Inter – laboratory comparison and Proficiency testing

September 11

2013

Total Coliform in water and waste water is an important parameter to assess the designated best uses of drinking water source and outdoor bathing water quality with respect to Primary Water Quality Criteria developed under the provision of Water Act, 1974. In order to assess uniformity in analytical procedure, an inter-laboratory comparison exercise for micro-biological testing of Total Coliform using MPN technique, is being undertaken by Bio-lab of CPCB, Delhi.

Analysis of Total Coliform by MPN Technique

Module for Inter –laboratory comparison and Proficiency testing for analysis of Total Coliform by MPN Technique

Bio science– Laboratory,Central Pollution Control Board, Delhi

The following module is to be used for the proficiency testing of participating laboratories, in the Bio science- Laboratory of Central Pollution Control Board, to understand the practical aptitude of the participant as well as to realize the participant' s eligibility for the laboratory work. The participant has to estimate the Total Coliform density in the given sample using the MPN technique for multiple tube fermentation method.

(Reference APHA, 22nd Edition)

Following observations are required to be made carefully:

1. Identification of physical attribute such as gas formation and turbidity.

2. Running control

3. Check no air bubble in the inverted Durham's tube or inverted fermentation vial prior to testing

4. Ensure exact amount of sample inoculation.

5. Ensure sterility of sample containers, glassware and equipment to be used for testing

Materials provided: First set

- Testtube stands containing 15 tubes in first set.
- First series of first set marked as A 1, A2, A3, A4, A5 Second series marked as B1, B2, B3, B4,B5, and third series marked as C1, C2, C3, C4, C5, for presumptive phase.
- Test tube stands containing 15 tubes in second set.
- First series of second set marked as a1, a2, a3, a4,a5, second series marked as b1,b2, b3, b4, b5, and third series marked as c1, c2, c3, c4, c5,) for confirmatory phase.
- One tube extra to be used as control.
- 5 tubes each containing 10ml of sterilized double strength (D.S.) Lauryl Tryptose broth each containing inverted Durham ‘s tube for Presumptive Phase (First series marked as A1,A2, A3, A4,A5,).
- 10 tubes each containing 10ml of satirized single strength (S.S)Lauryl Tryptose broth each containing inverted Durham’s tube or inverted fermentation vial for presumptive Phase (Second series

marked as B 1, B2, B3, B4, B5, B6, and third series Marked as C1, C2, C3, C4, C5,).

- 5 tube each containing 10ml of Sterilized Dilution Water (SDW) for dilution of waste water sample S2.

Second set

- 15 tubes each containing 10ml of sterilized single strength (S.S) Brilliant Green Lactose Bile broth each containing inverted Durham' s tube for Confirmatory Phase. (First series of 5 tubes marked as a1, a2,a3,a4,a5second series of five tubes marked as b1,b2, b3,b4,b5 and third series of five tubes marked as c1, c2, c3,c4, c5).

- 30 number of pre –sterilized plastic loops for inoculation from each tube of positive Presumptive Phase to respectiveBrilliant Green Lactose Bile broth tube of ConfirmatoryPhase.

- Two different samples(potable 150 ml and waste water 100ml) in each of 250 ml autoclaved Tarsonsbottle marked as S l and S2.

Third set

- First series of third set of LES Endo agar plate, secondary Layryl Tryptose broth, nutrient agar slant and Gram stained glass slides to be marked as a1, a2, a3, a4,a5, second series marked as b1,b2, b3, b4, b5, and third series marked as c1, c2, c3, c4, c5,) for completed phase.
- 10 gm LES Endo agarpowder in Tarson tube: Dispense 5.79 gm LES Endo agarpowder in 150ml distilled water, autoclave and prepare 15 LES Endo agarplate for Completed Phase.
- 10 gm Secondary LS broth powderin Tarson tube: Dispense 5.34 gm LS broth powder in 150ml distilled waterand prepare 15 autoclaved Secondary LS broth tubes containing inverted fermentation vials for Completed Phase.
- 10 gm Nutrient Agar powderin Tarson tube: Dispense 4.2gmNutrient Agar powder in 150ml distilled waterheat and prepare autoclaved Nutrient Agar slants for culture preparation.

Materials Required:

- Sterilized glass pipettes or micropipettes (each of 1ml) with sterilized micro tips (pre –autoclaved) for each serial dilutions and inoculation.
- 70% alcohol to be used as a disinfectant.
- Gram's staining kit
- Glass slides
- Microscope with 100X objective

Method:

- Two samples (Potable water sample Sl and waste water sample S2) will be provided to the participant in a 125 ml sample bottle at the initial point of testing.
- For direct inoculation of potable water sample S1, 10ml of sample S1 may be inoculated in first series of five tubes each containing 10 ml of double strength Lauryl Tryptose broth.
- 1ml of sample S1 may be inoculated in second series of five tubes each containing 10ml of single strength Lauryl Tryptose broth.
- 0.1 ml of sample S l may be inoculated in third series of five tubes each containing 10 ml of single strength Lauryl Tryptose broth for presumptive Phase Total Coliform, in Laminar flow.

