الشركة القابضة
لمياه الشرب والصرف الصحي

برنامج المسار الوظيفي
للعاملين بقطاع مياه الشرب والصرف الصحي

دليل
المتدرب

التحاليل المكروبيولوجية للصرف

كيمائي صرف- درجة ثالثة

تم إعداد المادة بواسطة الشركة القابضة لمياه الشرب والصرف الصحي
قطاع تنمية الموارد البشرية – الادارة العامة لتخطيط المسار الوظيفي
الإصدار الثاني-2020.

Contents

Chapter (1): Bacteriological analysis

Estimation of Total coliform bacteria by using most probable number (MPN) method

Introduction

- Total coliform includes a wide range of aerobic and facultatively anaerobic. Gram-negative, non-spore-forming bacilli.

Principle

- Total coliform bacteria are capable of growing in the presence of high bile salts concentrations with the fermentation of lactose (by me β-galactosidase enzyme) and production of acid or aldehyde within 24 hours at 35–37 °C.

Interference

- Mix sample to ensure equal bacterial cell distribution.
- High densities of non-coliform bacteria and the inhibitory nature of some MTF media, e.g. Lauryl Tryptose broth, may prevent gas formation. It's recommended to treat all tubes with turbidity, indicating growth, regardless of gas production, as presumptive total coliform-positive tubes.

Scope

- The procedure is used for estimation of Coliforms by Multiple-Tube Fermentation technique (MTF), also called Most Probable Number (MPN) procedure, in 96 hours, or less, in water samples on the basis of the production of gas and acid from fermentation of lactose.

Personal Responsible

- Chemist is responsible for the analysis.

Hazards and Precaution

- Follow the normal safety procedures required in microbiology laboratory.
- Mouth-pipetting is prohibited.
- Use a separate sterile tip for each sample.
- Replace the tip with a sterile one if becomes contaminated before transfers are completed.
- Do not insert tips more than 2.5 cm below the surface of sample when removing the sample volume.
- Avoid contamination when removing sterile tips from the container by not dragging tip across lips and necks of dilution bottles.
- Avoid picking up any membrane or scum on the needle while transferring by inclining the fermentation tube.
- Insert end of loop or needle into the liquid in tube to a depth of approximately 0.5 cm.
- Decontaminate all the used tubes, plates, and materials at the end of the analyses.

Chemical and Reagents

- Lauryl Tryptose broth.
- Brilliant Green broth.

- Phosphate buffered dilution water.
- Bromcresol purple.

Equipment and Supplies

- 16 × 100 mm tubes, borosilicate glass, with heat-resistant plastic caps.
- 16 × 150 mm tubes, borosilicate glass, with heat-resistant plastic caps.
- Durham tubes, borosilicate glass.
- Test tubes racks to hold sterile culture tubes.
- Automatic pipettes and associated sterile long tips capable of delivering the selected sample volume.
- Incubator: 35°C ± 0.5°C.
- Water bath Incubator: 44.5°C ± 0.2°C.
- Sterile disposable applicator stick.
- Sterile glass/ plastic petri dishes 90 mm.
- Sterile glass/ plastic petri dishes 50 mm.
- Indelible ink marker for labeling plates.
- Autoclave.

Sampling

Sample Collection

- As examined in sampling sheet

Sample Handling

- Analyze samples on day of receipt whenever possible. The time between sample collection and the placement of sam
- ples in the incubator must not exceed 30 hours.
- If arrival is too late for processing on same day, refrigerate overnight as long as holding time conditions still be met i.e. preservation temperature <10.0°C and time did not exceed 6 h.

Selection of sample size

- Arrange fermentation tubes in rows of five or ten tubes each in a test tube rack.
- The number of rows and the sample volumes selected depend upon the quality and character of the water to be examined. For non-potable water use five tubes per dilution (of 10, 1, 0.1 ml, etc.)

Procedures:

Media Preparation:

- Prepare and handle media and reagents appropriately, and carry out suitable performance test to ensure homogeneity, sterility, and suitability for each prepared batch.

Phosphate buffered dilution water

- Prepare a stock solution by dissolving 34 grams of KH_2PO_4 in 500 ml distilled water,
- Adjusting the pH to 7.2 with 1 N NaOH and dilute to one liter.
- Prepare dilution water by adding 1.25 ml of the stock phosphate buffer solution and 5.0 ml magnesium sulfate (50 grams $MgSO_4.7H_2O$ dissolved in one liter of water) to 1liter distilled water.
- This solution can be dispersed into various size dilution blanks or used as a sterile rinse for the membrane filter test.

Lauryl Tryptose broth

- Prepare and sterilize medium according to manufacturer instructions.
- Dispense 10 ml medium, before sterilization, in fermentation tubes with an inverted Durham tube.
- Alternatively, and only in case of unavailability of Durham tubes, add 0.01 g/Lbromcresol purple to medium to determine acid production.
- Close tubes with heat-resistant plastic caps.
- Ensure, after sterilization, that media cover inverted tube with at least one-half totwo-thirds, and completely filled with media i.e. no air space inside inverted Durham tubes.
- Use tubes within two weeks (if loose plastic cap used) and within three months (for tight screw cap tubes).

Samples Dilutions

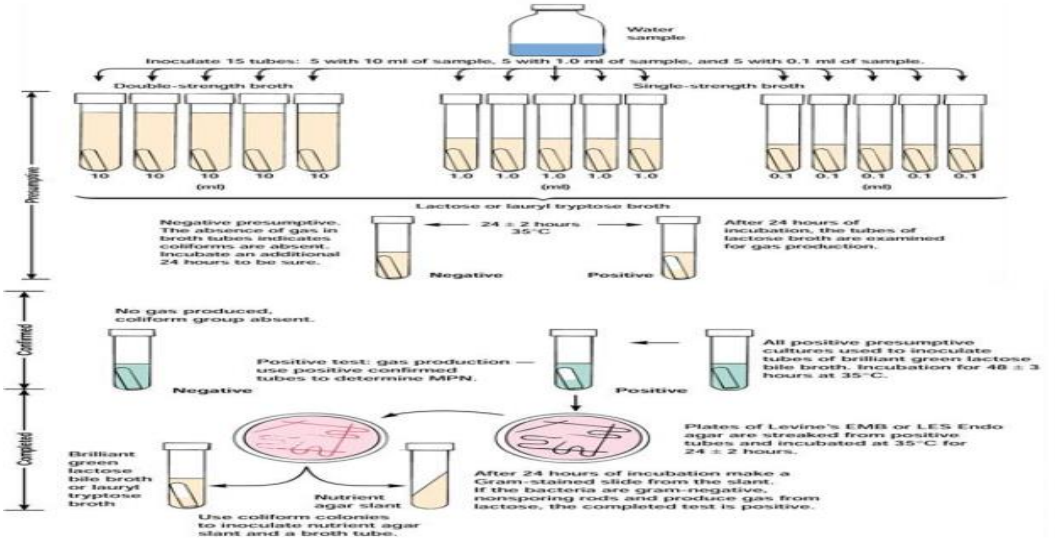
- Mix the sample by vigorously shakes the bottle.
- Use a sterile tip to transfer 1 ml of sample to 9 ml of sterile dilution water bottle, cap, and mix. 1 ml of this dilution is considered 1-10 of the original sample.
- Repeat the previous step to prepare further dilutions.

Presumptive phase

Media Preparation

- Use lauryl tryptose broth
- Arrange fermentation tubes in rows.
- Prepare five sets of tubes (e.g.10,1.0, 0.1, 0.01, 0.001 ml) using five tubes per set.

- Shake sample or dilutions vigorously.
- Inoculate each tube in the set with sample or dilution volume.
- Mix test portions in the medium by gentle agitation.
- Incubate inoculated tubes at 35 ± 0.5°C.
- After 24 ± 2 h swirl each tube gently and examine it for growth, gas, and acidic reaction (shades of yellow color). If inner vial is omitted, growth with acidity (yellow color) is signifies a positive presumptive reaction
- If no gas or acidic reaction is evident, re incubate and reexamine at the end of 48 ± 3 h.
- Record presence or absence of growth, gas, and acid production.



Interpretation

- Production of an acidic reaction and/or gas in the tubes within 48 ± 3 h constitutes a positive presumptive reaction.
- Submit tubes with a positive presumptive reaction to the confirmed phase.
- The absence of acidic reaction and/or gas formation at the end of 48 ± 3 h of incubation constitutes a negative test.

Confirmed Phase

Media Preparation

- Brilliant green broth (For Total Coliform):
- Prepare and sterilize medium according to manufacturer instructions.
- Dispense 10 ml medium, before sterilization, in fermentation tubes with an inverted Durham tube.

- Close tubes with heat-resistant plastic caps.
- Ensure, after sterilization, that media cover inverted tube with at least one-half totwo-thirds, and completely filled with media i.e. no air space inside inverted Durham tubes.
- Use tubes within two weeks (if loose plastic cap used) and within three months (for tight screw cap tubes) after preparation.
- Submit tubes with a positive presumptive reaction to the confirmed phase within 24 ± 2 h of incubation to the confirmed phase.
- If additional presumptive tubes showed active fermentation or acidic reaction at the end of 48 ± 3 h incubation period, submit to the confirmed phase.
- Gently rotate positive presumptive tubes (bottle) to resuspend the organisms.
- Use a sterile loop 3.0 mm in diameter to transfer three loopfuls from positive presumptive tube to a brilliant green tube for confirmation.
- Repeat for all other positive presumptive tubes.
- Incubate the inoculated tubes at 35 ± 0.5°C for 48 ± 3 h.

Interpretation

- Formation of gas in any amount, in Durham tube, of the brilliant green tubes, at any time, within 48 ± 3 h constitutes a positive confirmed result.

Estimation of bacterial density

- Table reading and recording of most probable number.
- Record coliform concentration as MPN/100 ml values of positive and negativetube combination and the sample volumes indicated as in table below which illustrates the MPN values for combination of positive and negatives results when five 1 ml and five 0.1 ml sample portion volumes of non-potable water.
- Select MPN value from the table for the combination of positive and negative results and calculate according to the following formula
- MPN/100ml=((table MPN / 100ml) ×10)/V

Where:

V: volume of sample portion at lowest collected dilution

Unit: The result is recorded as MPN/100ml.

Rules for different dilution

- When more than three dilutions are used in a decimal series of dilutions, use the following guidelines to select the three most appropriate dilutions and refer to Table

9221: IV. Several illustrative examples (A throughG) of combinations of positives are shown in Table 9221: V.

- First, remove the highest dilution (smallest sample volume) if it has all negative tubes and at least one remaining dilution has a negative tube.
- Next, remove the lowest dilution (largest sample volume) if it has all positive tubes and at least one remaining dilution has a positive tube.

