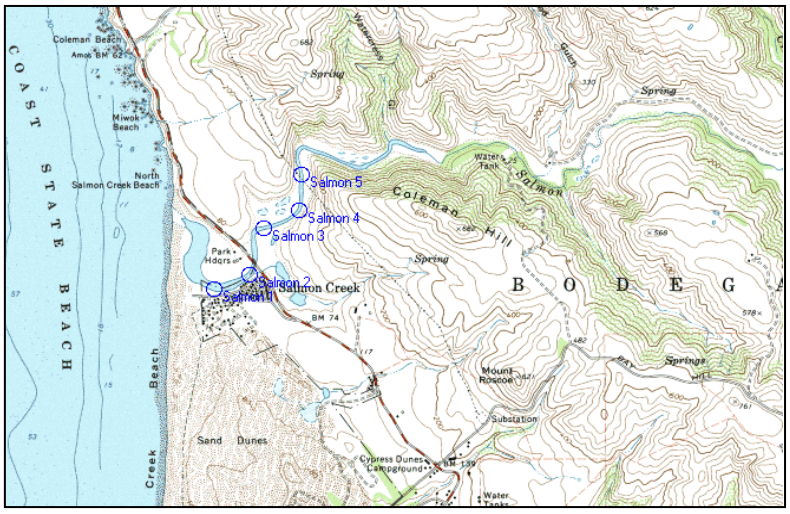
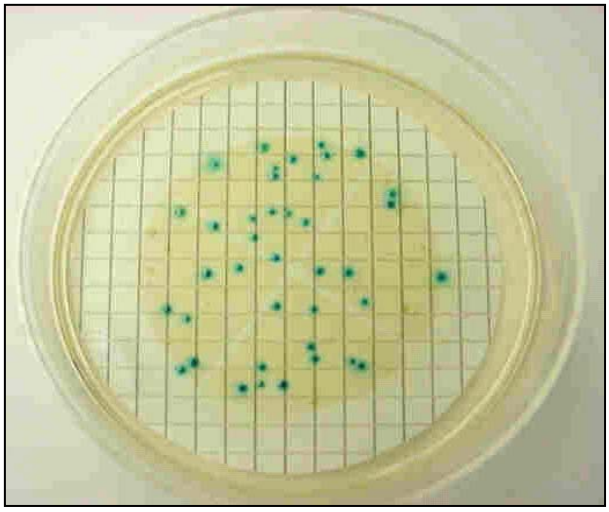
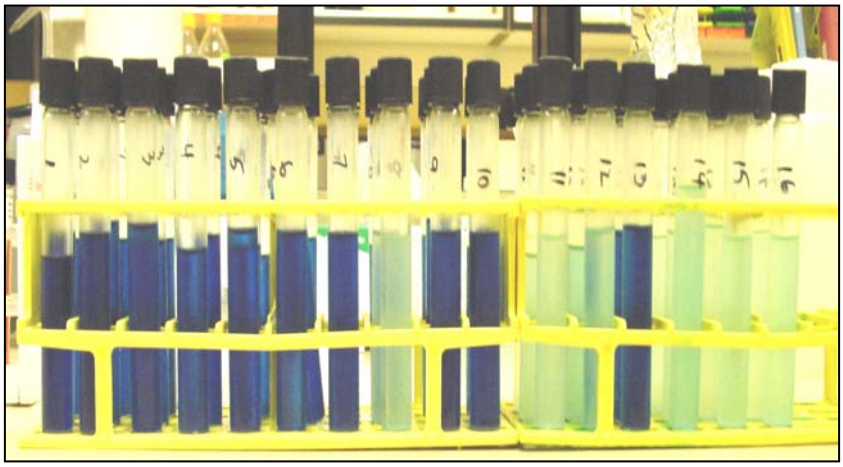
Protocol Considerations for Monitoring Fecal Coliform in Northern California Estuaries







prepared by

School of Veterinary Medicine
University of California, Davis

And

U.C. Cooperative Extension
Sonoma County

PROJECT TEAM

Rob Atwill, Professor/Specialist, School of Veterinary Medicine, University of California-Davis

David Lewis, Watershed Management Advisor, Marin, Mendocino, and Sonoma Counties, University of California Cooperative Extension

Ronald F. Bond, Laboratory Assistant, Veterinary Medicine Extension, School of Veterinary Medicine, University of California-Davis

Maria das Graças C. Pereira, Staff Research Associate, Veterinary Medicine Extension, School of Veterinary Medicine, University of California-Davis

Miguel Huerta, Field Technician, Marin, Mendocino, and Sonoma Counties, University of California Cooperative Extension

Samantha B. Ogata, Laboratory Assistant, Veterinary Medicine Extension, School of Veterinary Medicine, University of California-Davis

Cover photographs: In clockwise order, Salmon Creek Estuary (California Coastal Records Project), plated sample for enumeration of indicator bacteria, processed sample for enumeration of indicator bacteria by multiple tube method, and map of Salmon Creek Estuary (MapTech, Inc).

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Disclaimer: Representation of a specific manufacturer, make, or model for discussed equipment is not an endorsement of that product over others that are available to measure the same parameter or perform the same laboratory procedure.

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INTRODUCTION AND PURPOSE

Microbial contamination of California's bays, estuaries, and near-shore marine environments continues to impact the beneficial uses of these waters. Beneficial uses of the aquatic resources along the California coast range from shellfish harvesting and body contact recreation to recovery of threatened and endangered aquatic species. All of these beneficial uses are vulnerable to microbial contamination from freshwater inflows with elevated bacterial levels and if occurring, resuspension of bacteria laden riverine, estuarine, and bay sediments. A central challenge for resource agencies such as the Regional Water Quality Control Boards is to accurately monitor and interpret water quality data in order to reduce bacterial contamination and protect beneficial uses for these dynamic freshwater-coastal interfaces.

There are numerous standard operating procedures and protocols for water quality monitoring available to assist resource managers in developing and implementing monitoring protocols for water quality. These include both Federal and State agency water quality monitoring manuals and programs such as United States Environmental Protection Agency's *Volunteer Estuary Monitoring: A Methods Manual* (Ohrel and Register, 2006), U.S. Geological Survey Techniques of Water-Resources Investigations, book 9, National Field Manual for the Collection of Water-Quality Data (Wilde et al., various dates), and the California State Water Resources Control Board Surface Water Ambient Monitoring Program (SWRCB, 2005). Similarly, there are accepted and approved methods for constituent analysis such as *Method 1600: Enterococci in Water By Membrane Filtration using memberane-Enterococcus Indoxy – ß – D - Glucoside Agar (mEI)* (USEPA, 2006) and others (Clesceri et al. 1998).

In 2004 and 2005, the University of California conducted an in-depth study of microbial concentrations in water and sediment of five such estuaries in the Tomales Bay watershed. Studied estuaries included the Russian River, Salmon Creek, Estero Americano, Walker Creek, and Lagunitas Creek. The five selected estuaries represent a variety of environmental conditions across the coast of Marin and Sonoma Counties. In a general sense, these estuaries have similar a climate, and hydrology. Specifically, their similarity is the result of the Mediterranean climate in California, with cool wet winters and dry hot summers. As a result, all five estuaries experience an inflow of freshwater during the winter. The five estuaries differ in size, area of contributing watershed, and land use including agriculture, urbanization, and recreation. Additionally, the Russian River, Salmon Creek and Estero Americano are bar built estuaries while the Walker and Lagunitas Creeks are part of the low inflow system of Tomales Bay. These differences and similarities provide a good context for understanding how these factors interact with bacteria movement within these estuaries. For each of the five studied estuaries, we sampled a 15-point grid once per month for 10 months. This provided for the enumeration of waterborne and sediment-borne bacteria concentrations and loads for a dry season base flow, wet season base flow, and wet season storm flow conditions.
The preliminary results have been compiled and presented to the North Coast Regional Water Quality Control Board in a separate report (Atwill et al 2007).

The primary goal of that study and this document was to facilitate the development of an improved water quality monitoring protocol for quantifying sources and transport processes of bacterial contamination for estuaries and to extend this information to regulatory staff, conservation organizations, and allied watershed groups. By design this document is written to support monitoring water quality for microbial pollution in Northern California's estuaries. Using

the results and approach from the referenced study, this guide provides detailed procedures for replicating the field and laboratory methods used in that study. It also presents potential influences and bias in these estuarine environments that are important for a monitoring program to consider when designing a water quality monitoring program or interpreting the resulting data. In this way, this document compliments the standard operating procedures and monitoring protocols already in existence and further supports monitoring efforts in the highly dynamic coastal systems that are Northern California's estuaries.

SAMPLE COLLECTION

Field Equipment

Field equipment should be calibrated before use and post use for most instruments and calibrated by company specifications. The measurements from each calibration should be recorded, and each calibration point should not be out of range of the instrument. Representation of a specific manufacturer, make, or model for discussed equipment is not an endorsement of that product over others that are available to measure the same parameter.

DO/Temperature Meter

Many makes and models of dissolved oxygen meters are available. The model displayed below was proficient for getting dissolved oxygen and temperature reading.

www.YSI.com

Calibration

1. Ensure that the sponge inside the instrument's calibration chamber is wet. Insert the probe into the calibration chamber.
2. Turn the instrument on by pressing the ON/OFF button on the front of the instrument. Wait for the dissolved oxygen and temperature readings to stabilize. (usually 15 minutes is required after turning the instrument on)
3. To enter the calibration menu, use two fingers to press and release both the UP ARROW and DOWN ARROW keys at the same time.
4. The LCD will prompt you to enter the local altitude in hundreds of feet. Use the arrow keys to increase or decrease the altitude. EXAMPLE: Entering the number 12 designates 1,200 feet of elevation.
5. When the proper altitude appears on the LCD, press the ENTER key. The Model 55 should now display CAL in the lower left of the display, the calibration value should be displayed in the lower right of the display and the current DO reading (before calibration) should be on the main display.

6. Make sure that the DO reading (large display) is stable, then press the ENTER button. The LCD will prompt you to enter the approximate salinity of the water you are about to analyze. You can enter any number from 0 to 40 parts per thousand (PPT) of salinity. Use the arrow keys to increase or decrease the salinity setting. When the correct salinity appears on the LCD (zero for fresh water), press the ENTER key. The instrument will return to normal operation.
(https://www.ysi.com/DocumentServer/DocumentServer?docID=WQS_55_MANUAL)

Flow Meter

At each site where a water sample was taken, instantaneous flow was measured using a water flow probe as displayed below. In addition to this measurement, a secondary measurement of the sample transect cross-sectional area must be taken. These measurements were used in area-velocity method (velocity x channel width x channel depth) to calculate flow volume in cubic feet a second and cubic meters per second (Mosley and McKercher, 1993).

www.globalw.com/products/flowprobe.html

Maintenance

1. Probe Handle (Picture 2): When the Flow Probe expansion joint becomes submerged, water will enter the Probe handle. After use, dry the probe by separating the two handle sections, draining the water inside the probe handle, and letting the handle dry out in a warm place before reassembling. The flow probe handle can be cleaned with mild soap and water. DO NOT submerge the top of the pole and the computer. If the computer becomes submerged, remove it from the flow probe, and DRY IMMEDIATELY with a soft cloth; remove the battery and place in a warm place overnight to dry.
2. Battery Replacement: The computer is held onto the head of the Probe by a twist lock connection. To remove, turn computer ¼ turn to the left and pull off. To remove the battery, use a small coin to twist the battery cover on the back of the computer ¼ turn to the left. Replace battery, + side toward battery cover, using a CR2032, 3 volt lithium cell. After replacing battery the calibration numbers will require resetting.
3. Cleaning: Make sure the turbo prop turns freely before and after your measurements. Blow on the prop in the direction of flow. The prop should turn freely. If not, rinse the probe in clean water and remove any visible strings or hair materials from the prop

bearing. This should correct the problem. If the prop still does not turn freely, remove the prop screw and the prop, and wash them in clean water or soap and water. Replace prop and screw. Tighten screw firmly but make sure prop still spins freely. (http://www.globalw.com/downloads/flowprobe/flowprobe_manual.pdf)

Nephlometer (Turbidity)

Turbidity or water clarity can be measured by any number of nephlometer makes and models. One example is the displayed below.

www.lamotte.com

Calibration

1. Rinse a clean tube (0290) three times with the blank. Below 1 NTU (Nephelometric Turbidity Units) – The meter should be blanked with a 0 NTU primary standard or prepared turbidity-free (<0.1 NTU) water. For the most accurate results, use the same tube for the blank and the sample.
2. Fill the tube to the fill line with the blank. Pour the blank down the inside of the tube to avoid creating bubbles.
3. Dry the tube with a lint-free cloth. Put on a dry positioning ring. Cap the tube. Wipe the tube thoroughly again with a lint-free cloth.
4. Rinse a clean tube (0290), or the same tube, three times with the water to be tested. Avoid spilling water on the outside of the tube. IMPORTANT: While the tube is inverted, wipe the lip of the tube to remove droplets of liquid that may be present. This will prevent liquid from being trapped under the ring when the tube is returned to an upright position.
5. Fill the tube to the fill line with the sample. Pour the sample down the inside of the tube to avoid creating bubbles. http://www.lamotte.com/pages/common/pdf/instruct/1979.pdf

Salinity

Salinity can be measured by a number of means. We opted to use a refractometer because of its reliability and repeatability. There are many makes and models are available including the one displayed below.

