®, with dechlorinating agent, 180 mL	100/pkg	2075333
Battery eliminator	each	2580400
Battery pack, rechargeable, for portable incubator 12 VDC	each	2580300
Bottle, sample, sterilized, 100-mL, disposable with dechlorinating agent	12/pkg	2599112
Bottle, sample, sterilized, 100-mL, disposable with dechlorinating agent	50/pkg	2599150
Bottle, sample, sterilized, 100-mL, disposable	12/pkg	2495012
Bottle, sample, sterilized, 100-mL, disposable	50/pkg	2495050
Dechlorinating Reagent Powder Pillows	100/pkg	1436369
Dish, Petri, 47-mm, sterile, disposable	100/pkg	1485299
Dish, Petri, 47-mm, sterile, disposable	500/pkg	1485200
Filter Funnel Manifold, aluminum, 3-place (use with 13529-00)	each	2486100
Filter Unit, sterile, disposable with gridded membrane (use with 2656700)	12/pkg	2656600
Filtration Support (for field use), stainless steel	each	2586200
Funnels, Push-Fit and membrane filters (use with 2586200)	72/pkg	2586300
Germicidal Cloths	50/pkg	2463200
Incubator, portable, 12 VDC	each	2569900
Pump, vacuum/pressure, portable, 115 VAC, 60 Hz	each	2824800
Pump, vacuum/pressure, portable, 220 VAC, 50 Hz	each	2824802
Stopper, rubber, one hole, No. 8	6/pkg	211908
Tubing, rubber, 0.8 cm ID	3.7 m (12 ft)	56019
Sterilization Indicator, Sterikon®	15/pkg	2811115
Sterilization Indicator, Sterikon®	100/pkg	2811199
Syringe, 140-mL, polypropylene (use with 2586200)	each	2586100
Wicks, replacement, for alcohol burner 2087742	10/pkg	2097810

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Edition 7

United States
Environmental Protection
Agency

Method 1600: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI)

December 2009

U.S. Environmental Protection Agency
Office of Water (4303T)
1200 Pennsylvania Avenue, NW
Washington, DC 20460

EPA-821-R-09-016

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Volunteer Research Laboratories

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Volunteer Verification Laboratories

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Appendices A and B are taken from Microbiological Methods for Monitoring the Environment, Water and Wastes (Reference 18.7).

Appendix A: Part II (General Operations), Section A (Sample Collection, Preservation, and Storage).

Appendix B: Part II (General Operations), Sections C.3.5 (Counting Colonies) and C.3.6 (Calculation of Results).

Method 1600: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI)

December 2009

1.0 Scope and Application

1.1 Method 1600 describes a membrane filter (MF) procedure for the detection and enumeration of the enterococci bacteria in water. This is a single-step method that is a modification of EPA Method 1106.1 (mE-EIA). Unlike the mE-EIA method, it does not require the transfer of the membrane filter to another medium. The modified medium has a reduced amount of triphenyltetrazolium chloride (TTC) and includes indoxyl β-D-glucoside, a chromogenic cellobiose analog used in place of esculin. In this procedure, β-glucosidase-positive enterococci produce an insoluble indigo blue complex which diffuses into the surrounding media, forming a blue halo around the colony.

1.2 Enterococci are commonly found in the feces of humans and other warm-blooded animals. Although some strains are ubiquitous and not related to fecal pollution, the presence of enterococci in water is an indication of fecal pollution and the possible presence of enteric pathogens.

1.3 Epidemiological studies have led to the development of criteria which can be used to promulgate recreational water standards based on established relationships between health effects and water quality. The significance of finding enterococci in recreational fresh or marine water samples is the direct relationship between the density of enterococci and the risk of gastrointestinal illness associated with swimming in the water (References 18.1 and 18.2).

1.4 For method application please refer to Title 40 Code of Federal Regulations Part 136 (40 CFR Part 136).

2.0 Summary of Method

2.1 Method 1600 provides a direct count of bacteria in water based on the development of colonies on the surface of the membrane filter (Reference 18.4). A water sample is filtered through the membrane which 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 ± 0.5°C. All colonies greater than or equal to (≥) 0.5 mm in diameter (regardless of color) with a blue halo are recorded as enterococci colonies. A fluorescent lamp with a magnifying lens is used for counting to give maximum visibility of colonies.

3.0 Definitions

3.1 In Method 1600, enterococci are those bacteria which produce colonies greater than or equal to 0.5 mm in diameter with a blue halo after incubation on mEI agar. The blue halo should not be included in the colony diameter measurement. Enterococci include *Enterococcus faecalis (E. faecalis), E. faecium, E. avium, E. gallinarium,* and their variants. The genus *Enterococcus* includes the enterococci formerly assigned to the Group D fecal streptococci.

4.0 Interferences

4.1 Water samples containing colloidal or suspended particulate materials can clog the membrane filter and prevent filtration, or cause spreading of bacterial colonies which could interfere with enumeration and identification of target colonies.

5.0 Safety

5.1 The analyst/technician must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents, and materials, and while operating sterilization equipment.

5.2 The selective medium (mEI) and azide-dextrose broth used in this method contain sodium azide as well as other potentially toxic components. Caution must be exercised during the preparation, use, and disposal of these media to prevent inhalation or contact with the medium or reagents.

5.3 This method does not address all of the safety issues associated with its use. It is the responsibility of the laboratory to establish appropriate safety and health practices prior to use of this method. A reference file of material safety data sheets (MSDSs) should be available to all personnel involved in Method 1600 analyses.

5.4 Mouth-pipetting is prohibited.

6.0 Equipment and Supplies

6.1 Glass lens with magnification of 2-5X or stereoscopic microscope

6.2 Lamp, with a cool, white fluorescent tube

6.3 Hand tally or electronic counting device

6.4 Pipet container, stainless steel, aluminum or borosilicate glass, for glass pipets

6.5 Pipets, sterile, T.D. bacteriological or Mohr, glass or plastic, of appropriate volume

6.6 Sterile graduated cylinders, 100-1000 mL, covered with aluminum foil or kraft paper

6.7 Sterile membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil or kraft paper

6.8 Ultraviolet unit for sanitization of the filter funnel between filtrations (optional)

6.9 Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source (In an emergency or in the field, a hand pump or a syringe equipped with a check valve to prevent the return flow of air, can be used)

6.10 Flask, filter, vacuum, usually 1 L, with appropriate tubing

6.11 A filter manifold to hold a number of filter bases (optional)

6.12 Flask for safety trap placed between the filter flask and the vacuum source

6.13 Forceps, straight or curved, with smooth tips to handle filters without damage

6.14 Ethanol, methanol or isopropanol in a small, wide-mouth container, for flame-sterilizing forceps

6.15 Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing loops and needles

6.16 Thermometer, checked against a National Institute of Standards and Technology (NIST) certified thermometer, or one that meets the requirements of NIST Monograph SP 250-23

6.17 Petri dishes, sterile, plastic, 9 x 50 mm, with tight-fitting lids; or 15 x 60 mm with loose fitting lids; or 15 x 100 mm with loose fitting lids

6.18 Bottles, milk dilution, borosilicate glass, screw-cap with neoprene liners, 125 mL volume

6.19 Flasks, borosilicate glass, screw-cap, 250-2000 mL volume

6.20 Membrane filters, sterile, white, grid marked, 47 mm diameter, with 0.45 µm pore size

6.21 Platinum wire inoculation loops, at least 3 mm diameter in suitable holders; or sterile plastic loops

6.22 Incubator maintained at 41°C ± 0.5°C

6.23 Waterbath maintained at 50°C for tempering agar

6.24 Test tubes, 20 x 150 mm, borosilicate glass or plastic

6.25 Caps, aluminum or autoclavable plastic, for 20 mm diameter test tubes

6.26 Test tubes, screw-cap, borosilicate glass, 16 x 125 mm or other appropriate size

6.27 Autoclave or steam sterilizer capable of achieving 121°C [15 lb pressure per square inch (PSI)] for 15 minutes

7.0 Reagents and Standards

7.1 Purity of Reagents: Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (Reference 18.5). The agar used in preparation of culture media must be of microbiological grade.

7.2 Whenever possible, use commercial culture media as a means of quality control.

7.3 Purity of reagent water: Reagent-grade water conforming to specifications in: *Standard Methods for the Examination of Water and Wastewater* (latest edition approved by EPA in 40 CFR Part 136 or 141, as applicable), Section 9020 (Reference 18.6).

7.4 Phosphate buffered saline (PBS)

7.4.1 Composition:

Sodium dihydrogen phosphate (NaH_2PO_4)	0.58 g
Disodium hydrogen phosphate (Na_2HPO_4)	2.5 g
Sodium chloride (NaCl)	8.5 g
Reagent-grade water	1.0 L

7.4.2 Dissolve the reagents in 1 L of reagent-grade water and dispense in appropriate amounts for dilutions in screw cap bottles or culture tubes, and/or into containers for use as rinse water. Autoclave after preparation at 121°C (15 PSI) for 15 min. Final pH should be 7.4 ± 0.2.

7.5 mEI Agar

7.5.1 Composition:

Peptone	10.0 g
Sodium chloride (NaCl)	15.0 g
Yeast extract	30.0 g
Esculin	1.0 g
Actidione (Cycloheximide)	0.05 g
Sodium azide	0.15 g
Indoxyl β-D-glucoside	0.75 g
Agar	15.0 g
Reagent-grade water	1.0 L

7.5.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Autoclave at 121°C (15 PSI) for 15 minutes and cool in a 50°C water bath.

7.5.3 After sterilization add 0.24 g nalidixic acid (sodium salt) and 0.02 g triphenyltetrazolium chloride (TTC) to the mEI medium and mix thoroughly.

Note: The amount of TTC used in this medium (mEI) is less than the amount used for mE agar in Method 1106.1.

7.5.4 Dispense mEI agar into 9 × 50 mm or 15 × 60 mm petri dishes to a 4-5 mm depth (approximately 4-6 mL), and allow to solidify. Final pH of medium should be 7.1 ± 0.2. Store in a refrigerator.

7.6 Tryptic soy agar (TSA)

7.6.1 Composition:

Pancreatic digest of casein	15.0 g
Enzymatic digest of soybean meal	5.0 g
Sodium chloride (NaCl)	5.0 g
Agar	15.0 g
Reagent-grade water	1.0 L

7.6.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Autoclave at 121°C (15 PSI) for 15 minutes and cool in a 50°C waterbath. Pour the medium into each 15 × 60 mm culture dish to a 4-5 mm depth (approximately 4-6 mL), and allow to solidify. Final pH should be 7.3 ± 0.2.

7.7 Brain heart infusion broth (BHIB)

7.7.1 Composition:

Calf brains, infusion from 200.0 g	7.7 g
Beef heart, infusion from 250.0 g	9.8 g
Proteose peptone	10.0 g
Sodium chloride (NaCl)	5.0 g
Disodium hydrogen phosphate (Na_2HPO_4)	2.5 g
Dextrose	2.0 g
Reagent-grade water	1.0 L

7.7.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Dispense in 10-mL volumes in screw cap tubes, and autoclave at 121°C (15 PSI) for 15 minutes. Final pH should be 7.4 ± 0.2.

7.8 Brain heart infusion broth (BHIB) with 6.5% NaC1

7.8.1 Composition:

BHIB with 6.5% NaC1 is the same as BHIB above (Section 7.7), but with additional NaC1.

7.8.2 Add NaCl to formula provided in Section 7.7 above, such that the final concentration is 6.5% (65 g NaCl/L). Typically, for commercial BHIB media, an additional 60.0 g NaCl per liter of medium will need to be added to the medium. Prepare as in Section 7.7.2.

7.9 Brain heart infusion agar (BHIA)

7.9.1 Composition:

BHIA contains the same components as BHIB (Section 7.7) ,with the addition of 15.0 g agar per liter of BHIB.

7.9.2 Add agar to formula for BHIB provided in Section 7.7 above. Prepare as in Section 7.7.2. After sterilization, slant until solid. Final pH should be 7.4 ± 0.2.

7.10 Bile esculin agar (BEA)

7.10.1 Composition:

Beef Extract	3.0 g
Pancreatic Digest of Gelatin	5.0 g
Oxgall	20.0 g
Esculin	1.0 g
Ferric Citrate	0.5 g
Bacto Agar	14.0 g
Reagent-grade water	1.0 L

7.10.2 Add reagents to 1 L reagent-grade water, heat with frequent mixing, and boil 1 minute to dissolve completely. Dispense 10-mL volumes in tubes for slants or larger volumes into flasks for subsequent plating. Autoclave at 121°C (15 PSI) for 15 minutes. Overheating may cause darkening of the medium. Cool in a 50°C waterbath, and dispense into sterile petri dishes. Final pH should be 6.8 ± 0.2. Store in a refrigerator.

7.11 Azide dextrose broth (ADB)

7.11.1 Composition:

Beef extract	4.5 g
Pancreatic digest of casein	7.5 g
Proteose peptone No. 3	7.5 g
Dextrose	7.5 g
Sodium chloride (NaCl)	7.5 g
Sodium azide	0.2 g
Reagent-grade water	1.0 L

7.11.2 Add reagents to 1 L of reagent-grade water and dispense in screw cap bottles. Autoclave at 121°C (15 PSI) for 15 minutes. Final pH should be 7.2 ± 0.2.

7.12 Control cultures

7.12.1 Positive control and/or spiking organism (either of the following are acceptable)

- Stock cultures of *Enterococcus faecalis* (*E. faecalis*) ATCC #19433
- *E. faecalis* ATCC #19433 BioBalls (bioMérieux Inc., Durham NC)

7.12.2 Negative control organism (either of the following are acceptable)

- Stock cultures of *Escherichia coli* (*E. coli*) ATCC #11775
- *E. coli* ATCC #11775 BioBalls (bioMérieux Inc., Durham NC)

8.0 Sample Collection, Handling, and Storage

8.1 Sampling procedures are briefly described below. Detailed sampling methods can be found in Reference 18.7 (see Appendix A). Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Samples not collected according to these rules should not be analyzed.

8.1.1 Sampling techniques

Samples are collected by hand or with a sampling device if the sampling site has difficult access such as a dock, bridge, or bank adjacent to a surface water. Composite samples should not be collected, since such samples do not display the range of values found in individual samples. The sampling depth for surface water samples should be 6-12 inches below the water surface. Sample containers should be positioned such that the mouth of the container is pointed away from the sampler or sample point. After removal of the container from the water, a small portion of the sample should be discarded to allow for proper mixing before analyses.