According to guidelines:

- The three dilutions in (Example A) are selected by removal of the highest (0.001 ml) and the lowest (10 ml) dilutions.
- If the lowest dilution does not have all positive tubes, and several of the highest dilutions have all negative tubes, and then remove the highest negative dilutions (Example B).
- More than three dilutions may remain after removal of the lowest dilution with all positive tubes and high dilutions with all negative tubes. In this case, if the highest dilution with all positive tubes is within two dilutions of the highest dilution with any positive tubes, then use the highest dilution with any positive tubes and the two immediately lower dilutions. In (Example C), the highest dilution with all positive tubes is 0.1 ml, which is within two dilutions of 0.001 ml, which has one positive tube.
- In Example D, the highest dilution with all positive tubes is 0.01 ml, which is within two decimal dilutions of 0.001 ml.
- If, after removal of the lowest dilution with all positive tubes, no dilution with all positive reactions remains, then select the lowest two dilutions and assign the sum of any remaining dilutions to the third dilution. (Example E).
- If no dilution has all positive tubes (Example F), select the lowest two dilutions, corresponding to 10- and 1-ml sample. For the third dilution, add the number of positive tubes in the remaining dilutions (0.1, 0.01, and 0.001 ml sample), to yield a final combination of 4-3-2. If the third dilution is assigned more than five positive tubes, then the selected combination will not be in Table 9221: IV.
- If the three dilutions selected are not found in Table 9221: IV, then something in the serial dilution was unusual. In this case, the usual methods for calculating the MPN, presented here, may not apply. If a new sample cannot be collected and an MPN value is still desired, use the highest dilution with at least one positive tube and the two dilutions immediately lower as the three selected dilutions. In (Example G), the first

selection, 4-3-6 (the outcome from the highest three dilutions), is not in Table 9221: IV because 6 is greater than 5. The second selection, according to the above guidelines, would be 3-2-1. If this second set of selected dilutions is not in Table 9221: IV

TABLE 9221:V. EXAMPLES FOR CHOICE OF THREE COMBINATIONS OF POSITIVES FROM FIVE DILUTIONS

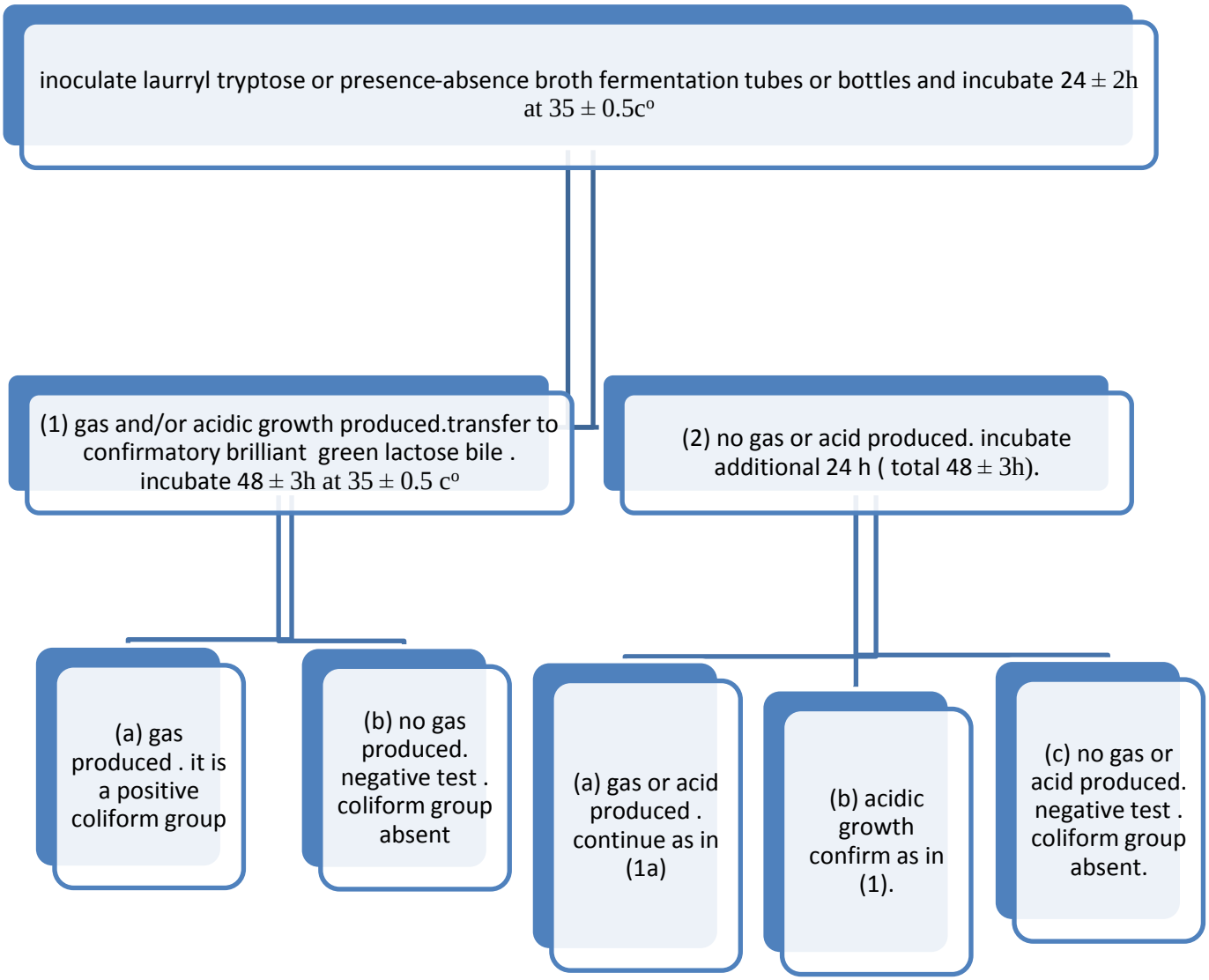
	Volume *mL*						
Example	10	1	0.1	0.01	0.001	Combination of Positives	MPN Index *No./100 mL*
A	5	5	1	0	0	x-5-1-0-x	330
B	4	5	1	0	0	4-5-1-x-x	48
C	5	2	5	2	1	x-x-5-2-1	7000
D	4	5	4	5	1	x-x-4-5-1	4800
E	5	4	4	0	1	x-4-4-1-x	400
F	4	3	0	1	1	4-3-2-x-x	39
G	4	3	3	2	1	x-x-3-2-1	1700

TABLE 9221:IV. MPN INDEX AND 95% CONFIDENCE LIMITS FOR VARIOUS COMBINATIONS OF POSITIVE RESULTS WHEN FIVE TUBES ARE USED PER DILUTION (10 mL, 1.0 mL, 0.1 mL)*

Combination of Positives	MPN Index/100 mL	Confidence Limits		Combination of Positives	MPN Index/100 mL	Confidence Limits	
		Low	High			Low	High
0-0-0	<1.8	—	6.8	4-0-3	25	9.8	70
0-0-1	1.8	0.090	6.8	4-1-0	17	6.0	40
0-1-0	1.8	0.090	6.9	4-1-1	21	6.8	42
0-1-1	3.6	0.70	10	4-1-2	26	9.8	70
0-2-0	3.7	0.70	10	4-1-3	31	10	70
0-2-1	5.5	1.8	15	4-2-0	22	6.8	50
0-3-0	5.6	1.8	15	4-2-1	26	9.8	70
1-0-0	2.0	0.10	10	4-2-2	32	10	70
1-0-1	4.0	0.70	10	4-2-3	38	14	100
1-0-2	6.0	1.8	15	4-3-0	27	9.9	70
1-1-0	4.0	0.71	12	4-3-1	33	10	70
1-1-1	6.1	1.8	15	4-3-2	39	14	100
1-1-2	8.1	3.4	22	4-4-0	34	14	100
1-2-0	6.1	1.8	15	4-4-1	40	14	100
1-2-1	8.2	3.4	22	4-4-2	47	15	120
1-3-0	8.3	3.4	22	4-5-0	41	14	100
1-3-1	10	3.5	22	4-5-1	48	15	120
1-4-0	10	3.5	22	5-0-0	23	6.8	70
2-0-0	4.5	0.79	15	5-0-1	31	10	70
2-0-1	6.8	1.8	15	5-0-2	43	14	100
2-0-2	9.1	3.4	22	5-0-3	58	22	150
2-1-0	6.8	1.8	17	5-1-0	33	10	100
2-1-1	9.2	3.4	22	5-1-1	46	14	120
2-1-2	12	4.1	26	5-1-2	63	22	150
2-2-0	9.3	3.4	22	5-1-3	84	34	220
2-2-1	12	4.1	26	5-2-0	49	15	150
2-2-2	14	5.9	36	5-2-1	70	22	170
2-3-0	12	4.1	26	5-2-2	94	34	230
2-3-1	14	5.9	36	5-2-3	120	36	250
2-4-0	15	5.9	36	5-2-4	150	58	400
3-0-0	7.8	2.1	22	5-3-0	79	22	220
3-0-1	11	3.5	23	5-3-1	110	34	250
3-0-2	13	5.6	35	5-3-2	140	52	400
3-1-0	11	3.5	26	5-3-3	170	70	400
3-1-1	14	5.6	36	5-3-4	210	70	400
3-1-2	17	6.0	36	5-4-0	130	36	400
3-2-0	14	5.7	36	5-4-1	170	58	400
3-2-1	17	6.8	40	5-4-2	220	70	440
3-2-2	20	6.8	40	5-4-3	280	100	710
3-3-0	17	6.8	40	5-4-4	350	100	710
3-3-1	21	6.8	40	5-4-5	430	150	1100
3-3-2	24	9.8	70	5-5-0	240	70	710
3-4-0	21	6.8	40	5-5-1	350	100	1100
3-4-1	24	9.8	70	5-5-2	540	150	1700
3-5-0	25	9.8	70	5-5-3	920	220	2600
4-0-0	13	4.1	35	5-5-4	1600	400	4600
4-0-1	17	5.9	36	5-5-5	>1600	700	—
4-0-2	21	6.8	40				

Schematic Outline

- **Schematic outline of presumptive and confirmed phases for total coliform detection**



Detection and Enumeration of Total Coliform by Membrane Filter Technique:

Principle

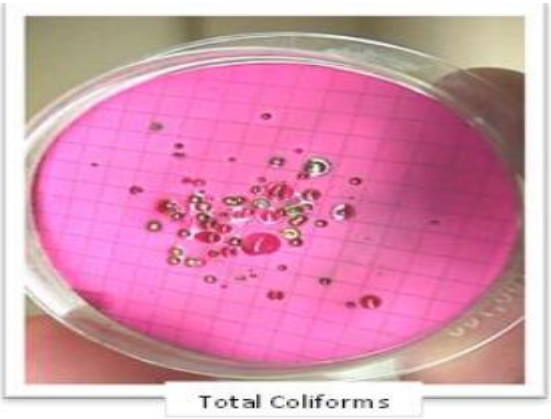
- Volume of water to be tested is filtered through 0.45 µm and the membrane is placed on Endo type medium. The selectivity of medium is due to sodium lauryl sulphate and sodium deoxycholate that acting as inhibitor of gram-positive bacteria. Coliform ferment lactose, produce acetaldehydes that reacts with sodium sulfite / basic fuchsin compound to form red colony. The development of metallic sheen occurs when the organism produce aldehyde with the rapid fermentation of lactose.
- If the inoculum is too heavy, the sheen will be suppressed. Lactose non-fermenting bacteria form clear, colorless colonies.

Interference

- Non-coliform bacteria may interfere with the recovery of coliforms when using a lactose-based medium. Data showed that the recovery of total coliforms using the MF technique decreased as the concentration of HPC bacteria increased. The greatest reduction occurred when the HPC densities exceeded 500 colony-forming units (CFU/ml). It should be noted that most water supplies maintaining a total chlorine residual of 0.2 mg/L have an HPC below 500 CFU/ml.
- Another data demonstrated that Pseudomonas aeruginosa (30 CFU/ml) and
- Aeromonas hydrophila (2 CFU/ml) caused significant reductions in sheen
- Production by coliforms on m-Endo LES agar. Flavobacterium sp. and Bacillus sp., in contrast, were not inhibitory, even at concentrations above 1000 CFU/ml.
- Samples with high turbidity caused by algae, particulate, or other interfering material can clog the membrane filter, thereby preventing filtration, or cause spreading of bacterial colonies which could interfere with identification of target colonies.
- Some members of the total coliform group may produce dark red, mucoid, or nucleated colonies without a metallic sheen. When verified these are classified as atypical coliform colonies.
- Pink (non-mucoid), blue, white, or colorless colonies lacking sheen are considered non-coliforms by this technique.
- Also, do not use the MF technique to test wastewater containing high levels of toxic metals or toxic organic compounds (e.g. phenol) because such substances may be concentrated by the filter and inhibit coliform growth.

