"Capacity Building and Strengthening Institutional Arrangement"

Analysis and sampling of water and water pollution

The modern technology for water analysis (Chemical and Microbiological)

Microbiological tests, short introduction to ecotoxicological tests

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APAT

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Introduction – Indicators organisms

- Coliforms and Faecal Coliforms
- Faecal streptococci
- Clostridium perfringens

PATHOGEN

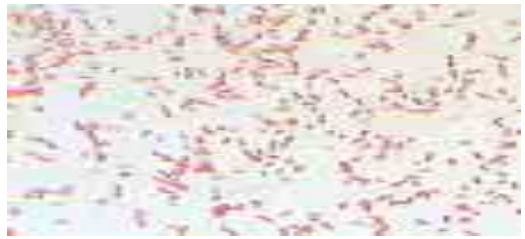
a. *Salmonella* spp

b. *Enteroviruses*

c. *Rotaviruses*

d. Intestinal Nematodes

The focus of this presentation are: Coliforms and Faecal Coliforms, Faecal Streptococci and Clostridium perfringens



Coliform and faecal coliforms

Coliform group of bacteria includes mainly species of the genera *Citrobacter, Enterobacter, Escherichia, Klebsiella* and Faecal Coliforms, of which *Escherichia coli* is the predominant species.

Coliforms are classified in two groups:

- The first one is able to grow outside of the intestine, especially in hot climates, consequently their counts can be unsuitable for monitoring wastewater
- The second one Faecal Coliform grow mainly in the intestine consequently are used for wastewater monitoring (as *E. coli)*

Faecal Streptococci

This group of organisms includes:

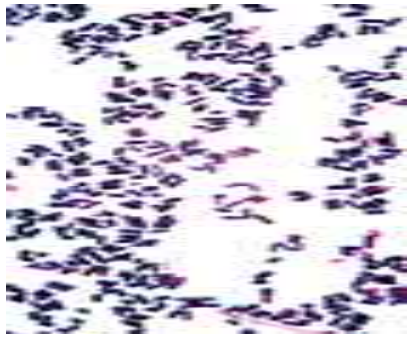
- species mainly associated with animals (Streptococcus bovis and S. equinus),
- species as S. faecalis and S. faecium, which occur both in man and in other animals;
- S. faecalis var liquefaciens, S. faecalis (biotypes) that hydrolyzes starch, which appear to be ubiquitous.

The enumeration of Faecal Streptococci in effluents is a simple routine procedure but has the following limitations:

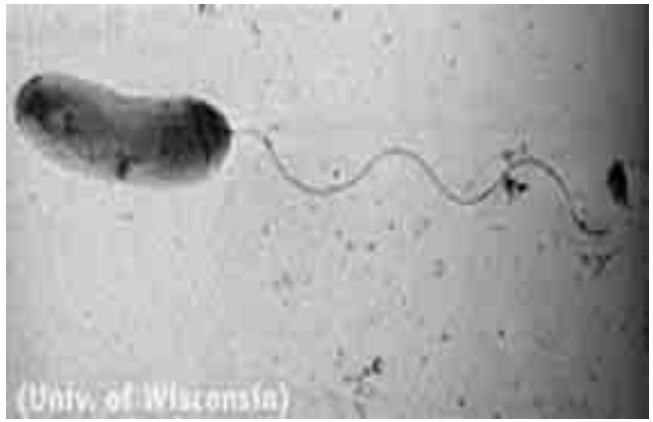
- the possible presence of the non-faecal biotypes which are part of the natural microflora on plants;
- the poor survival of Faecal Streptococci at high and at low temperatures.

Clostridium perfringens

This bacterium is only a faecal spore-forming anaerobic. This is normally used to detect intermittent or previous pollution of water, due to the prolonged survival of its spores.



Pathogens



Salmonella spp

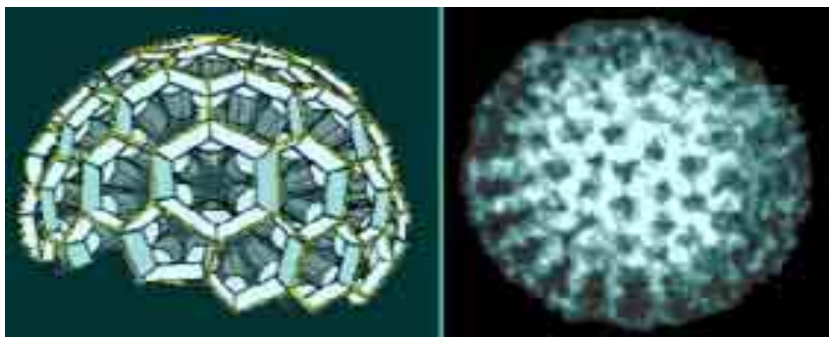
Several species of *Salmonellae* may be present in raw sewage from an urban community in a tropical country.