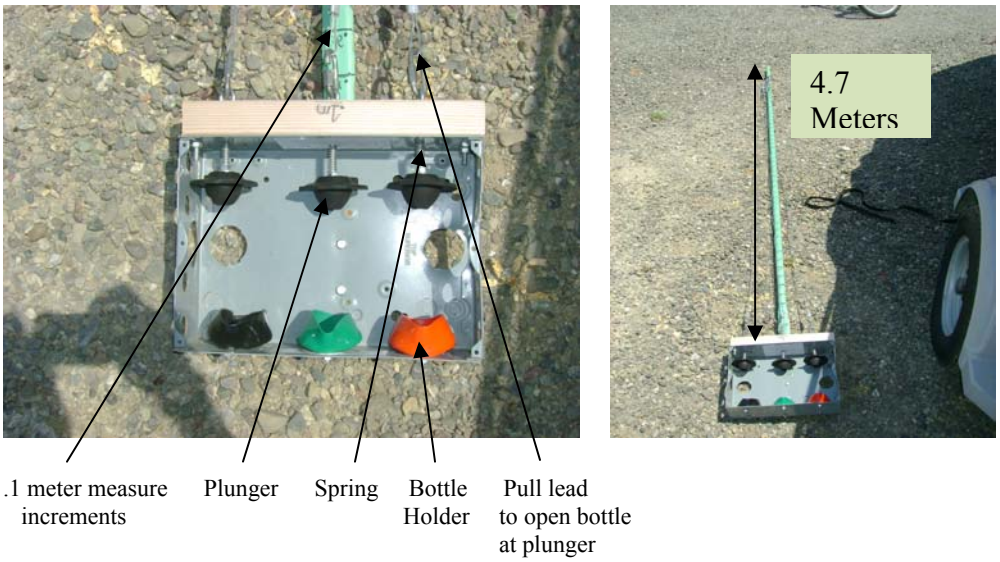
www.new.fishersci.com/

Calibration

1. The refractometer is be used in the laboratory to measure salinity in units of parts per thousand (ppt).
1. 2. Calibrate by placing a few drops of distilled water on the face of the prism and read just as you would a sample.
2. Read the units by viewing the blue line, and determine what ppt unit the line crosses.

Drop Sampler

Collecting water samples at prescribed depths requires a "drop sampler." To achieve collection of three samples from the same water column we developed the Huerta Lewis Atwill Bond (HLAB) drop sampler displayed below. This is constructed of materials available from local hardware stores. The HLAB is a PVC coated iron pipe with attached empty electrical box and three cable pull system attached to three spring loaded rubber stoppers. The top of the three sample bottles are closed while being lowered in a vertical position through the water column until the stoppers are opened at a discrete depth and reclosed before being drawn back through the water column.



Sediment Sampler

To collect estuarine bottom sediment we used an Eckman Dredge ™ displayed below. This type samples works well for materials ranging from silts and fine sand to gravel. Larger cobble and boulder material requires a different sampling method, instrument, and even laboratory analysis approach.

1. Operates in relatively shallow water on a cable or line triggered with a messenger, which should be clear from obstruction so messenger can reach dredge.
2. The housing should be cleaned with high pressure water hose and wiped down after each use.

Field Methods

Collection Considerations

1. When approaching the sample site in a boat it may be necessary to idle the engine and approach the site from a downstream direction so as not to disturb the bottom sediment.
2. Anchors should be lowered fore and aft of the boat to keep position over the sample site. Field parameters and meteorological data (dissolved oxygen, temperature, wind speed, etc.) should be taken at this time in cases of that the anchor was difficult to set and the bottom sediment has been disturbed.
3. For the USGS "6 tenths method", the flow probe is placed at the center of the subsection at a depth from the surface of 0.6 of the total depth. The flow probe is held in place and the average velocity is obtained over a period of 40 seconds. The 0.6 depth is assumed to be the average velocity point for the vertical profile. Therefore, this average is similar to that obtained in technique 2 (above) however; we feel that technique 2 is more accurate. http://www.globalw.com/downloads/flowprobe/flowprobe_manual.pdf
4. When discrete samples are desired from a specific depth, and the parameters to be measured do not require Teflon® coated samplers, an HLAB Modified Dip Sampler may be used. After removing sample bottles from the sampler housing, the rubber stoppers are sterilized with 70% ethanol and dried before next use. With multiple depth samples, care should be taken not to stir up the bottom sediment and thus bias the sample.

Collection Steps

1. Using a compact Fishfinder with Global Positioning Satellite (GPS) capability to determine exact site location and position boat over sample area.
2. Prior to anchoring turn DO meter on to ensure proper functionality of electrode and, subsequent to anchoring, lower DO probe into the water column until value on the display is stable and record the DO and temperature value.
3. Place flow meter into the water column at approximately the 60% of total water depth in order to get a representation of column flow. (See USGS 6 Tens Rule) The average flow velocity should be recorded after a minute in the water column. Given the proximity of the estuary to tidal influences the direction of flow should be recorded by placing a 360 degree pivoting object (string or vane) on the flow meter at the surface to determine the correct direction of flow.

Procedures for collecting water with an HLAB

1. Unscrew the lids and load sterile 1L sterile Nalgene bottle into place, making sure to test plunger for headspace to allow water to flow into bottle. Lids should be in a dry sterile place facing up.
2. Lower vertically the HABL into the water column to the correct depth marked on the lowering rod.
4. Once at the correct depth, pull up on the handle attached to the trigger lines to disengage plungers and hold for 10 seconds, then release trigger lines to reengage plungers.

5. Pull the sampler out of the water vertically to avoid contamination and place on deck so that the bottles are vertical.
6. Take the bottles out of the HLAB and make sure there is headspace for proper homogenization in when shaken, replace the tops.
7. Place sample bottle upright in a cooler of ice and drain excess water from bottom to ensure there is no sinking of sample over the cap line.

Procedures for collecting sediment with an Eckman Dredge:

1. Thread a sturdy nylon rope or stainless steel cable through the bracket, or secure the extended handle to the bracket with machine bolts.
2. Attach springs to both sides. Arrange the Eckman Dredge sampler so that the jaws are in the open position and trip cables are positioned over the release studs.
3. Lower the sampler to a point just above the sediment surface.
4. Drop the sampler sharply onto the sediment.
5. Trigger the jaw release mechanism by lowering a messenger down the line, or by depressing the button on the upper end of the extended handle.
6. Raise the sampler and slowly decant any free liquid through the top of the sampler. Be careful to retain fine sediments.
7. Open the dredge and transfer the sediment into a stainless steel or plastic bucket. Continue to collect additional sediment until sufficient material has been secured. Thoroughly mix sediment to obtain a homogeneous sample, and then transfer to the appropriate sample container. www.epa.gov/earth1r6/6pd/qa/qadevtools/mod5_sops/sediment_sampling/r9sedimentsample_gui.pdf

Collection and Chain of Custody

Water samples were analyzed within 6 to 96 hours after collection. Mean hour of analysis was 41 hours, with the extended times due to re-plating samples with too numerous to count colonies. Each sample set was accompanied by a written record of time and date and environmental data which is duplicated for various other lab services.

LABORATORY ANALYSES

Laboratory Equipment and Materials

Equipment:

Autoclave
Petri dishes, 60 X 100-mm, 85 X 15mm
Pipets, 2-, 10-, 25-, 50-mL
Micropipette, 20-200 µl, 100-1000µl
Micropipette tips, 20-200 µl, 100-1000µl
Laboratory Balances with optional printers
Vacuum manifold (stainless steel or PVC)
Magnetic filter funnels, for 47mm filters
Nitrocellulose filters, .47mm .45µm pore size
UV Filter Funnel Sterilizer
1.5, 2.0, 50, 250 mL centrifuge tubes
2.0 mL, 250 mL centrifuge rack
Centrifuge (1.5 to 2.0 ml rotor and 250 ml tube rotor)
Erlenmeyer flasks (2 L)
Disposable test tubes (16 X 150)
Autoclavable test tube caps (16 X 150)
Vacuum pump or in-house vacuum
Incubators, set at 37.0-, 44.5˚C
Pasteur glass pipets 5 inches
Media Tempering bath
Microwave
Nitrile Disposable gloves
Forceps
90% Ethel Alcohol
Permanent Marker

Autoclave and Sterilization:

Autoclave should reach desired temperature of 121˚C within 30 minutes of cycle start.
Heat sensitive tape is used on all items to verify they have been subjected to sterilization temperatures.
Test for sterility are done on a monthly basis.
Sterilization temperature checks comparing autoclave internal gauges (noted on cycle printouts) against a certified autoclave maximum registering thermometer,* these are done for temperature quality assurance of the autoclave.
*This thermometer is a certified NIST (National Institute of Standards and Technology) traceable, and requires re-certification annually.

Balances and Calibration Procedure

Balances are used to weigh dehydrated media and other laboratory practices prescribed to help obtain laboratory objectives.

Maintenance

1. Balances are kept clean by using a brush or soft cloth.
2. Calibration of balances is required on an annual basis and preformed by a professional. Certificates of calibration are maintained for a specified period of time to satisfy quality control.
3. Balances require monthly checks with a class S weight at 150.1 grams. Corrective action/rejection and recalibration - The tolerance level is not to exceed 150.30 grams and no less than 149.70 (+ or - .30 grams)
4. Balances serviced in house by laboratory personnel (using the manual) are logged and if adjustments are, if needed, will be made on site.

Incubator and Water Bath

Dry air incubators are set at 37.0ºC and a second set at 44.5ºC. Both are considered online at all times. In addition, general operating instructions are listed in plain view. Certified digital or total immersion thermometers will be used according to project specifications. The total immersion thermometer must be immersed in liquid within 2 cm of the target temperature. Do not exceed the target temperature as the liquid will skew the visual reading. (In addition, order non-mercury thermometers when applicable or purchase mercury thermometers that are coated in Teflon®.)

Maintenance

1. Monitor temperature with thermometers (37ºC, 44.5ºC) located one on the top shelf and one on the bottom shelf inside the incubator daily. The thermometers must have gradients with 0.5°C increments.
2. Adjust temperature, only when needed, using the manufactures adjustment procedure.
3. Keep circulating fans in use if possible. Keep a backup fan on hand in case of failure.
4. Use trays of deionized or distilled water inside the incubators to create a humid environment that is better able to maintain desired temperature.
5. Clean up any spills quickly and properly.
6. Do not use flammable liquids inside incubator.
7. Temperatures should read plus or minus 0.5ºC.

Rejection criteria/corrective action plan for 37ºC and 44.5ºC air incubators– Sometimes an incubator may read low following it being opened repeatedly. Wait 30 minutes and recheck. If, at the recheck, the unit is still >0.5ºC in arrears, the unit is considered offline until the problem is addressed. Any sample in that incubator must be removed (if time allows) or discarded.

Centrifuges

Each rotor on the large capacity centrifuge should carry samples from 5 mL to 500 mL, depending on capacity of tubes used. For smaller volumes, a micro-centrifuge is used for tubes that carry smaller volumes from 10µl to 2 mL.

Maintenance

1. For each cycle of samples, the rotors are balanced in order for the machine to get to the correct speed and avoid damage. Opposing rotors must carry the same weight to assure proper balance.
2. Before each cycle, the lid is to be tightly closed and locked.
3. The rotors must be at a complete stop before removing samples.
4. To clean the chamber, unplug the machine and remove the rotor. Using a clean rag, wipe down chamber with cleaner and let dry with hood up. Place rotor back in place and run an empty cycle to ensure stability.

Centrifuge Bottles and Tubes

Reusable 250 mL or 500 mL centrifuge tubes are used to carry higher volume samples and disposable 2.0 mL and 1.5 mL centrifuge tubes are used to carry smaller volume samples. To clean reusable large tubes, rinse tubes in hot tap water until pellet is removed from conical and any resultant sediment is washed from side walls of tube. Let tubes dry without capping. In separate containers, autoclave tubes for 15 minutes at 121°C (Manufacturer indicates that autoclaving tubes is only to be done several times then discarded to avoid breakdown of the plastic). Cap and store in conical racks.

Bio-safety Vertical Flow Hood

A vertical flow bio-safety hood is used for dispensing media and performing various controlled environmental experiments. A Laminar flow hood can also be used for dispensing media or running experiments. These hoods require a professional inspection annually and have the filters changed out. The date of inspection is recorded on the service tag which is then attached to the hood.

Protocol of Use:

1. Wipe entire hood surface with 70% Ethanol (EtOH).
2. Let blower run for 10 minutes before proceeding.
3. Turn blower off and switch to UV light for 15 min.
4. Switch to standard light for tasks inside the hood.
5. After use, wipe entire hood surface again with 70% Ethanol (EtOH).
6. Turn on UV light for 15 min and close hood.

Membrane Filtration Apparatus and Sterilization

3-place or 6-place manifolds and 47 mm magnetic filter funnels is used for membrane filtration of each sample (although other conventional methods can be employed). Each manifold is made of either stainless steel or PVC for autoclaving, which is attached to a 2L filtering flask with a side tube connected to a vacuum line or vacuum pump, and an effluent line attached at the top of the flask (see insert). The magnetic filter funnels are not autoclavable. Subsequent to each use of the filters funnels, it is necessary to place them into an ultraviolet sterilizer that is made to fit the 47 mm filter funnels and bases and sterilize for two minutes. Membrane filter used on the apparatus is the 47 mm filter with a 0.45µm pore size.

Care and Maintenance

1. All manifolds are washed after each use and the release valves are to be checked for ease of use.
2. All lines are checked for molding or corrosive properties.
3. Each filter funnel is soaked in deionized water weekly to prevent build-up on the surface of the filter.