Scope

- This is a detailed procedure for the detection and enumeration of Total Coliform(TC) by Membrane Filter (MF) technique in water samples in 24 hours or less on the basis of the production of aldehydes from fermentation of lactose.
- This procedure can be applied for different types of water, in microbiology laboratory it will be applied for the analysis of ground water, network distribution system (drinking water), and water from different treatment process.


Total Coliforms

Chemical and Reagents

- M-Endo agar
- Phosphate buffered dilution water

Equipment

- Stainless steel forceps.
- Sterile plastic or glass petri dishes 50 mm.
- Sterile membrane filtration units.
- Vacuum pump.
- Buffered rinse water screw cap bottles.
- Measuring 100 ml Cylinder class A.
- Sterile membrane filters (47 mm diameter, 0.45 µ ± 0.02 µm pore size, white, grid - marked).
- Sterile 47-mm diameter absorbent pads (used with broth).
- Electric Gas Burner.
- Incubator: 35°C ± 0.5°C.
- Colonies counter aid.
- Indelible ink marker for labeling plates.
- Sterile plastic loop, at least 3 mm diameter in suitable holders

Sampling

Sample collection

- As examined in sampling sheet.

Selection of sample size

- Select a volume of the wastewater sample to be examined about 50-100 ml. Use sample volume and dilution that will yield counts between 20 and 60 Total coliform colonies per membrane.
- When the bacterial density of the sample is unknown, filter several volumes or dilutions to achieve a countable density. Estimate volume and/or dilution expected to yield a countable membrane and select two additional quantities representing one-tenth and ten times this volume, respectively.

Procedure

Media preparation:

M-ENDO medium:

- The need for uniformity dictates the use of dehydrated media. Never prepare media from basic ingredients when suitable dehydrated media are available. Follow manufacturer's directions for rehydration the commercially prepared media (sterile ampoules) or powder.
- Rehydrate product in 1 L water. Heat to near boiling, promptly remove from heat, and cool to below 50 °C. Do not sterilize by autoclaving.
- Final pH should be 7.4 ± 0.2. Refrigerate finished medium, preferably in sealed plastic bags or other containers to reduce moisture loss, and discard unused broth after 96 h.

Phosphate buffered dilution water

- Prepare a stock solution by dissolving 34 grams of KH2PO4 in 500 ml distilled water, adjusting the pH to 7.2 with 1 N NaOH and dilute to one liter. Prepare dilution water by adding 1.25 ml of the stock phosphate buffer solution and 5.0 ml magnesium sulfate (50 grams MgSO4.7H2O dissolved in one liter of water) to 1-liter distilled water. This solution can be dispersed into various size dilution blanks or used as a sterile rinse for the membrane filter test.

Filtration of sample

- Label the bottom of plates with the sample identification number, analyst initials, and sample volume to be analyzed.

- Place a membrane filter (grid side up) using sterile forceps on the porous plate of the filter base.
- If you have difficulties in removing the separation papers from the filters due to static electricity, place a filter with the paper on top of the funnel base and turn on the vacuum, the separation paper will curl up, allowing easier removal.
- Attach the funnel to the base of the filter unit
- Shake the sample container vigorously.
- Measure the selected volume by using sterile measuring cylinder.
- Pour selected volume or dilution of the sample into the funnel.
- Turn on the vacuum pump and allow the vacuum to operate for a time enough to filtrate all the sample volume.
- Rinse the interior surface of funnel, with filter still in place, by filtering 20 to 30 ml portions of sterile dilution water. Alternatively, rinse funnel by a flow of sterile dilution water from a squeeze bottle.
- Turn off the pump then remove the funnel from the base of the filter unit.
- Hold the membrane filter at its edge with sterile forceps, gently lift and place the filter grid-side up on the medium plate.
- Slide the filter onto the medium, using a rolling action to avoid trapping air bubbles between the membrane filter and the underlying medium.
- Reseat the membrane if non-wetted areas occur due to air bubbles.
- Invert the plates, and incubate at 35 ± 0.5°C for 22 to 24 h.



Counting:

Calculation of Total Coliform Density:

- Generally, Compute the density from the sample quantities that produced MF counts within the desired range of 20 to 60 Total coliform colonies. This colony density range is more restrictive than the 20 to 80 total coliform range because of larger

colony size on M-ENDO medium. Calculate Total coliform density and record densities as Total coliforms per 100 ml.

- Bacteria/100 ml = (No.of Colonies Counted) / (Dilution factor)
- OR Bacteria/100 ml = (No.of Colonies Counted×100 ml) / (Sample Volume Filtered,ml in 100 ml)

Note:

- To aid in quality assurance, analyze samples in duplicate.
- Reporting:
- Report results as "Total Coliform / 100 ml of sample".
- If result was >500 CFU/ml, report as "Estimated" in case of positive total
- coliform, or "False Negative" in case of negative result.

If sample was delayed more than permissible time, write" Delayed" in report

Comment:

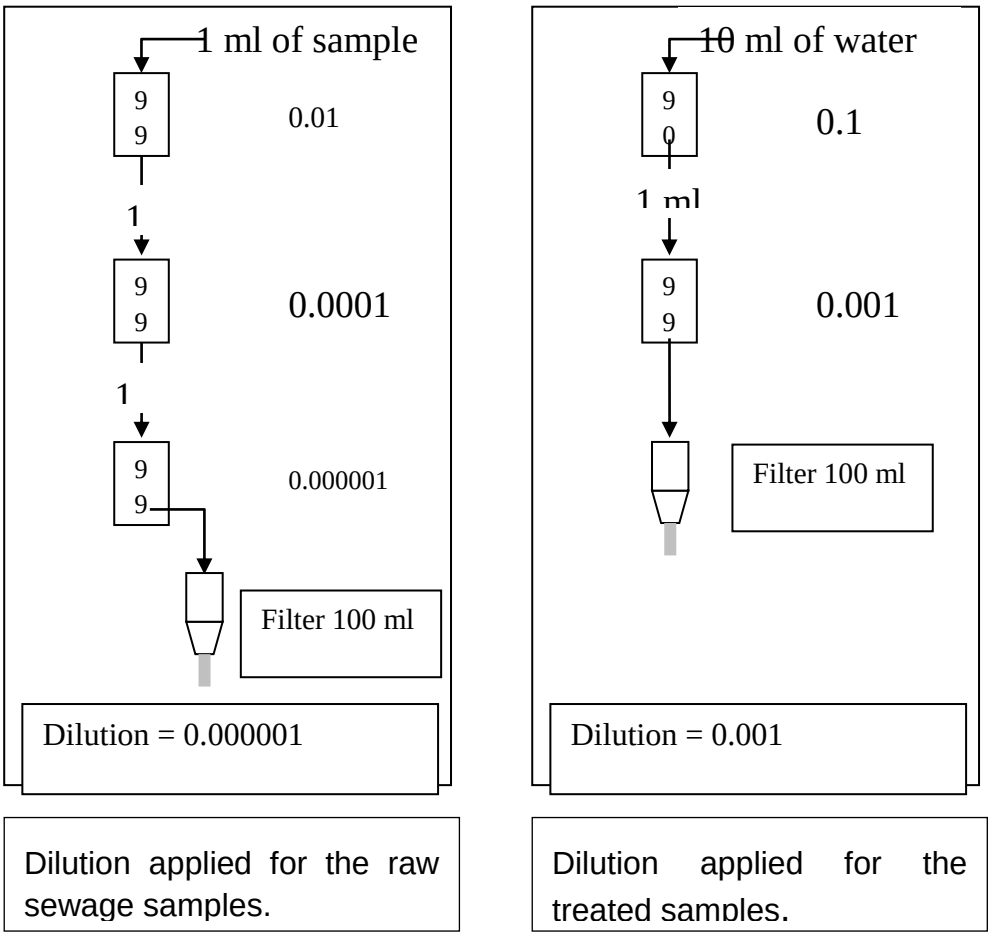
- If no coliform colonies are observed, report the coliform colonies counted as ''<1coliform/100 ml.''
- If the total number of bacterial colonies, coliforms plus non-coliforms, exceeds 200per membrane, report results as ''Too Numerous To Count'' (TNTC)
- If confluent growth occurs, covering either the entire filtration area of the membrane or a portion thereof, and colonies are not distinct enough for accurate counting, report results as ''confluent growth with (or without) coliforms.''
- Report confluent growth or TNTC without detectable coliform as 'Invalid''
- If sample was divided into two or more portions, Total the coliform counts on all filters and report the number of coliforms per 100 ml.
- Report the largest volume filter that has a coliform count falling in the ideal range; calculate final concentration value by multiplying the count by dilution factor (if present).
- If all volumes filters have a coliform count lower than the ideal range, disregard the rule and report the result from largest volume filter count; calculate final concentration value by multiplying the count by dilution factor (if present).
- If largest volume filter has a coliform count higher than the ideal range, choose next(second) dilution volume that falls inside ideal range and calculate final concentration value by multiplying the count by dilution factor.

- If next (second) dilution volume has coliform count lower than ideal range, and largest volume count is less than 100, use the count from the largest volume rather than the count from second volume; calculate final concentration value by multiplying the count by dilution factor.

Suggested samples volumes for membrane filter total coliform test

	Volume (X) To Be Filtered ml							
Water Source	100	50	10	1	0.1	0.01	0.001	0.0001
Lakes, reservoirs	X	X						
Wells, springs	X	X						
Water supply intake		X	X	X				
Natural bathing waters		X	X	X				
Sewage treatment plant			X	X	X			
Farm ponds, rivers				X	X	X		
Stormwater runoff				X	X	X		
Raw municipal sewage					X	X	X	
Feedlot runoff					X	X	X	
Sewage sludge						X	X	X

Dilution scheme:



Dilution applied for the raw sewage samples.

Dilution applied for the treated samples.

EXAPMLES OF TOTAL COLIFORM PLATES

- A – Plate shows the metallic sheen colonies of total coliforms growing on M-ENDO media. The number of colonies is perfect for counting. The sample portion and dilution used is correct.
- B – plate shows no colonies growing on M-ENDO media. The sample collected from filtered chlorinated water.

