Water Microbiology I

MPN test

Introduction

Water

- very essential factor needed by man (used for cooking, drinking, etc.)
- open and widely accessible, making it susceptible to contamination by chemicals and bacterial pathogens
- once contaminated, it would be harmful for human consumption.

Detection of m.o. in water

- Indicator and index m.o.
  - Coliform
  - faecal coliforms
  - *E. coli*
- Detection techniques
  - Multiple tube fermentation or MPN method
  - Membrane filtration method

Bacteria found in water

- Natural aquatic bacteria
  - mostly are Gram-negative bacteria e.g. *Pseudomonas*, *Acinetobacterium*, etc.
- Soil-dwelling bacteria
  - Enterobacteriaceae (e.g. *Enterobacter*), *Streptomyces*, *Bacillus*
- Intestinal m.o.
  - Coliform, *E. coli*, Faecal Streptococci

Introduction

Bacterial pathogens

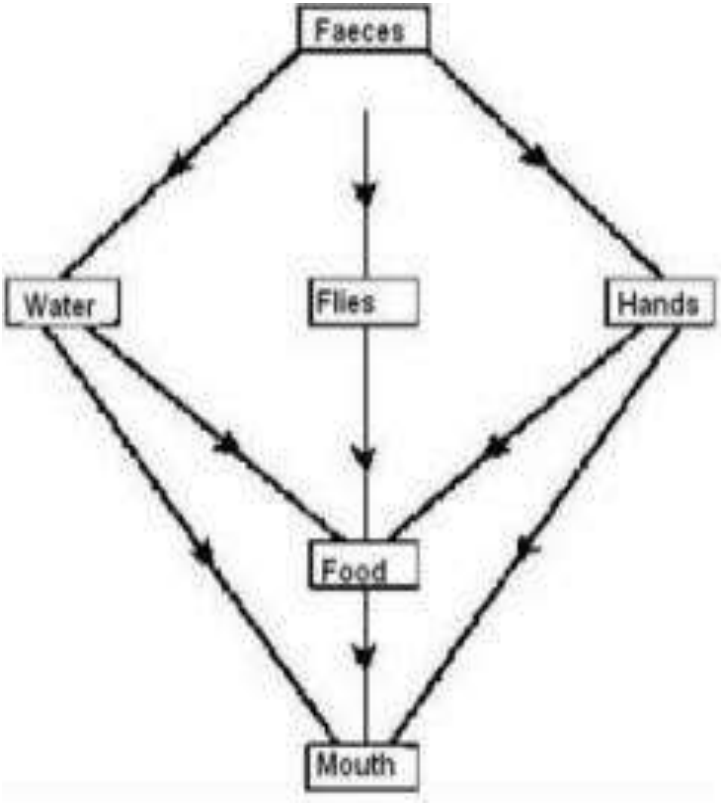
-may cause water-borne diseases such as Shigellosis, Malaria, Campylobacteriosis, Cholera, Giardiasis, etc.(bacteria ,parasite)

Prevalence:

In developing countries, 4/5 of all the illnesses are caused by these, with diarrhea being the leading cause of childhood deaths.

Water Borne Diseases

- Water-borne diseases are any illness caused by drinking water contaminated by human or animal faeces, which contain pathogenic microorganisms.
- The germs in the faeces can cause the diseases by even slight contact and transfer.



Water Borne Diseases

- **Bacterial infections**
- Botulism - Clostridium botulinum bacteria - gastro-intestinal food/water borne
- Campylobacteriosis
- Cholera - Vibrio cholerae bacteria - gastro-intestinal often waterborne
- Chronic granulomatous disease - caused by the Mycobacterium marinum infection and localized in skin, frequently occurred with aquarium keepers.[3]
- Diarrheal disease due to *E. coli*.

Water Borne Diseases

- Dysentery - Shigella/Salmonella bacteria - gastro-intestinal food/water
- Legionellosis - cause Pontiac fever and Legionnaires' disease
- Leptospirosis
- Otitis externa- "Swimmer's Ear"
- Typhoid - Salmonella typhi bacteria - gastro-intestinal water/food borne. Salmonellosis - due to many Salmonella species. Water/food/direct contact borne.
- Vibrio illness caused by the bacteria of Vibrio vulnificus, Vibrio alginolyticus and Vibrio parahaemolyticus commonly found in seafood and recreational water.

Viral Sources of Waterborne Disease

- Hepatitis A: inflammation and necrosis of liver
- Norwalk-type virus: acute gastroenteritis
- Rotaviruses: acute gastroenteritis, especially in children
- Enteroviruses: many types affect intestines and upper respiratory tract
- Reoviruses: infects intestines and upper respiratory tract

Objectives

1. To perform the Most Probable Number (MPN) Technique for testing the potability of different water samples.
2. To interpret results of water analyses using the MPN table.
3. To be familiar with common water-borne diseases and their causative reagents.

Problems when testing water

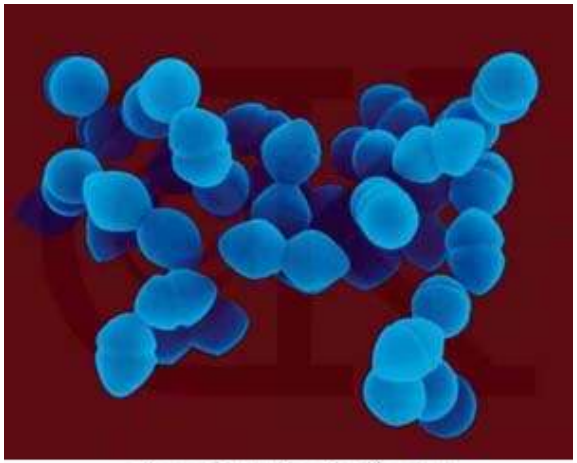
- Numerous water borne pathogens
- Individual pathogen numbers may be too low to detect in a reasonable sized water sample
- Isolation and detection of some pathogens can take several days, weeks, or months
- Absence of one particular pathogen does not rule out the presence of another.