8.1.2 Storage temperature and handling conditions

Ice or refrigerate water samples at a temperature of <10°C during transit to the laboratory. Do not freeze the samples. Use insulated containers to assure proper maintenance of storage temperature. Take care that sample bottles are not totally immersed in water during transit or storage.

8.1.3 Holding time limitations

Sample analysis should begin immediately, preferably within 2 hours of collection. The maximum transport time to the laboratory is 6 hours, and samples should be processed within 2 hours of receipt at the laboratory.

9.0 Quality Control

9.1 Each laboratory that uses Method 1600 is required to operate a formal quality assurance (QA) program that addresses and documents instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records storage and retrieval. Additional recommendations for QA and quality control (QC) procedures for microbiological laboratories are provided in Reference 18.7.

9.2 The minimum analytical QC requirements for the analysis of samples using Method 1600 include an initial demonstration of laboratory capability through performance of the initial precision and recovery (IPR) analyses (Section 9.3), ongoing demonstration of laboratory capability through performance of the ongoing precision and recovery (OPR) analysis (Section 9.4) and matrix spike (MS) analysis (Section 9.5, disinfected wastewater only), and the routine analysis of positive and negative controls (Section 9.6), filter sterility checks (Section 9.8), method blanks (Section 9.9), and media sterility checks (Section 9.11). For the IPR, OPR and MS analyses, it is necessary to spike samples with either laboratory-prepared spiking suspensions or BioBalls as described in Section 14.

Note: Performance criteria for Method 1600 are based on the results of the interlaboratory validation of Method 1600 in PBS and disinfected wastewater matrices. The IPR (Section 9.3) and OPR (Section 9.4) recovery criteria (**Table 1**) are valid method performance criteria that should be met, regardless of the matrix being evaluated, the matrix spike recovery criteria (Section 9.5, **Table 2**) pertain only to disinfected wastewaters.

9.3 Initial precision and recovery (IPR)—The IPR analyses are used to demonstrate acceptable method performance (recovery and precision) and should be performed by each laboratory before the method is used for monitoring field samples. EPA recommends but does not require that an IPR be performed by each analyst. IPR samples should be accompanied by an acceptable method blank (Section 9.9) and appropriate media sterility checks (Section 9.11). The IPR analyses are performed as follows:

9.3.1 Prepare four, 100-mL samples of PBS and spike each sample with *E. faecalis* ATCC #19433 according to the spiking procedure in Section 14. Spiking with laboratory-prepared suspensions is described in Section 14.2 and spiking with BioBalls is described in Section 14.3. Filter and process each IPR sample according to the procedures in Section 11 and calculate the number of enterococci per 100 mL according to Section 13.

9.3.2 Calculate the percent recovery (R) for each IPR sample using the appropriate equation in Section 14.2.2 or 14.3.4 for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.

9.3.3 Using the percent recoveries of the four analyses, calculate the mean percent recovery and the relative standard deviation (RSD) of the recoveries. The RSD is the standard deviation divided by the mean, multiplied by 100.

9.3.4 Compare the mean recovery and RSD with the corresponding IPR criteria in Table 1, below. If the mean and RSD for recovery of enterococci meet acceptance criteria, system performance is acceptable and analysis of field samples may begin. If the mean recovery or the RSD fall outside of the required range for recovery, system performance is unacceptable. In this event, identify the problem by evaluating each step of the analytical process, media, reagents, and controls, correct the problem and repeat the IPR analyses.

Table 1. Initial and Ongoing Precision and Recovery (IPR and OPR) Acceptance Criteria

Performance test	Lab-prepared spike acceptance criteria	BioBall™ acceptance criteria
Initial precision and recovery (IPR)		
• Mean percent recovery	31% - 127%	85% - 106%
• Precision (as maximum relative standard deviation)	28%	14%
Ongoing precision and recovery (OPR) as percent recovery	27% - 131%	78% - 113%

9.4 Ongoing precision and recovery (OPR)—To demonstrate ongoing control of the analytical system, the laboratory should routinely process and analyze spiked PBS samples. The laboratory should analyze one OPR sample after every 20 field and matrix spike samples or one per week that samples are analyzed, whichever occurs more frequently. OPR samples must be accompanied by an acceptable method blank (Section 9.9) and appropriate media sterility checks (Section 9.11). The OPR analysis is performed as follows:

9.4.1 Spike a 100-mL PBS sample with *E. faecalis* ATCC #19433 according to the spiking procedure in Section 14. Spiking with laboratory-prepared suspensions is described in Section 14.2 and spiking with BioBalls is described in Section 14.3. Filter and process each OPR sample according to the procedures in Section 11 and calculate the number of enterococci per 100 mL according to Section 13.

9.4.2 Calculate the percent recovery (R) for the OPR sample using the appropriate equation in Section 14.2.2 or 14.3.4 for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.

9.4.3 Compare the OPR result (percent recovery) with the corresponding OPR recovery criteria in Table 1, above. If the OPR result meets the acceptance criteria for recovery, method performance is acceptable and analysis of field samples may continue. If the OPR result falls outside of the acceptance criteria, system performance is unacceptable. In this event, identify the problem by evaluating each step of the analytical process, media, reagents, and controls, correct the problem and repeat the OPR analysis.

9.4.4 As part of the laboratory QA program, results for OPR and IPR samples should be charted and updated records maintained in order to monitor ongoing method performance. The laboratory should also develop a statement of accuracy for Method 1600 by calculating the average percent recovery (R) and the standard deviation of the percent recovery (s_r). Express the accuracy as a recovery interval from R - $2s_r$ to R + $2s_r$.

9.5 **Matrix spikes (MS)**—MS analysis are performed to determine the effect of a particular matrix on enterococci recoveries. The laboratory should analyze one MS sample when disinfected wastewater samples are first received from a source from which the laboratory has not previously analyzed samples. Subsequently, 5% of field samples (1 per 20) from a given disinfected wastewater source should include a MS sample. MS samples must be accompanied by the analysis of an unspiked field sample sequentially collected from the same sampling site, an acceptable method blank (Section 9.9), and appropriate media sterility checks (Section 9.11). When possible, MS analyses should also be accompanied by an OPR sample (Section 9.4), using the same spiking procedure (laboratory-prepared spiking suspension or BioBalls). The MS analysis is performed as follows:

9.5.1 Prepare two, 100-mL field samples that were sequentially collected from the same site. One sample will remain unspiked and will be analyzed to determine the background or ambient concentration of enterococci for calculating MS recoveries (Section 9.5.3). The other sample will serve as the MS sample and will be spiked with *E. faecalis* ATCC #19433 according to the spiking procedure in Section 14.

9.5.2 Select sample volumes based on previous analytical results or anticipated levels of in the field sample in order to achieve the recommended target range of enterococci (20-60 CFU, including spike) per filter. If the laboratory is not familiar with the matrix being analyzed, it is recommended that a minimum of three dilutions be analyzed to ensure that a countable plate is obtained for the MS and associated unspiked sample. If possible, 100-mL of sample should be analyzed.

9.5.3 Spike the MS sample volume(s) with a laboratory-prepared suspension as described in Section 14.2 or with BioBalls as described in Section 14.3. Immediately filter and process the unspiked and spiked field samples according to the procedures in Section 11.

Note: When analyzing smaller sample volumes (e.g, <20 mL), 20-30 mL of PBS should be added to the funnel or an aliquot of sample should be dispensed into a 20-30 mL dilution blank prior to filtration. This will allow even distribution of the sample on the membrane.

9.5.4 For the MS sample, calculate the number of enterococci (CFU / 100 mL) according to Section 13 and adjust the colony counts based on any background enterococci observed in the unspiked matrix sample.

9.5.5 Calculate the percent recovery (R) for the MS sample (adjusted based on ambient enterococci in the unspiked sample) using the appropriate equation in Section 14.2.2 or 14.3.4 for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.

9.5.6 Compare the MS result (percent recovery) with the appropriate method performance criteria in Table 2, below. If the MS recovery meets the acceptance criteria, system performance is acceptable and analysis of field samples from this disinfected wastewater source may continue. If the MS recovery is unacceptable and the OPR sample result associated with this batch of samples is acceptable, a matrix interference may be causing the poor results. If the MS recovery is unacceptable, all associated field data should be flagged.

9.5.7 Acceptance criteria for MS recovery (Table 2) are based on data from spiked disinfected wastewater matrices and are not appropriate for use with other matrices (e.g., ambient waters).

Table 2. Matrix Spike Precision and Recovery Acceptance Criteria

Performance test	Lab-prepared acceptance criteria	BioBall™ acceptance criteria
Percent recovery for MS	29% - 122%	63% - 110%

9.5.8 Laboratories should record and maintain a control chart comparing MS recoveries for all matrices to batch-specific and cumulative OPR sample results analyzed using Method 1600. These comparisons should help laboratories recognize matrix effects on method recovery and may also help to recognize inconsistent or sporadic matrix effects from a particular source.

9.6 Culture Controls

9.6.1 **Negative controls**—The laboratory should analyze negative controls to ensure that the mEI agar is performing properly. Negative controls should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should perform a negative control every day that samples are analyzed.

9.6.1.1 Negative controls are conducted by filtering a dilute suspension of viable *E. coli* (e.g., ATCC #11775) and analyzing as described in Section 11. Viability of the negative controls should be demonstrated using a non-selective media (e.g., nutrient agar or tryptic soy agar).

9.6.1.2 If the negative control fails to exhibit the appropriate response, check and/or replace the associated media or reagents, and/or the negative control, and reanalyze the appropriate negative control.

9.6.2 **Positive controls**—The laboratory should analyze positive controls to ensure that the mEI agar is performing properly. Positive controls should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should perform a positive control every day that samples are analyzed. An OPR sample (Section 9.4) may take the place of a positive control.

9.6.2.1 Positive controls are conducted by filtering a dilute suspension of viable *E. faecalis* (e.g., ATCC #19433) and analyzing as described in Section 11.

9.6.2.2 If the positive control fails to exhibit the appropriate response, check and/or replace the associated media or reagents, and/or the positive control, and reanalyze the appropriate positive control.

9.6.3 Controls for verification media—All verification media should be tested with appropriate positive and negative controls whenever a new batch of media and/or reagents are used. On an ongoing basis, the laboratory should perform positive and negative controls on the verification media with each batch of samples submitted to verification. Examples of appropriate controls for verification media are provided in **Table 3**.

Table 3. Verification Controls

Medium	Positive Control	Negative Control
Bile esculin agar (BEA)	*E. faecalis*	*E. coli*
Brain heart infusion broth (BHIB) with 6.5% NaCl	*E. faecalis*	*E. coli*
Brain heart infusion broth (BHIB) incubated at 45°C	*E. faecalis*	*E. coli*

9.7 **Colony verification**—The laboratory should verify 10 typical colonies (positive) and 10 atypical colonies (negative) per month or 1 typical colony and 1 atypical colony from 10% of all positive samples, whichever is greater. Verification procedures are provided in Section 12.0.

9.8 **Filter sterility check**—Place at least one membrane filter per lot of filters on a TSA plate, and incubate for 24 ± 2 hours at 35°C ± 0.5°C. Absence of growth indicates sterility of the filter. On an ongoing basis, the laboratory should perform a filter sterility check every day that samples are analyzed.

9.9 **Method blank**—Filter a 50-mL volume of sterile PBS and place the filter on a mEI agar plate and process according to Section 11.0. Absence of growth indicates freedom of contamination from the target organism. On an ongoing basis, the laboratory should perform a method blank every day that samples are analyzed.

9.10 **Filtration blank**—Filter a 50-mL volume of sterile PBS before beginning sample filtrations. Place the filter on a TSA plate, and incubate for 24 ± 2 hours at 35°C ± 0.5°C. Absence of growth indicates sterility of the PBS buffer and filtration assembly.

9.11 **Media sterility check**—The laboratory should test media sterility by incubating one unit (tube or plate) from each batch of medium (TSA, mEI agar, and verification media) as appropriate and observing for growth. Absence of growth indicates media sterility. On an ongoing basis, the laboratory should perform a media sterility check every day that samples are analyzed.

9.12 **Analyst colony counting variability**—Laboratories with two or more analysts should compare each analyst's colony counts from one positive field sample per month. Colony counts should be within 10% between analysts. Laboratories with a single analyst should have that analyst perform duplicate colony counts of a single membrane filter each month. Duplicate colony counts should be within 5% for a single analyst. If no positive field samples are available, a OPR sample may be substituted for these determinations.

10.0 Calibration and Standardization

10.1 Check temperatures in incubators twice daily with a minimum of 4 hours between each reading to ensure operation within stated limits.

10.2 Check thermometers at least annually against a NIST certified thermometer or one that meets the requirements of NIST Monograph SP 250-23. Check mercury columns for breaks.

10.3 Refrigerators used to store media and reagents should be monitored daily to ensure proper temperature control.

11.0 Procedure

11.1 Prepare the mEI agar as directed in Section 7.6.

11.2 Mark the petri dishes and report forms with sample identification and sample volumes.

11.3 Place a sterile membrane filter on the filter base, grid-side up and attach the funnel to the base so that the membrane filter is now held between the funnel and the base.

11.4 Shake the sample bottle vigorously about 25 times to distribute the bacteria uniformly, and measure the desired volume of sample or dilution into the funnel.

11.5 Select sample volumes based on previous knowledge of the enterococci level, to produce 20-60 enterococci colonies on membranes. It is recommended that a minimum of three dilutions be analyzed to ensure that a countable plate (20-60 enterococci colonies) is obtained.

11.6 Smaller sample size or sample dilutions can be used to minimize the interference of turbidity or for high bacterial densities. Multiple volumes of the same sample or sample dilutions may be filtered.

Note: When analyzing smaller sample volumes (e.g., <20 mL), 20-30 mL of PBS or phosphate-buffered dilution water should be added to the funnel or an aliquot of sample should be dispensed into a dilution blank prior to filtration. This will allow even distribution of the sample on the membrane.

11.7 Filter the sample, and rinse the sides of the funnel at least twice with 20-30 mL of sterile buffered rinse water. Turn off the vacuum and remove the funnel from the filter base.

11.8 Use sterile forceps to aseptically remove the membrane filter from the filter base, and roll it onto the mEI Agar to avoid the formation of bubbles between the membrane and the agar surface. Reseat the membrane if bubbles occur. Run the forceps around the edge of the filter outside the area of filtration, close to the edge of the dish, to be sure that the filter is properly seated on the agar. Close the dish, invert, and incubate at 41°C ± 0.5°C for 24 ± 2 hours.

