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Practical (Soil and Aquatic Microbiology) 2020-2021

المرحلة الرابعة ـ الدراستين الصباحية والمسائية
الفصل الدراسي الثاني

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Soil microbiology lab (1):

Soil microbiology

Soil is the material found on the surface of the earth that is composed of organic and inorganic material. Soil varies due to its structure and composition. Inorganic materials, or those materials that are not living, include weathered rocks and minerals. As rocks are broken down, they mix with organic materials, which are those materials that originate from living organisms. For example, plants and animals die and decompose, releasing nutrients back into the soil.

Types of Soil:

- there are **three** basic types of soil: sand, silt and clay. But, most soils are composed of a combination of the different types. How they mix will determine the texture of the soil, or, in other words, how the soil looks and feels.

Water content:

The water in the soil play a significant role, as it influences the metabolic activities of micro biota, soil water divided into two types:

1- **Free water (Gravitational water)** this water not bounded by colloidal material, which is affected by gravitation, so tend to drain quickly?

2- Bound water it's a thin water film on soil particles surface, because it's bounded by colloidal material, this type of water not affected by gravitation, so tend to drain slowly?

Water activity (**a**w)**:**

It's useful to express qualitatively the degree of water viability, microbiologist generally use **a**w, which is less amount of water required for microbial activities and growth, the water activity of a solution is 1/100 the humidity of a solution (expressed as a percentage).

Water activity of a solution or solid can be determined by different methods, the most current assay method is:

Drying method

Procedure:

1. Weight 10gm of soil in previously weighted clean Petri dish.
2. Dehydrate soil in hot air oven at 105° C for 3-4 hrs.
3. Reweight dried soil sample, and determine (aw) of sample according to the following.

Water content of soil sample = weight of moist soil - weight of dry soil

Weight of **dry** soil sample =l 0 - water content

$$\text{Weight of soil sample for } \mathbf{1\ gm\ dry\ soil} = \frac{\text{weight of dry soil}}{\text{weight of moist soil}}$$

Enumeration of soil Microorganisms

Soil microorganisms can be classified as: Bacteria, Actinomycetes, fungi, Algae and protozoa. Each of these groups has characteristics that define them and their functions in soil. Fertile soil contains a wide variety of M.O. but there is no particular isolation lab. Procedure can be giving a quiet and accurate microbial numbers in a soil sample?

Enumeration methods:

1. Direct slide count (Breed Method)

This method considered the quickest and simplest technique, although there are some disadvantages like:

- Estimate number of live and dead cells in sample.
- Bacteria are dominant in soil so it's difficult to distinguish other microbial cells that are present, such as microbial spores.
- can't distinguish microbes from soil particles.

Procedure:

1. Mark square (1 cm diameters) on clean glass slide.
2. Suspend 1 gm of soil sample in 9 ml of sterile diluted water, mix properly.
3. Spread loop full of soil dilution (0.01) in the square.
4. Stain with simple stain.
5. Count visible microbial cells in 10 microscopic fields and determine average no. of bacteria in soil sample from equation:

*No. of cells = **average** of cells in 10 fields x5000x100 x 10*

- To find the No. of cells in dried wt. of soil sample?

$$\textit{No. of cells in 1 gm of } \textbf{\textit{dry}} \textit{ soil sample} = \frac{\textit{No. of bacterial cells}}{\textbf{dry } \textit{wt of soil sample}}$$

Constant factors:
5000 = no. of fields in 1 cm^2
100 = (0.01) conversion of loop full volume to 1 ml.
10 = reciprocal of dilution

2 - **Dilution plate count (Viable Plate Count)**

This technique is widely used for determining approximate viable no. of soil bacteria, and it's applicable to any M.O. that will grow as colonies, but this method have some disadvantages**:**

- It ignores sampling error due to unequal distribution of the cells, especially in the high dilutions.
- There is no ideal culture medium for cultivation of most soil bacteria.
- Antagonistic activities of soil bacteria, such as production of antibiotics and some enzymes that inhibit the growth of another M.O.

No of cell / gm soil = No. of viable colonies x dilution factor x ?

$$\textbf{No of cell / gm of dried soil} = \frac{\textbf{No. of cell}}{\text{Wt. of 1gm dry soil}}$$

Lab (2):

Isolation of soil microorganisms

The most numerous microbes in soil are the bacteria followed by actinomycetes, the fungi, soil algae and soil protozoa but in our study we are going to concentrate on bacteria ,actinomycetes and fungi .