Indicator Organism Concept

- Widely used in determination and estimation of water contamination correlated to the presence of pathogens .
- **Why used:**
- Population large enough to isolate in small water samples (100 mL)
- Rapid
- Inexpensive
- Safe, not culturing pathogens

Bacterial-Indicator Organisms Common Groups

- Coliforms(recent fecal contamination)
  - Total coliforms
  - Fecal coliforms
  - *Escherichia coli*
- Streptococci
  - *fecal streptococci*
  - *Enterococci*
- Spore Formers(indication of old fecal contamination)
  - *Clostridium perfringens*



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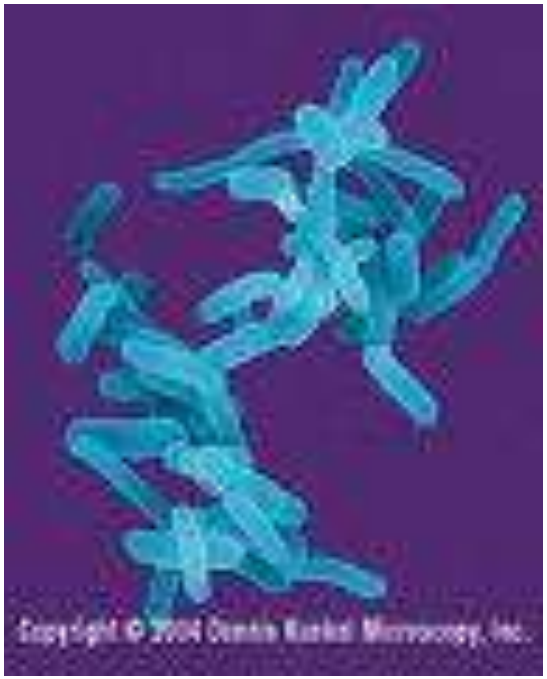
Characteristics of a Useful Indicator

- Useful for all water types
- Always present when pathogens are present
- Not present in the absence of the pathogen
- Correlated with degree of pollution
- More easily detectable than a pathogen
- Survive longer than the pathogen
- Not dangerous to work with



Coliforms

- Coliforms- refers to the various genera of the family *Enterobacteriaceae* which are lactose fermentors and are commonly found contaminants in water.
- Organisms that are under the genus *Escherichia, Enterobacter*, *Klebsiella*, *Serratia*, *Citrobacter*.
- Collectively, this group of Gram-negative bacilli are referred to as "coliforms" because they share similar morphological and biochemical characteristics.

Coliform Group (total coliform)

- *Enterobacteriaceae*
  - Facultative anaerobe
  - Gram negative
  - Non-spore forming
  - Rod shaped
  - Ferment glucose
  - Produce gas and acid within 48 h at 35 C

- Coliforms genera
  - *Enterobacter*
  - *Klebsiella*
  - *Citrobacter*
  - *Escherichia*

  - In addition to Ferment lactose

Coliforms

- Most of these organisms are members of the normal flora of humans and/or animals and are considered opportunistic pathogens.

- Most are found in the colon. Most of these organisms possess fimbriae that is used as appendages for adhesion purposes.

E.coli

- *E. coli* are found in intestine, their ability to survive for brief periods outside the body makes them an ideal indicator organism to test environmental samples for fecal contamination. Indicator organisms indicate that water received contamination of intestinal origin.

- *E. coli* are Gram negative bacterium that is commonly found in the lower intestine of warm-blooded animals, while other coliforms(*Enterobacter, Klebsiella)* can be found on plants and in soil.

Multiple Tube Fermentation Technique to determine Most Probable number

- uses a specified number of test tubes to statistically predict the number of organisms present (based on the expected population of organisms in the sample)
- tubes may also contain an inverted inner vial (Durham tube) for gas collection
- ideal for wastewater samples and non-potable samples, because the analyst can accommodate highly turbid samples by diluting prior to analysis