- C – plate shows a huge number of total coliform colonies due toincorrect dilution

Calculation:

$$\text{Bacteria/100 ml} = \frac{\text{No. of Colonies Counted}}{\text{Dilution factor}}$$

OR

$$\text{Bacteria/100 ml} = \frac{\text{No. of Colonies Counted} \times 100\text{ ml}}{\text{Sample Volume Filtered, ml in 100 ml}}$$

Example (1):

A total of 42 colonies grew after filtering 100 ml of a sample with dilution factor 1: 10.

$$\text{Bacteria/100 ml} = \frac{42}{0.1} = 420/100\text{ ml}$$

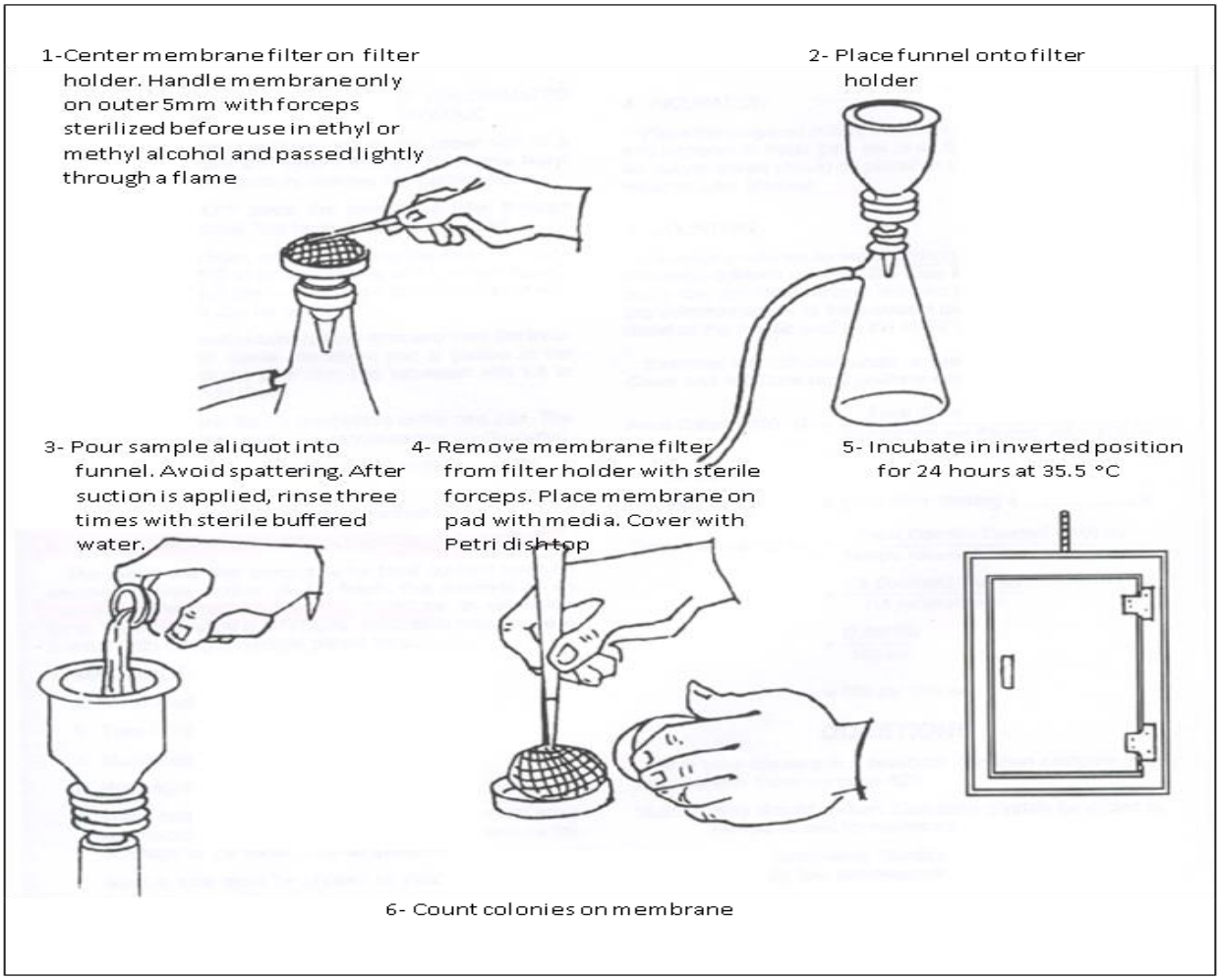
Example (2):

A total of 22 colonies grew after filtering a 10 ml sample.

$$= \frac{(22\text{ Colonies}) \times (100\text{ ml})}{10\text{ ml}}$$

= 220 per 100 ml

Schematic Outline



Detection and Enumeration of Thermotolerant (Fecal) Coliform by (MPN)

Principle

- Volumes of water sample to be tested are added to tubes, or bottles, containing the presumptive media with inverted vials (Durham Tube) or pH indicator and incubated at 35°C for 48 hours. The selectivity of media is due to sodium lauryl sulfate that acting as inhibitor of bacteria other than coliforms. After incubation, the tubes, or bottles, are examined for growth, gas, and/or acidic reaction (shades of yellow color).
- An additional confirmatory test is required to confirm the result.
- Total coliform bacteria (excluding E. coli) occur in both sewage and natural waters. Some of these bacteria are excreted in the faeces of humans and animals, but many coliforms are heterotrophic and able to multiply in water and soil environments.
- Thermo tolerant coliform (Fecal coliform) include species that may inhabit the intestines of warm-blooded animals and human. In most waters, the predominant genus is Escherichia, but some types of Citrobacter, Klebsiella and Enterobacter are also thermo tolerant. They are usually found in sewage and water recently subjected to fecal pollution.
- Thermo tolerant coliform (Fecal coliform) bacteria in this procedure are those facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 hours at 44.5°C.
- All sample analysis steps will be carried out under aseptic environmental conditions.

Interference

- Since the MPN indexes are based on a Poisson distribution, if the sample is not adequately mixed to ensure equal bacterial cell distribution before portions are removed, the MPN value will be a misrepresentation of the bacterial density.
- High densities of non-coliform bacteria and the inhibitory nature of some MTF media, e.g. Lauryl Tryptose broth, may prevent gas formation. It’s recommended to treat all tubes with turbidity, indicating growth, regardless of gas production, as presumptive total coliform-positive tubes.

Scope

- The procedure below is for estimation of Coliforms by Multiple-Tube Fermentation technique (MTF), also called Most Probable Number (MPN) procedure, in 96 hours, or less, in water samples on the basis of the production of gas and acid from fermentation of lactose.

Personal Responsible

- Microbiology staff is responsible for performing the analysis.

Hazard and Precautions

- Follow the normal safety procedures required in a microbiology laboratory.
- Mouth-pipetting is prohibited.
- Decontaminate all the used plates and materials at the end of the analyses.
- Use sterile filtration units at the beginning of each filtration series as a minimum precaution to avoid accidental contamination. A filtration series is considered to be interrupted when an interval of 30 min or longer elapses between samples filtrations.
- After such interruption, treat any further samples filtration as a new filtration series and sterilize all membrane filter holders in use.
- Avoid damaging or dislodging the membrane filter when attaching funnel to base of the filter unit.
- Do not expose poured plates with culture media to direct light; refrigerate in the dark, in a suitable tight container.

Chemical and Reagents

- Lauryl Tryptose broth.
- Brilliant Green broth.
- Ec broth
- Phosphate buffered dilution water.
- Nutrient agar.
- Gram Stain Reagent set.
- M-Endo LES agar.
- Bromcresol purple.

Equipment and Supplies

- 16 × 100 mm tubes, borosilicate glass, with heat-resistant plastic caps.
- 16 × 150 mm tubes, borosilicate glass, with heat-resistant plastic caps.
- Durham tubes, borosilicate glass.
- Test tubes racks to hold sterile culture tubes.
- Automatic pipettes and associated sterile long tips capable of delivering the selected sample volume.
- Incubator: 35°C ± 0.5°C.
- Water bath Incubator: 44.5°C ± 0.2°C. Colonies counter aid.

- Indelible ink marker for labeling plates.
- Sterile plastic loop, at least 3 mm diameter in suitable holders.
- Sterile disposable applicator sticks.
- Sterile glass/ plastic petri dishes 90 mm.
- Sterile glass/ plastic petri dishes 50 mm.
- Indelible ink marker for labeling plates.

Procedures

Sample Collection

- Analyze samples on day of receipt whenever possible. The time between sample collection and the placement of samples in the incubator must not exceed 6 hours.
- If arrival is too late for processing on same day, refrigerate overnight as long as holding time conditions still be met i.e. preservation temperature <10.0°C and time did not exceed 6 h.

Selection of sample size

- Arrange fermentation tubes in rows of five or ten tubes each in a test tube rack.
- The number of rows and the sample dilution selected depend upon the quality and character of the water to be examined.
- Use lauryl tryptose broth for presumptive phase.
- Arrange fermentation tubes in rows of five or ten tubes each in a test tube rack.
- Shake sample and dilutions vigorously about 25 times.
- Inoculate each tube in the set with sample volume.
- Mix test portions in the medium by gentle agitation.
- Incubate inoculated tubes (bottles) at 35 ± 0.5°C.
- After 24 ± 2 h swirl each tube (bottle) gently and examine it for growth, gas, and acidic reaction (shades of yellow color)
- If no gas or acidic reaction is evident, re-incubate and reexamine at the end of 48±3 h.
- Record presence or absence of growth, gas, and acid production.
- If the inner vial is omitted, growth with acidity signifies a positive presumptive reaction.

Presumptive Phase

Interpretation

- Production of an acidic reaction and/or gas in the tubes or bottles within 48 ± 3 h constitutes a positive presumptive reaction.
- Submit tubes or bottles with a positive presumptive reaction to the confirmed phase.
- The absence of acidic reaction and/or gas formation at the end of 48 ± 3 h of incubation constitutes a negative result.
- Submit drinking water samples demonstrating growth without a positive gas or acid reaction to the confirmed phase.

Confirmed Phase:

- Use EC broth (For Thermotolerant Coliform)
- Prepare and sterilize medium according to manufacturer instructions.
- Dispense 10 ml medium, before sterilization, in fermentation tubes with an inverted Durham tube.
- Close tubes with heat-resistant plastic caps.
- Ensure, after sterilization, that media cover inverted tube with at least one-half to two-thirds, and completely filled with media i.e. no air space inside inverted Durham tubes.
- Use tubes within two weeks (if loose plastic cap used) and within three months (for tight screw cap tubes) after preparation.
- Arrange EC tubes rows, in separate racks, in a similar manner to positive presumptive tubes (bottles).
- Gently rotate positive presumptive tubes (bottle) to resuspend the organisms.
- Use a sterile loop 3.0 mm in diameter to transfer three loopfuls from positive presumptive tube to a brilliant green tube and EC tube for confirmation.
- Repeat for all other positive presumptive tubes.
- Incubate EC (Fecal Coliform) tubes at 44.5 ± 0.2°C for 24 ± 2 h.

Interpretation

- Formation of gas in any amount, in Durham tube, of the brilliant green tubes, at any time, within 48 ± 3 h constitutes a positive confirmed result.
- Formation of gas in any amount, in Durham tube, of the EC tubes, at any time, within 24 ± 2 h constitutes a positive confirmed result.

- Calculate the MPN value of the number of positive brilliant green lactose bile tubes and EC tubes from MPN index
- In case of inoculating one bottle with 100 ml sample portion, report result as present or absent.

TABLE 9221:IV. MPN INDEX AND 95% CONFIDENCE LIMITS FOR VARIOUS COMBINATIONS OF POSITIVE RESULTS WHEN FIVE TUBES ARE USED PER DILUTION (10 mL, 1.0 mL, 0.1 mL)*