Isolation of Bacteria

We are going to isolate bacteria from soil by using Dilution plate count technique (Pouring plate count).

First, we have to add 1 gm of soil to 9 ml D.W to get 10^{-1} dilution, mix properly, farther dilutions are depend on the soil type as the following:

- domestic soil 8 dilutions
- Vegetable soil 6 dilutions
- citrus soil 5 dilutions
- Palm soil 4 dilutions
- Uncultured soil 2 dilutions

- Then transfer 0.1 ml from each 3 last dilutions to sterile Petri dishes, after that pour melted cold soil extract agar (pH 7) into dishes and thoroughly mix with still-fluid agar by a gentle horizontal rotation of dish.

- Finally incubate plates at 30 °C for 24 hr.

Note: count plates which show only about 30-300 colonies.

No of cell / gm soil = No. of viable colonies x dilution factor x ?

To find the No. of bacterial cells in dried wt. of soil sample

$$\text{No of cell / gm of dried soil} = \frac{\textbf{No. of cell}}{\text{Wt. of 1gm dry soil}}$$

Note: **For isolation and enumeration of spore forming soil bacteria (heat the last sample dilution at 80 ° C water bath for about 15 min), then transfer 0.1 ml to Petri dish and continue the same procedure as above.**

Lab (3): Isolation of Soil Fungi

Fungi typically divided into:

1- Molds: which are composed of branching filaments termed hyphae, that grow by apical extension to form mycelium. They are helpful but could also be harmful to plant and other soil microorganism.

2- Yeasts: which are unicellular and oval or round in shape and reproduce mainly a sexually by budding.

Isolation Procedure

1- Add 1 gm of soil sample to 9 ml D.W., and then make 10-fold serial dilution by D.W.

2- Transfer 1 ml from the last dilution to Petri-dish, then pour melted Malt extract agar medium (pH 5.5) or Rosebengal agar medium (pH 6) and thoroughly mix.

3- Incubate plates at 28 C^0 for week.

4- Examine formed colonies and recognize their color and morphology.

Slide preparation for mold

Add one drop of lactophenol in the middle of the slide , take apportion of colony , put cover slip, gently press the cover edge by loop and examine microscopically under high power.

- **Slide preparation for yeast**
- Simple stain.

3. Isolation of Actinomyces

Actinomycetes are a group of gram- positive bacteria which form branched filamentous hyphae like fungal hyphae but their hyphal diameter is less than fungal hyphae. Actinomycetes give soil its characteristic smell due to their production of volatile substances such as geosmin, Actinomyces prefer slightly alkaline environments.

Actinomycetes colonies are powdery mass form often pigmented with orange or red or yellow color.

Isolation procedure

1- Add1 gm of soil sample to 9 ml of D.W., and then makes serial 10-fold dilution with D.W.

2- Spread 1 ml of last dilution on Jensen's agar plates, pH8.5-9 (Actinomycetes selective medium), incubate at 28^0C for 3-7 days.

3- prepare slide using gram procedure, examine under high power, and observe Actinomycetes thin curly mycelia net.

Lab (4):

Role of Soil Microbes in Elements Recycling

Human & other organisms cells chemical composition can be described as a complex blend of organic compounds consist of different elements such as C , N , O and H in variety combinations , these elements which are essential component of all organism protoplasm undergo cyclical alteration between inorganic state free in nature and a combined organic state in living organisms .

Microorganisms play a major role in degradation (decomposition) of organic matter, that contribute in elements recycling in which maintain their balance & keep life on the earth from ceasing.

Carbon Cycle

The element carbon is the present in all living organisms. It's recycled through various processes, Transformation of carbon occurs constantly and ubiquitously, carbon is introduced into organic system from its most oxidized state Co_2 & it reduced primarily by photosynthesis and become part of photosynthetic organism's components as organic carbon in that temporarily. Immobilized until decomposition of cells by M.O.

Role of soil Micro Organisms in degradation of carbohydrates 1-

Degradation of cellulose

Cellulose….. Is a complex carbohydrate, which is part of plant structure polysaccharides, soil contain a rich deposit of M.O which produce extracellular cellulolase that break down cellulose into two to three glucose units called cellobiose and cellotriose respectively. These smaller compounds are readily degraded and assimilated as glucose.

Bacteria	*Cytophaga*	*Cellulomonas*	
Fungi	*Aspergillus*	*Penicilluim*	*Trichoderma*
Actionomycetes	*Nocardia*		

Isolation of Soil Cellulytic Microorganism

Procedure:

1- Suspend 1 gm of soil sample in 9 ml of **special isolation broth** (the medium contains all required nutrients except carbon source?)