Multiple Tube Fermentation Methods

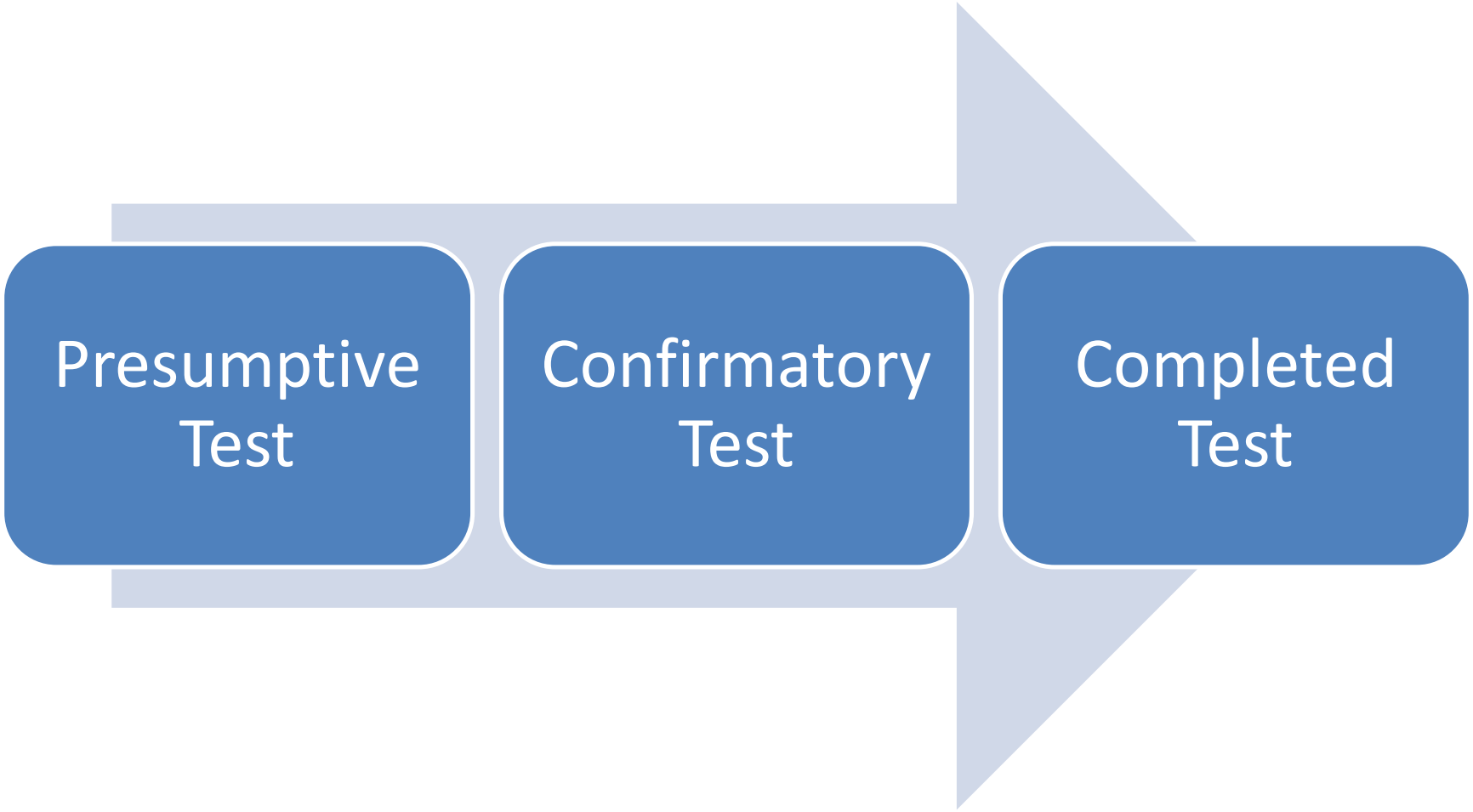
- Specific dilution is made
- Inoculate multiple tubes (3 or 5) of media with water sample
- Incubate
  - 35 C or
  - 44.5 C
- Count positive growth tubes
- Use Most-Probable-Number (MPN) table to estimate density

Methodology

Sampling for Tap Water Samples

1. Clean the tap. Let water flow for 1-2 minutes. Sterilize tap with flame and collect sample
2. Analyze water samples not more than 6hours after sampling or 24hours if chilled.
3. Multiple Tube Fermentation Technique

Multiple Tube Fermentation Technique



Presumptive Test

1

- **Prepare and sterilize 3 Ds Mac broth(10ml) with Durham tubes, 6 Ss Mac broth (5ml) with Durham tubes**

2

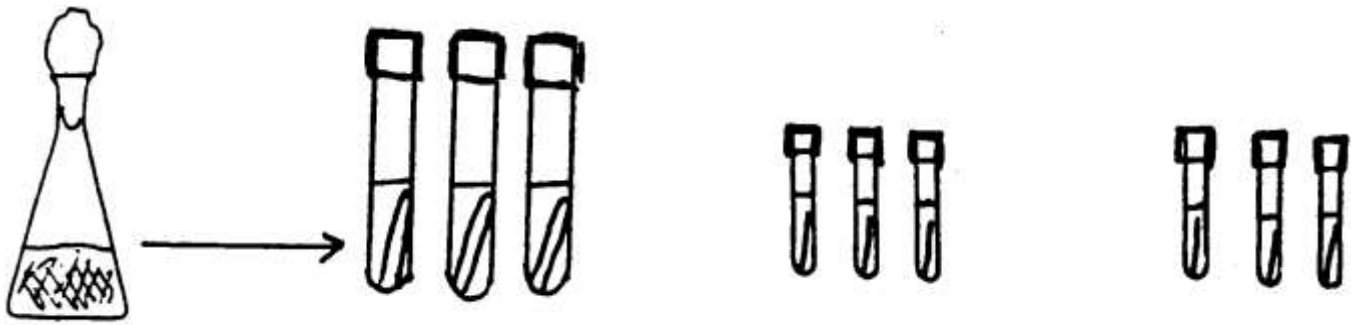
- **Inoculation: first 3 tubes with 10ml of the original sample, next 3 with 1ml of the of the original sample, next 3with 0.1ml of the original sample.**

3

- **Incubation for 35-37 ˚C for 48 hours. Observe for gas production, turbidity, and change in color to yellow. If positive proceed to Confirmatory Test.**