Wrist Shaker

A 12-place wrist shaker with timing control (Picture 8) is appropriate to homogenize water and sediment samples, in 250 mL conical tubes.

Media Tempering Bath

A tempering bath is used to temper agar that has been autoclaved or brought to a boil and needs to cool down before dispensing into Petri dishes or test tubes. The temperature of the tempering bath is maintained at 50°C plus or minus 5°C, to avoid solidification of agar. Agar is to be plated, in a bio-safety hood (laminar or vertical flow), after being tempered to avoid contamination.

Composition and Preparation of Differential Media

The media is listed in alphabetical order. Preparation is performed according to the manufacturer's instructions. Catalog number for each respective manufacture is provided in parenthesis.

1) CHROMagar (CHROMagar Microbiology, catalog number *EC168)*

Composition:

a. Agar	15.0 g
b. Chromogenic mix	9.0 g
c. Peptone,yeast and meat extracts	8.3 g
d. Sodium chloride	5.0 g
Adjust pH to 6.0 ±0.2	

Preparation:

Measure 37.3 grams of powder for 1L of purified water. Disperse powder slowly into a sterile flask containing purified water until swelling of the agar. Bring to a boil (100^0C) in a microwave, take flask out, stir gently, and return flask to oven for short repeated heating until complete fusion of agar grains. Place flask into a 50°C agar tempering bath. Dispense 6 mL quantities into 60 x 15mm sterile Petri plates and allow to gel and dry. Store plates in the dark under refrigeration (2-8^0C) for 2 weeks if properly prepared and protected from light and dehydration.

DEVTryptophan broth (EMD, catalog number *1.10694.0500)*

Composition:

a. Peptone from meat	10.0 g
b. DL-Tryptophan	1.0 g
c. Sodium chloride	5.0 g
pH 7.2 ± 0.2 at 25°C	

Preparation:

Measure 16 grams of Tryptophan broth for 1L of purified water. Dispense into suitable tubes and autoclave for 15 min at 121°C. Allow tubes to cool down at room temperature and store at 2-8°C.

MacConkey II Agar (Becton Dickinson/Difco, Catalog number *212306)*

Composition:

a. Pancreatic Digest of Gelatin	17.0 g
b. Pancreatic Digest of Casein	1.5 g
c. Peptic Digest of Animal Tissue	1.5 g
d. Lactose	10.0 g
e. Bile Salts	1.5 g
f. Sodium Chloride	5.0 g
g. Agar	13.5 g
h. Neutral Red	0.03 g
i. Crystal Violet	0.01 g

Preparation:

Measure 50 g of powder in 1L of purified water. Mix thoroughly in a flask. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave media at 121°C for 15 minutes. Place flask into a 50°C tempering bath. Dispense 20 mL quantities into 100 x 15mm sterile petri plates and allow to gel and dry. Store plates at 2-8°C.

mFC Agar (Difco, catalog number *267720)*
Rosalic Acid (Difco, catalog number *232281)*

Composition:

a. Agar	15.0 g
b. Pancreatic Digest of Casein	6.0 g
c. Yeast Extract	3.0 g
d. Sodium chloride	5.0 g
e. Lactose	12.5 g
f. Proteose Peptone # 3	9.0 g
g. Bile salts # 3	1.5 g
h. Aniline Blue	0.1 g
pH 7.4	

Preparation:

Rosolic Acid: Prepare a 1% solution, dissolving 1 g in 100 mL of 0.2N of NaOH.

Preparation:

Measure 52 grams of powder in 1 L of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Add 10 ml of 1% solution of Rosolic Acid in 0.2 N NaOH. Continue heating for 1 minute. DO NOT AUTOCLAVE. Dispense 6 mL quantities into 60 x 15mm sterile petri plates and allow to gel and dry. Store plates at 2-8°C.

MR-VP Broth (EMD catalog number *1.05712.0500)*

Composition:

a. Peptone from meat	7.0 g
b. D(+) Glucose	5.0 g
c. Phosphate buffer	5.0 g
pH 6.9 ± 0.2 at 25°C	

Preparation:

Measure 17 g of powder in 1 L of purified water. Dispense into suitable tubes and autoclave for 15 min at 121°C. Allow tubes to cool down at room temperature and store at 2-8°C.
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Phosphate Buffered Saline (Sigma-Aldrich, catalog number *P3813)*

Composition (0.01 M):

a. NaCL	0.0138 M
b. KCl	0.0027 M
pH 7.4	

Preparation:

Autoclave 1 L of reagent grade water for 15 minutes at 121°C. Open premix carefully and pour contents into bottle or flask without touching the inside, and close or cover immediately. Shake well.

Simmons Citrate Agar (BBL, catalog number *211620)*

Composition:

a. Ammonium Dihydrogen Phosphate	1.0 g
b. Dipotassium Phosphate	1.0 g
c. Sodium Chloride	5.0 g
d. Sodium Citrate	2.0 g
e. Magnesium Sulfate	0.2 g
f. Agar	15.0 g
g. Bromthymol Blue	0.08 g
pH 6.9 ± 0.2 at 25°C	

Preparation:

Measure 24.2 g Simmons Citrate Agar to 1 L of purified water. Mix well. Heat with frequent agitation and boil for 1 minute to dissolve the powder completely. Dispense into screwcap tubes and autoclave at 121°C for 15 min. Allow to cool in slanted position.

Triple Sugar Iron Agar (Difco, catalog number *226540)*

Composition:

a. Beef Extract	3.0 g
b. Yeast Extract	3.0 g
c. Pancreatic Digest of Casein	15.0 g
d. Proteose Peptone No. 3	5.0 g
e. Dextrose	1.0 g
f. Lactose	10.0 g
g. Sucrose	10.0 g
h. Ferrous Sulfate	0.2 g
i. Sodium Chloride	5.0 g
j. Sodium Thiosulfate	0.3 g
k. Agar	12.0 g
l. Phenol Red	24.0 mg

Preparation:

Measure 65 grams powder in 1 L of purified water, mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Dispense into tubes and autoclave at 118-121°C for 15 minutes. Cool in a slanted position so that deep butts are formed.

Urea Agar Base (BBL, catalog number *211795)*
Agar-Agar (EMD 1.01614.0500)

Composition of Urea Agar Base:

a. Pancreatic Digest of Gelatin	1.0 g
b. Dextrose	1.0 g
c. Sodium Chloride	5.0 g
d. Potassium Phosphate	2.0 g
e. Urea	20.0 g
f. Phenol Red	12.0 mg

Preparation:
Measure 29 g of the Urea Agar Base in 100 mL of purified water. Mix thoroughly. Sterilize by filtration. Suspend 15 g of agar in 900 mL of purified water. Autoclave at 121°C for 15 minutes. Cool to 50°C and add 100 mL of the sterile Urea Agar Base. Mix thoroughly and dispense aseptically in sterile tubes. Cool tubed medium in a slanted position so that deep butts are formed.

Laboratory Methods

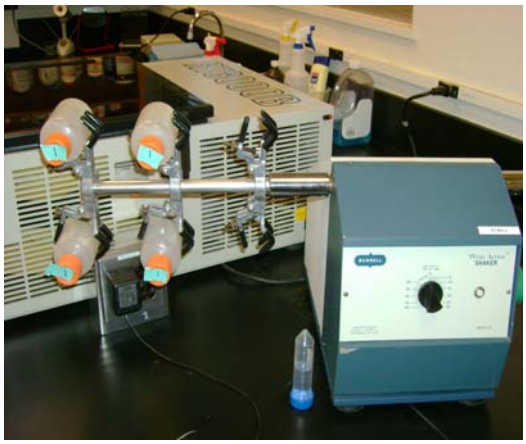
Water and Total Suspended Solids

Step 1

A. Homogenize 1 L of water sample by hand and pour aliquots of 250 mL into sterile conical centrifuge bottles displayed below.

Step 2

A. Place the bottles on an automated wrist shaker in a horizontal position (below left).
B. Set the wrist shaker arm at 45 degree angle.
C. Homogenize sample for 5 minutes at setting 7 using the control panel. (below right).



Wrist shaker with samples.

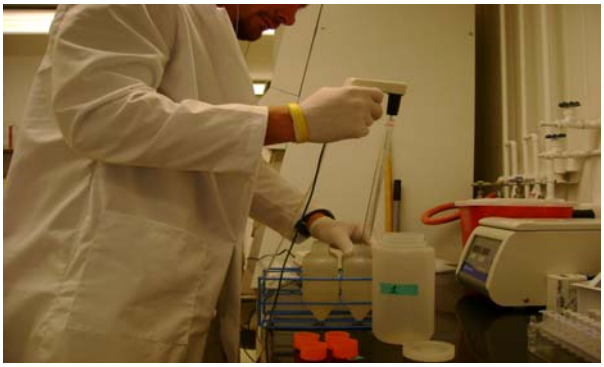
Wrist shaker controls.

Step 3

A. Centrifuge sample at 1,000 *g* for 10 minutes (below).

Step 4

A. Remove 200 ml of supernatant from each bottle using a sterile pipette (below).



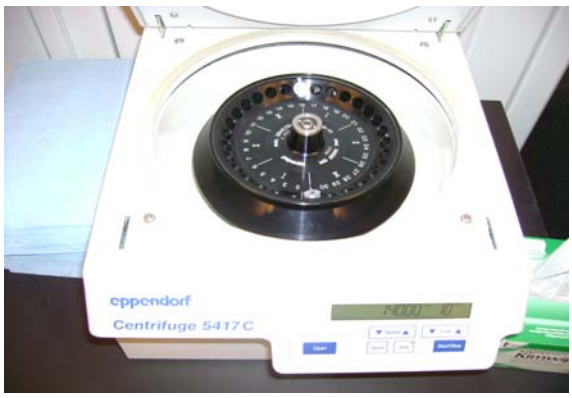
B. Dispense supernatants into a sterile bottle.
C. Discard the remaining supernatant by vacuum filtration without disturbing the pellet formed in the bottom of each conical centrifuge bottle.

Step 5

A. Pre-weigh a set of 2.0 mL centrifuge tube.
B. Record the weight.

Step 6

A. Resuspend the residual pellet from each of the four centrifuge bottles in 0.5 ml of purified water.
B. Combine the suspended residual pellet into the pre-weighed 2.0 ml micro-centrifuge tubes from step 5.
C. Centrifuge tubes at 14,000 *g* for 10 minutes (below).



Sample in micro-centrifuge with counterbalance

Step 7

A. Remove the supernatant without disturbing the pellet formed after centrifugation.
B. Weight the microcentrifuge tube containing the pellet.
D. Record the pellet weight.

Step 8

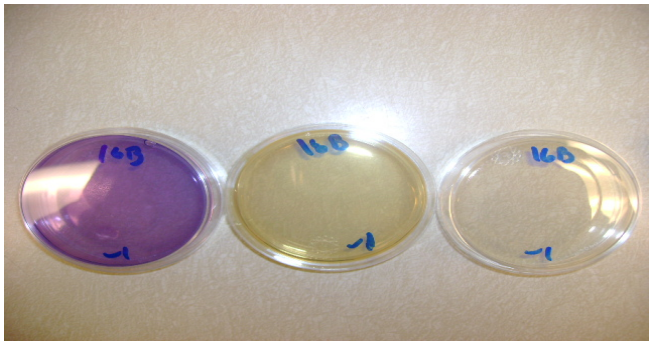
A. Resuspend the pellet in 1,200 µl of phosphate buffered saline.
B. Dispense 800 µl of resuspended pellet to 45 mL of sterile PBS (following page).
C. Dispense 400 µl of resuspended pellet to 45 mL of sterile PBS (following page).

Sediment Analysis

A. Dispense 90 mL of sterile PBS into 250 mL conical centrifuge tube.
B. Weight 20 grams of sediment.
C. Dispense 20 grams of sediment into 250 ml conical tube containing 90 mL of sterile PBS.
D. Mix suspension with an automated wrist shaker for 5 minutes at setting 7.
E. Centrifuge at 500 *g* for 10 minutes.
F. Divide supernatant into two 45 ml aliquots.

Membrane Filtration of Water, Total Suspended Solid and Sediment Samples

A. Disinfect bench area prior of sample filtration for bacteria enumeration.
B. Remove plates from refrigerator and allow them to warm at room temperature.
C. Label each plate bottom with sample number, dilution and necessary information (below).



Process for Membrane Filtration:

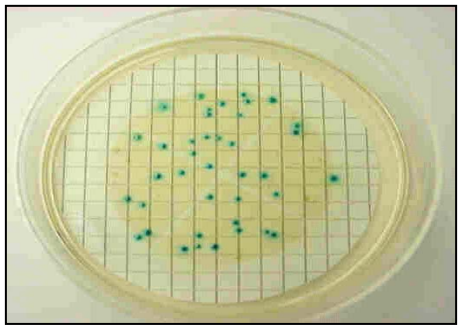
1. Shake sample bottle at a 1 ft. arc for 30 sec. until homogenized.
2. Dip smooth flat forceps, without corrugation in the inner side of the tip, into 90% ethyl or absolute methyl alcohol and flame.
3. Place membrane filters, without the waxy paper, onto pre-sterilized funnel (below).