2- Transfer 1 ml of soil suspension to other tube contain the same isolation medium + a **strip of filter paper** (as carbon source). Incubated tubes at 28^0C for 5 – 7days.

4- +ve result for cellulytic m.o yellow spots on filter paper strips (The cellulytic bacterial colonies colored by yellow to orange color due to formation of carotenoid or flexiratin pigments).

5- - ve result no color on the strip.

6- Prepare smear of detected spots on glass slide, stain with gram stain & examine under oil immersion.

2- Degradation of Pectin

Pectin….. is a homopolymer of D-galactronic acid found in middle lamella of plant cell wall & in blue green algae capsules .(in plant pectin solidify & support plant cells by their combination with calcium carbonate) .

Microbial pectinolytic enzymes (pectinase) are responsible for the lyses of pectin, which enable M.O to invade tissues of living plants causing soft rot as well as wet or dry necrosis & galls in economically important crops such as potatoes, carrot and cucumber.

Bacteria	*Erwinia*
Fungi	*Fusarium*

Isolation of soil Pectinolytic Microorganism

Procedure

1- Suspend 1 gm of soil sample in 9 ml of **special isolation broth** medium. 2- Transfer 1 ml of soil suspension to other tube contain the same isolation medium with **piece of potato** (as source of carbon), incubate at 28 ^{0}C for 5 – 7 days.

3- + ve result color change and soften of potato.

4- Prepare slide, stain with gram stain and examine under oil immersion.

3- Degradation of starch

Starch….. Consist of long chain of ∝ - glucosyl residues, Starch break down by amylases, which produced by a variety of living organisms, ranging from bacteria to plants & human. Bacteria & fungi secrete amylase to outside of their cells to carry out extracellular digestion, where they have broken down the soluble and products such as glucose and maltose are absorbed into their cells.

Amylases are classified based on how they break down starch molecules.

α- **Amylase (alpha–amylase)** : reduce the viscosity of starch by breaks the glucose glucose bonds down by removing two glucose units at a time therefore producing of maltose.

β- Amylase (Beta-amylase) : breaking down the bonds at random way. therefore producing varies

size of glucose chains.

Amyloglucosides (AMG) breaks successive bonds from the non-reducing ends of the straight chain therefore producing of glucose.

Amylase producer Microorganism

Although many M.O produce these enzymes, but the most common producers

Bacteria	*Bacillus* spp	*Clostridim* spp	*Micrococcus*
Fungi	*Aspergillus*	*Fusarium*	*Rhizopus*

Isolation of soil amylase producers Microorganisms

Procedure

1- Suspend 1 gm of soil sample in 9 ml of **special isolation broth** medium for isolation of starch hydrolysis M.O.
2- Incubate tubes at 28 C^0 for a week.
3- Transfer adequate volume of the medium to a test tube & add drops of gram's iodine solution.
4- + ve result yellow color confirm positive result, due to microbial hydrolysis of starch,

- ve result blue color confirm negative result .

Another detection procedure

For isolation of **amylolytic fungi…..** Spread 1 ml of soil suspension on potato dextrose agar plates PDA (with 0.1 mg/ml streptomycin sulfate), incubate at R.T for about 3-7 days.
For isolation of **soil amylolytic bacteria…..** Spread 1 ml of soil suspension on nutrient agar plate (1 % w/v soluble starch) at 30^0 C for 24 hr.
Starch hydrolyzing colonies will have an area of clearing around them, for confirming, flood plates with gram's iodine (area around the colony appear yellow, while area still contain starch appear ink – black color.

Lab (5): Nitrogen cycle

Is the biochemical cycle which nitrogen is converted into multiple chemical forms, consecutively passing from the atmosphere to soil to organism and back into the atmosphere?

The main stages of nitrogen cycle are:

1. Ammonification

2. Nitrification

3. Denitrification or nitrate reduction

4. Nitrogen fixation

1. Ammonification

It is a series of enzyme reactions resulting in release of ammonia (NH3) from complex organic nitrogenous compounds such as proteins and nucleic acids , usually occurs under aerobic condition.

Numerous microorganisms can release ammonia from organic compounds in the soil such as:

Proteus and *Micrococcus*

Isolaion and detection of ammonification M.O.

- Suspend 1 gm of the soil sample in 9 ml of Sodium casein broth medium (as source of protein) then incubate tubes at 28 Co for a week.
- Mix 1 ml microorganisms' suspension with few drops of Nessler reagent in clean test tube.