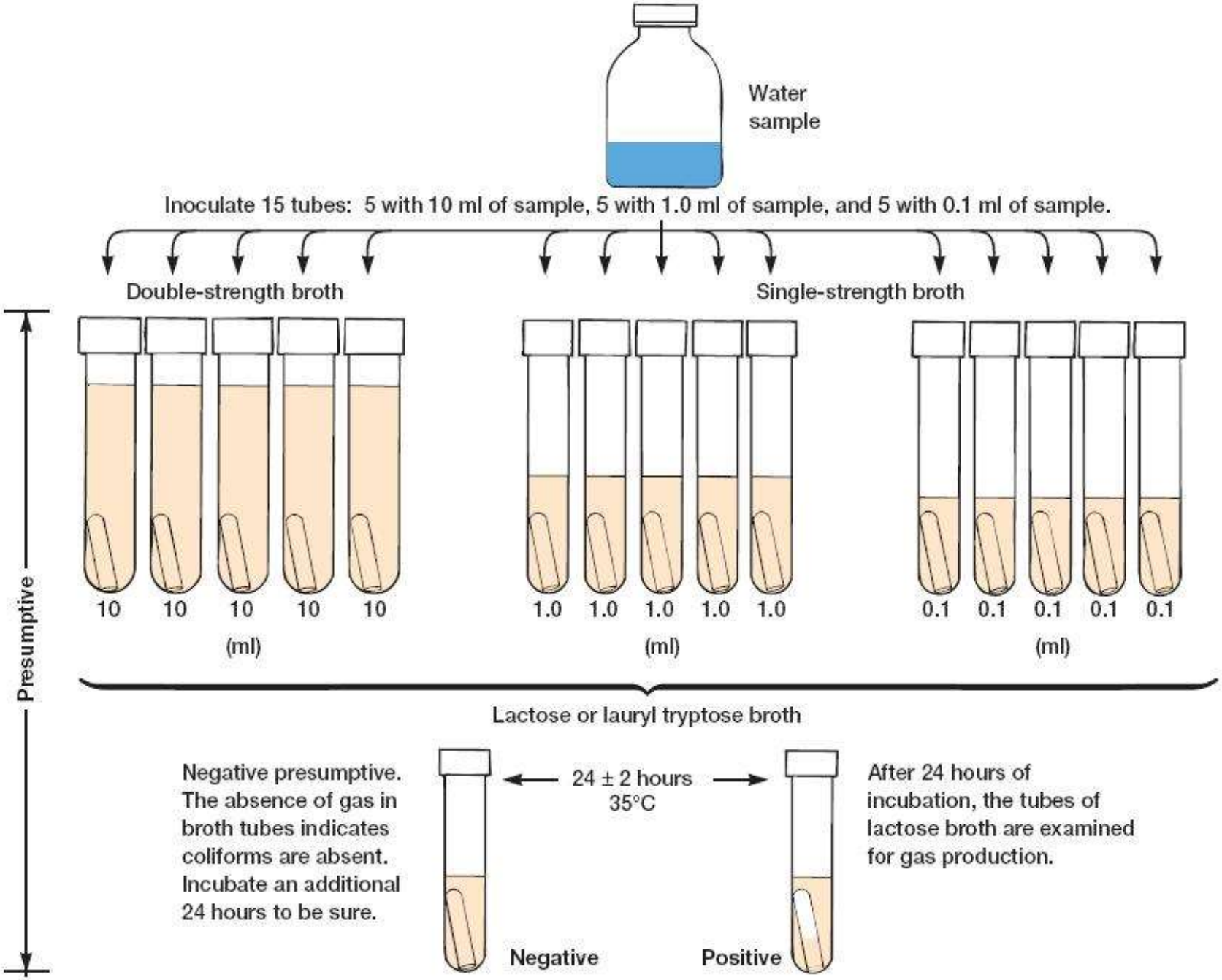
Bacteriological analysis of water:
Most Probable Number (MPN) technique

coliform: acid and gas from lactose <24 hours/37^{0}C
indicator organism: *E. coli*



Water sample	double strength	single strength	single strength
	10 ml sample	1.0 ml sample	0.1 ml sample

*Normally 5 Durham tubes are inoculated but this exercise is modified to three tubes in the interest of economy.


Water sample
Inoculate 15 tubes: 5 with 10 ml of sample, 5 with 1.0 ml of sample, and 5 with 0.1 ml of sample.
Double-strength broth
Single-strength broth
Presumptive
10 10 10 10 10
(ml)
1.0 1.0 1.0 1.0 1.0
(ml)
0.1 0.1 0.1 0.1 0.1
(ml)
Lactose or lauryl tryptose broth
Negative presumptive. The absence of gas in broth tubes indicates coliforms are absent. Incubate an additional 24 hours to be sure.
24 ± 2 hours
35°C
After 24 hours of incubation, the tubes of lactose broth are examined for gas production.
Negative
Positive

Presumptive test

Confirmatory Test

1

- **BGLB tubes (10ml) with Durham tubes were prepared and sterilized.**
- **Loopful of suspension from the positive presumptive tubes was inoculated and incubated at 35°C for 48hours.**

2

- **EMB plate is inoculated and incubated at 35C**
- **Loopful of suspension was inoculated and incubated at 44°C for 24 hours.**
- **Sometimes an IMViC reaction is inoculated.**