Note: If the medium is prepared in 15 × 60 mm loose lid petri dishes, they should be incubated in a tight fitting container (e.g., plastic vegetable crisper) containing a moistened paper towel to prevent dehydration of the membrane filter and medium.

11.9 After incubation, count and record colonies on those membrane filters containing, if practical, 20-60 colonies ≥0.5 mm in diameter with a blue halo regardless of colony color as an enterococci (see **Figure 1**). *Note:* When measuring colony size do not include the halo. Use magnification for counting and a small fluorescent lamp to give maximum visibility of colonies.

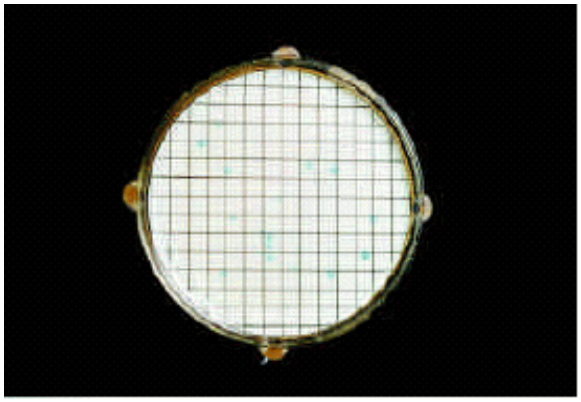

Figure 1. Enterococci colonies on mEI produce blue halos.

12.0 Verification Procedure

12.1 Colonies ≥0.5 mm in diameter of any color having a blue halo after incubation on mEI agar are considered to be "typical" enterococci colonies. Verification of colonies may be required in evidence gathering and it is also recommended as a means of quality control. The verification procedure follows.

Note: When evaluating wastewater using Method 1600, it is recommended that the false negative rate for each matrix be evaluated through biochemical confirmation and results adjusted accordingly, especially if large numbers of atypical colonies are observed in a particular matrix.

12.2 Using a sterile inoculating loop or needle, transfer growth from the centers of at least 10 well-isolated typical and at least 10 well-isolated atypical colonies into a BHIB tube and onto a BHIA slant. Incubate broth for 24 ± 2 hours and agar slants for 48 ± 3 hours at 35°C ± 0.5°C.

12.3 After a 24 hour incubation, transfer a loopful of growth from each BHIB tube to BEA, BHIB, and BHIB with 6.5% NaCl.

12.3.1 Incubate BEA and BHIB with 6.5% NaCl at 35°C ± 0.5°C for 48 ± 3 hours.

12.3.2 Incubate BHIB at 45°C ± 0.5°C for 48 ± 3 hours.

12.4 Observe all verification media for growth.

12.5 After 48 hour incubation, perform a Gram stain using growth from each BHIA slant.

12.6 Gram-positive cocci that grow and hydrolyze esculin on BEA (*i.e.*, produce a black or brown precipitate), and grow in BHIB with 6.5% NaCl at 35°C ± 0.5°C and BHIB at 45°C ± 0.5°C are verified as enterococci.

12.7 Alternately, commercially available multi-test identification systems (e.g., Vitek®) may be used to verify colonies. Such multi-test identification systems should include esculin hydrolysis and growth in 6.5% NaCl.

13.0 Data Analysis and Calculations

Use the following general rules to calculate the enterococci count per 100 mL of sample:

13.1 If possible, select a membrane filter with 20-60 colonies ≥0.5 mm in diameter (regardless of colony color) with a blue halo. Calculate the number of enterococci per 100 mL according to the following general formula:

$$\text{Enterococci / 100 mL} = \frac{\text{Number of enterococci colonies}}{\text{Volume of sample filtered (mL)}} \times 100$$

13.2 See general counting rules in Reference 18.7 (see Appendix B).

13.3 Report results as enterococci per 100 mL of sample.

14.0 Sample Spiking Procedure

14.1 Method 1600 QC requirements (Section 9.0) include the preparation and analysis of spiked reference (PBS) and matrix samples in order to monitor initial and ongoing method performance. For the IPR (Section 9.3), OPR (Section 9.4), and MS (Section 9.5) analyses it is necessary to spike samples with either laboratory-prepared spiking suspensions (Section 14.2) or BioBalls (Section 14.3) as described below.

14.2 Laboratory-Prepared Spiking Suspensions

14.2.1 Preparation

14.2.1.1 Stock Culture. Prepare a stock culture by inoculating a TSA slant (or other non-selective media) with *E. faecalis* ATCC #19433 and incubating at 35°C ± 3°C for 20 ± 4 hours. This stock culture may be stored in the dark at room temperature for up to 30 days.

14.2.1.2 Undiluted Spiking Suspension. Prepare a 1% solution of azide dextrose broth (ADB) by combining 99 mL of sterile phosphate buffered saline and 1 mL of sterile single strength azide dextrose broth in a sterile screw cap bottle or re-sealable dilution water container. From the stock culture of *E. faecalis* ATCC #19433 in Section 14.2.1.1, transfer a small loopful of growth to the 1 % azide dextrose broth solution and vigorously shake a minimum of 25 times. Disperse the inoculum by vigorously shaking the broth culture and incubate at 35°C ± 3°C for 20 ± 4 hours. This culture is referred to as the undiluted spiking suspension and should contain approximately 1.0×10^6 - 1.0×10^7 *E. faecalis* colony forming units (CFU) per mL of culture.

14.2.1.3 Mix the undiluted spiking suspension (Section 14.2.1.2) thoroughly by shaking the bottle a minimum of 25 times and prepare a series of dilutions (4 total) in the following manner:

14.2.1.3.1 Dilution "A"—Aseptically transfer 1.0 mL of the undiluted spiking suspension to 99 mL of sterile PBS and mix thoroughly by shaking the bottle a minimum of 25 times. This is spiking suspension dilution "A" and 1 mL contains 10^{-2} mL of the original undiluted spiking suspension.

14.2.1.3.2 Dilution "B"—Aseptically transfer 1.0 mL of dilution "A" to 99 mL of sterile PBS and mix thoroughly by shaking the bottle a minimum of 25 times. This is spiking suspension dilution "B" and 1 mL contains 10^{-4} mL of the original undiluted spiking suspension.

14.2.1.3.3 Dilution "C"—Aseptically transfer 11.0 mL of dilution "B" to 99 mL of sterile PBS and mix thoroughly by shaking the bottle a minimum of 25 times. This is spiking suspension dilution "C" and 1 mL contains 10^{-5} mL of the original undiluted spiking suspension.

14.2.1.3.4 Dilution "D"—Aseptically transfer 11.0 mL of dilution "C"

to 99 mL of sterile PBS and mix thoroughly by shaking the bottle a minimum of 25 times. This is spiking suspension dilution "D" and 1 mL contains 10^{-6} mL of the original undiluted spiking suspension.

14.2.2 Sample spiking

14.2.2.1 Add 3.0 mL of the spiking suspension dilution "D" (Section 14.2.1.3.4) to 100 mL or PBS or appropriate volume of sample and mix thoroughly by shaking the bottle a minimum of 25 times. The volume of undiluted spiking suspension added to each 100 mL sample is 3.0×10^{-6} mL, which is referred to as $V_{\text{spiked per 100 mL sample}}$ in Section 14.2.4.1 below. Filter the spiked sample and analyze the filter according to the procedures in Section 11.

14.2.3 Enumeration of spiking suspension

14.2.3.1 Prepare TSA spread plates, in triplicate, for spiking suspension dilutions "B", "C", and "D".

Note: Agar plates must be dry prior to use. To ensure that the agar surface is dry, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.

14.2.3.2 Mix dilution "B" by shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution "B" onto the surface of each TSA plate in triplicate.

14.2.3.3 Mix dilution "C" by shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution "C" onto the surface of each TSA plate in triplicate.

14.2.3.4 Mix dilution "D" by shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution "D" onto the surface of each TSA plate in triplicate.

14.2.3.5 Use a sterile bent glass rod or spreader to distribute the inoculum over the surface of plates by rotating the dish by hand or on a turntable.

Note: Ensure that the inoculum is evenly distributed over the entire surface of the plate.

14.2.3.6 Allow the inoculum to absorb into the medium of each plate completely. Invert plates and incubate at 35°C ± 0.5°C for 20 ± 4 hours.

14.2.3.7 Count and record number of colonies per plate. The number of enterococci (CFU / mL) in the undiluted spiking suspension will be calculated using all TSA plates yielding counts within the countable range of 30 to 300 CFU per plate.

14.2.4 Recovery calculations for spiked samples

14.2.4.1 Calculate the concentration of enterococci (CFU / mL) in the undiluted spiking suspension (Section 14.2.1.2) according to the following equation. Example calculations are provided in **Table 4**, below.

$$\textbf{Enterococci}_{\text{undiluted spike}} = (CFU_1 + CFU_2 + ... + CFU_n) / (V_1 + V_2 + ... + V_n)$$

Enterococci $_{\text{undiluted spike}}$ = Enterococci (CFU / mL) in undiluted spiking suspension

Where,

CFU = Number of colony forming units from TSA plates yielding counts within the countable range of 30 to 300 CFU per plate

V = Volume of undiluted sample on each TSA plate yielding counts within the countable range of 30 to 300 CFU per plate

n = Number of plates with counts within the countable range of 30 to 300 CFU per plate

Note: The example calculated numbers provided in the tables below have been rounded at the end of each step for simplification purposes. Generally, rounding should only occur after the final calculation.

Table 4. Example Calculations of Laboratory-prepared Enterococci Spiking Concentration

Examples	CFU / plate (triplicate analyses) from TSA plates			Enterococci CFU / mL in undiluted spiking suspension (Enterococci $_{\text{undiluted spike}}$) [a]
	10^{-5} mL plates	10^{-6} mL plates	10^{-7} mL plates	
Example 1	94, 106, 89	9, 11, 28	1, 0, 4	$(94+106+89) / (10^{-5}+10^{-5}+10^{-5}) =$ $289 / (3.0 \times 10^{-5}) = 9{,}633{,}333 =$ **9.6×10^6 CFU / mL**
Example 2	32, 55, 72	8, 5, 3	0, 0, 0	$(32+55+72) / (10^{-5}+10^{-5}+10^{-5}) =$ $159 / (3.0 \times 10^{-5}) = 5{,}300{,}000 =$ **5.3×10^6 CFU / mL**

[a] Enterococci undiluted spike is calculated using all plates yielding counts within the countable range of 30 to 300 CFU per plate

14.2.4.1 Calculate true concentration of spiked enterococci (CFU / 100 mL) according to the following equation. Example calculations are provided in **Table 5,** below.

$$T_{\text{spiked Enterococci}} = (\textbf{Enterococci}_{\text{undiluted spike}}) \times (V_{\text{spiked per 100 mL sample}})$$

Where,

$T_{\text{spiked Enterococci}}$ = Number of spiked Enterococci (CFU / 100 mL)
$\textbf{Enterococci}_{\text{undiluted spike}}$ = Enterococci (CFU / mL) in undiluted spiking suspension
$V_{\text{spiked per 100 mL sample}}$ = mL of undiluted spiking suspension per 100 mL sample

Table 5. Example Calculations for Determination "True" Spiked Enterococci Concentration

$\textbf{Enterococci}_{\text{undiluted spike}}$	$V_{\text{spiked per 100 mL sample}}$	$T_{\text{spiked Enterococci}}$
9.6×10^6 CFU / mL	3.0×10^{-6} mL per 100 mL of sample	$(9.6 \times 10^6$ CFU / mL) x $(3.0 \times 10^{-6}$ mL / 100 mL) = **28.8 CFU / 100 mL**
5.3×10^6 CFU / mL	3.0×10^{-6} mL per 100 mL of sample	$(2.8 \times 10^6$ CFU / mL) x $(3.0 \times 10^{-6}$ mL / 100 mL) = **8.4 CFU / 100 mL**

14.2.4.2 Calculate percent recovery (R) of spiked enterococci (CFU / 100 mL) according to the following equation. Example calculations are provided in **Table 6**, below.

$$R = 100 \times \frac{(N_s - N_u)}{T}$$

Where,

R = Percent recovery
N_s = Enterococci (CFU / 100 mL) in the spiked sample (Section 13)
N_u = Enterococci (CFU / 100 mL) in the unspiked sample (Section 13)
T = True spiked enterococci (CFU / 100 mL) in spiked sample (Section 14.2.4.1)

Table 6. Example Percent Recovery Calculations for Lab-prepared Spiked Samples

N_s (CFU / 100 mL)	N_u (CFU / 100 mL)	$T_{\text{spiked Enterococci}}$ (CFU / 100 mL)	Percent recovery (R)
42	<1	28.8	100 x (42 - 1) / 28.8 = **142%**
34	10	28.8	100 x (34 - 10) / 28.8 = **83%**
10	<1	8.4	100 x (10 - 1) / 8.4 = **107%**

14.3 BioBall™ Spiking Procedure

14.3.1 Aseptically add 1 BioBall™ to 100 mL of PBS or appropriate volume of sample and mix by vigorously shaking the sample bottle a minimum of 25 times. Analyze the spiked sample according to the procedures in Section 11.

14.3.2 Recovery calculations for samples spiked with BioBalls—Calculate percent recovery (R) of spiked enterococci (CFU / 100 mL) according to the following equation. Example calculations are provided in **Table 7,** below.

$$R = 100 \times \frac{(N_s - N_u)}{T}$$

Where,

R = Percent recovery
N_s = Enterococci (CFU / 100 mL) in the spiked sample (Section 13)
N_u = Enterococci (CFU / 100 mL) in the unspiked sample (Section 13)
T = True spiked enterococci (CFU / 100 mL) in spiked sample based on the lot mean value provided by manufacturer

Table 7. Example BioBall™ Percent Recovery Calculations

N_s (CFU / 100 mL)	N_u (CFU / 100 mL)	T (CFU / 100 mL)	Percent recovery (R)
24	<1	32	100 × (24 - 1) / 32 = **72%**
36	10	32	100 × (36 - 10) / 32 = **81%**

15.0 Method Performance

15.1 Performance Characteristics (Reference 18.4)

15.1.1 Precision - The degree of agreement of repeated measurements of the same parameter expressed quantitatively as the standard deviation or as the 95% confidence limits of the mean computed from the results of a series of controlled determinations. The precision among laboratories for marine water and surface water was 2.2% and 18.9%, respectively.