Combination of Positives	MPN Index/100 mL	Confidence Limits		Combination of Positives	MPN Index/100 mL	Confidence Limits	
		Low	High			Low	High
0-0-0	<1.8	—	6.8	4-0-3	25	9.8	70
0-0-1	1.8	0.090	6.8	4-1-0	17	6.0	40
0-1-0	1.8	0.090	6.9	4-1-1	21	6.8	42
0-1-1	3.6	0.70	10	4-1-2	26	9.8	70
0-2-0	3.7	0.70	10	4-1-3	31	10	70
0-2-1	5.5	1.8	15	4-2-0	22	6.8	50
0-3-0	5.6	1.8	15	4-2-1	26	9.8	70
1-0-0	2.0	0.10	10	4-2-2	32	10	70
1-0-1	4.0	0.70	10	4-2-3	38	14	100
1-0-2	6.0	1.8	15	4-3-0	27	9.9	70
1-1-0	4.0	0.71	12	4-3-1	33	10	70
1-1-1	6.1	1.8	15	4-3-2	39	14	100
1-1-2	8.1	3.4	22	4-4-0	34	14	100
1-2-0	6.1	1.8	15	4-4-1	40	14	100
1-2-1	8.2	3.4	22	4-4-2	47	15	120
1-3-0	8.3	3.4	22	4-5-0	41	14	100
1-3-1	10	3.5	22	4-5-1	48	15	120
1-4-0	11	3.5	22	5-0-0	23	6.8	70
2-0-0	4.5	0.79	15	5-0-1	31	10	70
2-0-1	6.8	1.8	15	5-0-2	43	14	100
2-0-2	9.1	3.4	22	5-0-3	58	22	150
2-1-0	6.8	1.8	17	5-1-0	33	10	100
2-1-1	9.2	3.4	22	5-1-1	46	14	120
2-1-2	12	4.1	26	5-1-2	63	22	150
2-2-0	9.3	3.4	22	5-1-3	84	34	220
2-2-1	12	4.1	26	5-2-0	49	15	150
2-2-2	14	5.9	36	5-2-1	70	22	170
2-3-0	12	4.1	26	5-2-2	94	34	230
2-3-1	14	5.9	36	5-2-3	120	36	250
2-4-0	15	5.9	36	5-2-4	150	58	400
3-0-0	7.8	2.1	22	5-3-0	79	22	220
3-0-1	11	3.5	23	5-3-1	110	34	250
3-0-2	13	5.6	35	5-3-2	140	52	400
3-1-0	11	3.5	26	5-3-3	170	70	400
3-1-1	14	5.6	36	5-3-4	210	70	400
3-1-2	17	6.0	36	5-4-0	130	36	400
3-2-0	14	5.7	36	5-4-1	170	58	400
3-2-1	17	6.8	40	5-4-2	220	70	440
3-2-2	20	6.8	40	5-4-3	280	100	710
3-3-0	17	6.8	40	5-4-4	350	100	710
3-3-1	21	6.8	40	5-4-5	430	150	1100
3-3-2	24	9.8	70	5-5-0	240	70	710
3-4-0	21	6.8	40	5-5-1	350	100	1100
3-4-1	24	9.8	70	5-5-2	540	150	1700
3-5-0	25	9.8	70	5-5-3	920	220	2600
4-0-0	13	4.1	35	5-5-4	1600	400	4600
4-0-1	17	5.9	36	5-5-5	>1600	700	—
4-0-2	21	6.8	40				

* Results to two significant figures.

Calculations:

- The MPN values, for variety of positive and negative tubes combinations, are given in previous table
- If the sample portion volumes used are those found in the tables, report the value corresponding to the number of positive and negative results in the series as the MPN/100ml.

Reporting Results

- Report results as “fecal Coliform / 100 ml of sample”.

Detection and Enumeration of Thermotolerant (Fecal) Coliform by Membrane Filter Technique

Introduction

- Thermotolerant coliform (Fecal Coliform) bacteria in this method are those facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that produce blue colony within 24 h incubation at 44.5 ± 0.2°C on m FC medium.

Principle

- Volume of water to be tested is filtered through 0.45 µm and the membrane is placed on m-FC medium.
- The selectivity of medium is due to Bile salt No.3 that acting as inhibitor of gram-positive bacteria in addition to elevated incubation temperature (44.5± 0.2°C). Aniline blue is pH indicator turning into blue color in acidic medium.
- Thermotolerant coliform (Fecal Coliform) ferment lactose, produce acid that change pH of the medium into acidic, which results in coloring colonies with blue.
- Total coliform bacteria that are able to ferment lactose at 44.5 °C are known as thermo-tolerant coliforms. Thermo-tolerant coliforms were traditionally called fecal coliforms, but they also have been documented in organically rich waters or tropical climates in the absence of recent fecal contamination. So, testing for E. coli, a specific indicator of fecal contamination, is recommended
- In most waters, the predominant genus is Escherichia, but some types of Citrobacter, Klebsiella and Enterobacter are also thermotolerant. They are usually found in sewage and water recently subjected to fecal pollution.
- Populations of thermo-tolerant coliforms are composed predominantly of E. coli; as a result, this group is regarded as a less reliable but acceptable index of faecal pollution. The presence of thermo-tolerant coliform (fecal coliform) provides evidence of recent fecal pollution.
- Membrane Filter technique is highly reproducible technique which depends on sample filtration through a 47-mm, 0.45 µm pore size cellulose membrane filters that retains the bacteria present in the sample. It can be used to test relatively large sample volumes and usually yields numerical results more than multiple-tube fermentation procedure.

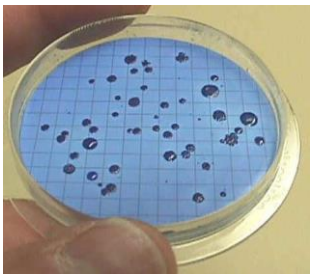
Interference

- Samples of storm water collected during the first runoff (initial flushing) after a long dry period may have a background growth that will interfere with the recovery of fecal coliform. To eliminate such interference, add 1 % rosolic acid salt reagent to the prepared media.
- Samples with high turbidity caused by algae, particulate, or other interfering material can clog the membrane filter, thereby preventing filtration, or cause spreading of bacterial colonies which could interfere with identification of target colonies.
- Non-fecal coliform colonies, a gray to cream-colored, may be observed on m FC media.

Scope

- This method describes a detailed procedure for detection and enumeration of Thermotolerant coliform (Fecal Coliform) by Membrane Filter (MF) technique in water samples in 24 hours on the basis of fermentation of lactose at elevated temperature.

Personal Responsible



- Chemist is responsible for the analysis.

Hazards and precaution

- Follow the normal safety procedures required in a microbiology laboratory.
- Mouth-pipetting is prohibited.
- Decontaminate all the used plates and materials at the end of the analyses.
- Use sterile filtration units at the beginning of each filtration series as a minimum precaution to avoid accidental contamination. A filtration series is considered to be interrupted when an interval of 30 min or longer elapses between samples filtrations.
- After such interruption, treat any further samples filtration as a new filtration series and sterilize all membrane filter holders in use.
- Avoid damaging or dislodging the membrane filter when attaching funnel to base of the filter unit.
- Do not expose poured plates with culture media to direct light; refrigerate in the dark, in a suitable tight container.

Chemicals and Reagents:

- M-FC medium.
- Lauryl Tryptose broth.
- EC broth.
- Phosphate buffered rinse water.
- Ethyl alcohol 95% in small wide-mouth vials.

Equipment and Supplies

- Stainless steel forceps.
- Sterile plastic petri dishes 50 mm.
- Sterile membrane filtration units.
- Vacuum pump.
- Buffered rinse water screwed cap bottles.
- Measuring 100 ml Cylinder class A.
- Sterile membrane filters (47 mm diameter, 0.45µ ± 0.02 µm pore size, white, grid-marked).
- Sterile 47-mm diameter absorbent pads (used with broth).
- Electric Gas Burner.
- Incubator: 44.5°C ± 0.2°C.
- Incubator: 35°C ± 0.5°C.
- Colonies counter aid.
- Indelible ink marker for labeling plates.
- Sterile plastic loop, at least 3 mm diameter in suitable holders.

Procedures:

Sample collection:

- Analyze samples on day of receipt whenever possible. The time between sample collection and the placement of samples in the incubator must not exceed 30 hours.
- If arrival is too late for processing on same day, refrigerate overnight as long as holding time conditions still be met i.e. preservation temperature <8.0°C and time did not exceeded 6 hr.

Selection of sample size:

- The official volume for regulation purpose to be filtered is 100 ml.
- Use sample volumes that will yield counts between 20 and 60 fecal coliform colonies per membrane. Recommended volumes will be 100 and 50 ml.
- Analyze waters by filtering desired volume in same funnel, or by filtering replicate smaller sample volumes e.g. duplicate 50-ml or four replicates of 25-ml portions.
- For special monitoring purposes, such as troubleshooting water quality problems or identification of fecal coliform breakthrough in low concentrations from treatment barriers; it can be test up to 1-L samples. If particulates prevent filtering a 1-L sample through a single filter, divide sample into four portions of 250 ml for analysis.

- the fecal coliform counts on each membrane to report the number of fecal coliforms per 100 ml.

Media preparation

- Prepare and handle media and reagents appropriately, and carry out suitable performance test to ensure homogeneity, sterility, and suitability for each prepared batch.

M-FC medium

- Prepare and sterilize medium according to manufacturer instructions.
- Dispense in 5 to 7 ml quantities into lower section of 50 mm Petri dishes, if dishes of any other size are used, adjust quantity to give an equivalent depth of 4 to 5 mm.
- If liquid medium is used, place a pad in the culture dish and saturate with about 2.0 ml broth medium and carefully remove excess medium by decanting the plate.
- If plates are previously prepared and stored in the refrigerator, allow them to warm at room temperature. The crystals that form on agar plates after refrigeration will disappear as the plates warm up.
- Use plates within two weeks after preparation.

Lauryl tryptose broth

- Prepare and sterilize medium according to manufacturer instructions.
- Dispense 10 ml medium, before sterilization, in fermentation tubes with an inverted Durham tube.
- Alternatively, and only in case of unavailability of Durham tubes, add 0.01 g/L bromcresol purple to medium to determine acid production.
- Close tubes with heat-resistant plastic caps.
- Ensure, after sterilization, that media cover inverted tube with at least one-half to two-thirds, and completely filled with media i.e. no air space inside inverted Durham tubes.
- Use tubes within two weeks (if loose plastic cap used) and within three months (for tight screw cap tubes).

EC broth

- Prepare and sterilize medium according to manufacturer instructions.
- Dispense 10 ml medium, before sterilization, in fermentation tubes with an inverted Durham tube.
- Close tubes with heat-resistant plastic caps.
- Ensure, after sterilization, that media cover inverted tube with at least one-half to two-thirds, and completely filled with media i.e. no air space inside inverted Durham tubes.

- Use tubes within two weeks (if loose plastic cap used) and within three months (for tight screw cap tubes).

Sample Filtration

- Label the bottom of plates with the sample identification, analyst initials, and sample volume to be analyzed.
- Place a membrane filter (grid side up) using sterile forceps on the porous plate of the filter base.
- If you have difficulties in removing the separation papers from the filters due to static electricity, place a filter with the paper on top of the funnel base and turn on the vacuum, the separation paper will curl up, allowing easier removal.
- Attach the funnel to the base of the filter unit
- Shake the sample container vigorously.
- Measure the selected volume by using sterile measuring cylinder.
- Pour selected volume or dilution of the sample into the funnel.
- Turn on the vacuum pump and allow the vacuum to operate for a time enough to filtrate all the sample volume.
- Rinse the interior surface of funnel, with filter still in place, by filtering three 20 to 30 ml portions of sterile dilution water. Alternatively, rinse funnel by a flow of sterile dilution water from a squeeze bottle.
- Turn off the pump then remove the funnel from the base of the filter unit.
- Hold the membrane filter at its edge with a sterile forceps, gently lift and place the filter grid-side up on the medium plate.
- Slide the filter onto the medium, using a rolling action to avoid trapping air bubbles between the membrane filter and the underlying medium.
- Reseat the membrane if non-wetted areas occur due to air bubbles.
- Invert the plates, and incubate at 44.5 ± 0.2°**C** for 24 ± 2 h.

Counting:

- To determine colony count on membrane filters, use a suitable optical device with a cool white fluorescent light source to provide optimal viewing of sheen.
- Count colonies with various shades of blue as typical fecal coliform, grey to green colonies as atypical fecal coliform. Non-fecal coliform colonies are gray to cream-colored.
- Count both typical and a typical fecal coliform colony.