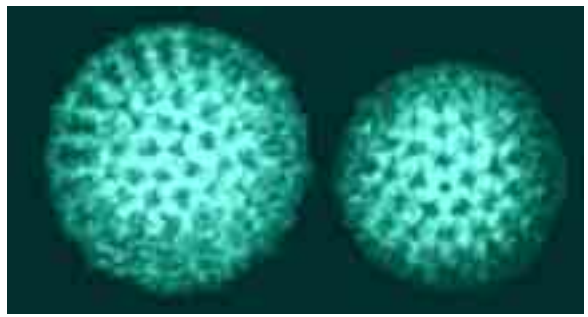
Health risk from Enteroviruses

May give rise to severe diseases, such as Poliomyelitis and Meningitis, or to a range of minor illnesses such as respiratory infections. Although there is no strong epidemiological evidence for the spread of these diseases via sewage irrigation systems, there is some risk and it is desirable to know to what extent viruses are removed by existing and new treatment processes, especially under tropical conditions. Virus counts can only be undertaken in a dedicated laboratory, as the cell culture techniques required are very susceptible to bacterial and fungal contamination.

Helth risk from Rotaviruses

These viruses are known to cause gastro-intestinal problems and, though usually present in lower numbers than enteroviruses in sewage, they are known to be more persistent, so it is necessary to establish their survival characteristics relative to enteroviruses and relative to the indicator organisms in wastewaters. It has been claimed that the removal of viruses in wastewater treatment occurs in parallel with the removal of suspended solids, as most virus particles are solids-associated. Hence, the measurement of suspended solids in treated effluents should be carried out as a matter of routine.





Sampling for microbiological analysis

Sampling method for microbiological analysis

Sampling activity in microbiology must be carried out with clean/sterilised containers.

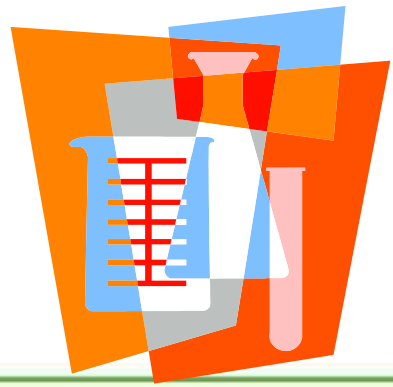
Sterilised containers are needed in sampling of superficial waters (sterilised bottles must be wrapped before sterilisation, handled with pincers or with other fit for purpose systems);

Clean containers can be used for waste waters;

Sampling equipment for microbiological characteristics

For the majority of samples, sterilized glass or plastics bottles are suitable (see ISO 5667-2). To collect samples considerably below the water surface, as in lakes and reservoirs, various deep sampling devices are available and the sampling devices are described in ISO 5667-2.

All apparatus used, including pumps and pumping equipment, has to be free from contamination (e.g. by flushing) and should not introduce new micro-organisms.



Relevant standards

ISO 5667-1:1980, Water quality – Sampling Part 1: Guidance on the design of sampling programmes.

ISO 5667-5, Water quality – Sampling Part 5: Guidance on sampling of drinking water used for food and beverage processing

ISO 5667-2, Water quality – Sampling – Part 3: Guidance on the preservation and handling of samples.

ISO 17994, Water quality – Criteria for establishing equivalence between microbiological methods

The choice of representative sampling

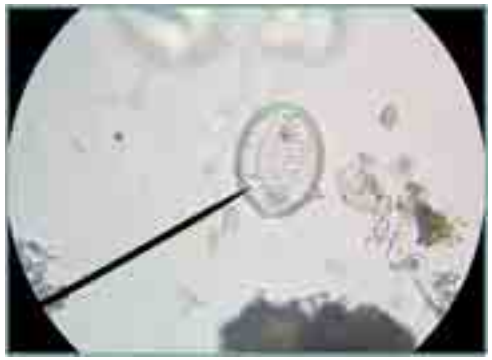
The choice of representative sampling points, frequency of sampling, type of samples taken, etc. is dependent on the objective of the study.

In general, the sampling approach for chemical analysis is compatible with the purpose of biotesting.

Some tests, however, require the water and waste water to be handled and kept in a particular way.