15.1.2 Bias - The persistent positive or negative deviation of the results from the assumed or accepted true value. The persistent positive or negative deviation of the results from the assumed or accepted true value was not significant.

15.1.3 Specificity - The ability of a method to select and/or distinguish the target bacteria from other bacteria in the same water sample. The specificity characteristic of a method is usually reported as the percent of false positive and false negative results. The specificity for this medium as reported for various environmental water samples was 6.0% false positive and 6.5% false negative.

15.1.4 Multilaboratory variability - A collaborative study was conducted among fourteen collaborators at twelve laboratories to examine the interlaboratory reproducibility of the method. Reproducibility among laboratories (RSD_R) for freshwater, marine water, chlorinated secondary effluent, and non-chlorinated primary effluent ranged from 2.2% for marine water to 18.9% for freshwater with a low enterococcal density.

15.2 Interlaboratory Validation of Method 1600 in Disinfected Wastewater (Reference 18.3)

15.2.1 Twelve volunteer participant laboratories, two enterococci verification laboratories, and two research laboratories participated in the U.S. Environmental Protection Agency's (EPA's) interlaboratory validation study of EPA Method 1600. The purposes of the study were to characterize method performance across multiple laboratories and disinfected wastewater matrices and to develop quantitative quality control (QC) acceptance criteria. A detailed description of the of the study and results are provided in the validation study report (Reference 18.3). Results submitted by laboratories were validated using a standardized data review process to confirm that results were generated in accordance with study-specific instructions and the September 2002 version of EPA Method 1600.

15.2.2 Recovery - Method 1600 was characterized by mean laboratory-specific recoveries of enterococci from disinfected wastewater samples spiked with BioBalls™ ranging from 77.1% to 114.9%, with an overall mean recovery of 90.8%. Mean laboratory-specific recoveries of enterococci from PBS samples spiked with BioBalls ranged from 88.0% to 105.1%, with an overall mean recovery of 95.4%.

15.2.3 Precision - Method 1600 was characterized by laboratory-specific relative standard deviations (RSDs) from disinfected wastewater samples spiked with BioBalls™ ranging from 0% to 69.5%, with an overall pooled, within-laboratory RSD of 22.6%. For PBS samples spiked with BioBalls, laboratory-specific RSDs ranged from 3.1% to 13.7%, with an overall pooled, within-laboratory RSD of 8.1%.

15.2.4 False positive confirmation rates - Method 1600 laboratory-specific false positive confirmation rates for unspiked disinfected/secondary results combined, ranging from 0.0% to 10.0%. For secondary wastewater (excluding disinfected results), only 2 of 123 typical colonies submitted to verification were non-enterococci, resulting in a false positive confirmation rate of 1.6%. For disinfected wastewater (excluding secondary results), none of the 66 typical colonies submitted to verification were non-enterococci, resulting in a false positive confirmation rate of 0.0%. Since all 2184 typical colonies observed during the study could not be submitted to confirmation, the percent of total colonies that would have resulted in a false positive result was estimated (see Table 6, Reference 18.3). It is estimated that 0.0% and 1.2% of the total colonies would have resulted in a false positive for disinfected wastewater and secondary wastewater, respectively.

15.2.5 False negative rates - Method 1600 laboratory-specific false negative rates laboratory-specific false negative confirmation rates for unspiked disinfected/secondary results combined, ranged from 28.6% to 100.0%. For secondary wastewater (excluding disinfected results), 62 of 79 atypical colonies submitted to verification were identified as enterococci, resulting in a false negative confirmation rate of 78.5% for secondary wastewater. For disinfected wastewater (excluding secondary results), eight of eight atypical colonies submitted to verification were identified as enterococci, resulting in a false negative confirmation rate of 100.0% for disinfected wastewater. Since all 839 atypical colonies observed during the study could not be submitted to confirmation, the percent of total colonies that would have resulted in a false negative result was estimated. It is estimated that 21.2% and 22.8% of the total colonies would have resulted in a false negative for disinfected wastewater and secondary wastewater, respectively. The false positive and negative assessments are provided in **Table 8**.

Table 8. False Positive and False Negative Assessment for Unspiked Disinfected and Unspiked Secondary Wastewater Effluents

Matrix	Total colonies		False positive (FP) assessment				False negative (FN) assessment			
	Typical	Atypical	Typical colonies submitted	No. FP colonies	FP confirmation rate (%) [a]	Estimated % of total colonies that would have been a FP [b]	Atypical colonies submitted	No. FN colonies	FN confirmation rate (%) [c]	Estimated % of total colonies that would have been a FN [d]
Disinfected	391	105	66	0	0.0	0.0	8	8	100.0	21.2
Secondary	1793	734	123	2	1.6	1.2	79	62	78.5	22.8
Disinfected + Secondary	2184	839	189	2	1.1	0.8	87	70	80.5	22.3

[a] False positive confirmation rate = number of false positive colonies / number of typical colonies submitted

[b] Percent of total colonies estimated to be false positives = [(total typical colonies FP confirmation rate) / (total number of typical and atypical colonies observed); e.g., [(1793 ×(2/123))/(1793+734)] × 100 = 1.2%

[c] False negative confirmation rate = number of false negative colonies / number of atypical colonies submitted

[d] Percent of total colonies estimated to be false negatives = [(total atypical colonies* FN confirmation rate) / (total number of typical and atypical colonies observed)] × 100; e.g., [(734 ×(62/79))/(1793+734)] × 100 = 22.8%

15.2.6 During evaluation of the study results, it was noted that many of the false negatives (atypical colonies submitted to verification which were identified as enterococci) were pink to red in color but simply lacked a blue halo. The predecessor to EPA Method 1600 for enterococci is EPA Method 1106.1 which uses mE and EIA media. For EPA Method 1106.1, pink to red colonies on mE, which produce a brown precipitate after transfer to EIA are considered positive for enterococci. Tetrazolium chloride (TTC), the reagent responsible for producing pink to red enterococci colonies on mE, is also included as a reagent in mEI. A follow-on study was conducted, for which pink to red colonies without halos from unspiked secondary wastewaters were submitted to verification. For pink to red colonies without halos that were ≥0.5 mm colony size, 54 of 90 colonies submitted were identified as enterococci, resulting in a 60.0% verification rate.

Results of the verification analyses from the initial study were assessed with pink to red colonies without halos being counted as enterococci. When pink to red colonies without halos are counted as enterococci, the estimated percent of total colonies that would have resulted in false positives increases slightly from 0.8% to 2.7%, for combined disinfected and secondary results. More importantly, the estimated percent of total colonies that would have resulted in false negatives decreased from 22.3% to 7.0% for combined disinfected and secondary results and from 21.2% to 2.9% for disinfected wastewater. The re-assessment of false positive and false negative initial study results with pink to red colonies without halos counted as enterococci are provided in **Table 9**.

Table 9. Re-Assessment of False Positive and False Negative Initial Study Results with Pink to Red Colonies without Halos Counted as Enterococci

Matrix (sample no.)	Total colonies		False positive (FP) assessment				False negative (FN) assessment			
	Typical	Atypical	Typical colonies submitted	No. FP colonies	FP confirmation rate (%) [a]	Estimated % of total colonies that would have been a FP [b]	Atypical colonies submitted	No. FN colonies	FN confirmation rate (%) [c]	Estimated % of total colonies that would have been a FN [d]
Disinfected (Samples 1-4)	477	19	69	0	0.0	0.0	4	3	75.0	2.9
Secondary (Samples 5, 6)	2291	236	166	7	4.2	3.8	32	27	84.4	7.9
Disinfected & Secondary (Samples 1-6)	2768	255	235	7	3.0	2.7	36	30	83.3	7.0

a False positive confirmation rate = number of false positive colonies / number of typical colonies submitted

b Percent of total colonies estimated to be false positives = [(total typical colonies × FP confirmation rate) / (total number of typical and atypical colonies observed)] × 100; e.g., [(2291×(7/166))/(2291+236)] × 100 = 3.8%

c False negative confirmation rate = number of false negative colonies / number of atypical colonies submitted

d Percent of total colonies estimated to be false negatives = [(total atypical colonies × FN confirmation rate) / (total number of typical and atypical colonies observed)] × 100; e.g., [(236 ×(27/32))/(2291+236)] × 100 = 7.9%

16.0 Pollution Prevention

16.1 The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.

16.2 Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

17.0 Waste Management

17.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the biohazard and hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.

17.2 Samples, reference materials, and equipment known or suspected to have viable enterococci attached or contained must be sterilized prior to disposal.

17.3 Samples preserved with HCl to pH <2 are hazardous and must be neutralized before being disposed, or must be handled as hazardous waste.

17.4 For further information on waste management, consult "The Waste Management Manual for Laboratory Personnel" and "Less Is Better: Laboratory Chemical Management for Waste Reduction," both available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street NW, Washington, DC 20036.

18.0 References

18.1 Cabelli, V. J., A. P. Dufour, M. A. Levin, L. J. McCabe, and P. W. Haberman, 1979. *Relationship of Microbial Indicators to Health Effects at Marine Bathing Beaches.* Amer. Jour. Public Health. 69:690-696.

18.2 Dufour, A.P. 1984. *Health Effects Criteria for Fresh Recreational Waters,* EPA-600/1-84-004. Office of Research and Development, USEPA.

18.3 USEPA. 2004. *Results of the Interlaboratory Validation of EPA Method 1600 (mEI) for Enterococci in Wastewater Effluent.* December 2004. EPA 821-R-04-008.

18.4 Messer, J.W. and A.P. Dufour. 1998. *A Rapid, Specific Membrane Filtration Procedure for Enumeration of Enterococci in Recreational Water*. Appl. Environ. Microbiol. 64:678-680.

18.5 ACS. 2000. *Reagent Chemicals, American Chemical Society Specifications*. American Chemical Society, New York. For suggestions of the testing of reagents not listed by the American Chemical Society, see *AnalaR Standards for Laboratory Chemicals*, BDH, Poole, Dorset, UK and the United States Pharmacopeia.

18.6 APHA. 1998. *Standard Methods for the Examination of Water and Wastewater*. 20th Edition. American Public Health Association, Washington D.C.

18.7 Bordner, R., J.A. Winter, and P.V. Scarpino (eds.). *Microbiological Methods for Monitoring the Environment: Water and Wastes*, EPA-600/8-78-017. Cincinnati, OH: U.S. Environmental Protection Agency, 1978.

Appendix A:
Part II (General Operations), Section A (Sample Collection, Preservation, and Storage)

Sample Collection[1]

1.0 Sample Containers

1.1 **Sample Bottles:** bottles must be resistant to sterilizing conditions and the solvent action of water. Wide-mouth borosilicate glass bottles with screw-cap or ground-glass stopper or heat-resistant plastic bottles may be used if they can be sterilized without producing toxic materials (see examples A and C in Figure 1). Screw-caps must not produce bacteriostatic or nutritive compounds upon sterilization.

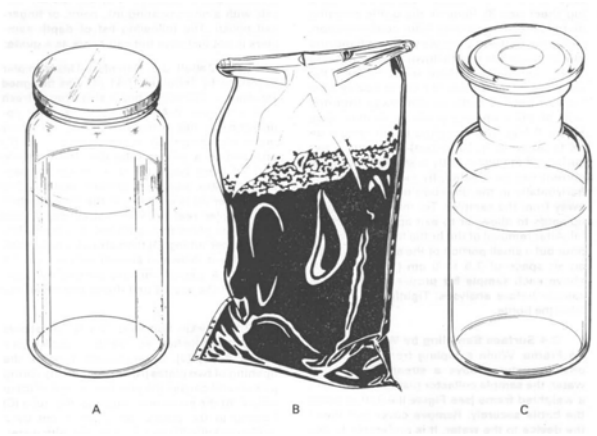

Figure 1. Suggested sample containers.

1.2 **Selection and Cleaning of Bottles:** Samples bottles should be at least 125 mL volume for adequate sampling and for good mixing. Bottles of 250 mL, 500 mL, and 1000 mL volume are often used for multiple analyses. Discard bottles which have chips, cracks, and etched surfaces. Bottle closures must be water-tight. Before use, thoroughly cleanse bottles and closures with detergent and hot water, followed by a hot water rinse to remove all trace of detergent. Then rinse them three times with laboratory-pure water.

1.3 **Dechlorinating Agent:** The agent must be placed in the bottle when water and wastewater samples containing residual chlorine are anticipated. Add sodium thiosulfate to the bottle before sterilization at a concentration of 0.1 mL of a 10% solution for each 125 mL sample volume. This concentration will neutralize approximately 15 mg/L of residue chlorine.

1.4 **Chelating Agent:** A chelating agent should be added to sample bottles used to collect samples suspected of containing >0.01 mg/L concentrations of heavy metals such as copper, nickel or zinc, etc. Add 0.3 mL of a 15% solution of ethylenediaminetetraacetic acid (EDTA) tetrasodium salt, for each 125 mL sample volume prior to sterilization.

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[1]The text is taken from Part II, Section A, of the EPA publication "Microbiological Methods for Monitoring the Environment" EPA-600/8-78-017, December 1978.

1.5 **Wrapping Bottles:** Protect the tops and necks of glass stoppered bottles from contamination by covering them before sterilization with aluminum foil or kraft paper.

1.6 **Sterilization of Bottles:** Autoclave glass or heat-resistant plastic bottles at 121°C for 15 minutes. Alternatively, dry glassware may be sterilized in a hot oven at 170°C for not less than two hours. Ethylene oxide gas sterilization is acceptable for plastic containers that are not heat-resistant. Sample bottles sterilized by gas should be stored overnight before being used to allow the last traces of gas to dissipate.

1.7 **Plastic Bags:** The commercially available bags (Whirl-pak) (see example B in Figure 1) are a practical substitute for plastic or glass samples bottles in sampling soil, sediment, or biosolids. The bags are sealed in manufacture and opened only at time of sampling. The manufacturer states that such bags are sterilized.

2.0 Sampling Techniques

Samples are collected by hand or with a sampling device if the sampling site has difficult access such as a bridge or bank adjacent to a surface water.

2.1 **Chlorinated Samples:** When samples such as treated waters, chlorinated wastewaters or recreational waters are collected, the sample bottle must contain a dechlorinating agent (see section 1.3 above).