Calculations

- Compute the count, using membrane filters with 20 to 60 fecal coliform colonies by the following equation:

$$\text{Fecal coliform / 100 mL} = \frac{\text{Colonies counted} \times 100}{\text{ML sample filtered}}$$

Reporting Results

- Report results as “Fecal Coliform / 100 ml of sample”
- If sample was delayed more than permissible time, write “Delayed” in report comment.
- If no thermotolerant coliform (fecal coliform) colonies are observed, report the fecal coliform colonies as ‘‘<1 fecal coliform/100 ml.’’
- If the total number of thermotolerant coliform (fecal coliform) colonies exceeds 60 per membrane, report results as ‘‘Too Numerous to Count with (or without) fecal coliforms’’ (TNTC).
- If confluent growth occurs, covering either the entire filtration area of the membrane or a portion thereof, and colonies are not distinct enough for accurate counting, report results as ‘‘confluent growth with (or without) fecal coliforms.’’
- Report confluent growth or TNTC without detectable fecal coliform as ‘Invalid’’.
- If sample was divided into two or more portions, Total the fecal coliform counts on all filters and report the number of fecal coliforms per 100 ml.
- Report the largest volume filter that has a fecal coliform count falling in the ideal range; calculate final concentration value by multiplying the count by dilution factor (if present).
- If all volumes filters have a fecal coliform count lower than the ideal range, disregard the rule and report the result from largest volume filter count. Calculate final concentration value by multiplying the count by dilution factor (if present).

- If largest volume filter has a fecal coliform count higher than the ideal range, choose next (second) dilution volume that falls inside ideal range and calculate final concentration value by multiplying the count by dilution factor.
- If next (second) dilution volume has fecal coliform count lower than ideal range, and largest volume count is less than 100, use the count from the largest volume rather than the count from second volume; calculate final concentration value by multiplying the count by dilution factor.

Examples	100 ml	50 ml	Reported Value
01	54	18	54
02	10	5	10
03	89	52	104
04	90	18	90 or TNTC
05	101	18	36
06	TNTC	90	180
07	TNTC	TNTC	TNTC

Chapter (2): Microscopic examination

Introduction

- The microscope provides the wastewater treatment plant operator with a special tool for process control and troubleshooting of activated sludge process.
- The microscope may be used on a routine or as needed basis to determine the impact of various operational conditions on the biomass and the treatment efficiency of the activated sludge process.
- A method was developed around 1975, by which important quality characteristics of the biomass in activated sludge plans could be determine in a quick and easy manner through microscopic investigation.
- Microscopic examination of the MLSS can be a significant aid in the evaluation of the activated sludge process. The presence of various microorganisms within the sludge floc can rapidly indicate good or poor treatment.
- The most important of these microorganisms are the heterotrophic and autotrophic bacteria, which are responsible for purifying the wastewater. In addition, protozoa play an important role in clarifying the wastewater and act as indicators of the degree of treatment.

Principle

The microscope enables an operator to see the organisms in treatment process as follow:

- Correlate healthy and unhealthy biota to operational conditions.
- Correlate healthy and unhealthy biota to industrial discharges.
- Evaluate the impact of changes in the mode of operation.
- Identify factors responsible for loss of settleability.
- Identify factors responsible for foam production.
- Identify appropriate process control measures.
- Monitor and regulate process control measures.
- Microscopes are used to see indicator bugs and other microorganisms.
- The activated sludge process contains a large number and diversity of organisms. The major organisms by number and roles performed in the activated sludge process are bacteria and protozoa (unicellular). The minor organisms are the metazoa (multicellular), additional organisms that are found in the activated sludge process include algae, fungi, immature insects and water flea.

Interference

- Record the date, time, temperature, and location of sample on the worksheet.
- A minimum of three slides per sample should be examined.

Test methods:

- Generally, two types of methods are used to sample for protozoa in wastewater treatment process: direct sampling and use of artificial solid support media.

  A-Artificial support media: For the isolation of every species

  B- Direct Sampling: This procedure describes the direct sampling technique.

Note:

- Polyurethane foam has been used successfully for sampling Protozoa in activated sludge units. Small "1 cm^3" units of foam are left immersed in an aeration tank for several days, and these are squeezed to release the protozoa.

Scope

The procedure is used for investigation and estimation of microorganisms in activated sludge process.

Personal Responsible

The biologist is responsible for investigation and estimation of microorganisms activated sludge.

Hazards and precaution

- Laboratory coats must be worn at all times.
- Eye protection and a fume cupboard must be used when handling concentrated acids.
- The biological room of the lab should be clean and dry.
- All chemical containers must be clearly labeled.
- After samples and reagents have been used; any residual material adhering to the outside of the bottle should be wiped or rinsed off to prevent contact with the hands during future handlings.
- The habit of placing discarded pipettes on tabletops, laboratory carts, or in sinks without adequate decontamination presents an unnecessary health risk to the laboratory personnel.
- Disinfectant solutions in the discard container should be replaced each morning to ensure maximum disinfection.
- Using disposable laboratory items will eliminate many washing problems.

- When hands are contaminated, they should be thoroughly washed in warm water using mild soap or detergent.

Chemical and Reagents

- Water: Distillation should be carried out behind a safety screen.
- 1% Nickel sulphate: To slow down rapidly moving protozoa, you can use one drop of 1% nickel sulphate, on the slide.
- Methylene blue stains Solution: Dissolve 0.01 g of Methylene blue in 100 ml of absolute alcohol.
- Lugol's iodine stains solution: Dissolve 10 g of Potassium iodide 5 g of Iodine crystals in 100 ml distilled water.
- Mix reagent and filter into brown bottle.
- Stopper tightly and store away from light.

Equipment and Supplies

- Binocular microscope 10x and 40x objective
- Hemocytometers, Counting chambers
- Beakers 250 ml
- Pipette 1 ml
- Pasteur pipettes
- Slides
- Slide covers 22 x 22 mm cover slips.

Procedures

Sampling collection and Frequency

- The investigation is done with sludge collected from the discharge end of the aeration tank.
- Frequencies from daily, and weekly to once every mean cell residence time (MCRT) have been used.

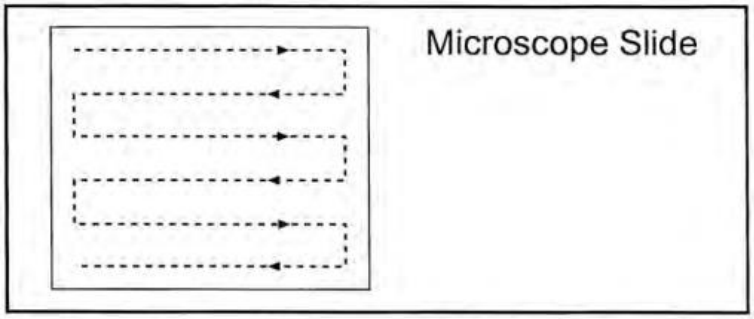
Sample Handling

- The bottles are one third filled with sludge, so as to maintain aerobic conditions in the sludge as long as possible.
- The sludge must not thicken.
- The investigation should be carried out with sludge that is as fresh as possible.
- Samples that cannot be analyzed directly must be kept cool (4-7) ^{o}C in open bottles, the sample must freeze.

- The contents of the sample bottle must mix by shaking it gently by hands.
- If an oil immersion objective is used, a drop of immersion oil must first be put on the cover slip.

Preparation of microscope slide

- Samples were prepared in triplicate and analysed at 100X, 200X, 400X and 1000X magnifications using a light microscope to determine the morphological characteristics of the microorganism's present.
- A drop of the fresh activated sludge is placed on glass slide, a cleaned cover slip placed on the drop, the slide ready and can be observed, the droplet is neither too big nor too small.
- Methylene blue or lugol's iodine stain can be used to aid the distinguished of microorganisms, add one drop of the stain to a drop of sludge on the slide and cover with cover slip.
- Examine the slide under the microscope
- Observed of slid must be systematically follow a back-and-forth pattern, moving down the slide as the following figure
- Provide a mark for each type of microorganisms on the worksheet
- Types of microorganism, which is normally found in activated sludge



Schematic outline of the manner in which a slide should be viewed.

The microscopic investigation

- The eye sees a mixture of irregularly shaped conglomerates(=Floc) through microscope, organisms that move around can be seen here and there, while filamentous structure are sometimes present between the flocs, the color of the floc components can vary from gray-yellow to brown-black

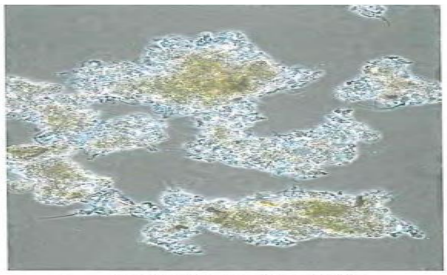
Characteristics of activated sludge flocs

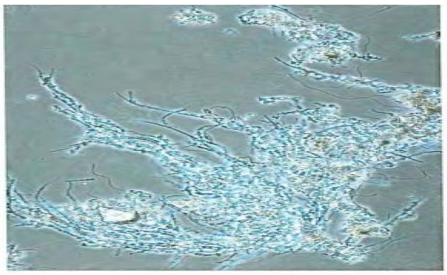
Structure

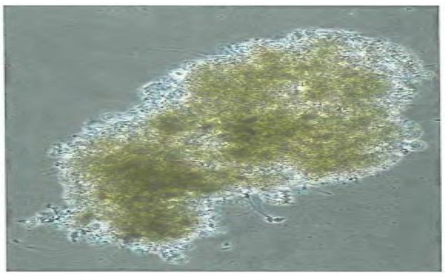
- Compact flocs: the floc mostly brown and flocs settle faster, leaving a clear supernatant.
- Pin floc tiny very compact floc, often with few bacteria that may or may not settle well, but leaves floc suspended in the supernatant.
- Open flocs (Straggler floc loose floc structure): in which water can flow through the floc particles which is characterized by large, amorphous floc which may or may not have filamentous bacteria. Generally, settles slowly.
- Bulking floc usually characterized by filamentous bacteria. Filamentous bacteria extend between floc particles, connecting them together, and thus preventing them from compacting and settling well.
- Dispersed floc loose small floc structure, very little organization. Poor settling usually has a very turbid supernatant.

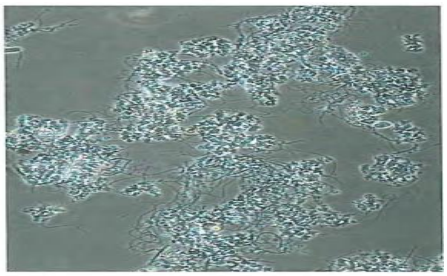
Strength

- Firm floc: surrounding liquid is distinctly separated.
- Weak floc: interference between floc and the liquid and can also be damaged.


Rounded sludge flocs (150×).


Irregularly shaped flocs (150×).


A compact sludge floc (150×).


An open floc (150×).