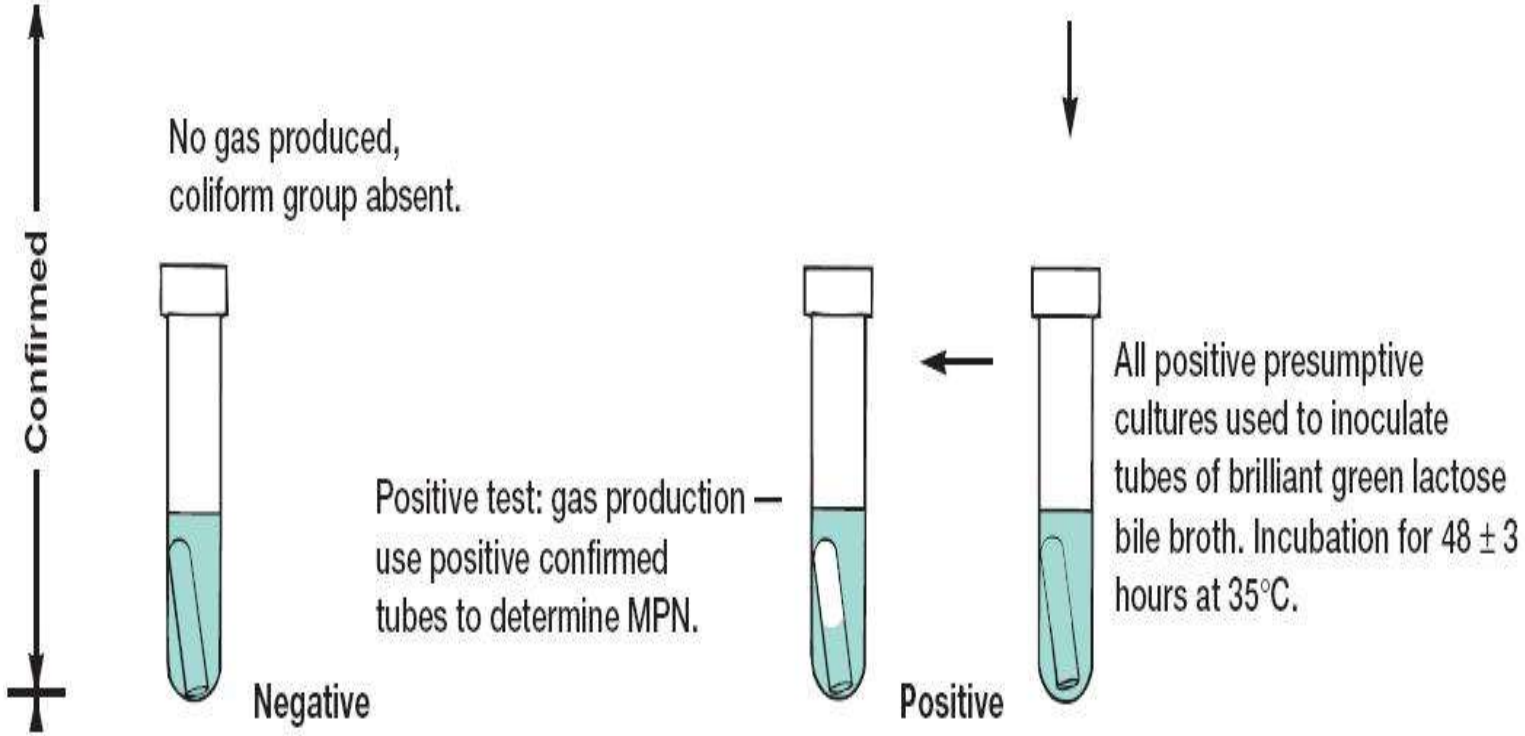
3

- **BGBL : Turbidity and gas production is observed.**
- **EMB: Green metallic sheen growth is observed on the plate.**
- **IMViC reaction : ++--**
- **Those positive for the confirmatory test were subjected to the completed test.**

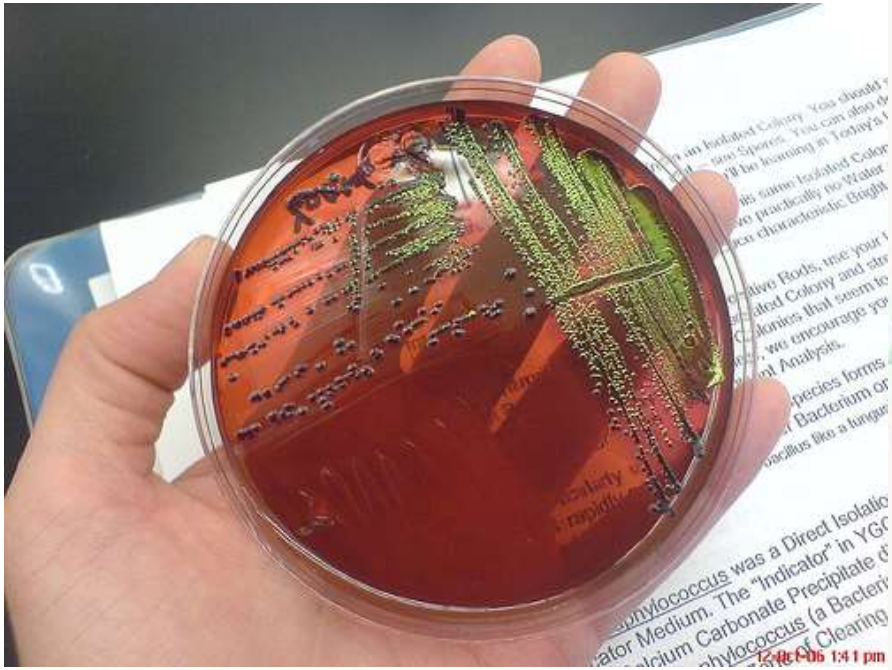
BGLB components

- Peptone: a source of nitrogen, vitamins and minerals.
- Lactose: fermentable carbon source
- Oxgall (bile) and brilliant green: inhibitor of gram-positive bacteria and most gram-negative bacteria except coliforms
- Basic fuchsin and erioglaucine: pH indicators
- Monopotassium phosphate: buffering agent.