4. For water samples with low turbidity, pour 100 mL and/or additional serial dilutions of 45 mL PBS. For TSS and sediment samples, pour samples re-suspended in 45 mL of PBS and/or any additional serial dilution into the filter unit**.** Use sterile funnels for each volume to be tested**.**
5. Wet the membrane filter with approximately 10 ml of sterile water.
6. Turn on vacuum line and manifold dial counter clockwise to open valve.
7. Filter the sample and rinse the inside of each funnel with about 50 mL sterile water to ensure the entire sample has been filtered.
8. Use sterile forceps to remove membrane filters and roll it, side up, onto selective media. Air spaces between the filter and media are to be avoided.
9. Place CHROMagar (EC) plates in a 35°C incubator for 2 hr resuscitation period and transfer to 44.5°C incubator for 24 hr ±4 hr.
10. Place mFC (FC) plates directly into a 44.5°C incubator for 24 hr ±4 hr.
11. Monitor incubator temperature 24 hrs before, during, and after samples arrive in lab for analysis. Record each temperature in the morning and in the afternoon.

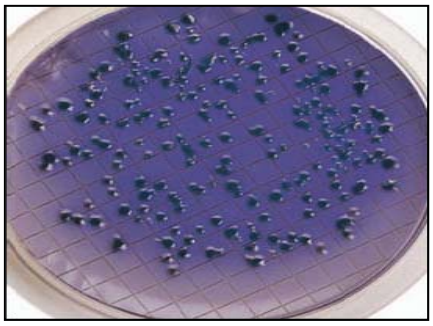
12. Sterilize filter funnels for 2 minutes in an ultraviolet sterilizer.
13. Repeat procedures 1-12 for additional samples.

Interpretation

1. Colonies appear BLUE on CHROMagar (below left).
2. Fecal coliform colonies appear BLUE on m FC agar (below right).



Typical *E. coli* on CHROMagar EC



Typical Fecal Coliform on mFC agar

Estimation of Colony Forming Unit (CFU)

1. Select plates between 20-80 colonies.
2. Record as TNTC for "Too numerous to count" plates above 80 colonies.
3. Report as < 1 fecal coliform or /100 mL if no colony is present.

CFU estimation for water samples:

$$\text{Total (cfu/100 mL)} = \frac{(\text{Number of colonies})\,(100)}{\text{Volume of sample (mL)}}$$

CFU estimation for Total Suspended Solids:

$$\text{Total (cfu/g)} = \frac{\text{Number of colonies}}{\text{Mass of negative pellet (g)}}$$

CFU estimation for Sediment:

$$\text{Total (cfu/g)} = \frac{\text{Number of colonies}}{\text{Mass of sediment processed (g)}}$$

Confirmation

1. Select typical colony observed on CHROMagar EC and streak onto MacConkey II agar plates.
2. Incubate plates at 37°C for 24 hr ±4 hr.

3. Select typical colony produced on MacConkey II (below) upon ability of the isolate to ferment the carbohydrate. Colorless or pink to red colonies should be expected on this medium.

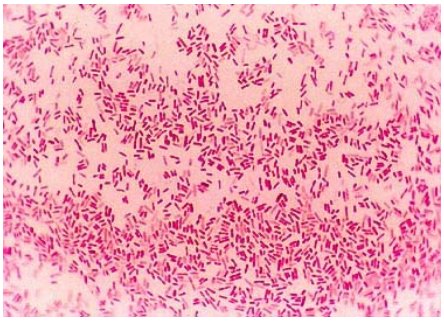
Isolated *E. coli* on MacConkey II agar
http://www.emlab.com/m/media/Ecoli_Feb07.jpg

4. Select a single colony to perform biochemical and morphology confirmation.
5. Streak selected colony onto Sheep blood agar and incubate at 37°C for 24 hr ±4 hr and perform Gram stain.

Gram Stain Procedure:

1. Use a wax pencil to draw circles or to divide a clean slide into sections.
2. Make a dot on the upper right-hand corner of the slide for slide orientation.
3. Add a drop or large loopful of purified water onto selected area of the slide.
4. Flame a straight wire or loop and let it cool.
5. Touch part of the culture to be examined with your loop or wire.
6. Touch the drop of water on the slide with the bit of culture and mix the culture into the water.
7. Spread the suspension into a thin layer over the slide.
8. Allow smear to air dry.
9. Heat slide by passing over a flame 2-3 times.
10. Add crystal violet drop by drop until the smear is covered.
11. Let crystal violet for 1 minute.
12. Rinse the slide very gently with water and blot.
13. Cover smear with Gram's iodine for 1 minute.
14. Drain off the iodine and wash gently with 95% ethanol. The ethanol wash can be done by gently dripping the ethanol down the slide held in a tilt position. This procedure should take 10-20 seconds.
15. Rinse the smear very gently with water and blot.
16. Cover smear with safranin for 30 seconds.
17. Rinse the smear gently with water and blot dry.
18. Place a drop of immersion oil directly onto the stain and examine with the 100X objective using bright field illumination.

19. *E. coli* is gram negative rod. (below).



Typical E coli under microscope.
http://student.ccbcmd.edu/courses/bio141/lecguide/unit1/prostruct/images/gnrod.jpg

Biochemical Reactions

Indole test (DEVTryptophan broth)

Procedure: Inoculate a loopful of bacteria into tryptone broth. Incubate for 24 hours at 37^0C.

Interpretation: Add 0.2 – 0.3 mL (4-6 drops) of Kovac's reagent and shake gently. Watch the tube for 10 minutes for the appearance of a dark red ring at the top of culture. Color can also be developed immediately after the addition of Kovac's reagent and then fade. Some bacteria split tryptophan into indole and pyruvic acid using tryptophanase. This test is very important in differentiating (indole positive) from some closely related enteric bacteria.

MRVP test (MRVP broth)

Procedure: Inoculate a loopful of bacteria into MRVP broth. Incubate for 48 hours at 37^0C

1) Methyl Red test

Interpretation: Remove 1 mL sample of the MRVP broth culture and transfer to a sterile tube test. Add several drops of methyl red. A red color is a positive test and a yellow color is a negative test. produce a mixed acid reaction which is detected by addition of methyl red.

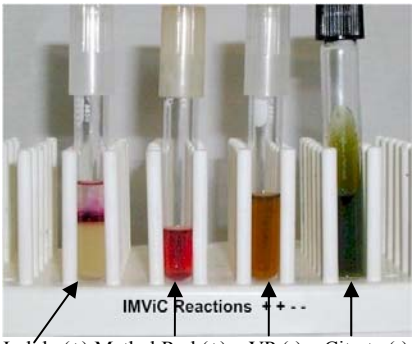
2) Voges-Proskauer Test

Interpretation: Remove another 1 mL sample from the MRVP broth and transfer to a sterile tube test. Add 0.6 mL (12 drops) of the VP reagent A and 0.2 mL (4 drops) of the 40% KOH, VP reagent B and shake gently to mix. Let the tube stand undisturbed for 2 hours and check from time to time for the appearance of a pink to red color. The color is indicative of acetoin which is an intermediate in the formation of butylene glycol. A negative result can either produce a gray color or no change of color. This test is negative for *E. coli*.

Citrate test (Simmon's Citrate)

Procedure: Streak a loop full of bacteria onto a citrate agar slant. Incubate for 48 hours at 37^0C.

Interpretation: A positive reaction is indicated by a slant with a blue color. A negative slant will have no growth of bacteria and will remain green. Citrate can be the sole source of carbon and energy source for growth. *E. coli* will not grow in this medium.

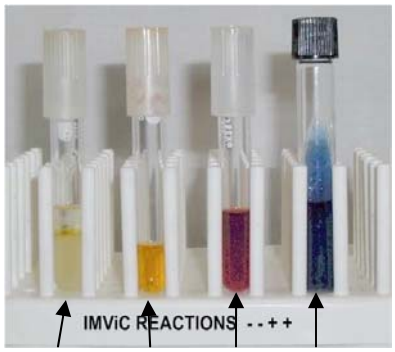


Indole (+) Methyl Red (+) VP (-) Citrate (-)

Picture 22

http://science.nhmccd.edu/biol/wellmeyer/media/IMViC.htm

IMVC test - Typical result



Indole (-) Methyl Red (-) VP (+) Citrate (+)

Picture 23

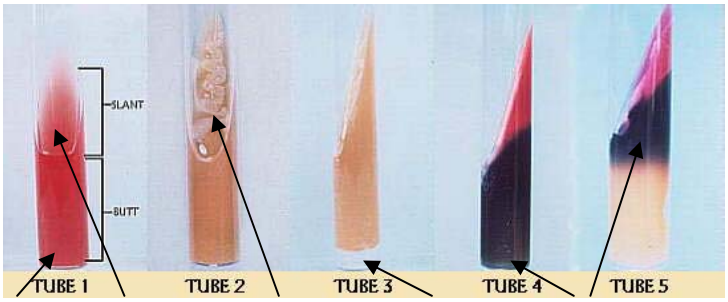
http://science.nhmccd.edu/biol/wellmeyer/media/IMViC.htm

IMVC test - Typical *E. aerogenes* result

Triple Sugar Iron Agar (TSI)

Procedure: Stab one isolated colony into the butt of the tube, and then streak back and forth along the surface of the slant. Incubate with caps loosened at 35ºC for 24 hours.

Interpretation: Examine after 18-24 hours for carbohydrate fermentation, gas production and hydrogen sulfide (H_2S) production. Any combination of these reactions may be observed. Typical *E. coli* will produce yellow butt, yellow slant, gas production, and growth on slant (below)



Urease Reaction (Urea Agar)

Procedure: Streak the surface of a urea agar slant with an isolated colony of the organism to be tested. The butt will serve as a color control. Leave the cap of the tube on loosely and incubate the tube for 8-24 hours at 35ºC. A negative test can be re-incubated for up to 6 days.

Interpretation: Ammonia is formed during incubation and makes the media alkaline, producing a pink color. *E. coli* should produce a negative result by this test (below).

Decay Curve Laboratory Methods

Differences in the number of hours needed to sample each estuary resulted in different intervals of time between sample collection and processing at the analytical laboratory. In order to adjust bacterial indicator (fecal coliforms,) enumerations to a single time-duration standard of

24 hours, we conducted a time-dependent decay analysis for fecal coliforms and (as described above in the "Membrane Filtration of Water, TSS, and Sediment Samples" section). For each estuary (n=5) and for each season (n=3, dry baseflow, wet baseflow, wet stormflow), two water and two sediment samples were collected on different dates (n=2). For each of these 30 sampling dates, fecal coliforms and were enumerated in residual water, suspended solids, and sediment supernatant using membrane filtration as described above at approximately 4, 8, 24, 30, 48, and 54 hours post-collection in order to generate the necessary raw data for statistical modeling of the decay coefficients in our source water.

STATISTICAL ANALYSES

Decay Curve Estimation Procedures

A linear mixed effects regression model (Pinheiro and Bates, 2000) was used to estimate the magnitude and significance of the time-dependent decay coefficients for fecal coliforms and in our source water. The $\log_{10}$ concentration of each bacterial indicator was used as the outcome variable, site, season and time (duration in hours between water collection in the field and bacterial enumeration in the laboratory) were set as fixed effects, and the group of enumeration for the same sample date were set as a repeated measure (group random effect) to control for potential lack of independence of bacterial concentration. In addition, given marked heteroscedasticity of the error term across time, an exponential variance term was included in the model (Pinheiro and Bates, 2000). Level of significance for the various terms was set at P-value<0.05, based on either a likelihood ratio test for interaction terms or a conditional t-test for main terms. Results of this analysis identified significant decay coefficients for in water and sediment related to season and estuary (Table 1).

Table 1: Estimated time-dependent decay coefficients for adjusting fecal coliforms and to a 24 hour duration standard, stratified by matrix (residual water, suspended solids, sediment) and indicator bacteria (fecal coliforms,).

Factors	Coefficient[c]	P-value[b]
Water - Fecal coliforms	N.S.	>0.10
Water -		
Duration (hr)	-0.00021	0.92
Duration H Season interaction		
wet-storm[a]	0.0	--
wet-base	-0.0045	0.02
dry-base	-0.0078	0.0001
Duration H Site interaction		
Russian River[a]	0.0	--
Walker Creek	0.0023	0.37
Salmon Creek	-0.00034	.90
Estero Americano	0.0072	0.005
Lagunitas Creek	0.00063	0.82
Suspended solids - Fecal coliforms	N.S.	>0.10
Suspended solids -	N.S.	>0.10
Sediment - Fecal coliforms	N.S.	>0.10
Sediment -		
Duration (hr)	0.0043	0.04
Duration H Season interaction		
wet-storm[a]	0.0	--
wet-base	-0.0026	0.32
dry-base	-0.0054	0.03

[a] Referent condition for the categorical variable. [b] Adjusted for potential lack of independence due to repeated sampling of high use areas across storms.

In order to adjust the bacterial indicator (BI) concentration in each sample matrix (residual water, suspended solids, sediment) tested *x* hours (t=x) after initial time of collection (t=0) to a single 24-hour standard (t=24), we first assumed the following basic model,

$$\log_{10}(BI_{t=x}) = \log_{10}(BI_{t=0}) + \beta(t = x) \quad (1)$$

whereby $\log_{10}(BI_{t=x})$ is the observed $\log_{10}$ concentration of the bacterial indicator determined x hours (t=x) after initial time of collection, $\log_{10}(BI_{t=0})$ is the modeled $\log_{10}$ concentration of the bacterial indicator at the initial time of collection (t=0), and $\exists(t=x)$ is the fitted decay coefficient generated by the linear mixed effects model described above. The decay process is for samples held at approximately 4°C. Once $\exists(t=x)$ is obtained, equation (2) is used to adjust each sample to a single 24-hour standard (t=24), which is derived as follows,

$$\log_{10}(BI_{t=24}) = \log_{10}(BI_{t=0}) + \beta(24)$$
$$\log_{10}(BI_{t=24}) = \log_{10}(BI_{t=x}) - \beta(x) + \beta(24)$$
$$\log_{10}(BI_{t=24}) = \log_{10}(BI_{t=x}) + \beta(24 - x)$$
$$BI_{t=24} = (BI_{t=x})\, 10^{\beta(24-x)} \quad (2)$$

whereby $BI_{t=24}$ is the fitted or expected concentration of the bacterial indicator at a 24-hour standard, $BI_{t=x}$ is the observed concentration of the bacterial indicator determined x hours (t=x) after initial time of collection, and $10^{\exists(24-x)}$ is the expected decay coefficient adjustment factor raised to the power of 10 which allows us to model concentrations of the bacterial indicator directly instead of $\log_{10}$ concentration values.