2.2 **Composite Sampling:** In no case should a composite sample be collected for bacteriologic examination. Data from individual samples show a range of values. A composite sample will not display this range. Individual results will give information about industrial process variations in flow and composition. Also, one or more portions that make up a composite sample may contain toxic or nutritive materials and cause erroneous results.

2.3 **Surface Sampling by Hand:** A grab sample is obtained using a sample bottle prepared as described in (1) above. Identify the sampling site on the bottle label and on a field log sheet. Remove the bottle covering and closure and protect from contamination. Grasp the bottle at the base with one hand and plunge the bottle mouth down into the water to avoid introducing surface scum (Figure 2). Position the mouth of the bottle into the current away from the hand of the collector and, if applicable, away from the side of the sampling platform. The sampling depth should be 15-30 cm (6-12 inches) below the water surface. If the water body is static, an artificial current can be created, by moving the bottle horizontally in the direction it is pointed and away from the sampler. Tip the bottle slightly upwards to allow air to exit and the bottle to fill. After removal of the bottle from the stream, pour out a small portion of the sample to allow an air space of 2.5-5 cm (1-2 inches) above each sample for proper mixing of the sample before analyses. Tightly stopper the bottle and place on ice (do not freeze) for transport to the laboratory.

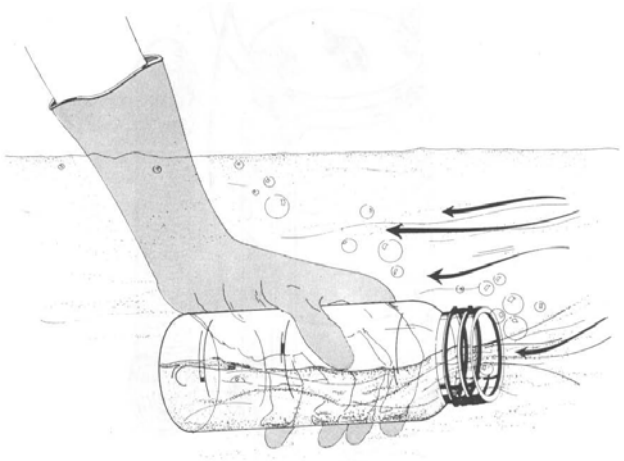

Figure 2. Grab sampling technique for surface waters.

3.0 Selection of Sampling Sites and Frequency

These will be described for streams, rivers, estuarine, marine, and recreational waters as well as domestic and industrial wastewaters.

3.1 **Stream Sampling:** The objectives of the initial survey dictate the location, frequency and number of samples to be collected.

3.1.1 Selection of Sampling Sites: A typical stream sampling program includes sampling locations upstream of the area of concern, upstream and downstream of waste discharges, upstream and downstream from tributary entrances to the river and upstream of the mouth of the tributary. For more complex situations, where several waste discharges are involved, sampling includes sites upstream and downstream from the combined discharge area and samples taken directly from each industrial or municipal waste discharge. Using available bacteriological, chemical and discharge rate data, the contribution of each pollution source can be determined.

3.1.2 Small Streams: Small streams should be sampled at background stations upstream of the pollution sources and at stations downstream from pollution sources. Additional sampling sites should be located downstream to delineate the zones of pollution. Avoid sampling areas where stagnation may occur (e.g., backwater of a tributary) and areas located near the inside bank of a curve in the stream which may not be representative of the main channel.

3.1.3 Large Streams and Rivers: Large streams are usually not well mixed laterally for long distances downstream from the pollution sources. Sampling sites below point source pollution should be established to provide desired downstream travel time and dispersal as determined by flow rate measurements. Particular care must be taken to establish the proper sampling points. Occasionally, depth samples are necessary to determine vertical mixing patterns.

3.2 **Estuarine and Marine Sampling**: Sampling estuarine and marine waters requires the consideration of other factors in addition to those usually recognized in fresh water sampling. They include tidal cycles, current patterns, bottom currents and counter-currents, stratification, seasonal fluctuations, dispersion of discharges and multi-depth samplings.

The frequency of sampling varies with the objectives. When a sampling program is started, it may be necessary to sample every hour around the clock to establish pollution loads and dispersion patterns. The sewage discharges may occur continuously or intermittently.

When the sampling strategy for a survey is planned, data may be available from previous hydrological studies done by the Coast Guard, Corps of Engineers, National Oceanic and Atmospheric Administration (NOAA), U.S. Geological Survey, or university and private research investigations. In a survey, float studies and dye studies are often carried out to determine surface and undercurrents. Initially depth samples are taken on the bottom and at five feet increments between surface and bottom. A random grid pattern for selecting sampling sites is established statistically.

3.2.1 Estuarine Sampling: When a survey is made on an estuary, samples are often taken from a boat, usually making an end to end traverse of the estuary. Another method involves taking samples throughout a tidal cycle, every hour or two hours from a bridge or from an anchored boat at a number of fixed points.

In a large bay or estuary where many square miles of area are involved, a grid or series of stations may be necessary. Two sets of samples are usually taken from an area on a given day, one at ebb or flood slack water, and the other three hours earlier, or later, at the half tidal interval. Sampling is scheduled so that the mid-sampling time of each run coincides with the calculated occurrence of the tidal condition.

In location sampling sites, one must consider points at which tributary waters enter the main stream or estuary, location of shellfish beds and bathing beaches. The sampling stations can be adjusted as data accumulate. For example, if a series of stations half mile apart consistently show similar values, some of these stations may be dropped and other stations added in areas where data shows more variability.

Considerable stratification can occur between the salt water from the sea and the fresh water supplied by a river. It is essential when starting a survey of an unknown estuary to find out whether there is any marked stratification. This can be done by chloride determinations at different locations and depths. It is possible for stratification to occur in one part of an estuary and not in another.

On a flood tide, the more dense salt water pushing up into the less dense fresh river water will cause an overlapping with the fresh water flowing on top. A phenomenon called a salt water wedge can form. As a result, stratification occurs. If the discharge of pollution is in the salt water layer, the contamination will be concentrated near the bottom at the flood tide. The flow or velocity of the fresh water will influence the degree of stratification which occurs. If one is sampling only at the surface, it is possible that the data will not show the polluted underflowing water which was contaminated at the point below the fresh water river. Therefore, where stratification is suspected, samples at different depths will be needed to measure vertical distribution.

3.2.2 Marine Sampling: In ocean studies, the environmental conditions are most diverse along the coast where shore, atmosphere and the surf are strong influences. The shallow coastal waters are particularly susceptible to daily fluctuations in temperature and seasonal changes.

Sampling during the entire tidal cycle or during a half cycle may be required. Many ocean studies such as sampling over the continental shelf involve huge areas and no two areas of water are the same.

Selection of sampling sites and depths are most critical in marine waters. In winter, cooling of coastal waters can result in water layers which approach 0°C. In summer, the shallow waters warm much faster than the deeper waters. Despite the higher temperature, oxygen concentrations are higher in shallow than in deeper waters due to greater water movement, surf action and photosynthetic activity from macrophytes and the plankton.

Moving from the shallow waters to the intermediate depths, one observes a moderation of these shallow water characteristics. In the deeper waters, there is a marked stabilization of conditions. Water temperatures are lower and more stable. There is limited turbulence, little penetration of light, sparse vegetation and the ocean floor is covered with a layer of silts and sediments.

3.3 Recreational Waters (Bathing Beaches): Sampling sites at bathing beaches or other recreational areas should include upstream or peripheral areas and locations adjacent to natural drains that would discharge stormwater, or run-off areas draining septic wastes from restaurants, boat marinas, or garbage collection areas. Samples of bathing beach water should be collected at locations and times of heaviest use. Daily sampling, preferably in the afternoon, is the optimum frequency during the season. Weekends and holidays which are periods of highest use must be included in the sampling program. Samples of estuarine bathing waters should be obtained at high tide, ebb tide and low tide in order to determine the cyclic water quality and deterioration that must be monitored during the swimming season.

3.4 Domestic and Industrial Waste Discharges: It is often necessary to sample secondary and tertiary wastes from municipal waste treatment plants and various industrial waste treatment operations. In situations where the plant treatment efficiency varies considerably, grab samples are collected around the clock at selected intervals for a three to five day period. If it is known that the process displays little variation, fewer samples are needed. In no case should a composite sample be collected for bacteriological examination. The National Pollution Discharge Elimination System (NPDES) has established wastewater treatment plant effluent limits for all dischargers. These are often based on maximum and mean values. A sufficient number of samples must be collected to satisfy the permit and/or to provide statistically sound data and give a fair representation of the bacteriological quality of the discharge.

Appendix B:
Part II (General Operations), Sections C.3.5 (Counting Colonies) and C.3.6 (Calculation of Results)

Counting Colonies[1]

1.0 Counting Colonies

Colonies should be counted using a fluorescent lamp with a magnifying lens. The flourescent lamp should be nearly perpendicular to the membrane filter. Count colonies individually, even if they are in contact with each other. The technician must learn to recognize the difference between two or more colonies which have grown into contact with each other and single, irregularly shaped colonies which sometimes develop on membrane filters. The latter colonies are usually associated with a fiber or particulate material and the colonies conform to the shape and size of the fiber or particulates. Colonies which have grown together almost invariably show a very fine line of contact.

2.0 Calculation of Results

2.1 Select the membrane filter with the number of colonies in the acceptable range and calculate count per 100 mL according to the general formula:

Count per 100 mL = (No. of colonies counted/Volume of sample filtered, in mL) x 100

2.2 Counts Within the Acceptable Limits

The acceptable range of colonies that are countable on a membrane is a function of the method. Different methods may have varying acceptable count ranges. All examples in this appemdix assume that the acceptable range of counts is between 20-60 colonies per membrane.

For example, assume that filtration of volumes of 50, 15, 5, 1.5, and 0.5 mL produced colony counts of 200, 110, 40, 10, and 5, respectively.

An analyst would not actually count the colonies on all filters. By inspection the analyst would select the membrane filter with the acceptable range of target colonies, as defined by the method, and then limit the actual counting to such membranes.

After selecting the best membrane filter for counting, the analyst counts colonies and applies the general formula as in section 2.1 above to calculate the count/100 mL.

2.3 More Than One Acceptable Count

2.3.1 If there are acceptable counts on replicate plates, carry counts independently to final reporting units, then calculate the arithmetic mean of these counts to obtain the final reporting value.

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[1]The text is largely taken from Part II, Section C, of the EPA publication "Microbiological Methods for Monitoring the Environment" EPA-600/8-78-017, December 1978. Some examples were kindly provided by Kristen Brenner, US EPA.

Example, if the counts are 24 and 36 for replicate plates of 100 mL each, then the arithmetic mean is calculated as follows:

$$\frac{(24\ \text{CFU}/100\ \text{mL} + 36\ \text{CFU}/100\ \text{mL})}{2} = 30\ \text{CFU}/\ 100\ \text{mL}$$

2.3.2 If there is more than one dilution having an acceptable range of counts, independently carry counts to final reporting units, then average for final reported value.

For example, if volumes of 100, 10, 1 and 0.1 mL produced colony counts of Too Numerous To Count (TNTC), 55, 30, and 1, respectively, then two volumes, 10 mL and 1 mL, produced colonies in the acceptable counting range.

Independently carry each MF count to a count per 100 mL:

$$\frac{55}{10} \times 100 = 550\ \text{CFU}/100\ \text{mL}$$

and

$$\frac{30}{1} \times 100 = 3000\ \text{CFU}/100\ \text{mL}$$

Calculate the arithmetic mean as in section 2.3.1 above:

$$\frac{(550\ \text{CFU}/100\ \text{mL} + 3000\ \text{CFU}/100\ \text{mL})}{2} = 1775\ \text{CFU}/100\ \text{mL}$$

Report this as 1775 CFU/100 mL.

2.4 If all MF counts are below the lower acceptable count limit, select the most nearly acceptable count.

2.4.1 For example, sample volumes of 100, 10 and 1 mL produced colony counts of 17, 1 and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of the most nearly acceptable plate count, 17, and report as 17 CFU/100 mL.

Note that in this case, because no calculations were done (i.e. this is the count for 100 mL), the count is reported as 17 CFU/100 mL rather than an "estimated count of 17 CFU/100 mL"

2.4.2 As a second example, assume a count in which sample volumes of 10 and 1 mL produced colony counts of 18 and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of the most nearly acceptable plate count, 18, and calculate as in section 2.3.2 above.

$$\frac{18}{10} \times 100 = 180 \text{ CFU /100 mL}$$

Report this as an estimated count of 180 CFU/100 mL.

2.5 If counts from all membranes are zero, calculate using count from largest filtration volume.

For example, sample volumes of 25, 10, and 2 mL produced colony counts of 0, 0, and 0, respectively, and no actual calculation is possible, even as an estimated report. Calculate the number of colonies per 100 mL that would have been reported if there had been one colony on the filter representing the largest filtration volume. In this example, the largest volume filtered was 25 mL and thus the calculation would be:

$$\frac{1}{25} \times 100 = 4 \text{ CFU /100 mL}$$

Report this as < (less than) 4 CFU/100 mL.

2.6 If all membrane counts are above the upper acceptable limit, calculate count using the smallest volume filtered.

For example, assume that the volumes 1, 0.3, and 0.01 mL produced colony counts of TNTC, 150, and 110 colonies, respectively. Since all colony counts are above the acceptable limit, use the colony count from the smallest sample volume filtered and estimate the count as:

$$\frac{110}{0.01} \times 100 = 1{,}100{,}000 \text{ CFU /100 mL}$$

Report this as estimated count 1.1×10^6 CFU/100 mL

2.7 If typical colonies are too numerous to count (TNTC), use upper limit count with smallest filtration volume.

For example, assume that the volumes 1, 0.3, and 0.01 mL all produced too many typical colonies, and that the laboratory bench record indicated TNTC.

Use the upper acceptable count for the method (60 colonies in this example) as the basis of calculation with the smallest filtration volume and estimate the count as:

$$\frac{60}{0.01} \times 100 = 600{,}000 \text{ CFU /100 mL}$$

Report this as > (greater than) 6×10^5 CFU/100 mL

2.8 If colonies are both above and below the upper and lower acceptable limits (i.e., no counts are within the acceptable limits), select the most nearly acceptable count.

2.8.1 For example, sample volumes of 100, 10 and 1 mL produced colony counts of 64, 6 and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of the most nearly acceptable plate count, 64, and report as 64 CFU/100 mL.