Positive result

Golden -orange deposit demonstrates releasing of ammonia

2. Nitrification

Oxidation of ammonia (produced from the degradation of organic compounds in ammonification) to nitrate by a specialized group of strictly aerobic chemolithotrophes.

Oxidation of ammonia occurs in two steps:

In the first step: ammonia is oxidized to nitrite (NO_2^-) (nitrosofication)

The most involved microorganisms in this process are: *Nitrosomonas* and *Nitrosococcus*

Isolation & detection of Nitrosofication M.O

1. Suspend 1 gm of the soil sample in 9 ml of Allen I broth medium (Contains $(NH_4)_2 SO_4$ as ammonia source), incubate tubes at 28 C^o for a week.

2. Mix 1ml microbial suspension with an equal volume of reagent A and reagent B let them react for a few seconds.

Positive result: Formation of a red colored deposit indicates releasing of NO_2 as a result of the nitrification process.

2. In the second step is oxidized NO_2 to nitrate (NO_3),the *Nitrobacter* mainly carry out the second step.

Isolation and detection Nitrite Oxidizing microorganisms

Follow procedure as in previous but use Allen II broth as a medium contains $NaNO_2$ as nitrite source. For detection of released NO_3, mix 1 ml of microorganism's suspension with drops of nitrate reagent which consists of Diphenylamine (DPA) and sulfuric acid (H_2SO_4) .

Positive result

Formation of blue deposit indicates releasing of No_3.

Note: If deposit not observed, that does not mean absence of nitrifying microorganisms in taken soil sample rather it mean ammonia oxidation continued.

3. Denitrification or nitrate reduction

Reduce nitrate to nitrite, ammonia and return to the atmosphere as nitrogen gas (N2↑), under anaerobic conditions.

The reaction mediated by nitrate reductase enzyme that used nitrate as an electron acceptor in anaerobic respiration.

This process is performed by bacterial species such as *Pseudomonas* and *Clostridium* .

Denitrification detection procedure

Suspend 1 gm of the soil sample in Allen l8 broth medium (the medium contain KNO_3 as source of NO_3) in test tube. Fill tubes completely with medium to create anaerobic condition incubate at 28 C^o for a week.

Result

Read the result by the following procedures:

- NO_3 detection reagent: formation of blue deposit detects absence of denitrifying microorganisms.
- NO_2 detection reagent: formation of red colored deposit detects reduction of NO_3 to NO_2.
- Nessler reagent: formation of golden –orange deposit detects complete reduction of NO_3 to ammonia.

Lab (6): Nitrogen fixation

Is a process by which nitrogen gas is converted into ammonia or related nitrogenous compounds in soil by soil microorganisms.
Nitrogenase is the most important enzyme involved in nitrogen fixation.

Two kinds of nitrogen-fixing microorganisms are recognized:

- Non symbiotic N_2 fixer: Those are capable of converting N_2 to cellular nitrogen independently of other living organism's so-called free-living nitrogen-fixing bacteria.
- The main involved M.O. *Azotobacter* : large gram-negative motile rods that may be ovoid or coccoidal in shape (pleomorphic), aerobic and cells form cysts as the culture ages.

(Macroscopically)
Azotobacter has grown on solid agar media as large convex, mucoid colonies with white, brownish color, colorless and any other color depend on bacterial species, as soon as culture aged brownish colonies pigmented with dark chocolate color.

Isolation of *Azotobacter*

1. Suspend 1 gm of the soil sample in 9 ml of D.W.

2-Spread 1 ml of soil suspension on Ashby's agar medium pH 7.6, (selective *Azotobacter* medium), includes sodium molybdenum, incubate the plates at $28C^0$ for a week.
Azotobacter characterized as mucoid and brownish large colonies.

Symbiotic N2 fixers
Symbiotic N_2 fixers microorganisms live in the roots of legume family plants and other plants . the fixation process results from a mutualistic association between legumes plants and bacteria.
main symbiotic N2 fixer bacteria is *Rhizobium.*
Which are G-ve rods to pleomorphic, motile with variably placed flagella.
Rhizobium invades susceptible plant roots and from visible nodules which live and fix N2 directly from the air.

Isolation of *Rhizobium* from soil
1. Cut roots of any legumes, wash with tap water.
2. Select and cut visible large pink nodule , transfer to clean glass slid, crush it carefully by pressing it by forceps , until seeing a milky suspension then mix with water drop , stain with gram stain , examine under oil immersion *Rhizobium* appears look like Latin letters Y X Z T , also bacterial can be detected as G-ve bands.