Regression Analyses for Estimating Biases and Influences

Linear mixed effects regression (Pinheiro and Bates, 2000) was used to identify factors such as transect location, depth of water sample, and season and flow regime classification (dry season base flow, wet season base flow, wet season storm flow) cumulative annual rainfall, and tidal factors associated with indicator bacteria values. Fecal coliforms and concentrations and instantaneous loads functioned as the outcome variables, with each sample location (estuary, transect, position, depth) as a group effect to adjust the *P*-values for repeated sampling at the same sites. We employed a forward stepping approach in final modeling to develop the multivariate regression model, with $P<0.05$ set as the criterion for inclusion of predictor variables in the final model.

BIASES AND INFLUENCES

The terms biases and influences represent factors introduced through sampling decisions and field conditions, respectively, that have a significant influence on California estuarine water bacteria values. This discussion begins with the site conditions or **influences** that exist in these estuaries because they are the context in which a monitoring program is developed and conducted. With these background influences established, the discussion will present **biases** or the ways in which sample collection decisions within the estuary or water column can influence bacterial enumeration in water from these systems.

Season

California's Mediterranean climate predetermines that an estuary monitoring program will generally encounter three hydrologic conditions or seasons:

- **Wet Season Stormflow** during periods of high rainfall and resulting increased runoff and maximum delivery of freshwater from a respective estuary's contributing watershed and drainage area.
- **Wet Season Baseflow** between storms during the wet winter months from November through April.
- **Dry Season Baseflow** during the dry summer months from approximately May through September or October.

As indicated, there is an environmental gradient across these seasons from maximum to minimum hydrologic connectivity of an estuary to its surrounding uplands and drainage area. Rainfall that results in runoff in area streams, may only have a delay of hours or days before it moves through an estuary during wet season stormflow. Conversely, little to no upland surface water reaches the estuary because of intermittent or ephemeral conditions that dominate these systems during dry season baseflow.

The magnitude of difference in general estuary water quality between the three seasons is profound and therefore must be accounted for in a monitoring program. There can easily be a three order of magnitude difference in fecal coliform and values for example (Figure 4).

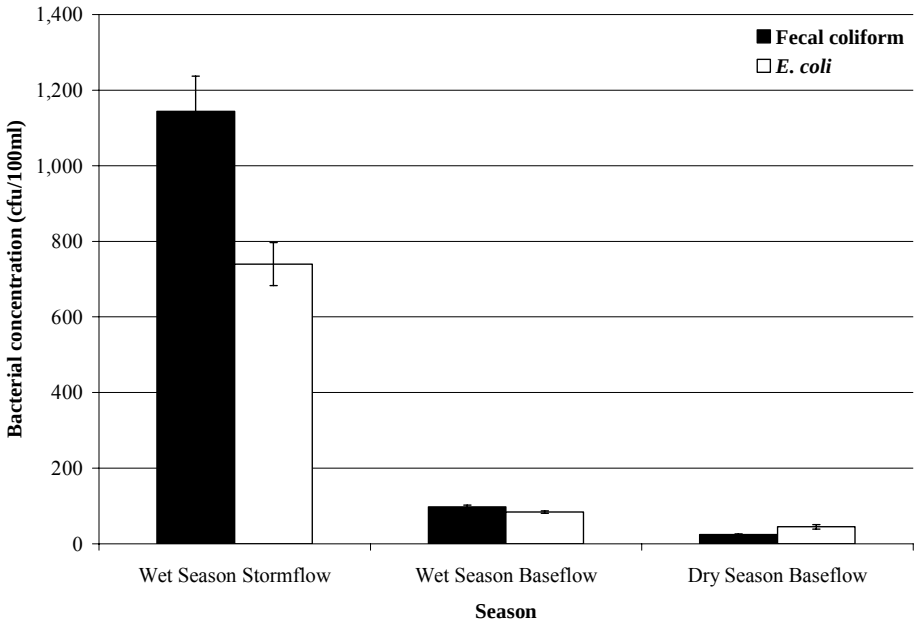


Figure 4: Concentrations of Fecal coliform and in five studied estuaries during three respective seasons, wet season stormflow, wet season baseflow, and dry season baseflow.

Placing each collected sample into one of these three seasons will contribute to forming the context needed to correctly interpret results. This is done through annual and storm

hydrograph analysis or separation (Brooks et al. 1997; Hewlett and Hibbert, 1967) that can be easily performed using stage height and flow data available through the United States Geologic Survey for California (http://waterdata.usgs.gov/ca/nwis/rt). An example of the available discharge data is provided in Figure 5. This is the daily discharge in the Russian River at the Guerneville, California gauging station from October 1, 2005 to September 30, 2006.

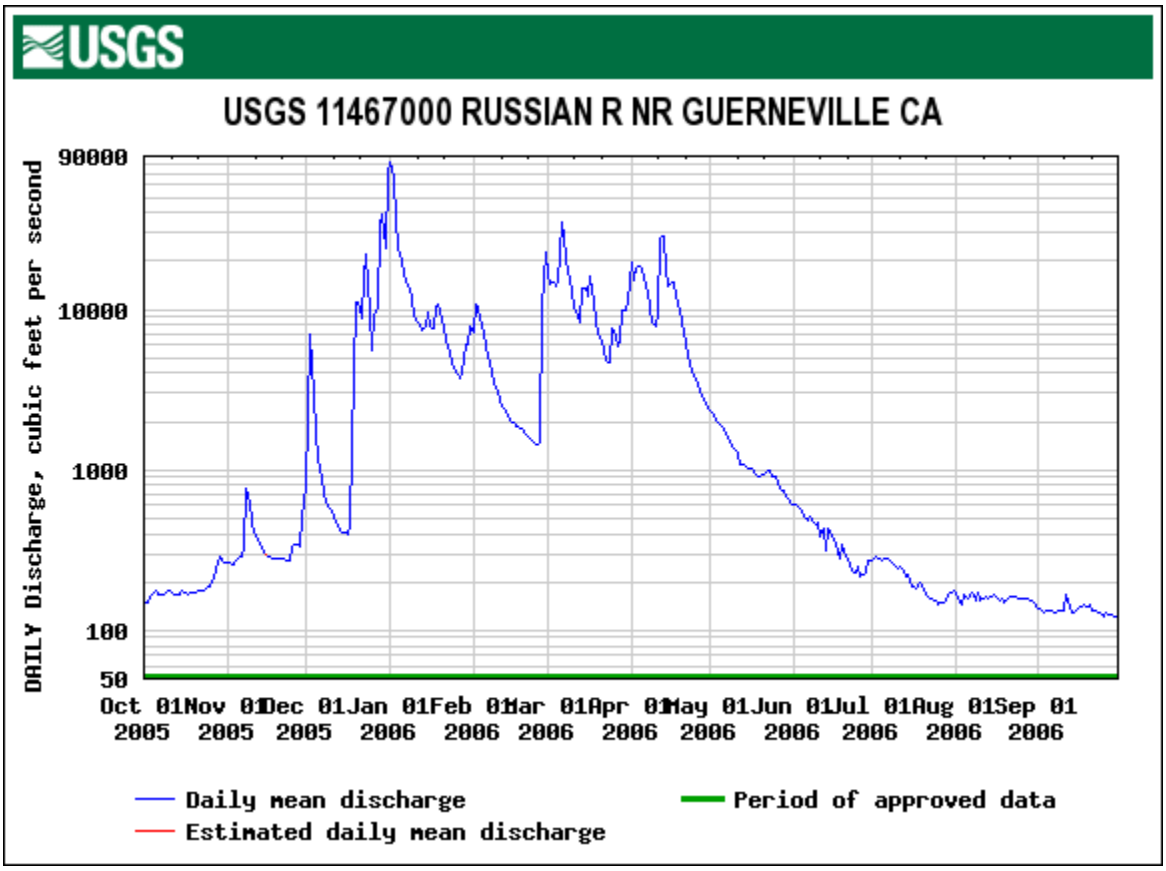


Figure 5: Figure of daily discharge for the Russian River at the Gurneville Bridge in Gurneville, California that was generated from the United States Geologic Survey web based resource for discharge data.

This type of data is generally available for a site near a studied estuary and can be downloaded as the graph presented or as table for further analysis such as that demonstrated in Figure 6. By comparing monitoring program sample dates to this data, samples can be placed into three broad seasonal groups. This includes the lower baseflow discharge values during the dry season, high stormflow discharge values indicated by rapid increases in discharge during the wet season, and baseflow between the peak discharges identified as the valleys in the hydrograph in between storms during the wet season (Figure 6).

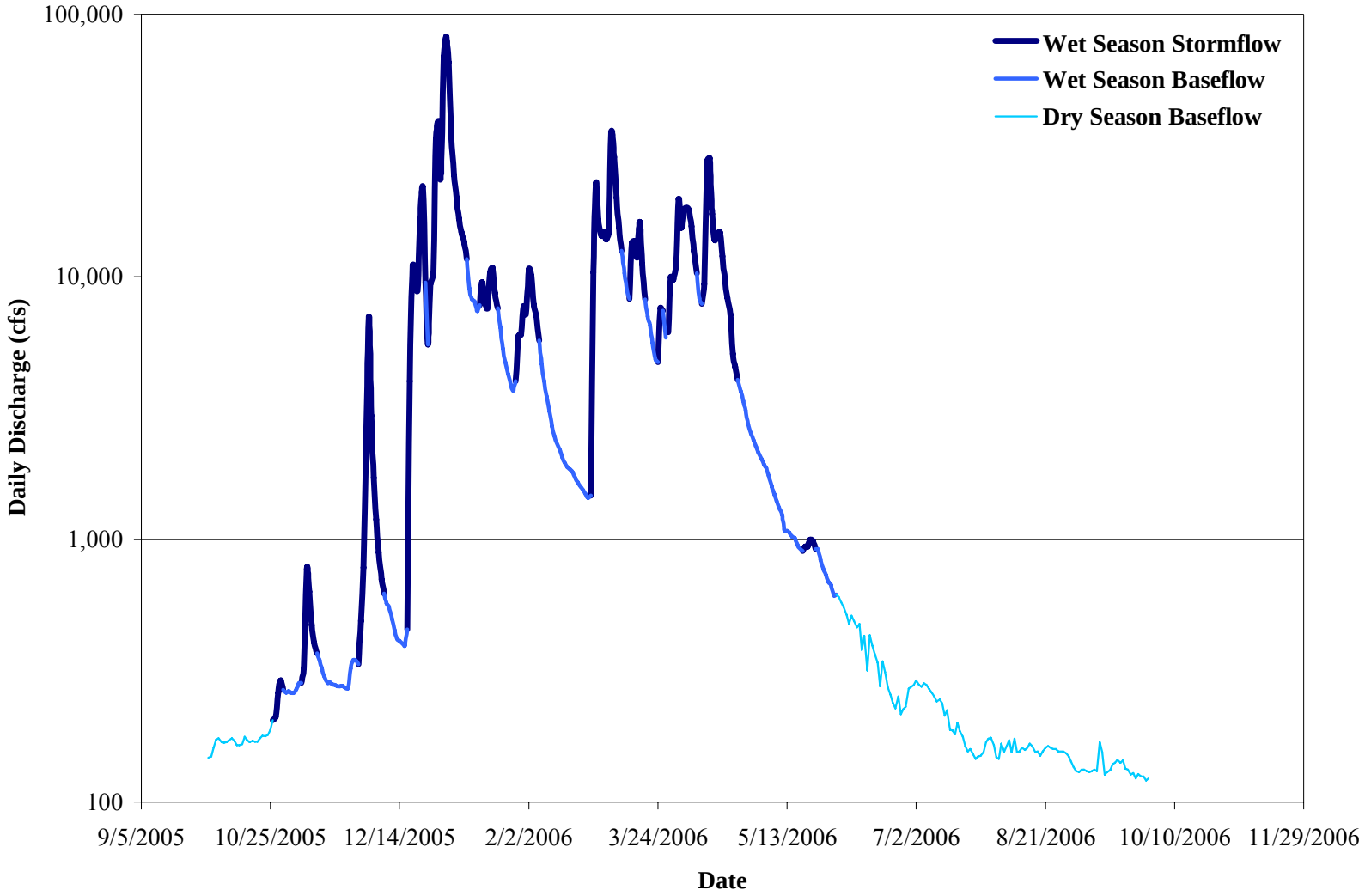


Figure 6: Analysis of the annual hydrograph of daily discharge in the Russian River at Gurneville, California. Daily discharge values have been categorized into one of three seasons, wet season stromflow, wet season baseflow, or dry season baseflow.

Salinity

Ocean water salinity is typically 33 to 37 ppt compared to stream water at 3 to 5 ppt. Salinity within these systems is variable seasonally and from the mouth inland based upon the interface between these two sources of water. The important to point to keep mind is that the position of that interface is moving back and forth through the estuary. During storms and high stream discharge the entire estuary can be virtually a freshwater body. Comparatively, summer periods of low discharge allow tides and the salinity of ocean water to dominate the location of the freshwater/saltwater interface. And in extreme cases such as bar built estuaries, hypersaline conditions can be experienced during the summer.