Note that in this case, because no calculations were done (i.e. this is the count for 100 mL), the count is reported as 64 CFU/100 mL rather than an "estimated count of 64 CFU/100 mL"

2.8.2 As a second example, assume a count in which sample volumes of 100, 10 and 1 mL produced colony counts of 98, 18, and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of the most nearly acceptable plate count, 18, and calculate as in section 2.3.2 above.

$$\frac{18}{10} \times 100 = 180 \text{ CFU /100 mL}$$

Report this as estimated count 180 CFU/100 mL.

2.9 If there is no result because of a confluent growth, > 200 atypical colonies (TNTC), lab accident, etc., report as No Data and specify the reason.

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Designation: D1253 – 08

StandardTest Method for Residual Chlorine in Water[1]

This standard is issued under the fixed designation D1253; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method covers the determination of residual chlorine in water by direct amperometric titration.

1.2 Within the constraints specified in Section 6, this test method is not subject to commonly encountered interferences and is applicable to most waters. Some waters, however, can exert an iodine demand, usually because of organic material, making less iodine available for measurement by this test method. Thus, it is possible to obtain falsely low chlorine readings, even though the test method is working properly, without the user's knowledge.

1.3 Precision data for this test method were obtained on estuary, inland main stem river, fresh lake, open ocean, and fresh cooling tower blowdown water. Bias data could not be determined because of the instability of solutions of chlorine in water. It is the user's responsibility to ensure the validity of the test method for untested types of water.

1.4 In the testing by which this standard was validated, the direct and back starch-iodide titrations and the amperometric back titration, formerly part of this standard, were found to be unworkable and were discontinued in 1986. Historical information is presented in Appendix X1.

NOTE 1—Orthotolidine test methods have been omitted because of poor precision and accuracy.

1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1129 Terminology Relating to Water
D1193 Specification for Reagent Water
D2777 Practice for Determination of Precision and Bias of Applicable Test Methods of Committee D19 on Water
D3370 Practices for Sampling Water from Closed Conduits
D5847 Practice for Writing Quality Control Specifications for Standard Test Methods for Water Analysis

3. Terminology

3.1 *Definitions:* For definitions of terms used in this test method, refer to Terminology D1129.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *combined residual chlorine, n*—residual consisting of chlorine combined with ammonia nitrogen or nitrogenous compounds.

3.2.2 *free available chlorine residual, n*—residual consisting of hypochlorite ions, hypochlorous acid, or a combination thereof.

3.2.3 *total residual chlorine (chlorine residual), n*—the amount of available chlorine-induced oxidants present in water at any specified period, subsequent to the addition of chlorine.

NOTE 2—Chlorine present as chloride is neither included in these terms nor determined by this test method.

NOTE 3—Bromine, bromine combined with ammonia or nitrogenous compounds, and chlorine dioxide are not distinguished by this test method from the corresponding chlorine compounds.

4. Summary of Test Method

4.1 This is an amperometric titration test method utilizing phenylarsine oxide as the titrant. When the titrator cell is immersed in a sample containing chlorine, current is generated. As phenylarsine oxide is added, the chlorine is reduced and the generation of current ceases. When chlorine is present as a chloramine, potassium iodide is added, releasing iodine, which

[1] This test method is under the jurisdiction of ASTM Committee D19 on Water and is the direct responsibility of Subcommittee D19.05 on Inorganic Constituents in Water.

Current edition approved Oct. 1, 2008. Published October 2008. Originally approved in 1953. Last previous edition approved in 2003 as D1253 – 03. DOI: 10.1520/D1253-08.

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

***A Summary of Changes section appears at the end of this standard**

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is titrated in a similar manner. The iodine content is calculated in terms of free chlorine.

5. Significance and Use

5.1 Chlorine is used to destroy or deactivate a variety of unwanted chemicals and microorganisms in water and wastewater.

5.2 An uncontrolled excess of chlorine in water, whether free available or combined, can adversely affect the subsequent use of the water.

6. Interferences

6.1 This test method is not subject to interferences from temperature, color, or turbidity of sample.

6.2 Values of pH above 8.0 interfere by slowing the reaction rate. Buffering the sample to pH 7.0 or less eliminates the interference.

6.3 Erratic behavior of the apparatus in the presence of cupric ions has been reported.

6.4 Cuprous and silver ions tend to poison the electrode of the titrator.

6.5 Nitrogen trichloride and some N-chloro compounds are often present as products of the chlorination of wastewaters and will titrate partially as free available chlorine and partially as combined residual chlorine. This error can be avoided only in the determination of total residual chlorine.

6.6 Exposure to high concentrations of free available chlorine causes a film-type polarization that reverses very slowly. This can be avoided by diluting the sample with water to less than 10 mg/L of free available chlorine.

6.7 If chlorine dioxide is present, an unknown portion titrates as free available chlorine. Total chlorine dioxide titrates as total residual chlorine.

6.8 Depending upon final pH, chlorination of waters containing ammonia or nitrogenous organic compounds can produce high concentrations of dichloramine. This compound produces four to five times as much current as monochloramine. The current produced by as little as 5 mg/L of dichloramine can cause the microammeter pointer to read offscale even at the end point in the titration of free available chlorine. This may be overcome by use of an opposing voltage in the apparatus' circuitry. The instrument's manufacturer should be consulted in this regard.

7. Apparatus

7.1 *Amperometric Titration Apparatus*[3,4]—Refer to Fig. 1.

NOTE 4—When the titrator has been out of service for a day or more, check the electrode for sensitivity by noting the rapidity of the pointer deflection. If the pointer responds slowly after the addition of KI solution, add a small amount of biiodate. If it responds slowly to free available chlorine, sensitize it by adding chlorine.

[3] *Water and Sewage Works*, May 1949, p. 171, and *Journal American Water Works Association*, Vol 34, 1942, pp. 1227–1240.

[4] Amperometric titrators are available commercially from most laboratory supply houses.

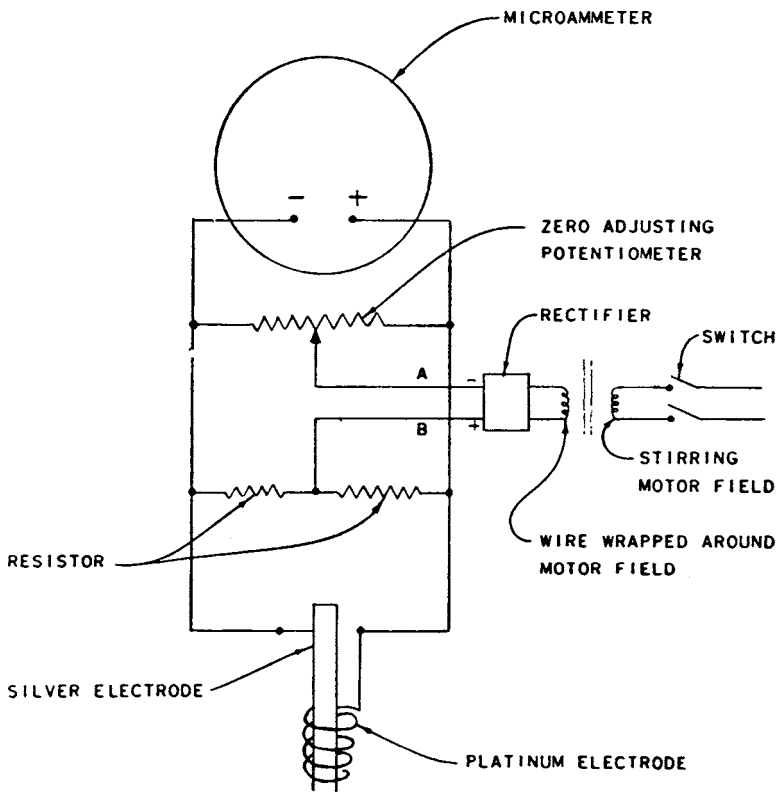

FIG. 1 Wiring Diagram of Amperometric Titrator

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7.2 *Glassware*—Condition with water containing at least 10 mg/L of residual chlorine for at least 2 h prior to use and then rinse thoroughly.

8. Reagents and Materials

8.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society.[5] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

8.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D1193, Type III, further treated to be free of chlorine demand. Other reagent water types (Type I) may be used provided it is first ascertained that the water is of sufficiently high purity to permit its use without adversely affecting the bias and precision of the test method. Type III water was specified at the time of round robin testing of this method. A suggested method for preparation of chlorine demand-free water is to add approximately 20 mg/L of available chlorine to Type III water, let it stand for about a week in darkness, and then expose it to sunlight until no chlorine remains. Filtration through a carbon filter is an alternative process which requires less time to remove chlorine.

8.3 *pH 4.0 Buffer Solution*—Dissolve 243 g of sodium acetate trihydrate and 480 g of glacial acetic acid in water and dilute to 1 L.

8.4 *pH 7.0 Buffer Solution*—Dissolve 25.4 g of monobasic potassium phosphate and 86 g of dibasic sodium phosphate in water and dilute to 1 L.

8.5 *Biiodate, Solution Standard* (0.0282*N*)—Dissolve 0.9163 g of potassium biiodate in water and dilute to 1 L in a volumetric flask. Store in an amber glass-stoppered bottle.

8.6 *Phenylarsine Oxide, Solution Standard* (0.00564 *N*)—Dissolve 0.8 g of phenylarsine oxide in 150 mL of sodium hydroxide solution (12 g/L). After settling, decant 110 mL of this solution, add 800 mL of water, and bring to a pH of 9.0 by adding hydrochloric acid (1 + 1). This should require about 2 mL of HCl (1 + 1). Continue acidification with HCl (1 + 1) until a pH of 6 to 7 is reached, as indicated by a glass-electrode system; then dilute to a total volume of 1L. Standardize to 0.00564 *N* against 0.0282 *N* biiodate solution using the titrator (7.1) as the end-point indicator. Add 1 mL of chloroform for preservation.

8.7 *Potassium Iodide Solution* (50 g/L)—Dissolve 50 g of KI in water and dilute to 1 L. Add 1 g of sodium bicarbonate to stabilize the solution. Store in an amber bottle and avoid direct exposure to sunlight.

9. Sampling

9.1 Collect the sample in accordance with Practices D3370. Take care that the sample is representative and keep it away from direct sunlight prior to analysis.

9.2 All tests should be made as soon as possible after collection of the sample (not more than 5 min) because the residual chlorine may diminish with time, due to the chlorine demand of the sample. Where time of contact is important, the elapsed time between the addition of chlorine and the determination of chlorine should be taken into account.

10. Procedure

10.1 For residual chlorine concentrations of 2.0 mg/L or less, use a 200-mL sample. For greater concentrations, use a 100-mL sample. It is preferable that the size of the sample be such that not more than 2 mL of titrant will be required to complete the titration.

10.2 *Determination of Total Residual Chlorine:*

10.2.1 Add 1 mL of KI solution to a 200-mL sample and immediately add 1 mL of pH 4.0 buffer solution.

10.2.2 Immerse the electrodes in the sample and start the stirrer. Adjust the microammeter pointer of the potentiometer to the right or high current side of the scale so the pointer can deflect counterclockwise during the analysis.

10.2.3 Titrate using standard phenylarsine oxide solution, adding the titrant in small increments, and noting the deflection of the microammeter pointer. Plot the progress of the titration on linear graph paper with current on the vertical axis and titrant volume on the horizontal axis. Add a small volume of titrant, wait a few seconds, and plot the current-volume point on the graph.

10.2.4 Readjust the potentiometer several times during the titration, if necessary, to bring the pointer back on scale.

10.2.5 Continue the analysis by determining at least three points spread over the downward sloping titration curve and at least three points after the equivalence or end point. The latter points will indicate practically no change in current. Points just before the end point shall be disregarded in its determination. The millilitres of titrant at the end point defined by the intersection of the two linear sections of the titration curve should be recorded.

10.3 *Determination of Free Available Chlorine Residual:*

10.3.1 Add 1 mL of pH 7.0 buffer solution to a 200-mL sample.

10.3.2 Repeat the phenylarsine oxide titration beginning with 10.2.2.

10.3.3 Note a rapid deflection of the pointer for each increment of titrant indicates the presence of free available chlorine. Slight counterclockwise movements of the pointer after addition of individual drops of titrant is a drift effect and does not indicate the presence of free available chlorine.

10.4 *Determination of Combined Available Chlorine Residual:*

[5] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For Suggestions on the testing of reagents not listed by the American Chemical Society, see *Annual Standards for Laboratory Chemicals,* BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary,* U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

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10.4.1 Complete the titration for the determination of free available chlorine residual as in 10.3.

10.4.2 To the same sample, add 1 mL of KI solution and 1 mL of pH 4.0 buffer solution and repeat the titration as in 10.2.

11. Calculation

11.1 Calculate the various types of chlorine residual, in milligrams per litre, as follows:

$$\text{Chlorine residual, mg/L} = 200\,A/V$$

where:

A = phenylarsine oxide solution (0.00564 N) required for the titration of 10.2, 10.3, or 10.4, depending on the specific type of chlorine residual determined, mL, and
V = sample used, mL.

12. Precision and Bias[6]

12.1 The overall precision (S_t) and the single operator precision (S_o) of this test method for free available chlorine (FAC) and for total residual chlorine (TRC) were determined by eight or nine qualified cooperators each with analysis equipment and reagents at each of five sites. Each site constituted a different chlorinated cooling water matrix: estuary, inland main stem river, fresh lake, open ocean, and fresh cooling tower blowdown. Each site water was chlorinated up to nine levels. Samples were collected simultaneously and analyzed within 5 min of collection by all eight or nine cooperators. Duplicate sampling and analysis runs were made at each level.

12.2 The S_t and S_o for FAC was found to vary linearly with the mean concentration of FAC, X, in mg/L, over the range for X from 0.0 to 1.0.

12.2.1 For the pooled results from all of the matrices tested:

$$S_t = 0.025 + 0.199\,X\ (n = 37,\ r = 0.848)$$

$$S_o = 0.008 + 0.081\,X\ (n = 35,\ r = 0.638)$$

where:

n = number of runs, and
r = correlation coefficients.

12.3 The S_t and S_o for TRC was found to vary linearly with the mean concentration of TRC, Y, in mg/L, over the range for Y from 0.0 to 3.5.

12.3.1 For the pooled results from all of the matrices tested:

$$S_t = 0.022 + 0.098\,Y\ (n = 39,\ r = 0.865)$$

$$S_o = 0.012 + 0.024\,Y\ (n = 38,\ r = 0.695)$$

12.4 The bias of the test method could not be determined since the instability of solutions of chlorine in water does not permit the determination of an acceptable true value for TRC and FAC in the samples.