BGBL medium

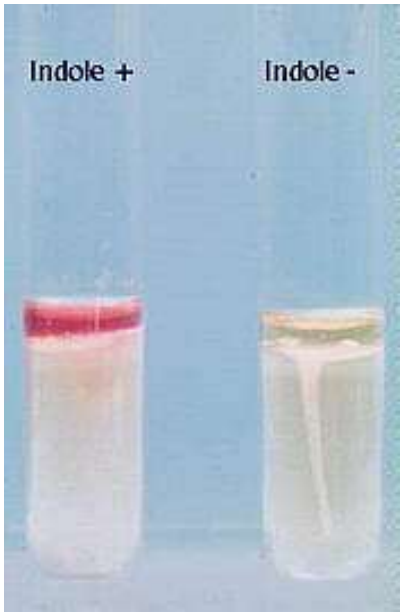

Confirmed
No gas produced,
coliform group absent.
Negative
Positive test: gas production –
use positive confirmed
tubes to determine MPN.
Positive
All positive presumptive
cultures used to inoculate
tubes of brilliant green lactose
bile broth. Incubation for 48 ± 3
hours at 35°C.

Confirmatory Test

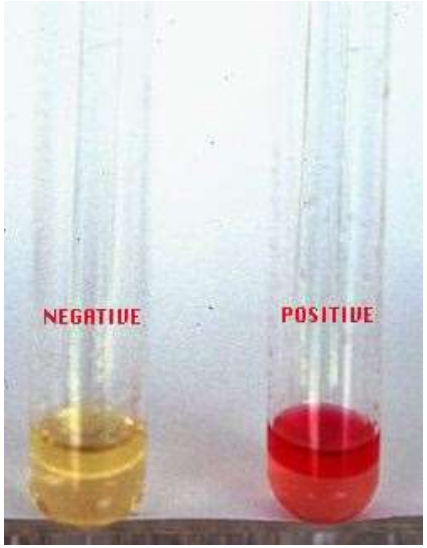


E. coli vs E. aerogenes

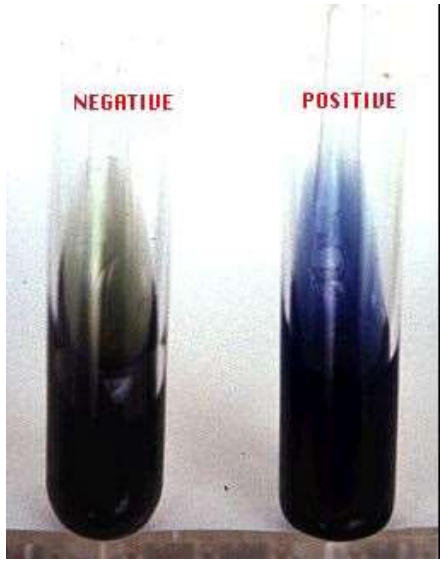
IMViC Test



Indole



MR- VP



Citrate

MPN Table

10mL tubes Positive	1-mL tubes positive	0.1 mL tubes positive	MPN/100 mL
0	0	0	0
0	0	1	2
0	0	2	4
0	1	0	2
0	1	1	4
0	1	2	6
0	2	0	4
0	2	1	6
0	3	0	6
1	0	0	2
1	0	1	4
1	0	2	6
1	0	3	8
1	1	0	4
1	1	1	6

Completed Test

1

- **Representative colonies were chosen,**
- **inoculated onto Lactose broth , and incubated for 35°C for 24hours. (Look for gas production)**

2

- **Perform gram-staining**

3

- **Culture on NA slant**

Reading results

- Presumptive test:
- Any + ve tube is given a value of 1
- Any –ve tube is given a value of 0
- Add result of 3 tubes of 10ml Ds Mac broth ,then 3 tubes of 1ml Ss Mac broth, then 3 tubes of 0.1 ml Ss Mac broth
- Get value of 3 Numbers e.g. 1 0 3 look it in MPN table ,the index is 8 coliforms /100ml of water.
- If confirmed ,then it should be reported.

Directions for collection

- Bottle is pre-sterilized. Do not open or remove cap or touch inside of bottle.
- Do not rinse bottle .It contains sodium thiosulfate to neutralize the bactericidal effect of chlorine.
- When collecting tap or well water ,allow water to run ,or pumped out for several min.
- This is to provide a representative sample from source

Directions for collection

- Lake , river etc. select a good place to obtain sample , and extend bottle away from body.
- Best volume is 100ml , 50 ml is OK.
- The sample should be tested as soon as possible .If not possible it should be refrigerated until testing.

Table 1.Table of Most Probable Numbers (MPN) Per 100 ML of Sample using Three Tubes of Each Dilution

Number of positive tubes in dilutions			MPN per	Number of positive tubes in dilutions			MPN per
10 ml	1 ml	0.1 ml	100 ml	10 ml	1 ml	0.1 ml	100 ml
0	0	0		2	0	0	9.1
0	1	0	3	2	0	1	14
0	0	2	6	2	0	2	20
0	0	3	9	2	0	3	26
0	1	0	3	2	1	0	15
0	1	1	6.1	2	1	1	20
0	1	2	9.2	2	1	2	27
0	1	3	12	2	1	3	34
0	2	0	6.2	2	2	0	21
0	2	1	9.3	2	2	1	28
0	2	2	12	2	2	2	35
0	2	3	16	2	2	3	42
0	3	0	9.4	2	3	0	29
0	3	1	13	2	3	1	36
0	3	2	16	2	3	2	44
0	3	3	19	2	3	3	53
1	0	0	3.6	3	0	0	23
1	0	1	7.2	3	0	1	39
1	0	2	11	3	0	2	64
1	0	3	15	3	0	3	95
1	1	0	7.3	3	1	0	43
1	1	1	11	3	1	1	75
1	1	2	15	3	1	2	120
1	1	3	19	3	1	3	160
1	2	0	11	3	2	0	93
1	2	1	15	3	2	1	150
1	2	2	20	3	2	2	210
1	2	3	24	3	2	3	290
1	3	0	16	3	3	0	240
1	3	1	20	3	3	1	460
1	3	2	24	3	3	2	1100
1	3	3	29				