There is a strong indirect and significant ($p>0.05$) relationship between salinity and concentrations of Fecal coliform and in these estuaries (Figure 7). By measuring salinity simultaneously to sample collection for bacterial enumeration, a monitoring program will firstly be able to create reasonable expectations for microbial concentrations. Accordingly, when bacterial values are anomalous to this relationship the monitoring program can use these finding to identify if field laboratory methods have been comprised or if the findings are pointing to microbial delivery and transport of significant note.

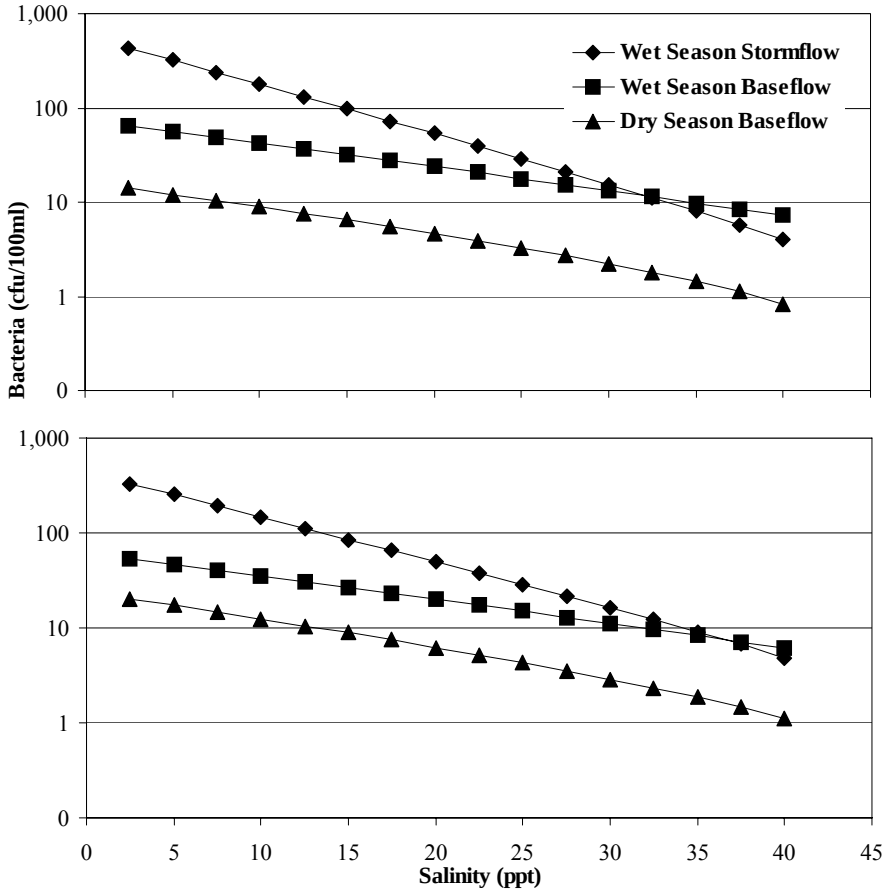


Figure 7: Results of a data driven model that estimate the concentrations of fecal coliform (top) and (bottom) as function of salinity in estuarine waters.

Precipitation

As discussed in earlier in the section on seasons, California coastal climate is predominately Mediterranean. The result is that over 80 percent of the annual rainfall occurs between October and April. The effect of this precipitation distribution is a flushing from the uplands and surrounding drainage area to a respective estuary. This flushing occurs at multiple time steps including annual, five-day, and 24-hour periods (Figure 8).

On an annual basis fecal coliform and concentrations in estuarine waters increase with annual rainfall to approximately 20 total inches of precipitation. At that juncture microbial concentrations decrease. The implication is that bacteria sources within the contributing watershed are limited in the amount of bacteria available for delivery to the estuary. Similar dynamics and flushing occur for 5-day and 24-hour cumulative rainfall.

Because of this flushing, tracking cumulative rainfall amounts for each sample collected will strengthen the interpretation of such results. One option for this is to instrument the estuary and watershed with self-recording rain gages. Alternatively, rainfall data is from existing gages in the area can be used to meet this purpose. A good source for such data is the California Data Exchange Center (http://cdec.water.ca.gov/).

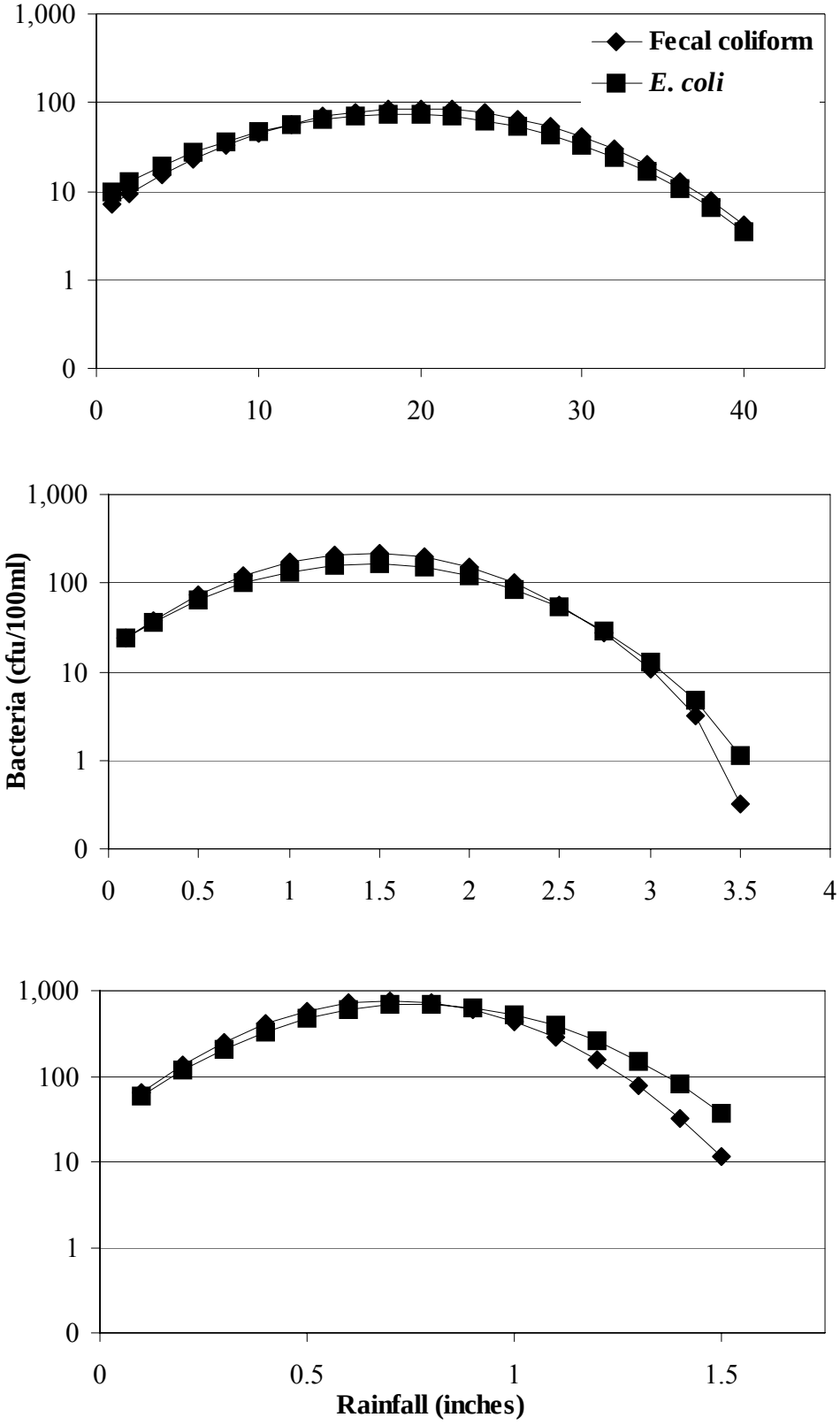


Figure 8: Results of a data driven model that estimate the concentrations of Fecal coliform and in estuarine waters as functions of annual (top), 5-day (middle), and 24-hourl (bottom) cumulative precipitation.

Depth

Three depth group categories were used that corresponded to the depths at which water samples were collected. The *shallow* designation corresponds to samples collected 0.1 m below the water's surface. Samples collected at the mid-point of the water column were grouped into the *intermediate* category. And those samples collected at 0.1 m above the estuary bottom were grouped into the *deep* category.

Results from this analysis indicate a decreasing trend in indicator bacteria concentration with depth. In contrast, values for both salinity and turbidity increase with depth. Regression analyses confirms that these patterns are significant ($p<0.05$).

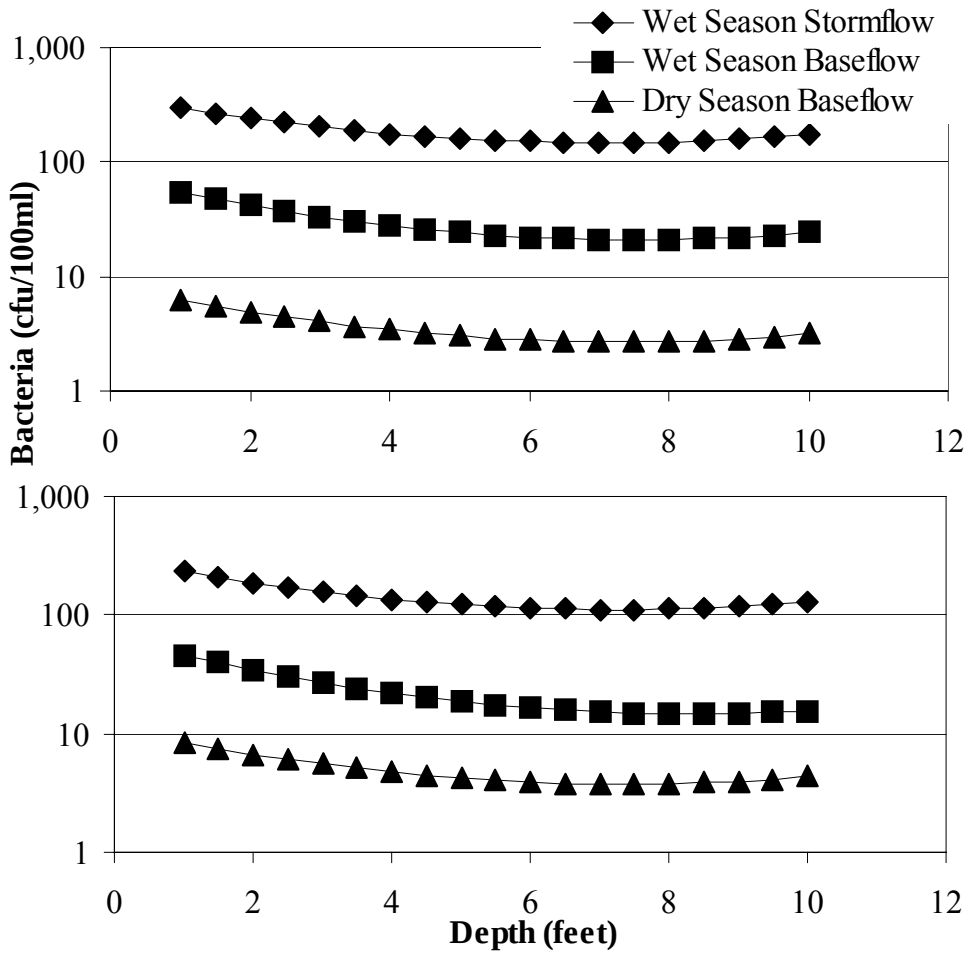


Figure 9: Results of a data driven model that estimate the concentrations of Fecal coliform (top) and (bottom) in estuarine waters as a function of depth in water column at which a sample is collected

Location

Graphical presentation of bacteria concentrations to each of the five transect locations indicates that they are greatest at transect three (Figure 10). This is likely because of the pattern of bacteria concentrations in the Estero Americano wherein the highest concentrations were consistently at transect three. Regression modeling identified a significant increase ($p>0.05$) in bacteria concentrations from transect one to transect five, or moving inland from the mouth of the estuary to its contributing stream or river. Similarly salinity values decreased significantly moving inland from transect one to transect five. Turbidity values, in contrast, increased to transect four and then decreased.