Aquatic Microbiology Lab (7):

Water is the elixir of life. It is an essential part of protoplasm and creates a state for metabolic activities to occur smoothly. Therefore, there is no life can exist without water. Also there are thousands of m.o. which live in water so can do diseases.

Water receives m.o. from air, soil, sewage, organic wastes, dead plants and animals…etc.

Water Microorganisms ….. large number of m.o. both saprophytes and pathogens are found in water (bacteria, fungi, algae, protozoa, nematodes several human and animal viruses) are transferred by water.

The natural water such as: lakes, streams, rivers, contain several amount of nutrients that support the growth of m.o.

There are different ways by which m.o. enters water supply, for example broken sewage lines, congested centers, inappropriate waste treatment, lack of awareness among people , Unhygienic environments at water public places, People suffering from diseases also discharge pathogenic microbes in water through their excrete, for ex. amoeba dysentery, typhoid fever, bacillary dysentery and poliomyelitis …etc.

Water Sanitary Tests

Feacal Bacterial Indicators

The water potability (suitability for drinking) is considered as one of the basis in microbiological examination of water.

1- fecal coliforms

Intestinal bacteria in water generally can't survive in aquatic environment due to physiological stress but if they can enter human in the meanwhile, they can cause serious disease.

The characteristics groups of intestinal bacteria are the **coliforms**, (facultative anaerobes, G-ve, non-spore former, rod , ferment lactose with gas formation within 48hr at 30ºC).

The coliform groups are present in water due to fecal contamination i.e. discharge of feces by human and animal in water. coliforms are members of the family Enterobacteriaceae which includes: *E.coli*, *Enterobacter*, *salmonella* and *klebsiella pneumonia***.**

These bacteria make up about 10% of intestinal m.o. of human and animal therefore was used as **bacterial indicator** of fecal water pollution.

If such bacteria cannot detected in water /**100** ml, the water can consider as **potable water.**

Sanitary Tests for coliforms

The standard multiply tube fermentation technique (most probable number **M.P.N**.) are used for coliform detection.
(This method involves 3 routine standards tests):

1) The presumptive test.
2) The confirmed test.
3) The complete test.

1. Presumptive test

A serial dilution of fermentation tubes (macConkey broth),are inoculated with water sample. These tubes are incubated for 24 - 48hr at 37ºC.

Procedure

1. Make serial dilution (10^{-x}) of water samples as the following:

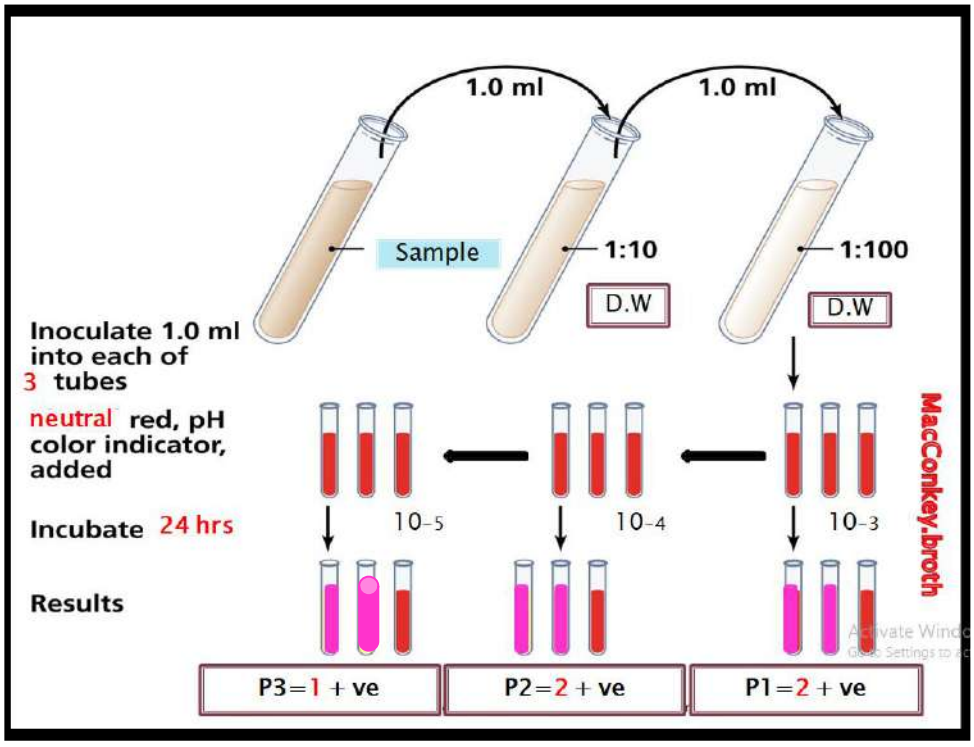
Sewage water	**9** dilutions
River water	**8** dilutions
Stagnant water	**6** dilutions
Brooklet water	**5** dilutions
Tank water	**4** dilutions
Drinking water	**3** dilutions