Typical Water Quality Standards

- Water standards when coliforms are used as pollution indicator
- Drinking Water and swimming pool water
  - No coliforms contamination acceptable less than 3 coliforms /100ml of sample
- Recreational water
  - 100- 200 fecal coliforms /100 ml
- Fish and wildlife habitat
  - 5000 fecal coliforms/100 ml

False +ve and –ve

- **False positive:**
- *Enterobacter aerogenes - a soil* organism; use the IMViC test for Distinction

- **False negative:**
- *A case of Salmonella typhi in* Riverside in 1965 in USA

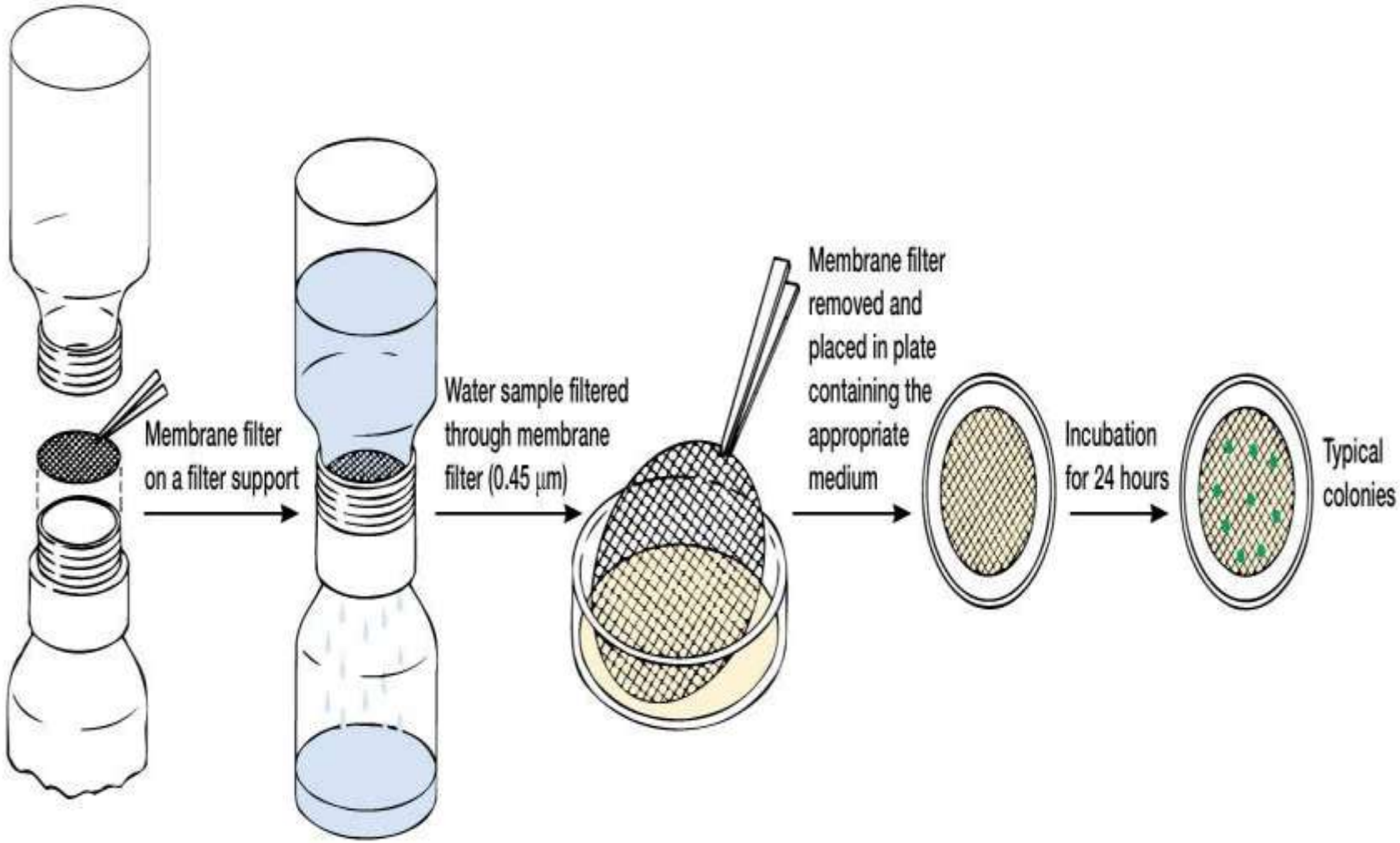
Water Microbiology II

Membrane filtration method

Membrane Filter Methods

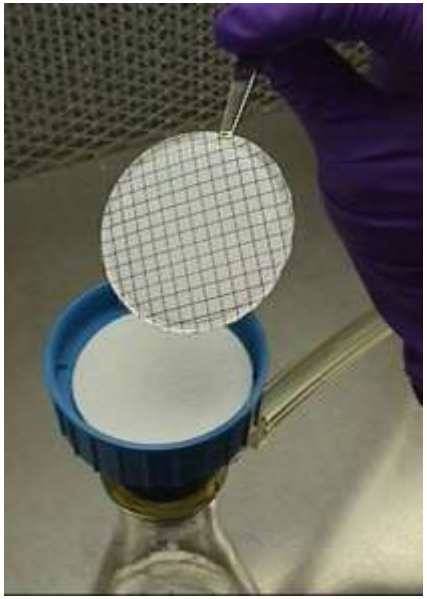
- Filter water through a 0.45 or 047 µm membrane filter(Bacterial cells can not pass through)
- Place membrane on selective media(EMB)
- Incubate
  - 35 C total Coliform
  - 44.5 C fecal Coliform
- Count colonies