Reporting:

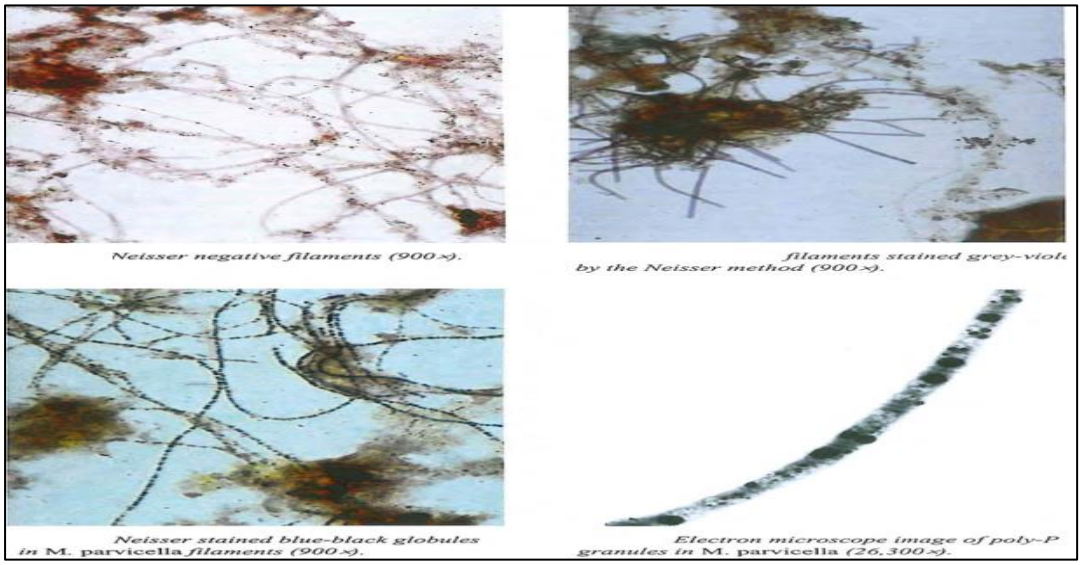
- Record the date, time, temperature, and location of sample on the worksheet.
- A minimum of three slides per sample should be examined.
- Provide a mark for each microorganism on the worksheet.
- Provide a mark for each type of microorganisms on the worksheet.
- Types of microorganism, which is normally found in activated sludge.
- Report results as “microorganism / ml of sample”

Common Microorganisms in activated sludge sample

1-Bacteria

- Bacteria are the major work force in waste treatment.
- There have many different strange names but most fit in the category called heterotrophs. Under the microscope, bacteria will appear as very small black dots some will be stationary others will be motile.
- Filamentous bacteria are normal components of activated sludge biomass.
- Filamentous bacteria grow in long thread-like strands, whose cells do not separate from each other after cell division and therefore grow in the form of filaments.
- Then, they connect with each other to form a mesh that is the most important part in floc formation and causes the separation of a fluid or removal of sediment from a fluid.
- Filamentous bacteria can provide a support structure for other bacteria to attach to as they form floc and it serves as the backbone of floc formation and settling.

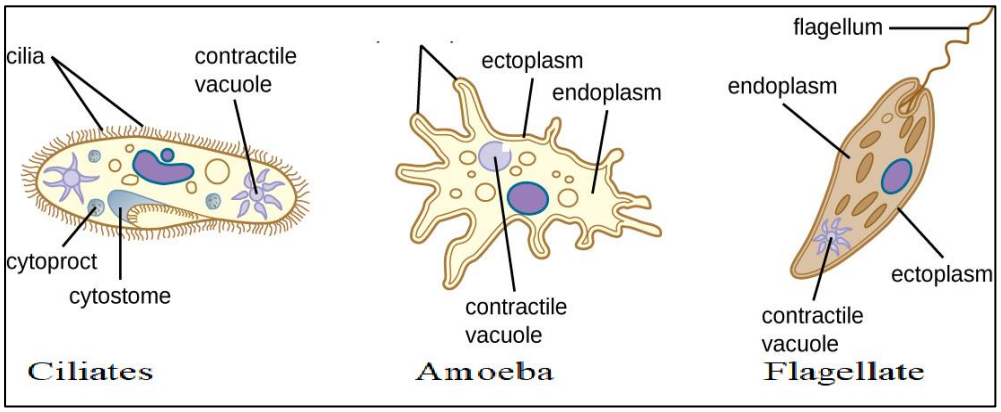
The excessive growth of these filamentous bacteria can cause potential problems with the sludge settling that reduce the efficiency of the wastewater treatment plants



2-Protozoa:

- Protozoa are one-celled, animal like organisms e.g Amoebae, flagellates, ciliates, and Sporozoa.
- The most important of which is their removal of non-flocculated bacteria from wastewater through their feeding activities, they also decrease turbidity and BOD of the effluent.

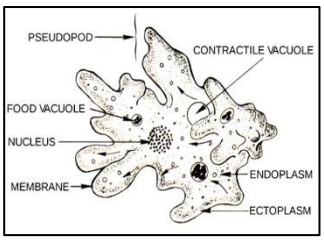
- Studies have shown species of protozoa that excrete specific materials to cause flocculation of bacteria and suspended solids in the wastewater.
- This suggests that protozoa play a role in effluent quality.
- The protozoa have an important role in maintaining a good balance in biological ecosystems by predation indirectly increases bacterial activity by preventing bacteria from reaching self-limiting numbers. Bacteria are thus kept in a state of prolonged youth and their rate of assimilation of organic materials is greatly increased.
- Protozoa may also function to break up large floc masses and encourage a more active biomass through their motility.
- This suggests that protozoa act as bio-indicators of the health of the sludge.
- Activated sludge protozoa have been placed in 3 groups in the activated sludge process, these groups include the amoebae, flagellates and ciliates.

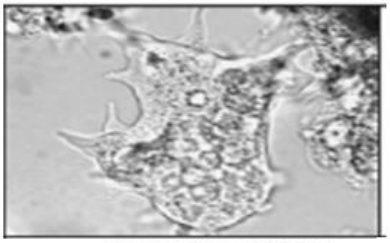


First: Amoebae

- Most primitive form of protozoa, there are two types of amoebae,

  A - Naked amoebae
- The naked amoebae do not have a protective covering and are motile by pseudopodia, also known as false feet.
- It was found that the Amoeba predominates during system start-up, from a toxic overload; it was present during unfavorable conditions only as low dissolved oxygen (DO) and high loading. It is believed that the amoeba has the ability to ingest the organic matter.

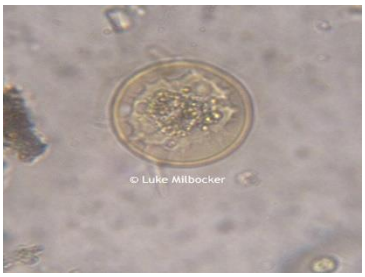


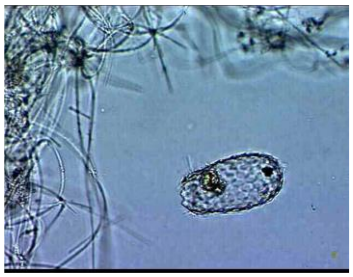


Amoebae (400x)

B - The testate amoebae

- Microscope magnification required to view: 400x.
- Free-living amoebae may be "testate" (enclosed within a hard shell).
- Testate amoebae are almost always present and often in large number in low loading and nitrifying conditions.





Second: Flagellates

Flagellates are with one or more *flagella*, whip-like organelles used for movement through the liquid, microscope magnification required to view: 400x, 1000x preferred, There are two types of flagellates,

A - Plant like flagellates

- Contain chloroplasts and are capable of photosynthesis, these flagellates also are known as motile algae.
- In the presence of excess phosphorus, pigmented flagellates may proliferate rapidly. Because pigmented flagellates are phototrophic, the swimming action toward the sunlight may cause bulking in secondary clarifiers when present in such large numbers e.g. Euglena.

B - Animal like flagellates

- Do not contain chloroplasts. The flagellates similar to the amoeba are dominant during recovery from a toxic discharge.
- This protozoon was present in higher numbers during the unfavorable conditions as low DO and high loading. e.g: Peranema.

Third: ciliates

- The ciliates are so named because of the cilia, small hairs that are distributed over the entire or part of the body and are generally ovoid or pear-shaped.
- The movement of the cilia forces the particles into the cell's gullet.
- Ciliates in three ecological categories depending on their nutrition strategies, type of locomotion and physical location within the floc:

A- Free swimming ciliates;

- Rapid, rhythmic cilia movement propels them through the liquid.
- Some are completely covered with cilia while others have cilia in rows or spirals around the cell.
- Colpidium, and Paramecium are common examples of free-swimming ciliates. Swim freely in the bulk solution, that is, they do not attach to floc particles.
- If these ciliates are the dominant protozoan group, this indicates that the bacterial population and DO concentration are high. It also indicates a wastewater environment that is not yet stabilised and a sludge that is intermediate in health.
- Some swimming ciliates have exhibited tolerance to the toxic and low dissolved oxygen conditions and their dominance has been associated with bad effluent quality.
- The free ciliates such as Chilodonella, Paramecium and Lionotus graze on free-swimming bacteria, so they clean the liquid phase of the activated sludge.
- Chillodonella can be correlated with low food-to-mass (F/M) ratio and long solid retention time (SRT), while Lionotus related with deficiently settling sludge.

B-Crawling (Grazing) ciliates;

- Ciliates have specialized cilia that look and function like legs, allowing them to crawl around on floc particles and “flick” up the bacteria so that they can consume them.
- Those moving on the floc surface, with flattened cells and specialized ventral cilia mainly hypotrichosis such as Euplotes and Aspidisca species.
- Aspidisca, Euplotes are well known bio-indicators of nitrification and usually occur under conditions of good floc formation and generally indicate good activated sludge operation.

C-Attached (Stalked or sessile) ciliates;

- Those firmly fixed to the floc through a stalk (as Vorticella or Epistylis), a lorica (as Thuricola sp. or Vaginicola sp.) and easily visible under 100x and 200x.
- Stalked ciliates can be seen in single organism form or can grow in colonies.
- Each “head” in a colony of stalked ciliates is considered one organism.
- Stalked ciliates usually attach themselves to a piece of floc but can occasionally be seen moving through the water, with or without the stalk.

- Some stalked ciliates have a contractile protein in their stalk that routinely contracts into a tightly wound coil, then springs out. This "spring action" stirs the water and helps the stalked ciliates gather food.
- Each species resembles a tulip or tube shape with cilia (small hairs) around the opening. The cilia trap bacteria, which are used as a food source, by creating a current that moves the bacteria toward the opening. The stalk ciliate then contracts in a quick motion, which pushes the food into the body where it can be utilized.
- These protozoa are generally a sign of stable, healthy activated sludge operation.
- If treatment conditions are bad, for example, low DO levels or toxicity, stalked ciliates will leave their stalks.
- The attached ciliates are said to grow best under rapidly flowing water and seems to enhance nitrification. It is generally found at the works with an effluent low BOD and good quality effluent.
- It can therefore be stated that stalked ciliates are very sensitive to any changes in the operating of the system.
- some of stalk ciliates have look like a pin cushion called suctorians which indicate to Low organic loading, ammonia and adequate dissolved oxygen

3- Metazoa:

- group of multicellular animals including nematodes, rotifers and oligochaete worms.
- they are strict aerobes and do not tolerate adverse operational conditions such as low dissolved oxygen concentration, high pollution, and toxicity.
- The most commonly observed metazoa in the activated sludge process are the rotifer and the free-living nematode although present in relatively small numbers

A-Rotifers:

- Rotifers are the simplest of the multicellular animals.
- They are found in many different types of water including aeration stabilization basin systems, activated sludge, and some freshwater systems.
- Rotifers range in size from 40 to 500 µm; they are in fact, microscopic crustaceans and move by swimming freely through the bulk water or crawling.
- They have a ciliated area at the anterior end (mouth opening) that resembles a “rotating wheel.” This group of cilia at the mouth aids in the feeding and movement of the rotifer.

- Many rotifers also have a posterior podite (foot) which allows them to attach to floc. It usually looks like a forked tail.
- The main role of rotifers in wastewater systems is the removal of bacteria., They also aid in floc formation.
- Rotifers thrive in conditions with plenty of oxygen and are an indicator species for low (BOD), low toxicity, and stable wastewater systems.

B-Nematodes:

- Their body none segmented and have long thin cylindrical bodies, with one end usually sharply pointed while the other end tapers to a blunt tip (little fire hoses).
- Which feed on bacteria, protozoa, fungus, other nematodes and floc, they tunnel through floc, slime, and biofilm, increasing oxygen penetration, preventing excess build up, and keeping it porous.
- Their presence, growth, and movement are bioindicators of long sludge age.
- They are sensitive to anoxic conditions, and their population decreases in warm temperatures.
- They along with other metazoa are usually seen at low F: M ratios and BOD.