- Prepare dilution water for waste water sample S2.
- Set of 5 tubes each containing 10ml of sterilized dilution water may be used for making the dilution series.
- The standard method of serial dilution may be used as Annexure 1.
- 1ml of sample will be transferred from the sample bottle to the first tube of the Dilution series, thus formulating 10^{-1} ml sample (0.1 ml).
- 1ml of sample from 10^{-1} ml sample is further transferred to the second dilution series tube to formulate 10^{-2} ml sample (0.01ml).
- The process of serial dilution is further continued till the 10^{th} dilution leading to formulation of 10^{-1} ml, 10^{-2}ml, 10^{-3}ml, 10^{-4} ml,10^{-5}ml, 10^{-6} ml, 10^{-7}ml, 10^{-8} ml, 10^{-9} ml, 10^{-10} ml etc. dilution series of sample is obtained.
- Inoculate 1ml each from the selected three dilution 10^{-2} , 10^{-3}, and 10^{-4} of the sample S2, to each of first , second and third series of five tubes each containing 10ml of single strength Lauryl Tryptose broth aseptically.
- Incubate the inoculated Lauryl Tryptose broth tubes at 35± 0.5^{0} C for 24-48±3 hours.

- Observe the turbidity and gas production caused due to fermentation in Durham's tube or inverted fermentation vial after 24-48±3 hours for positive tubes.
- Transfer a loop full of inoculum from each positive tube of presumptive Phase to set Brilliant Green Lactose Bile broth tubes for confirmatory Phase.
- Incubate the inoculated Brilliant Green Lactose Bile broth tubes at 35±0.5^{o}c for 24-48 ±3hours for Confirmatory phase of Total Coliform analysis
- Observe all inverted Durham's tube or inverted fermentation vial having gas formation for confirmation of positive tubes in each set of dilution and note it down (e.g. 5,5,4 positive results will mean 5 positive tubes for 10^{-1} / ml sample; 5 positive tubes for 10^{-2}/ ml sample and 4 positive tubes for 10^{-3} /ml sample).
- Completed Phase may be done for waste water S2 sample.

- For Completed phase of Total Coliform analysis, take a loop full of inoculum from each positive tube of Confirmatory phase of Brilliant Green Lactose Bile broth tubes and aseptically streak, with the help

of sterile loop, on each LES Endo agar plate. Incubate the inverted agar plates at 35±0.5^{0}C for 24±2 hours.

- Observe discrete colonies separated by at least 0.5cm on LES Endo agar plates
- Observe typical (pink to dark red with a green metallic sheen) or atypical (pink, red, white or colorless colonies without sheen).
- Pick up one or more typical or atypical colonies from each LES Endo agar plate and transfer to a single –strength secondary Lauryl Tryptose broth fermentation tube with inverted fermentation vials (Durham's tube or inverted fermentation vial) and simultaneously on to a nutrient agar slant.
- Incubate inoculated secondary Lauryl tryptose broth tubes and nutrient agar slants at 35±0.5^{0}C for 24± 2hours.
- If gas is not produced in secondary Lauryl Tryptose broth tubes in inverted fermentation vials within 24 ±2 hours, re-incubate it and examine again at 48±3hours.
- Prepare Gram stained slides of bacteria grown on nutrient agar slant as per Annexure 2

- Microscopically examine Gram-stained preparation from those 24 hour nutrient agar cultures corresponding to +ve secondary Laryl Tryptose tubes having gas in inverted fermentation vials.
- Formation of gas in the secondary tubes of Lauryl Tryptose broth within 48±3hours and demonstration of Gram –ve non-spore forming, rod shaped bacteria from the agar culture constitute positive results for the Completed Phase demonstrating the presence of members of the Coliform group.
- Make entries of the final results of Completed Phase in Result sheet no. 3.
- Compute/Calculate MPN / 100ml for Total Coliform by comparing the observed positive results with the MPN Index table/ Thomas formula given in APHA 22^{nd} edition (Table 1).
- If sample is diluted than multiply the obtained result (number) with the dilution factor.
- Dilution factor for 10^{-1} ml sample is 10; Dilution factor for 10^{-2} ml sample is 100; Dilution factor for 10^{-3} ml sample is 1000 and so forth with increasing dilutions (Annexure1).
- Express the final result in result sheet 1and 2 and 3 as MPN /100 ml for Total Coliform in the given sample S1 and S2.