2. Suspend 1 **ml** of water sample in 9 **ml** D.W.(**10^{-1}**),then transfer 1 ml of first dilution to another tube to prepare the second dilution(**10^{-2}**).

3. Prepare the last 3 dilutions for each water sample in **macConkey broth** tubes rather than D.W.

ex…. if the water sample require 5 dilutions (Brooklet water), the 1st and the 2nd dilutions use **D.W.** , while the 3 last dilutions use macConkey broth (suspending 1 ml of 2nd dilution in 9 ml macConkey broth (**10^{-3}**) and the other two remaining dilutions prepare in same way(**10^{-4}** and **10^{-5}**).

4. Incubate MacConkey tubes for 24hr at 37ºC.
5. read the result as the following:
(+ve)….. coliform change the color of macConkey broth from **red** to **pink** due to lactose fermentation (as macConkey broth contain neutral red indicator. change to **pink** in acidic environment).



6. Enumerate **+ve** test tubes for each P1,P2 and P3 .then find the MPN value of coliform by using **MPN standard table** .

No. of cells / ml =M.P.N.value × reverse of mid dilution×?

The number of coliform present is not absolute, but is a statistical estimate.

2. Confirmed test

The positive tubes in Presumptive test does not mean that fecal coliform are detected? other coliforms also can give false positive presumptive test because they also can ferment lactose. Therefore a confirmed test is necessary to identify the presence of fecal coliform.

Procedure

Transfer 1ml of any +ve tube of presumptive test to another **macConkey broth** tubes (contain Durham tubes) incubate for 24hr **at 44.5ºC**.

+ve result…… for **fecal coliform**: color change to_____+ gas in durham tube.

3. Complete test

The complete test is use to be ascertain about the presence of fecal coliform in water.

Procedure

1. Streak a loop full from the +ve tubes on macConkey agar or Eosin Methylene Blue agar (EMB).
2. Incubate plates at 37ºC for 48hr.
3. Macroscopically ……..coliform colonies on macConky agar are (pink with dark center and opaque) while on EMB agar (colonies nucleated with metallic sheen).

4. Make a slide from colony on glass slide with Gram stain.

Aquatic microbiology lab (8):

Feacal Bacterial Indicators

There are other groups of bacteria invariably present in human and animal feces:
2- Clostridium especially ***Clostridium perfringens***
3- Fecal streptococci, especially ***Streptococcus faecalis***
4- Certain species of anaerobic bacteria***, Bifidobacterium bifidus.***

These species are easily isolated from water by the use of relatively simple methods of selective cultivation and are readily identified. The first two species are used frequently as indices of fecal pollution in both water and foods.

2-*Clostridium*

Clostridia… are obligate anaerobic, gram-positive, endospore-bearing , large rods , with rounded ends. The majority of Clostridia are harmless and helpful saprophytes . some of them live in intestinal tract of human and animal, other cause serious diseases of human and animals.

The advantage of testing the presence f Clostridia especially ***Clostridium perfringens*** in water samples, is suggesting the **type of pollution**: an **old fecal** pollution, because the survival time of pollution indicator organisms in water (***Clostridium perfringens,*** resistant spores can survive in water).

The presence of this bacteria in the absence of the others, particularly ***E. coli,*** suggests pollution that may had presence for a considerable **long time (old pollution)**, but if both (Clostridia & *E. coli*) founded in water suggest relatively a few hours or days **(recent pollution)** .

(1) Presumptive test

1. Full a glass tube of tested water sample without any serial dilution (heat the tube in water bath for 10 min at 80 °C ?).
2. Transfer 1ml of heated water sample to tubes with selective medium **differential reinforced Clostridia medium (DRCM)** supplemented with sodium sulfate and ferrous citrate in equal volumes to great reducing state , incubate tubes an aerobically (anaerobic gar) at 37 °C for 48 hr.
3. +ve tubes detected by the formation of **black deposit** of ferrous sulphate **FeS** .