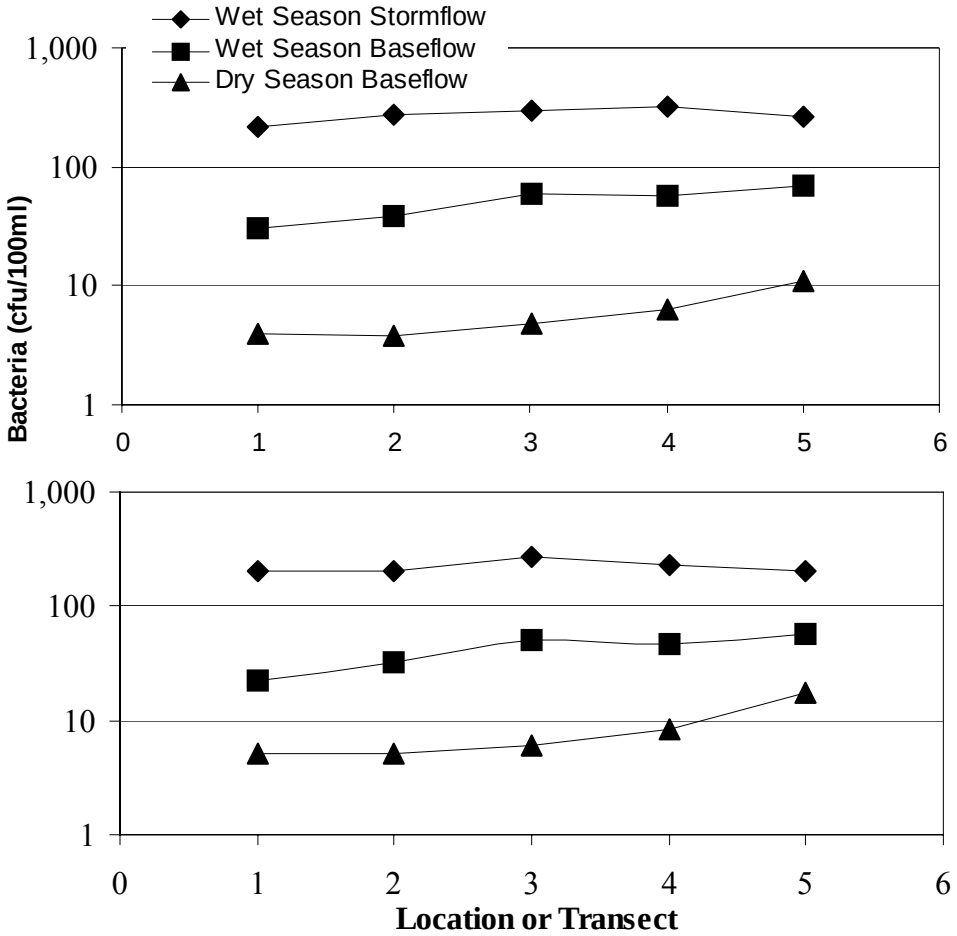


Figure10: Results of a data driven model that estimate the concentrations Fecal coliform (top) and (bottom) in estuarine waters as a function of location or transect within the estuary. Location 1 represents the mouth of the estuary and Location 5 the point furthest inland.

The relationship is relevant for monitoring in an estuary with limited access. For example it may only be possible to monitor from a public access point such as a bridge. Understanding and documenting the distance from the mouth of the estuary will contribute to a better intuition and interpretation of monitoring results.

Position

Concentrations of fecal coliforms and were not significantly associated with the differing sample positions of left, middle, and right within the channel.

Estuarine Sediment as an Environmental Reservoir

Estuary functions include the collection, storage, and release of sediment that can serve as an environmental reservoir for bacteria. Fecal coliform concentrations within the sediment of the five studied estuaries ranged from 10 to 320 cfu/gm and values were 8 to 340 cfu/gm. These concentrations translate to over 2 million fecal coliform and up to three quarters of a million on average in one square meter of estuary bottom 15 cms deep. If resuspended into the water column, through tidal and wind action, these sediments and associated bacteria have the potential to elevate water column bacterial values.

There is a similar seasonal influence on sediment bacteria values to that observed in water values. Specifically, the highest concentrations are in Wet Season Stormflow, followed by Wet Season Baseflow, followed by Dry Season Baseflow (Figure 11). Results from genetic analysis indicate that there is greater population variability within the water and the sediment fraction than there is relatedness between the two.

These materials can vary in there composition with regard to mineral and organic matter. They also can have varying size class distributions. This is important because percent fines in the sediments is directly related to the concentration of both fecal coliform and in these materials. This relationship is statistically significant for the two Wet Season flow periods but not during Dry Season Baseflow.

These observations combine to indicate bacteria is more likely being deposited or laid down through wet season transport processes and less likely to be resuspended into the water column and confound water bacteria values. It is worth noting, however, that there have been exceptions to this conclusion documented in other systems (Wu et al. 2007).

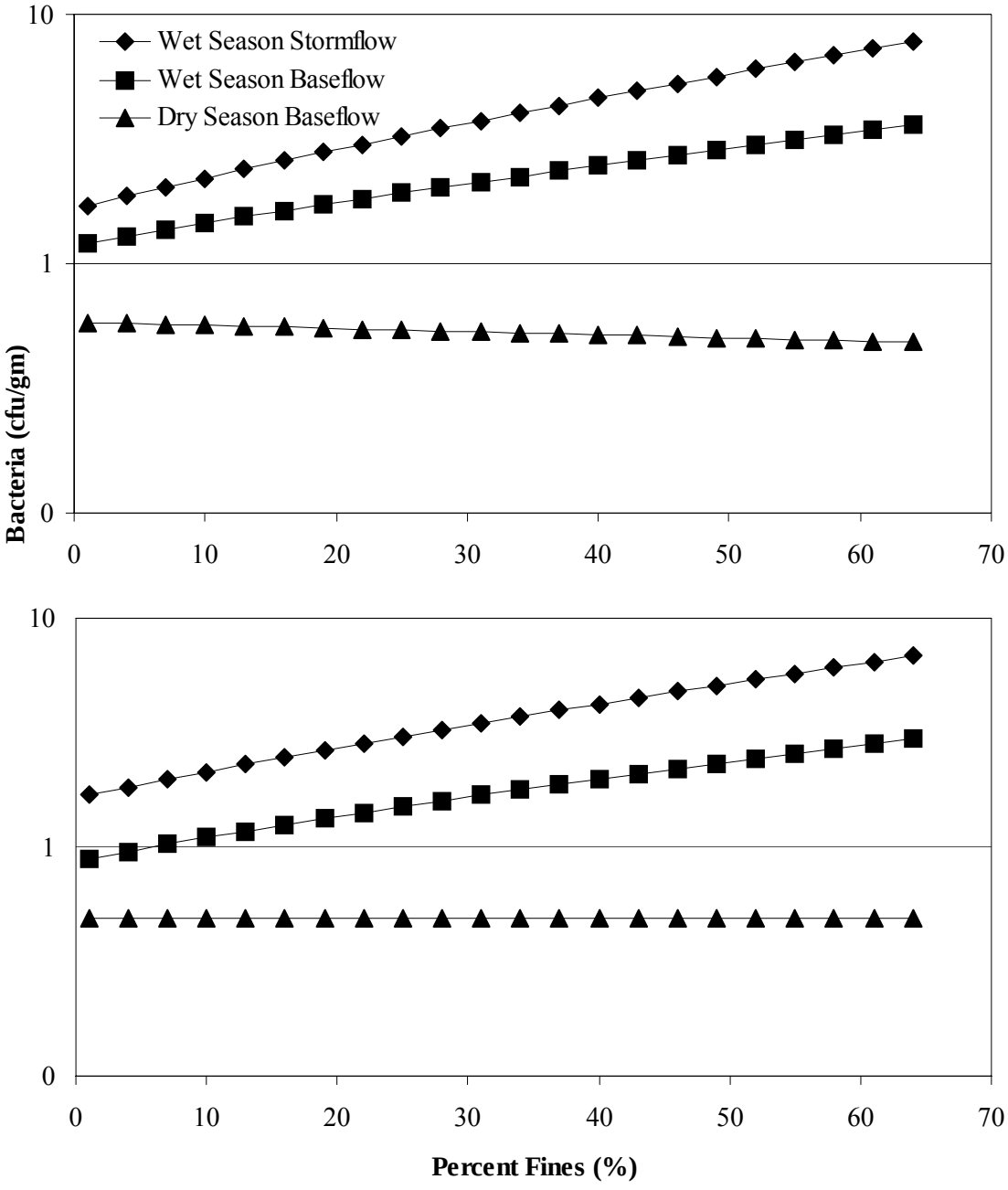


Figure 11: Results of a data driven model that estimate the concentrations of Fecal coliform (top) and (bottom) concentrations as a function of percent fines in estuarine bottom sediment during the Wet Season Stormflow, Wet Season Baseflow, and Dry Season Baseflow.

Summary

Concentrations of indicator bacteria in estuarine water are the result of estuary influences and monitoring biases (Table 3). The influences include season, with the highest concentrations occurring stormflow conditions. Water salinity or its indication of water derived by the ocean as opposed to fresh water upstream sources is indirectly related to bacteria concentrations. And cumulative precipitation on the annual, 5-day, and 24-hour basis all demonstrate a similar pattern of increasing concentrations or flushing, followed by decreasing concentrations.

Monitoring biases include sample collection depth, with concentrations decreasing the further below the water surface a sample is collected. Additionally, the location of sample collection from the mouth of the estuary to the end of the tidally influenced zone relate to increasing bacteria concentrations in the same direction. This generally parallels a similar trend in salinity. The position of sample collection, or where a sample is collected along the cross-section of the estuary did not significantly biases bacteria concentrations in sampled water.

Table 3: Role that estuary influences and monitoring biases have on indicator bacteria concentrations in sampled water.

Influences and Biases	Wet Season Stormflow	Wet Season Baseflow	Dry Season Baseflow
Salinity	↓	↓	↓
Precipitation			
Annual		↑ ≤ 20 inches ≥ ↓	
Five-day		↑ ≤ 1.5 inches ≥ ↓	
24-hour		↑ ≤ 0.75 inches ≥ ↓	
Depth	↓	↓	↓
Location (moving inland)	↑	↑	↑
Position	NS	NS	NS

INDICATOR RELATIONSHIPS

E. coli is one of the many species of bacteria that comprise the larger fecal coliform group. As such, we would expect that *E. coli* counts from a water sample would be tightly correlated with but in general less than fecal coliform counts from the same water sample. For example, if *X* represents bacteria counts (cfu) of *E. coli* and *Y* represents bacteria counts of all other species of bacteria (cfu) included in the fecal coliform group (e.g., *Klebsiella* spp., *Enterobacter* spp. *Citrobacter* spp.), it is reasonable to assume that $(X + Y) \exists (X)$ given that $Y \exists 0$. This same assumption underscores the observation that fecal coliform standards are larger in value than *E. coli* standards. The data from our project, using the methods described in our final report, suggests that this assumption holds true only for wet season stormflow conditions in that the regression coefficient for *E. coli* is greater than one (Table 4, Figure 12). For example, for every additional *E. coli* that was enumerated during these turbulent high flow conditions, there was on average 1.45 (45% more) additional fecal coliforms. These are the hydrological conditions when upstream sources of water are expected to dominate the water quality conditions measured within the downstream estuary due to high stream flows discharging into the location, especially at sampling locations at the upstream limits of the estuary. At baseflow conditions during the wet season (e.g., between storm events when stream flow returns to baseflow velocity) or dry season, we found that *E. coli* counts were either similar (winter) or exceeded (summer) fecal coliform counts (Table 4, Figures 13 and 14). Given that water quality standards for *E. coli* are lower than the standards for fecal coliforms, this would suggest that *E. coli* may be a more sensitive indicator of degraded water quality conditions during baseflow conditions compared to using fecal coliforms as the bacterial indicator of water quality.

Table 4: Association between concentrations of *E. coli* and fecal coliforms for five California estuaries, stratified by seasonal streamflow conditions (winter stormflow, winter baseflow, summer baseflow).

Untransformed bacterial counts (cfu / 100 ml)		
Factors	Coefficient[a]	*P*-value[b]
Wet Season Stormflow		
Intercept	68.9	0.15
concentration	1.45	<0.0001
Wet Season Baseflow		
Intercept	15.2	<0.0001
concentration	0.98	<0.0001
Dry Season Baseflow		
Intercept	7.9	<0.0001
concentration	0.51	<0.0001
concentration^2	-0.0002	<0.0001
Log-transformed bacterial counts ($\log_{10}$[cfu + 1 / 100 ml])		
Wet Season Stormflow		
Intercept	-0.0013	0.97
concentration	1.05	<0.0001
Wet Season Baseflow		
Intercept	0.49	<0.0001
concentration	0.61	<0.0001
concentration^2	0.077	0.0001
Dry Season Baseflow		
Intercept	0.035	0.11
concentration	0.84	<0.0001

[a] Adjusted for potential lack of independence due to repeated sampling of each sampling location.
[b] *P*-value based on a conditional t-test.