12.5 Precision for this test method conforms to Practice D2777 – 77, which was in place at the time of collaborative testing. Under the allowances made in 1.4 of Practice D2777 – 06, these precision data do meet existing requirements for interlaboratory studies of Committee D19 test methods.

13. Quality Control

13.1 In order to be certain that analytical values obtained using these test methods are valid and accurate within the confidence limits of the test, the following QC procedures shall be followed when analyzing residual chlorine.

13.2 *Calibration and Calibration Verification:*

13.2.1 Standardize the titrating solution against the potassium biiodate solution.

13.2.2 Verify titrating solution by analyzing a sample with a known amount of the residual chlorine. The amount of the sample shall fall within ±15 % of the known concentration.

13.2.3 If standardization cannot be verified, restandardize the solution.

13.3 *Initial Demonstration of Laboratory Capability:*

13.3.1 If a laboratory has not performed the test before, or if there has been a major change in the measurement system, for example, new analyst, new instrument, and so forth, a precision and bias study shall be performed to demonstrate laboratory capability.

13.3.2 Analyze seven replicates of a known solution prepared from an Independent Reference Material containing a known amount of residual chlorine. Each replicate shall be taken through the complete analytical test method including any sample preservation and pretreatment steps. The replicates may be interspersed with samples.

13.3.3 Calculate the mean and standard deviation of the seven values and compare to the acceptable ranges of bias in Section 12. This study should be repeated until the recoveries are within the limits given in Section 12. If an amount other than the recommended amount is used, refer to Practice D5847 for information on applying the F test and t test in evaluating the acceptability of the mean and standard deviation.

13.4 *Laboratory Control Sample (LCS):*

13.4.1 To ensure that the test method is in control, analyze an LCS containing a known amount of residual chlorine with each batch or 10 samples. If large numbers of samples are analyzed in the batch, analyze the LCS after every ten samples. The LCS shall be taken through all of the steps of the analytical method including sample preservation and pretreatment. The result obtained for the LCS shall fall within ±15 % of the known amount.

13.4.2 If the result is not within these limits, analysis of samples is halted until the problem is corrected and either all the samples in the batch must be reanalyzed, or the results must be qualified with an indication that they do not fall within the performance criteria of the test method.

13.5 *Method Blank:*

13.5.1 Analyze a reagent water test blank with each batch. The amount of residual chlorine found in the blank should be less than the analytical reporting limit. If the amount of residual chlorine is found above this level, analysis of samples is halted until the contamination is eliminated, and a blank shows no contamination at or above this level, or the results

[6] Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR:D19-1124. Contact ASTM Customer Service at service@astm.org.

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shall be qualified with an indication that they do not fall within the performance criteria of the test method.

13.6 *Matrix Spike (MS):*

13.6.1 Residual chlorine is not an analyte that can be feasibly spiked into samples.

13.7 *Duplicate:*

13.7.1 To check the precision of sample analyses, analyze a sample in duplicate with each batch. The value obtained shall fall within the control limits established by the laboratory.

13.7.2 Calculate the standard deviation of the duplicate values and compare to the precision in the collaborative study using an F test. Refer to 6.4.4 of Practice D5847 for information on applying the F test.

13.7.3 If the result exceeds the precision limit, the batch shall be reanalyzed or the results shall be qualified with an indication that they do not fall within the performance criteria of the test method.

13.8 *Independent Reference Material (IRM):*

13.8.1 In order to verify the quantitative value produced by the test method, analyze an Independent Reference Material (IRM) submitted as a regular sample (if practical) to the laboratory at least once per quarter. The amount of the IRM should be in the analytical range for the method chosen. The value obtained shall fall within the control limits established by the laboratory.

14. Keywords

14.1 amperometric; analysis; chlorine; water

APPENDIX

(Nonmandatory Information)

X1. RATIONALE FOR DISCONTINUATION OF TEST METHODS

X1.1 *Direct and Back Starch-Iodide Titrations and Amperometric Back Titration:*

X1.1.1 These two test methods were discontinued in 1986. These test methods may be found in *1985 Annual Book of ASTM Standards*, Vol 11.01. These test methods were originally issued in 1953.

X1.1.2 These test methods are biiodate solutions as titrating agents. Attempts to include these test methods in the round-robin testing were not successful because the reaction rate of the biiodate solution with phenylarsine oxide was slow and inconsistent. The little data obtained were widely varied, nonreproducible, and were not relatable to the values being tested.

X1.1.3 Field experience indicates that both test methods can work if iodine solution is used in place of biiodate solution as the titrating agent. Validation of these test methods through round-robin testing, however, has not been carried out.

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Attachment 3
Ambient TRC Bench
Scale Studies

PROPOSAL TO

NEW YORK DEPARTMENT OF ENVIRONMENTAL PROTECTION

FOR

Chlorine DEGRADATION STUDIES FOR TWO NYC DEP CSO FACILITIES

Prepared by:

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June 18, 2015

Brief Description of Planned CSO Degradation Studies

Receiving Water and CSO Samples

CSO-specific receiving water samples will be collected using GLEC equipment and will be hauled from the receiving water of interest to the GLEC mobile laboratory currently housed at 26th Ward WWTP, where the samples will be off-loaded into a storage tank. The CSO samples will be collected during a rain event using GLEC equipment, and will also be hauled to the GLEC mobile lab at the 26th Ward WWTP. Prior to any testing, the CSO and receiving water samples will be characterized for TSS, turbidity, ammonia, nitrite, nitrate and COD, as well as pH and temperature. In addition, both the effluents and receiving waters will be tested for initial fecal coliform and enterococcus densities prior to each test.

Chlorine Degradation Studies with Pathogen Inactivation

The CSO chlorine degradation studies will be carried using equipment in the GLEC mobile laboratory, as well as at space provided by the 26th Ward in-house laboratory. The studies will mimic the degradation studies that were developed under the TRC program for DEP in 2013/2014. Between four-six samples will be collected from each of the two selected CSO facilities during the study. Each sample will be tested for chlorine degradation and pathogen inactivation at 2 different dilutions and with 3 different applied chlorine doses. The selected dilutions will be determined in collaboration with DEP based on the specific CSO discharge location and the hydraulics of the receiving water location. The chlorine doses to be tested will range between 4 - 20 mg/l ; the specific doses to be tested will be informed by the on-going CSO disinfection studies. **Figure 1** shows the proposed set of testing conditions for a single CSO sample. These testing conditions will be used to run the collected samples in duplicate over a two-to-three day period after the CSO sample is collected. Each specific testing condition will be carried out in a batch system according to the dilution and sampling times shown in **Figure 2.**

Figure 1 – Degradation Testing Conditions

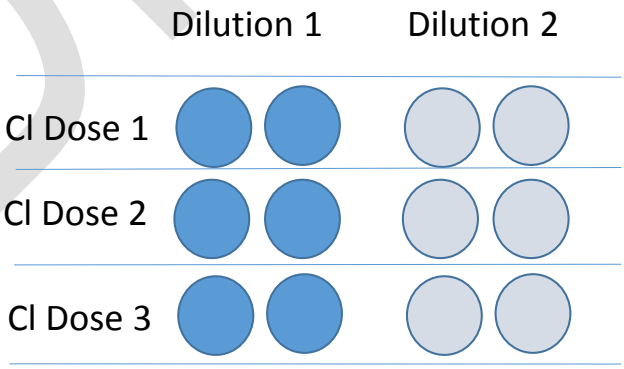

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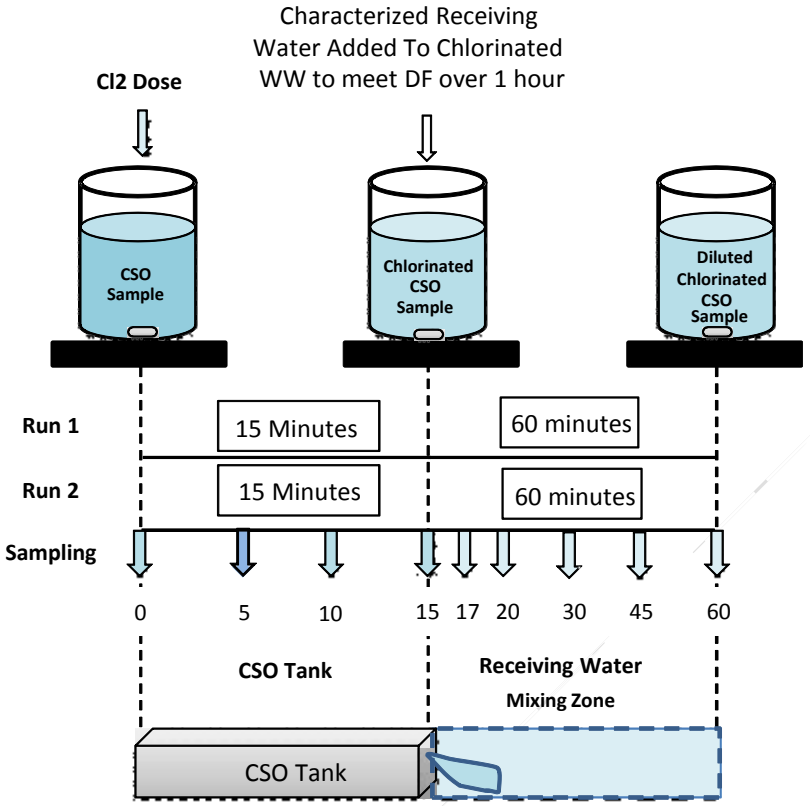
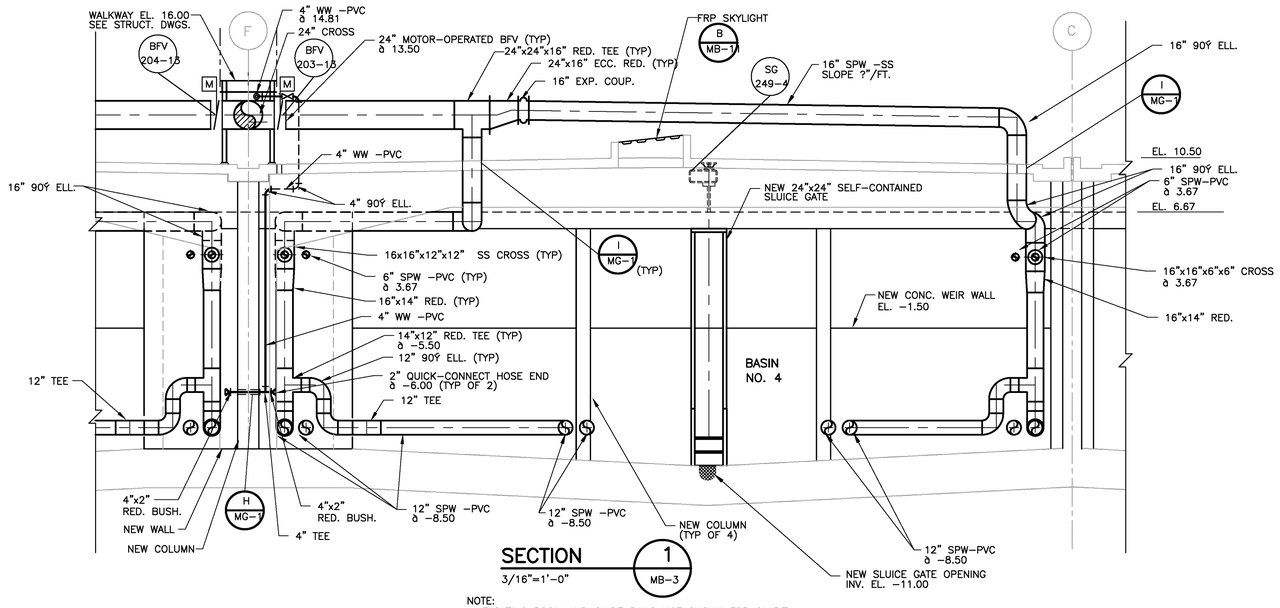
Figure 2 – CSO Chlorine Degradation Test with Specific Dilution and Sampling Times



The specific analyses that will be carried on each sample are shown in **Table 1.** CPO represents chemically-(chlorine) produced oxidant, which is how free and combined chlorine are measured using standard methods for wastewaters. EC/FC represents fecal coliform and *Entercoccus* counts. The Nitrogen series includes ammonia, nitrite and nitrate. The sampling times may be altered during the testing period to account for new information gained during the study

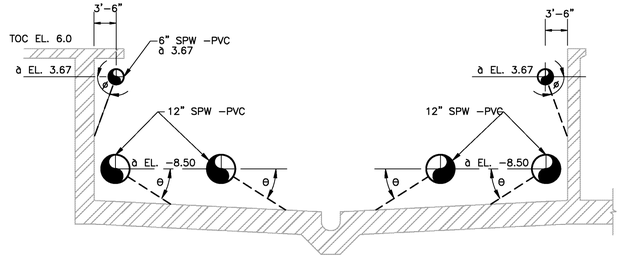
Table 1- Sampling Plan for Each Individual Degradation Test

Sample	CPO	FC/EC	N Series	pH	Turb	Temp	COD	UVT
CSO Sample	√	√	√	√	√	√	√	√
Receiving Water Sample	√	√	√	√	√	√	√	√
0 min.	√	√	√	√	√	√		
1 min.	√					√		
5 min.	√					√		
10 min.	√					√		
15 min.	√	√		√		√		
17 min.	√					√		
20 min.	√					√		
30 min.	√					√		
45 min.	√					√		
60 min.	√	√		√	√	√		



SECTION 1 — 3/16"=1'–0" — MB–3

NOTE:
1. FLOATING BOOM AND GUIDE RAILS NOT SHOWN FOR CLARITY.
2. REFER TO DETAIL A ON THIS SHEET FOR SPRAY WATER PIPE ORIFICE ORIENTATION.