(2) Confirmed test

The confirmed test is done to confirm the presence of ***Clostridium perfringens*** in positive tubes by using **Litmus Milk** (stormy fermentation phenomenon), since ***Clostridium perfringens*** ferment sugar in litmus milk(lactose to lactic acid) lead to greats extremely acidic condition which lead to coagulation of milk protein(Casein) and the gas that formed through sugar fermentation push protein clot to the surface.

Procedure

1. Transfer 1ml of +ve tubes to preheated test tubes filled with litmus milk medium (precaution: be carefully do not expose samples to air).
2. Incubate tubes an aerobically at 37° C for 48 hr.
3. Detect +ve tubes for stormy fermentation.
4. Make a slide, stain with gram stain and examine under oil immersion.

Aquatic microbiology Lab (9):

3-Fecal Streptococci

The fecal streptococci found in the feces of humans and other worm- blooded animals:

(a)- *Streptococcus faecalis*

(b)- *Streptococcus bovis*

(c)- *Streptococcus equines*

Because of **limited survival time outside** the intestinal tract their presence indicates **very recent pollution.**

Fecal streptococci data verify fecal pollution and many provide additional information concerning the recency and probable origin of pollution, In combination with data on coliform.

(FC/ FS) Ratio

The relation of the fecal coliform and fecal streptococci density may provide information on the potential source of contamination, (N. of **Fecal coliform /N.** of **Fecal streptococci) ,**if the ratio greater than 1 indicates human fecal pollution (domestic wastes), while if the ratio less than 1 indicates worm –blooded animals pollution .

Most probable number (M.P.N.) of fecal streptococci

1- Presumptive test

Procedure:

1- add **0.5** ml of water (different types of water) to 3- sterile tubes with **4.5** ml of **Azide dextrose broth** mixed well (**10^{-1}**) .

2-Transfer **0.5** ml from previous tubes to another 3- tubes of Azide dextrose broth mixed well (**10^{-2}**).

3- Finally transfer **0.5** ml from (**10^{-2}**) to another 3- test tubes with Azide dextrose broth and mixed well (**10^{-3}**).

Now we have 9-tubes contain **Azide dextrose broth** (10^{-1}), (10^{-2}), (10^{-3}).

Notes : The procedure include a few number of dilutions**?** because this bacteria do not grow easily in media.

4- Incubate at 37^0 C for 24-72 hr.

5- Record the results if the tubes negative or positive (**+ve**: turbid + change in color from purple to yellow.

6- calculate the number of cells for 1 ml (cell /ml) by using the value of (M.P.N.) from the table .

7- Compare the cell number of coliform(previous data) with the cell number of streptococci, to evaluate the ratio of **(FC/ FS)**.

2-Confirmed test

Confirmed test for streptococci is essential to Confirm the the presence of fecal streptococci especially ***Streptococcus faecalis*** .

Procedure:

1- Transfer **0.5** ml from +ve results of **Azide dextrose broth** (yellow tubes) to another tubes contain **4.5** ml of **Azide dextrose broth**, mixed well.

2- Incubate the tubes at **45^0 C** for 48 hr**.**

3- + ve result ….turbidity and color change from purple to **yellow** will confirm the presence of ***Streptococcus faecalis***

3- Complete test

Inoculate the selective solid media for Streptococci **Pfiezer selective agar (PSE agars)** with positive result of Confirm test . colonies of ***Streptococcus faecalis*** on this medium have black- brown color encircled by

Aquatic microbiology Lab (10):

Detection of pathogenic bacteria

Pathogenic bacteria come into the water mainly from domestic waste water and most of them cannot stay for a long time because soon they die, so the water-laden feces (Sewage) are the source of disease particularly pathogenic intestinal microorganisms.

The study and investigation of coliform bacteria is important for its close relationship with pathogenic bacteria, it is possible to isolate pathogenic bacteria in the case of presence of coliform bacteria in water , so this test is important always. But we will depend on previous tests have made to the coliform and investigated to other pathological types of bacteria i.e. ***Salmonella , Shigella , Vibrio*** .

Isolation and Identification of S*almonella and Shigella*

Salmonella

G-ve bacteria, bacilli, non- spore former, motile by peripheral flagella, mostly non lactose fermented, produce H_2S.

<u>Distinguishing from other Enterobacteriaceae by:</u>

1- Motility

2- Do not produce capsule

3- non-lactose fermenter through 18-24h when grow on Macconky or S.S.agar so they have pale colonie.