C-Aeleosoma Worm (Bristle Worm):

- Bristle worms are nicknamed for their bristle-like hairs throughout the body; many species of Oligochaete worms have a similar appearance. Except for difference in size, most of them resemble the common earthworm in external anatomy. It bears bristles called "setae" on most segments.
- Bristle worms are long and cylindrical with tapering ends. They are commonly with extend from its sides and burnt orange dots on its surface which can cause a reddish colour in your activated sludge.
- When reproducing, they “bud” off a segment of their length to produce a new worm. They also use their bristles to assist with movement.
- Bristle worms graze on the bacterial population in activated sludge flocs and biofilms and also consume organic sludge.
- In doing so they enhance oxygen penetration in the floc structures, and promote microbial activity. an abundance indicates an older sludge.
- The biggest problem with older sludge (very low F/M) is the increase in effluent turbidity and pin floc carryover in the secondary clarifiers

D-Tardigrades (water bear):

- Water bears have eight legs with claws and a gait (head) that resembles a bear and are one of the larger critters we see under the microscope in activated sludge processes (typically at 200x magnification).
- As a metazoa, water bears are higher up on the food chain in activated sludge processes, while bacteria are responsible for the majority of biological wastewater treatment. In wastewater, water bears may prey on bacteria, plant matter, algae, or may be carnivorous, preying on nematodes and rotifers.
- Some species of water bear are even known to be cannibalistic and prey on other water bears.
- The presence of tardigrades in wastewater indicates: higher sludge retention time and low ammonia concentrations (good nitrification)

Reporting Results

- Qualitative Assessment of Protozoa: When the data from the worksheet is entered into the computer spreadsheet, the computer will provide a qualitative assessment of the **activated sludge, as follows:**

Based on the five following categories:

- Absent
- Frequent
- Regular
- Occasional
- Absent

Make a qualitative assessment for each type of protozoa and mark the worksheet against one category.

Vitality: Based on the five following categories

- Very vigorous
- Vigorous
- Ok
- Stressed
- Very stressed

Make a qualitative assessment for:

- Stalked ciliated protozoa.

- Other protozoa and make a mark on the worksheet against one category for the two types of protozoa.

Flock strength

Based on the following assessment of floc size assign, a category of floc strength.

- Strong more than 500 µm
- Dense 400 – 500 µm
- Moderate 250 – 400 µm
- Weak 150 – 250 µm
- Very weak 50 – 150 µm
- Examples of the work sheets for protozoa:see appendix.

Filamentous bacteria:

- Make a qualitative assessment forfilamentous bacteria based on the following assessment
- Frequency and vitality
- Excellent
- Good
- Ok
- Bad
- Very bad

Floc strength

- Strong
- Dense
- Moderate
- Weak
- Very weak

Filamentous bacteria

- Within floc only
- Present
- Absent
- Protruding
- Dominating

An overall assessment will be made by the computer spreadsheet based on the three categories above as follows:

- Excellent

- Good
- Ok
- Bad
- Very bad
- Examples of the work sheets for filamentous:see appendix

Ascaris Examination

Introduction

- The Ascaris lumbricoides, common saying "round worm of man", is the largest of the intestinal nematodes parasitizing humans. It is the most common worm found in human.
- It is worldwide in distribution and most prevalent throughout the tropics, sub-tropics and more prevalent in the countryside than in the city.
- The incidence is several million infections annually. Of these cases, about 210 million are symptomatic. In some rural settings with poor sanitation, perhaps half the children of 2-12 years have ascariasis.

Morphology:

Adult:

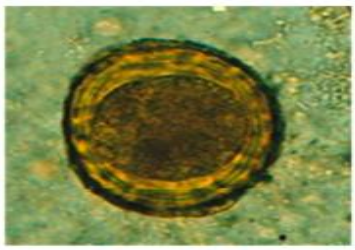
- The adults are cylindrical in shape, cream white or pinkish in color.
- The female averages 20-35cm in length, the largest 49 cm.
- The male is smaller, averaging 15-31 cm in length and distinctly more slender than the female.
- The typical curled tail with a pair sickle like copulatory spines.On the tip of the head there are three lips, arranged as a Chinese word.
- They have a complete digestive tract.
- Reproductive organs are tubular. Male has a single reproductive tubule. The female has two reproductive tubules and the vulva is ventrally located at the posterior part of the anterior 1/3 of the body.

Egg:

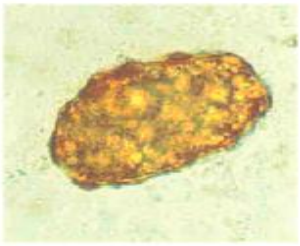
- There are three kinds of the eggs. They are fertilized eggs, unfertilized eggs and decorticated eggs. We usually describe an egg in 5 aspects: size, color, shape, shell and content.
- Fertilized eggs: broad oval in shape, brown in color, an average size 60× 45 μm. The shell is thicker and consists of ascaroside, chitinous layer, fertilizing membrane and mammillated albuminous coat stained brown by bile. The content is a fertilized ovum. There is a new moon (crescent) shaped clear space at the each end inside the shell as in shape (1).
- Unfertilized egg: Longer and slender than a fertilized egg. The chitinous layer and albuminous coat are thinner than those of the fertilized eggs without ascaroside and

fertilizing membrane. The content is made of many refract able granules various in size as in shape.

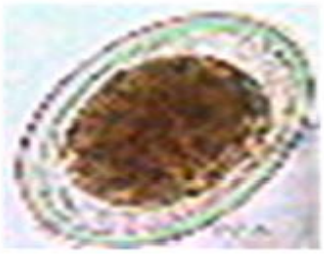
- Decorticated eggs: Both fertilized and unfertilized eggs sometimes may lack their outer albuminous coats and are colorless as in shape.



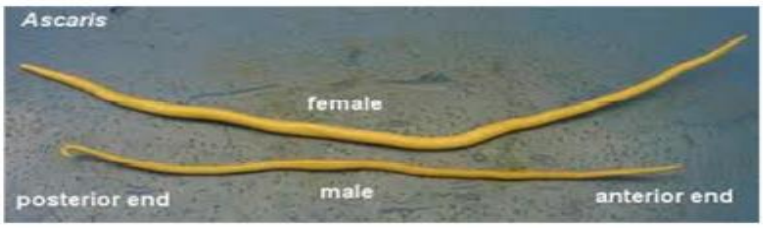
Fertilized eggs



Unfertilized egg



Decorticated eggs



Ascaris

Interference

- Long periods of time spent at the microscope are very tiring and can lead to errors. The time required to examine each McMaster slide is usually only 1-2 minutes, so that operator error is reduced.

Scope

- The procedure is used for examination of Ascaris for raw water, final treated effluents and sludge.

Personal Responsible

- Chemist is responsible for the analysis.

Hazard and precaution

- Ether is highly flammable and toxic.

Chemicals and Reagents

- Zinc sulfate solution (33%, relative density 1.18)
- Ether or ethyl acetate
- Acetoacetic buffer (PH=4.5) (15 gm sodium acetate trihydrate, 3.6 ml glacial acetic acid, made up to 1 liter with distilled water)
- Detergent solution (1 ml triton x-100 or tween 80, made up to 1 liter with tap water).

Equipment and Supplies

- Plastic containers for sample collection
- Centrifuge capable of generating 1000g
- Centrifuge tubes with lids
- Pasteur pipettes and teats
- McMaster counting slides
- Vortex mixer (not absolutely essential).
- Measuring cylinder or graduated pipette
- Hydrometer
- Microscope with manometer

Sampling

- Allow the sample to sediment for 1-2 hours and centrifuge the sediment.

Environment conditions:

- Keep the sample in clean bottles and tightly closed.

Procedures

- Suspend the pellet in an equal volume of acetoacetic buffer, pH 4.5 (if the volume ·of the pellet is 2 ml, add 2 ml of buffer). If the pellet is less than 2 ml, add buffer up to 4 ml to ensure that, after extraction with ethyl acetate, there is sufficient volume of buffer above the pellet to allow the ethyl acetate layer to be poured off without re suspension of the pellet.
- Leave the full McMaster slide to stand on a flat surface for 5 min before examination. This allows all the eggs to float to the surface.
- If more than one centrifuge tube has been used, transfer all the sediments to one tube (remember to rinse thoroughly with detergent solution to ensure that no sediment is discarded), and recentrifuged at 1000 g for 15 min.
- Mix the final flotation suspension thoroughly, preferably in a vortex mixer to ensure homogeneous mix.
- Quickly fill a Pasteur pipette and carefully run the solution into one compartment of the McMaster slide.
- Fill the whole compartment completely even though it is only the section under the grid that is to be counted.
- Work quickly and smoothly at this stage so that eggs do not start to float in the test-tube or in the pipette.
- Ensure that there are no air bubbles under the grid.

- The sample of wastewater of known volume (V liters), usually 1 liter for raw or partially treated wastewaters and 10 liters for final treated effluents. and for sludge prepare 1L of sludge suspension at concentration 5% from sludge dried weight.
- Allow the sample to sediment for 1-2 hours, depending on the size of the container.
- Remove 90% of the supernatant using a suction pump or siphon.
- Carefully transfer the sediment to one or more centrifuge tubes, depending on the volume, and centrifuge at 1000 g for 15 min. Remember to rinse the container well with detergent solution, and add the rinsing to the sediment.
- Remove the supernatant. If more than one centrifuge tube has been used, transfer all the sediments to one tube (remember to rinse thoroughly with detergent solution to ensure that no sediment is discarded), and re centrifuge at 1000 g for 15 min.
- Suspend the pellet in an equal volume of acetoacetic buffer, pH 4.5 (if the volume of the pellet is 2 ml, add 2 ml of buffer). If the pellet is less than 2 ml, add buffer up to 4 ml to ensure that, after extraction with ethyl acetate, there is sufficient volume of buffer above the pellet to allow the ethyl acetate layer to be poured off without re suspension of the pellet.
- Add two volumes of ethyl acetate or ether (i.e., 4 ml in the above example) and mix the solution thoroughly in a vortex mixer. The sample can also be shaken by hand. This is quite acceptable if a mechanical mixer is not available.
- Centrifuge the sample at 1000 g for 15 min. The sample will now have separated into three distinct phases (layer).
- All the non-fatty, heavier debris, including helminths eggs, larvae and protozoa, will be in the bottom layer.
- Above this will be the buffer, which should be clear.
- The fatty and other material moves into the ethyl acetate or ether and forms a thick dark plug at the top of the sample.
- Record the volume of the pellet containing the eggs, and then pour off the rest of the supernatant in one smooth action. It may be necessary to loosen the fatty plug first by running a fine needle around the side of the centrifuge tube.
- Resuspend the pellet in five volumes of zinc sulfate solution, (i.e., if the volume of the pellet is 1 ml, add 5 ml of ZnSO4). Record the volume of the final product (X ml).
- Mix the sample thoroughly, preferably using a vortex mixer. Note that a minimum of 1.5 ml is required to fill a two-chambered McMaster slide.

- Quickly remove an aliquot with a Pasteur pipette and transfer to a McMaster slide for final examination.
- Leave the full McMaster slide to stand on a flat surface for 5 min before examination. This allows all the eggs to float to the surface.
- Place the McMaster slide on the microscope stage and examine under 10× or 40× magnification. Count all the eggs seen within the grid in both chambers of the McMaster slide.
- For greater accuracy, the mean of two slides, or preferably three, should be recorded.

Calculation

- Calculate the number of eggs per liter from the equation:

$$N = AX/PV$$

where:

N = number of eggs per liter of sample
A = No. of eggs counted in the McMaster slide or the mean of counts from two or three slides
X = volume of the final product (ml)
P = volume of the McMaster slide (0.3 ml)
V = original sample volume (liters)

Reporting Results

- The result is recorded as number of eggs / liters

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قام بإعداد الإصدار الثانى من هذا البرنامج

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قام بالتنسيق الفنى والإخراج لهذا الإصدار

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للاقتراحات والشكاوى قم بمسح الصورة (QR)