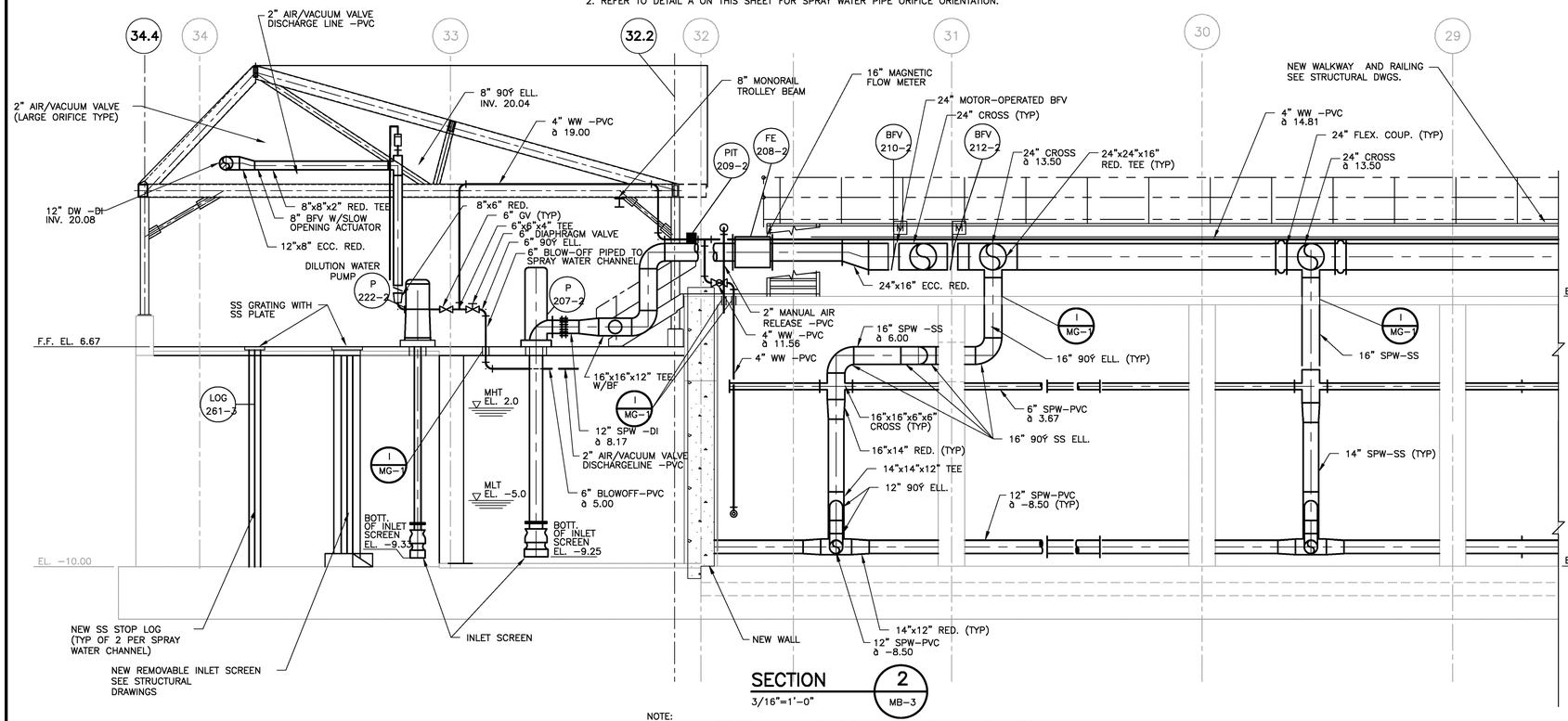


SPRAY ANGLE θ = APPROX. 60Ý
SPRAY ANGLE ∅ = APPROX. 70Ý

SPRAY WATER PIPING ORIFICE ORIENTATION

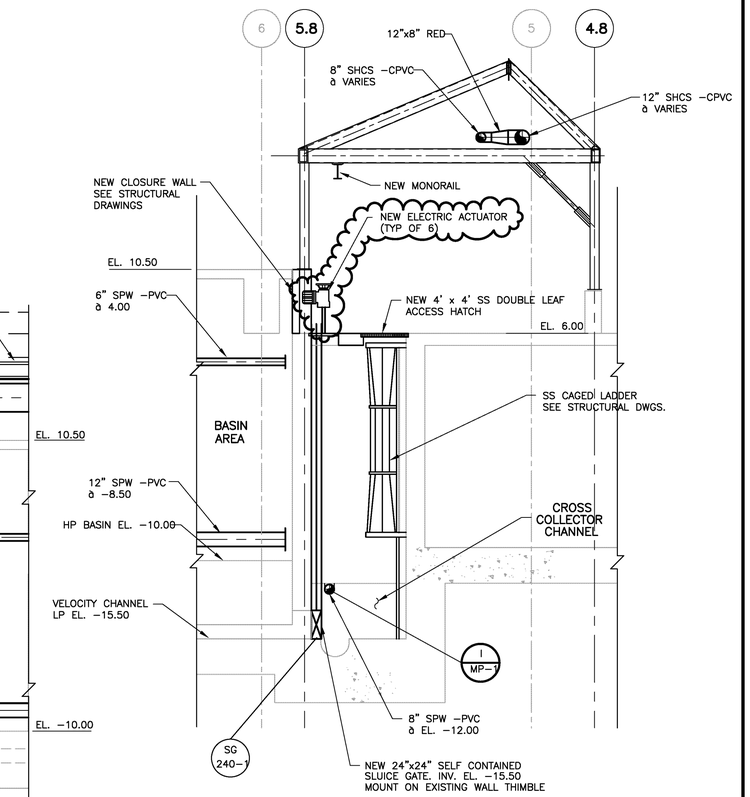
DETAIL A — NTS — MB–2

NOTES:
1. SIX INCH DIAMETER SPRAY WATER HEADER SHALL CONTAIN 40 1/2" DIAMETER ORIFICES SPACED ON ONE FOOT CENTERS TURNED APPROX. 70Ý FROM THE HORIZONTAL.
2. TWELVE INCH DIAMETER SPRAY WATER HEADERS SHALL CONTAIN 40 3/4" DIAMETER ORIFICES SPACED ON ONE FOOT CENTERS TURNED APPROX. 60Ý FROM THE HORIZONTAL.



SECTION 2 — 3/16"=1'–0" — MB–3

NOTE:
1. NEW WALL BETWEEN BASIN NO. 3 AND BASIN NO. 4 NOT SHOWN FOR CLARITY.
2. REFER TO DETAIL A ON THIS SHEET FOR SPRAY WATER PIPE ORIFICE ORIENTATION.



SECTION 1 — 3/16"=1'–0" — MB–2

WARNING
IT IS A VIOLATION OF SECTION 7209, SUB–DIVISION 2 OF THE NEW YORK STATE EDUCATION LAW FOR ANY PERSON UNLESS ACTING UNDER THE DIRECTION OF A LICENSED PROFESSIONAL ENGINEER OR LAND SURVEYOR TO ALTER IN ANY WAY PLANS, SPECIFICATIONS, PLATES OR REPORTS TO WHICH THE SEAL OF A PROFESSIONAL ENGINEER OR LAND SURVEYOR HAS BEEN ATTACHED.

AS-BUILT DRAWING

3/16" = 1'– 0"
4 0 4 8

NO.	DATE	DESCRIPTION	APPR'D
		REVISIONS	

DESIGNED BY:
DRAWN BY:
SHEET CHK'D BY:
CROSS CHK'D BY:
APPROVED BY:
DATE: OCTOBER 1999

CDM
Camp Dresser & McKee
100 Crossways Park West
Woodbury, NY 11797
Tel: (516) 496-8400
PICONE / McCULLAGH
JOINT VENTURE
31 GARDEN LANE
LAWRENCE, NY 11559
(516) 239–1600

AS–BUILT DATE:
OCTOBER, 2010

APPROVED FOR THE CITY OF NEW YORK

PROJECT MANAGER

CHIEF, DIVISION OF FAC. DES.N (S)

CITY OF NEW YORK
DEPARTMENT OF ENVIRONMENTAL PROTECTION
BUREAU OF ENVIRONMENTAL ENGINEERING
SPRING CREEK AWPCP UPGRADE
CAPITAL PROJECT WP-225

CONTRACT NO. SC -1G

BASIN BUILDING SECTIONS I

MECHANICAL

DATE: OCTOBER 1999
FILENAME: MBBSC001
SHT. NO: 185 OF 308
DWG. NO: AB-SC1-G-MB-9

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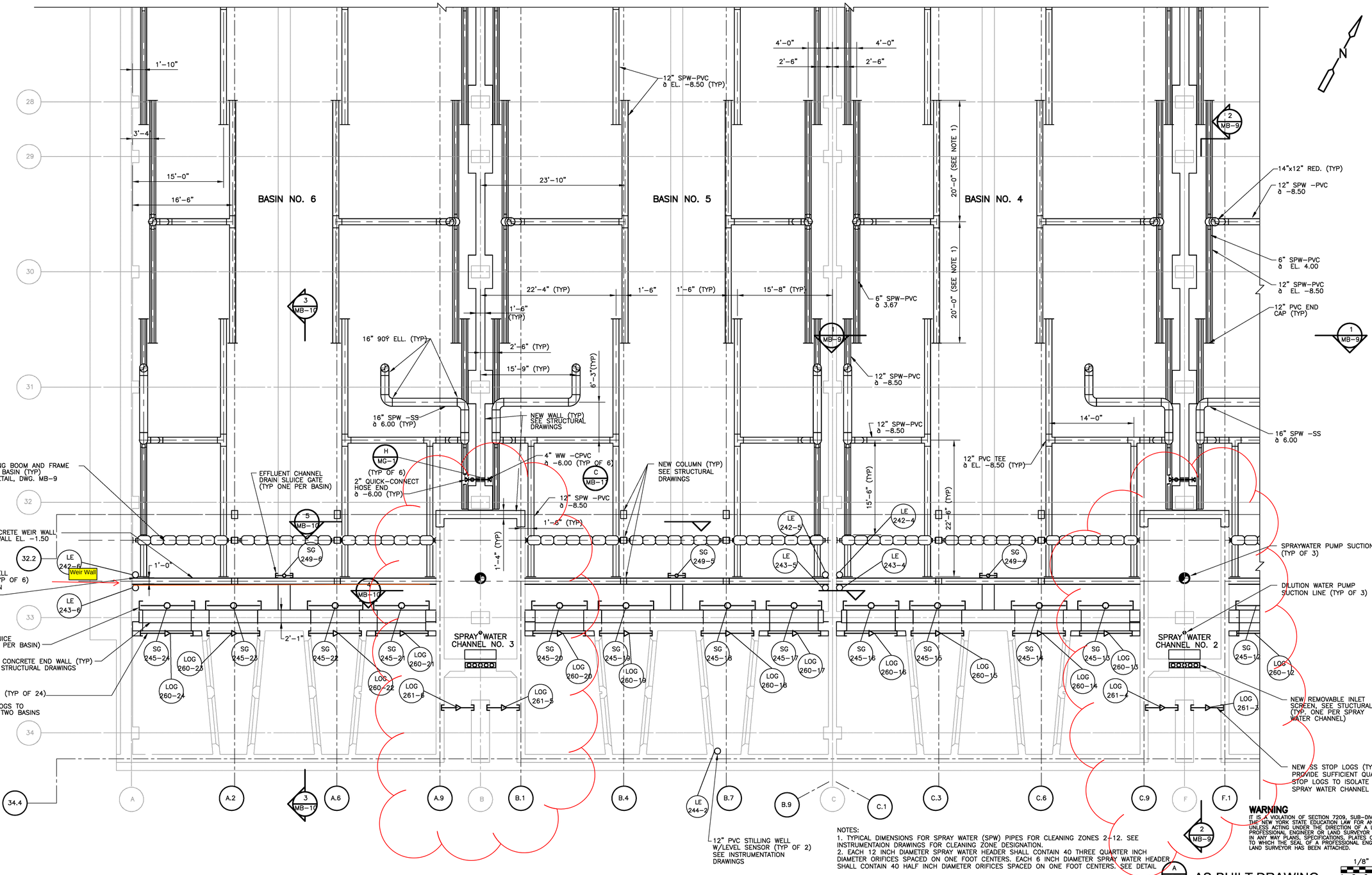

NOTES:
1. TYPICAL DIMENSIONS FOR SPRAY WATER (SPW) PIPES FOR CLEANING ZONES 2-12. SEE INSTRUMENTAION DRAWINGS FOR CLEANING ZONE DESIGNATION.
2. EACH 12 INCH DIAMETER SPRAY WATER HEADER SHALL CONTAIN 40 THREE QUARTER INCH DIAMETER ORIFICES SPACED ON ONE FOOT CENTERS. EACH 6 INCH DIAMETER SPRAY WATER HEADER SHALL CONTAIN 40 HALF INCH DIAMETER ORIFICES SPACED ON ONE FOOT CENTERS. SEE DETAIL
3. THE BASIN AREA AND EFFLUENT WING ARE SYMMETRICAL ABOUT COLUMN LINE F.

WARNING
IT IS A VIOLATION OF SECTION 7209, SUB-DIVISION 2 OF THE NEW YORK STATE EDUCATION LAW FOR ANY PERSON UNLESS ACTING UNDER THE DIRECTION OF A LICENSED PROFESSIONAL ENGINEER OR LAND SURVEYOR TO ALTER IN ANY WAY PLANS, SPECIFICATIONS, PLATES OR REPORTS TO WHICH THE SEAL OF A PROFESSIONAL ENGINEER OR LAND SURVEYOR HAS BEEN ATTACHED.

1/8" = 1'- 0"
4 2 0 4 8

AS-BUILT DRAWING

NO.	DATE	DESCRIPTION	APPR'D
		REVISIONS	

DESIGNED BY:
DRAWN BY:
SHEET CHK'D BY:
CROSS CHK'D BY:
APPROVED BY:
DATE: OCTOBER 1999

CDM
Camp Dresser & McKee
100 Crossways Park West
Woodbury, NY 11797
Tel: (516) 496-8400

PICONE / McCULLAGH JOINT VENTURE
31 GARDEN LANE
LAWRENCE, NY 11559
(516) 239-1600

AS-BUILT DATE: OCTOBER, 2010

APPROVED FOR THE CITY OF NEW YORK

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CHIEF, DIVISION OF FAC. DES.N (S)

CITY OF NEW YORK
DEPARTMENT OF ENVIRONMENTAL PROTECTION
BUREAU OF ENVIRONMENTAL ENGINEERING
SPRING CREEK AWPCP UPGRADE
CAPITAL PROJECT WP-225

CONTRACT NO. SC 1-G

BASIN BUILDING
PARTIAL PLAN SOUTH AT EL. -15.50

MECHANICAL

DATE: OCTOBER 1999
FILENAME: MBBPP004
SHT. NO: 179 OF 308
DWG. NO: AB-SC1-G-MB-3

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Attachment 4