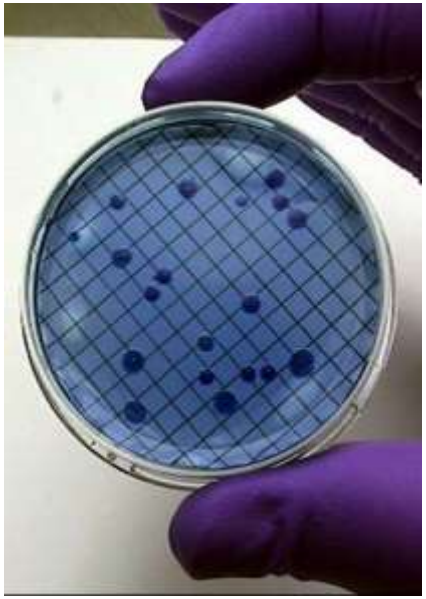
MF method


Membrane filter
on a filter support
Water sample filtered
through membrane
filter (0.45 μm)
Membrane filter
removed and
placed in plate
containing the
appropriate
medium
Incubation
for 24 hours
Typical
colonies

MF method

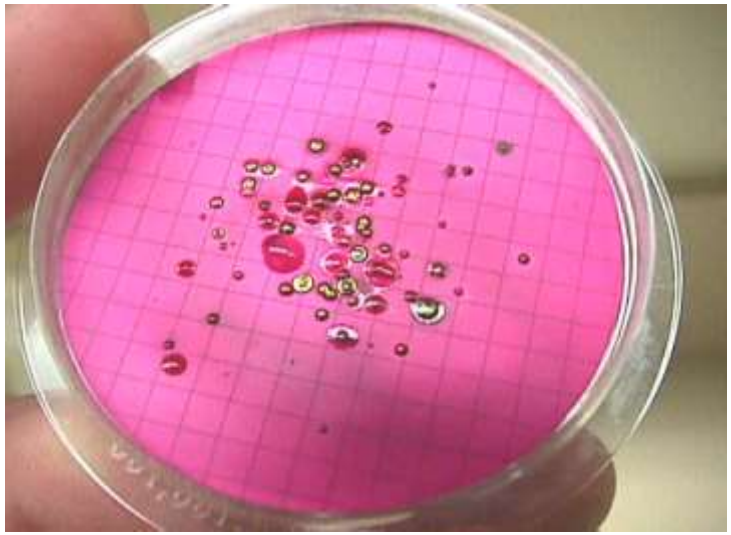
Filter water through a 0.45 µm membrane filter
Place membrane on selective media
Incubate
    35 C total Coliform
    44.5 C fecal Coliform
Count colonies

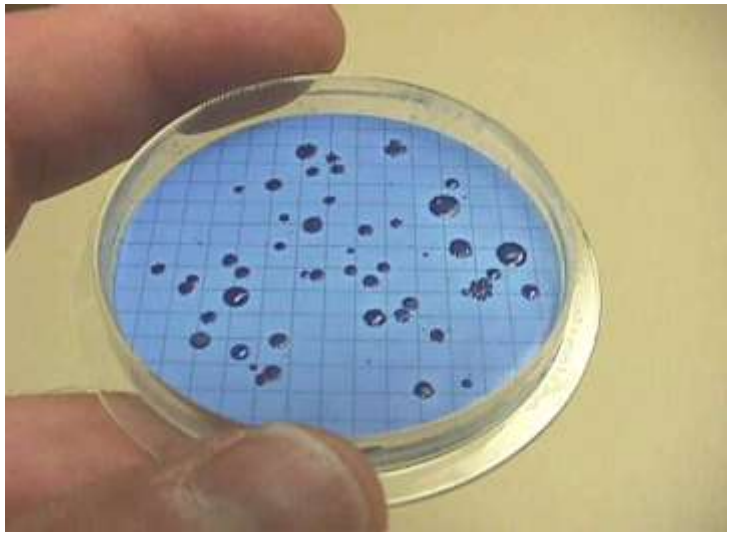




Membrane filtration method

Results:





Coliform bacteria produce colonies with a characteristic **"metallic green sheen"**

RESULTS

- **Quality limit**: less than 1 Coliform /100ml of water , the water is potable.
- **Action limit**: 4 coliforms/100ml of water, it means that the water company must take immediate action to remedy the problems that are responsible for the presence of coliforms in water.

Membrane filtration method Advantages

- More than 100ml samples can be tested
- Effective and acceptable technique. Used to monitor drinking water in government laboratories.
- Rapid
- Lower chance of contamination esp. on low scale
- More accurate

MPN Advantages

- It is relatively simple and cheap.
- It is the method of choice for determining fecal coliforms densities and timing of contamination
- ideal for wastewater samples and non-potable samples, because the analyst can accommodate highly turbid samples by diluting prior to analysis

Disadvantages

MPN test

- **labor intensive ,Large amount of glassware is required**
- **Its lack of precision, large errors**
- **still requires survival and culture of organisms in lab**

M F method

- **Not suitable for turbid or waste waters.**