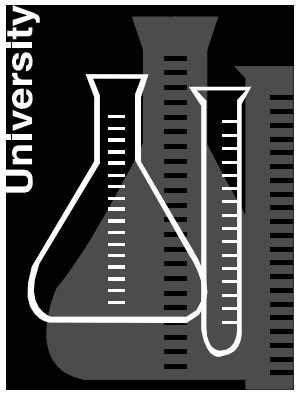
1) Brachionus

2) Diatomea



Volume, shape and material of the vessels

The volume, shape and material of the vessels are dependent on the nature of the sample (e.g. degradability/stability), the number of replicates, the volume required for these tests and the necessity of preserving and storing the samples prior to further processing.



Sampling and transport

During the sampling the following information should be collected:

Chemico-physical parameters (water temperature at the depth of sampling, pH, dissolved oxygen and chlorine concentrations)

Time and date of collection, sampling point identification, meteorological characteristics.

TRANSPORT

Collected samples must be stored at a temperature between 4 and 10 °C

Sample containers should be stored in darkness

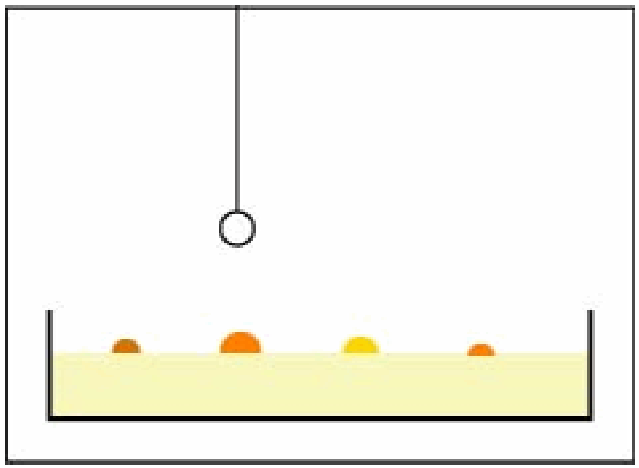
Storage and max time from sampling to analysis

Storage before analysis 4-10 °C

Max time from sampling to analysis

E. Coli and coliforms	12-18 h
Enterococci	12-18 h
Bacteria	12-18 h

Microbiological Analysis
Coliform Methods

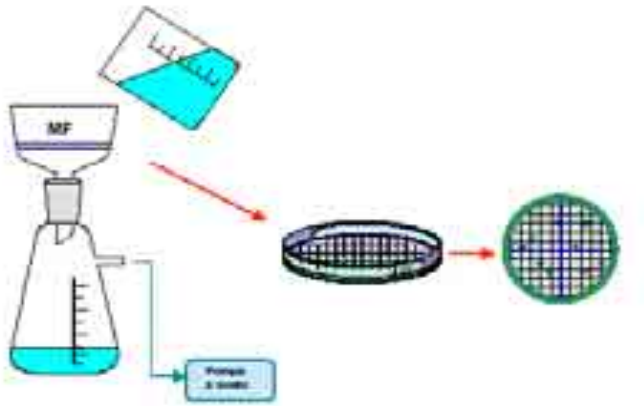


Determination of E.coli and coliform bacteria – Membrane filtration method (MF) (analytical process)

This test is based on membrane filtration, subsequent culture on a differential agar medium and calculation of the number of target organisms in the sample

Culture agar medium

Membrane filtration



Determination of E.coli and coliform bacteria – Membrane filtration method (MF) – Standard Test and Rapid Test (1/3)

Standard test

The Standard Test includes incubation on the membrane on a selective medium with subsequent further biochemical characterization of the typical lactose-positive colonies, leading to the detection and enumeration of coliform bacteria and E.coli within 2d to 3d.

Rapid test

The Rapid Test consists of two incubation steps allowing the detection and enumeration of E.coli within 21 ± 3h.

Determination of E.coli and coliform bacteria – Membrane filtration method (MF) – Standard Test and Rapid Test (2/3)

FILTRATION AND INCUBATION

Test portion of the sample is filtered through membranes which retain the bacteria (47 mm or 50 mm in diameter, nominal pore diameter 0.45 μm, preferably with grids).

Standard Test

One membrane is placed on a selective lactose agar medium which is incubated at (36 ± 2)°C for (21 ± 3)h

Rapid Test

One membrane on a casein (tryptic digest)-containing agar medium incubated at (36 ± 2)°C for 4h to 5h, followed by incubation at (44,0 ± 0,5)°C for 19h to 20h on a agar medium containing casein (tryptic digest) and bilesalts.