Shigella

G-ve bacteria, bacilli, non- spore former, non-motile, non-lactose fermenter, do not produce H_2S.

Characterized *Salmonella* from *Shigella*

1- Production of H_2S.

2- Produce gas and acidity in sugar solution.

3- Motile

4- Most of them do not have the ability to produce Indol .

Salmonella **….**caused infection of salmonellosis when dealing with contaminated food and drink.

Shigella. ….*Shigella* can cause dysentery through contaminated food and drink when it moves to the large intestine and begins to reproduce, the dysentery accompanied by diarrhea with blood and mucous materials, abdominal pain and neurological disorders.

Enrichment ..

The important steps to isolate the pathogenic bacteria, because:

1 - Pathogenic bacteria are few in number in the water

2 - Often the selective media toxic to pathogenic bacteria.

Procedure:

1 - Different water samples are taken

2 - Add the water sample to multiplier enrichment media with equal volume of enrichment media.

Enrichment media for ***Salmonella*** and ***Shigella***

1- Selenite broth

This medium allows the rapid proliferation of ***Salmonella*** and it should be incubated within a period of 24 hours; the growth indicated by turbidity and orange color.

Notes :

Increasing the incubation period of selenite broth allows the growth of coliform bacteria, which were inhibited during the early hours of incubation in this medium.

2- GN broth

This medium gives a good growth of the ***Shigella***, it inhibited coliform and fecal streptococci , but if the incubation period is more than 24 hours allows the growth of acidophilic bacteria-like ***Proteus , Pseudomonus aeroginosa***.

Note: The growth indicated by turbidity formed in the media.

Growth in Selective media

To separate pathogenic bacteria from non-pathogenic bacteria we should do the following:
1 - The temperatures and duration of incubation should be appropriate for pathogenic bacteria.

2 - Use appropriate media.
Classification of solid selective media:

(1) Differential medium

This media contain or does not contain materials inhibit the growth of microorganisms other unintended to grow on it, but contains materials intended to give the bacteria special characters that distinguish them from other bacteria, such as **(EMB agar)** allowing for growth of types and genus of bacteria other than the ***E. coli*** but, only ***E. coli*** give the phenomenon of **green metallic sheen**, other bacteria appear in red color.

(2) Selective media

This media contains inhibited materials, allows the growth of bacterial groups without the other, but not necessarily with material distinguish these groups from each other, so it could be selective and differential media at the same time for example:

1- **EMB agar… This media is consider as** selective medium because it contains substance inhibited the growth of G +ve bacteria and allows the growth of G-ve bacteria and it is differential media because it distinguishes the ***E. coli*** from the other enteric bacteria .

2- **Macconky agar… This media is consider** as selective and differential medium ; it is selective because it contain **crystal violet** and the **bile salt which** inhibit the growth of G+ve bacteria and allows the growth of G-ve enteric bacteria ,also it distinguish between their genus, the G-ve lactose fermenter bacteria appear as pink colonies while non- lactose fermenter bacteria appear as pale colonies.

3-**S.S. agar (*Salmonella* - *Shigella* agar)**

This media is consider as selective and differential medium, because it contains the bile salts which inhibit the growth of G +ve bacteria and it also contains lactose to differentiate between lactose fermenter and non- lactose fermenter bacteria , ***Salmonella*** and ***Shigella*** colonies appear as pale colonies because both of them cannot fermented lactose sugar also this medium distinguishes between ***Salmonella*** and ***Shigella*** , ***Salmonella*** produce of H_2S which reacts with iron, giving the FeS (Black precipitation) which is concentrated in the center of the colonies while ***Shigella*** colonies appear as pale colonies without a black center because it is not produce of H_2S, ,this media contain Neutral red as indicator .

Procedure:

1 - Notes that growth on GN broth appears as a turbid while the growth on Selenite broth appears as a turbid as well as the color changed to orange.

2- **S.S.agar** is prepared and the Petri- dish is divided into two equal halves by which the first half is inoculated from **Selenite broth (turbidity and orange color)** while the second half is inoculated from **GN broth (turbidity).**

3- Incubate the plate at 37C˚for 24h.

4- Notes the appearance of colonies.

5- Pale colonies + FeS ⟶ ***Salmonella***

Pale colonies without FeS ⟶ ***Shigella***

6- Make a slide and stained with Gram stain

Notes: We cannot distinguish between *Salmonella* and *Shigella* under the microscope because they have same features; G-ve , bacilli, non-spore former.