Result sheet 1 for:

Date of Sample received:

Date of analysis of sample:

Sample code	**Mark + to combination of positive tubes and – to negative tubes for presumptive Phase**			**Combination of positive tubes/ MPN Index value**	**Multiplication of Dilution factor**	**Final Result for presumptive PhaseMPN/100ml**
	10 ml	1ml	0.1ml			
Control						
S1	A1	B1	C1			
	A2	B2	C2			
	A3	B3	C3			
	A4	B4	C4			
	A5	B5	C5			
Sample code	**Mark + to combination of positive tubes and – to negative tubes for confirmatory Phase**			**Combination of positive tubes/MPN Index value**	**Multiplication of Dilution factor**	**Final Result for confirmatory Phase MPN/100ml**
	10 ml	1ml	0.1ml			
Control						
S1	a1	b1	c1			
	a2	b2	c2			
	a3	B3	c3			
	a4	b4	c4			
	a5	b5	c5			

Name & Signature of Analyst

Result sheet 2 for:

Date of Sample received:

Date of analysis of sample:

Sample code	**Mark + to combination of positive tubes and – to negative tubes for presumptive Phase**			**Combination of positive tubes/MPN Index value**	**Multiplication of Dilution factor**	**Final Result for presumptive PhaseMPN/100ml**
	0.01ml for 10^{-2}	0.001 ml for 10^{-3}	0.0001ml for 10^{-4}			
Control						
S2	A1	B1	C1			
	A2	B2	C2			
	A3	B3	C3			
	A4	B4	C4			
	A5	B5	C5			
Sample code	**Mark + to combination of positive tubes and – to negative tubes for confirmatory Phase**			**Combination of positive tubes/MPN Index value**	**Multiplication of Dilution factor**	**Final Result for confirmatory Phase MPN/100ml**
	0.01ml for 10^{-2}	0.001 ml for 10^{-3}	0.0001ml for 10^{-4}			
Control						
S2	a1	b1	c1			
	a2	b2	c2			
	a3	B3	c3			
	a4	b4	c4			
	a5	b5	c5			

Name & Signature of Analyst

Result sheet 3 for:

Date of Sample received:

Date of analysis of sample:

Sample code	**Mark + to combination of positive LES ENDO agar plates for Completed Phase**			**Combination of positive LES ENDO agar plates/MPN Index value**	**Multiplication of Dilution factor**	**Final Result for Completed Phase MPN/100ml**
	0.01ml for 10^{-2}	0.001 ml for 10^{-3}	0.0001ml for 10^{-4}			
Control						
S2	a1	b1	c1			
	a2	b2	c2			
	a3	B3	c3			
	a4	b4	c4			
	a5	b5	c5			
Sample code	**Mark + to combination of positive secondary LS broth tubes and – to negative tubes for Completed Phase**			**Combination of positive Secondary LS broth tubes/MPN Index value**	**Multiplication of Dilution factor**	**Final Result for Completed Phase MPN/100ml**
	0.01ml for 10^{-2}	0.001ml for 10^{-3}	0.0001ml for 10^{-4}			
Control						
S2	a1	b1	c1			
	a2	b2	c2			
	a3	B3	c3			
	a4	b4	c4			
	a5	b5	c5			
Sample code	**Mark + to combination of positive slides showing Gram –ve bacteria for Completed Phase**			**Combination of positive Gram –ve bacteria/MPN Index value**	**Multiplication of Dilution factor**	**Final Result for Completed Phase MPN/100ml**
	0.01ml for 10^{-2}	0.001 ml for 10^{-3}	0.0001ml for 10^{-4}			
Control						
S2	a1	b1	c1			
	a2	b2	c2			
	a3	B3	c3			
	a4	b4	c4			
	a5	b5	c5			

Name & Signature of Analyst

Table 1 : APHA -22ND EDITION

MULTIPLE – TUBE FERMENTATION TECHNIQUE for Estimation of Bacterial Density

MPN Index AND 95% CONFIDENCE LIMTS FOR VARIOUS COMBNATION OF POSTIVE RESULTS WHEN FIVE TUBES ARE USED PERDILUTION (10ML, 1.0ML, 0.1 ML)

Combination Of positives	MPN Index / 100mL	Confidence Limits		Combination of positives	MPN Index/100mL	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	5-1-3	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	400
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				

EXAMPLES FOR CHOICE OF THREE COMBINATIONS OF POSITIVE FROM FIVE DILUTIONS

Example	Volume in mL 10	1	0.1	0.01	0.001	Combination of positives	MPN Index No./100mL
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

For selected dilution use Thomas formula: MPN /100 mL(approx.) =100x $P/(N \times T)^{1/2}$

Where:

P = number of positive results,

N = volume of sample in all the negative portions combined, mL, and

T = total volume of sample in the selected dilutions, mL.