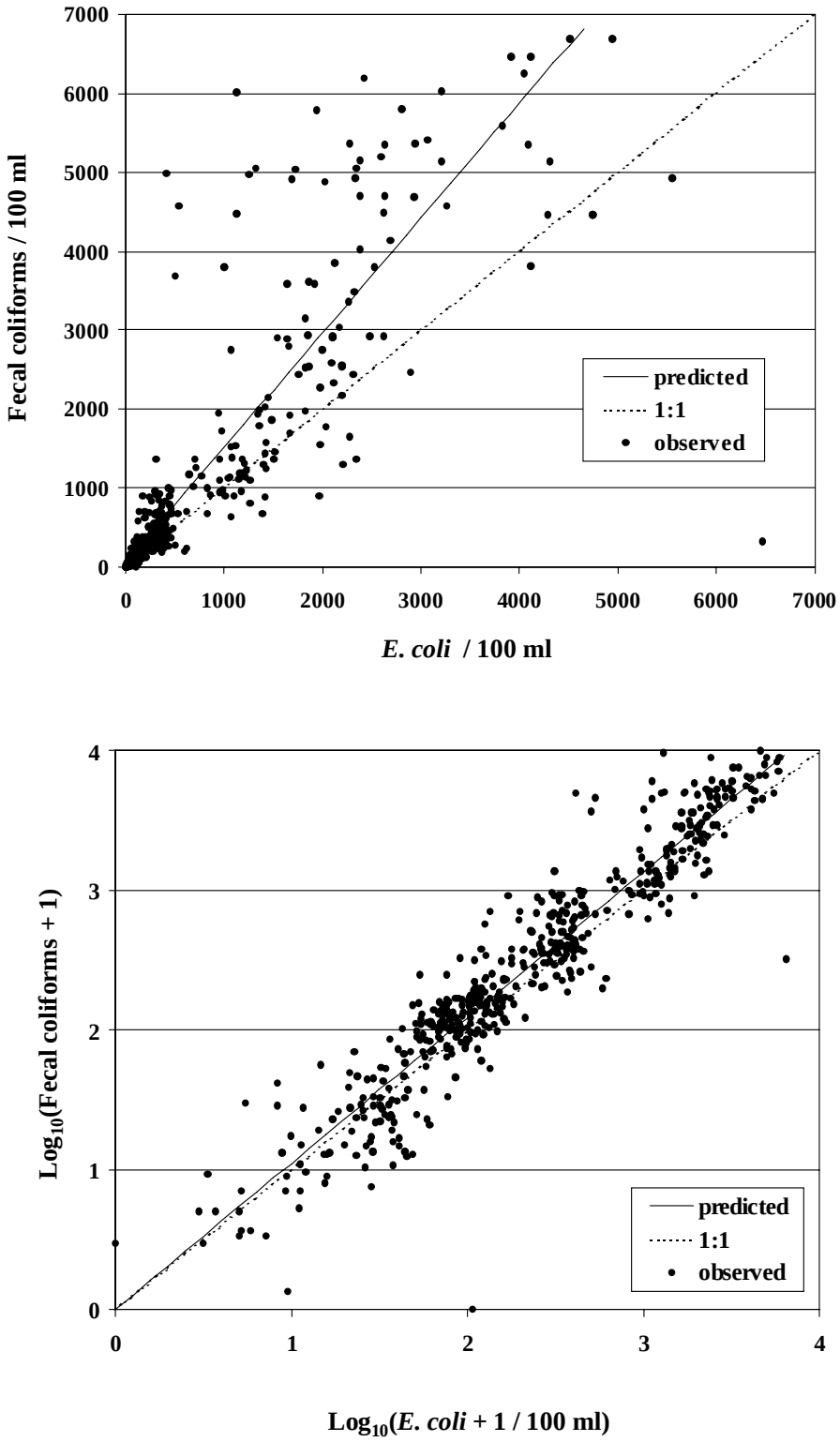


Figure 12: Relationship of concentration to fecal coliform concentration during wet season stormflow as untransformed (top) and Log_{10}+1 transformed data (bottom). Solid lines represent the data predicted relationship between and fecal coliform. Doted lines indicate the relationship of *E.coli* to fecal coliform if they occurred on a 1:1 or equal basis within each sample.

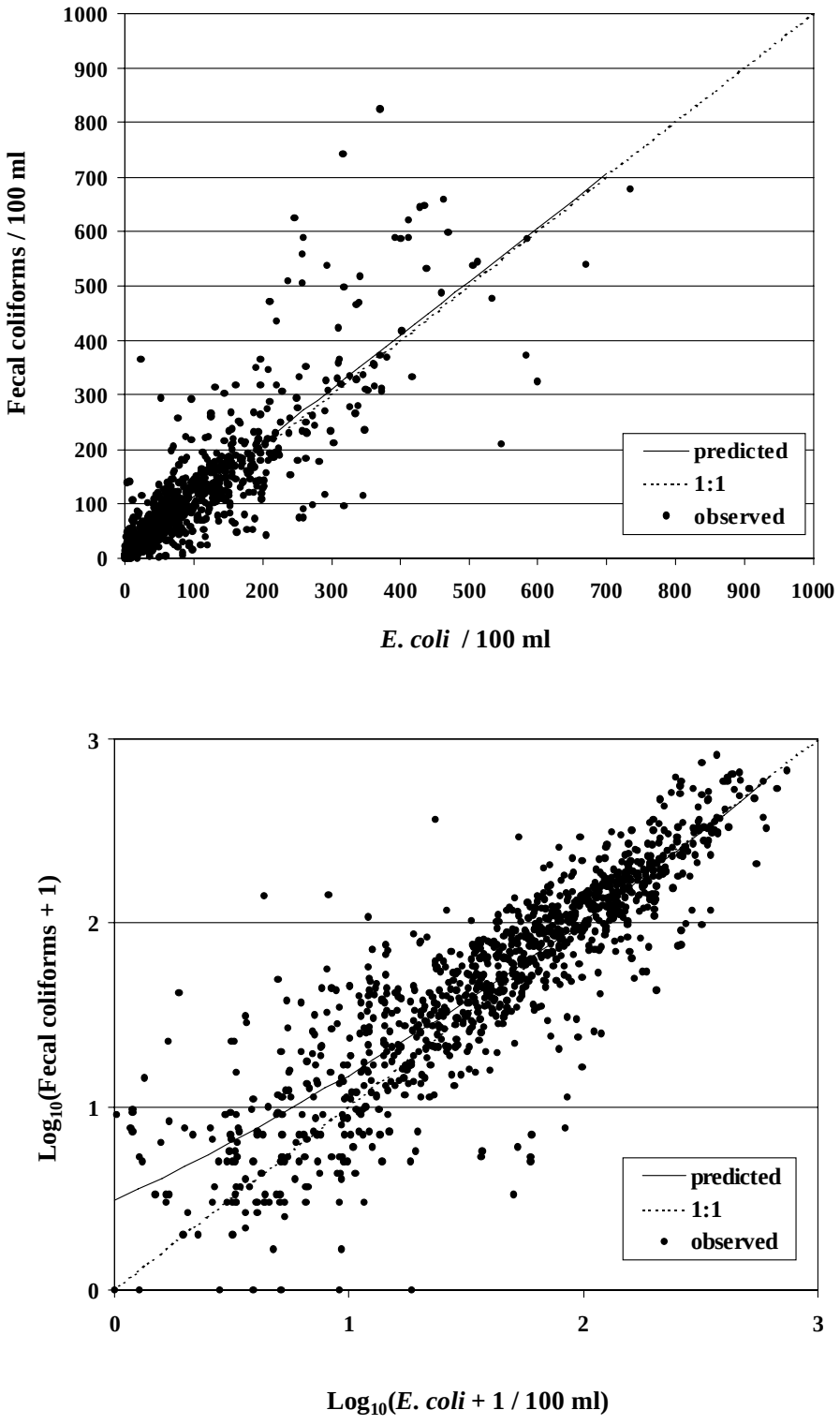


Figure 13: Relationship of concentration to fecal coliform concentration during wet season baseflow as untransformed (top) and Log_{10}+1 transformed data (bottom). Solid lines represent the data predicted relationship between and fecal coliform. Doted lines indicate the relationship of *E.coli* to fecal coliform if they occurred on a 1:1 or equal basis within each sample.

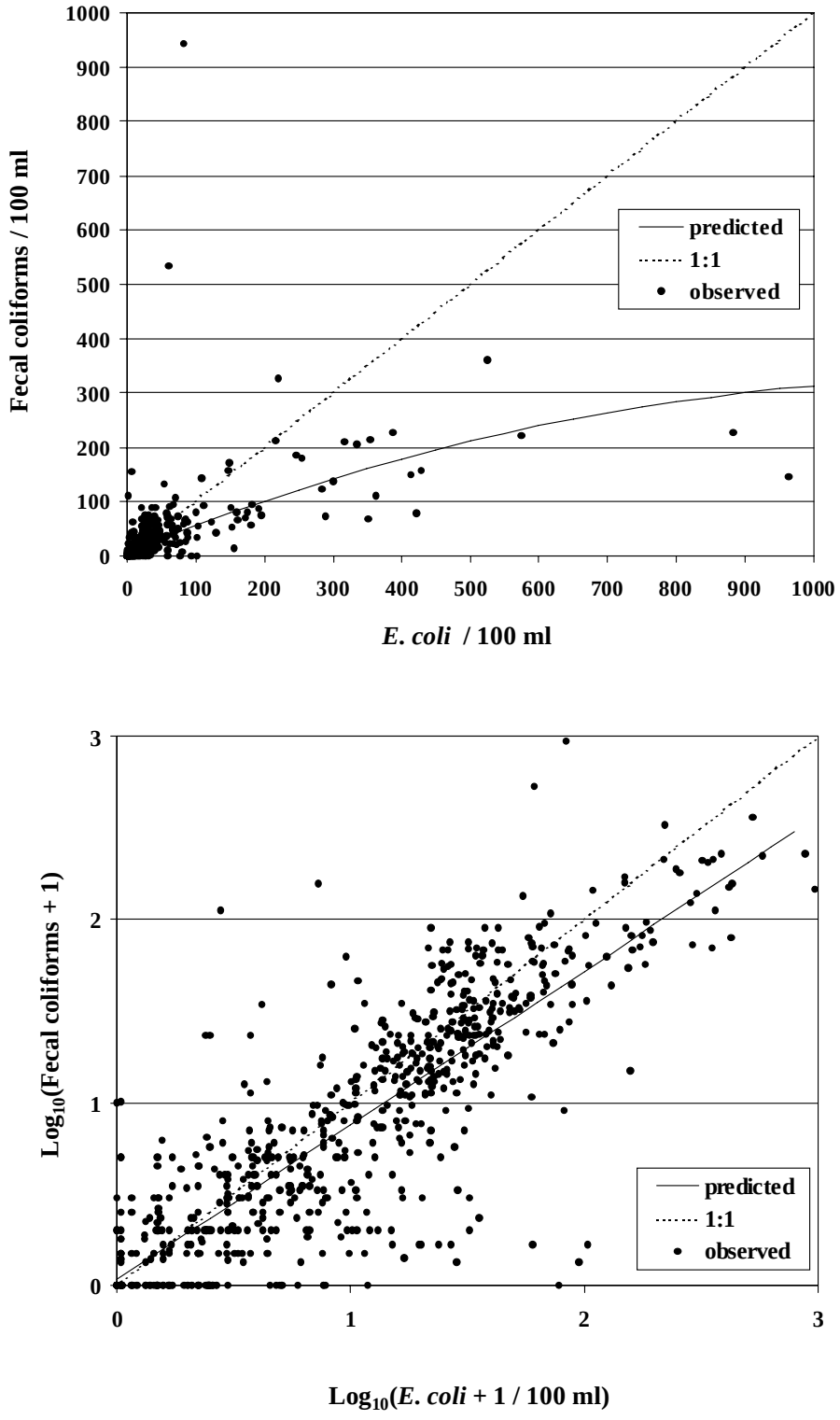


Figure 14: Relationship of concentration to fecal coliform concentration during dry season baseflow as untransformed (top) and Log_{10}+1 transformed data (bottom). Solid lines represent the data predicted relationship between and fecal coliform. Doted lines indicate the relationship of *E.coli* to fecal coliform if they occurred on a 1:1 or equal basis within each sample.

REFERENCES

Atwill, R., D. Lewis, M. das Gracas C. Pereira, M. Huerta, R. Bond, S.B. Ogata, and P. Bass. 2007. Characterizing Freshwater Inflows and Sediment Reservoirs of Fecal Coliform and at Five Estuaries in Northern California. University of California Cooperative Extension, Sonoma County and University of California School of Veterinary Medicine. Davis, California. 46 pgs.

Becton, Dickinson and Company, 2003. Manual of Microbiological Culture Media, Difco and BBL Manual. Sparks, Maryland

Clersci, L.S., A.E. Greenberg, and A.E. Eaton (Editors). 1998. Standard Methods for the Examination Water and Wastewater Protocols. 20th Edition. American Public Health Association, American Water Works Association, Water Environment Federation. Washington, DC.

CSWRCB. 2005. Comprehensive Monitoring and Assessment Strategy to Protect and Restore California's Water Quality. California State Water Resources Control Broad, Surface Water Ambient Monitoring Program. Sacramento, California. 142 pgs.

Brooks, K.N., P.F. Ffolliott, H.M. Gregersen, and L.R. DeBano. 1997. Hydrology and the management of watersheds. 2nd Edition. Iowa State University Press. Ames, Iowa. 502 pgs.

Hewlett, J. D. and A. R. Hibbert. 1967. Factors affecting the response of small watersheds to precipitation in humid areas. In: Forest Hydrology, Proceedings of the International Symposium of Forest Hydrology. Pennsylvania State University. pgs 272-286.

Mosley, M.P. and A.I. McKercher: 1993, 'Streamflow', In:Maidment, D.R. (Editor), 'Handbook of hydrology', McGraw Hill, Inc., New York, New York. pgs. 8.1-8.39.

Ohrel Jr., R.L., and K.M. Register. 2006. Volunteer Estuary Monitoring A Methods Manual. 2nd Edition. US Environmental Protection Agency and The Ocean Conservancy. Washington, DC. 396 pgs.

USEAP. 2006. Method 1600: Enterococci in Water By Membrane Filtration using membrane-Enterococcu Indoxy – ß – D - Glucoside Agar (mEI). Washington, DC. 42 pgs.

Wilde, F.D. et al. (various dates). U.S. Geological Survey Techniques of Water-Resources Investigations, book 9, National Field Manual for the Collection of Water-Quality Data. http://pubs.water.usgs.gov/twri9A/.

Wu, J., S. Dorner, and P. Rees. 2007. Fate and transport of potential pathogens: The contribution from sediments. In: Drewes, J.E., W.A. Bataglin, and D. Kolpin (Editors). Emerging contaminants of concern in the environment: Issues, Investigations, and Solutions. American Water Resources Association, Middleburg, Virginia, TPS-CD-ROM. 6 pgs.