Determination of E.coli and coliform bacteria – Membrane filtration method – Standard Test and Rapid Test (3/3)

EVALUATION AND CONFIRMATION

The characteristic colonies on the membrane are counted as lactose-positive bacteria. For coliform bacteria and E.coli, subculture is carried out of randomly selected characteristic colonies for confirmatory tests: oxidase and indole production. The numbers of lactose-positive coliform bacteria and E.coli likely to be present in 100 mL of the sample are counted.

NEGATIVE OXIDASE REACTION Coliform bacteria

NEGATIVE OXIDASE AND A POSITIVE INDOLE REACTION E.coli

Determination of coliform organisms, thermotolerant coliform organisms and presumptive E.coli (1/5)

This method is specific for the detection and enumeration of coliform organism, thermotolerant coliform organisms and presumptive Escherichia coli (presumptive E.coli) in water by culture in a liquid medium in multiple tubes and calculation of their most probable numbers (MPN) in the sample.

This method can be applied to all types of water, including those containing an appreciable amount of suspended matter.

Determination of coliform organisms, thermotolerant coliform organisms and presumptive E.coli (2/5)

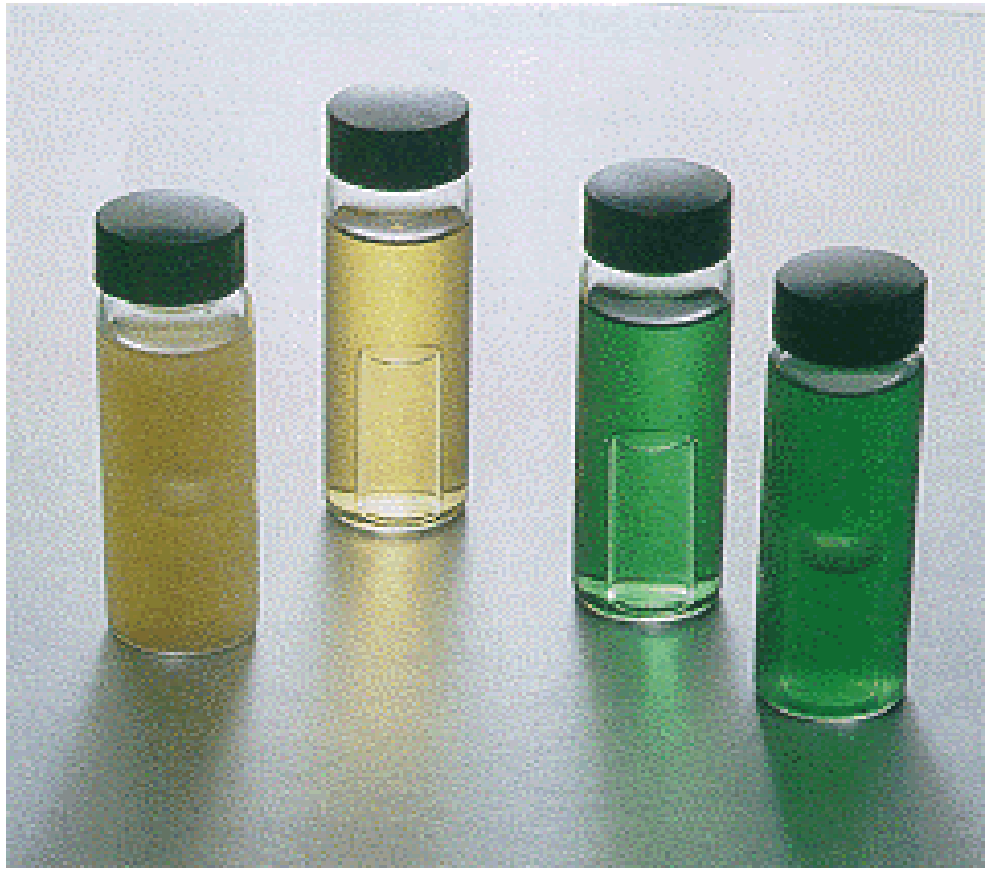
PRINCIPLE

Inoculation of test portion of the sample, diluted or undiluted, into a series of tubes of a enrichment liquid culture medium containing lactose (24h at 37°C)

Subculture from each tube showing turbidity with gas production into more selective confirmatory medium (Brillant-green lactose (bile) broth) at 44°C and 37°C.

Incubation at these confirmatory media for up to 48h at either 35°C or 37°C for the detection of coliform organisms, and at 44°C for up to 24h for thermotolerant coliform organism and presumptive E.coli

Multiple tube fermentation method (3/5)

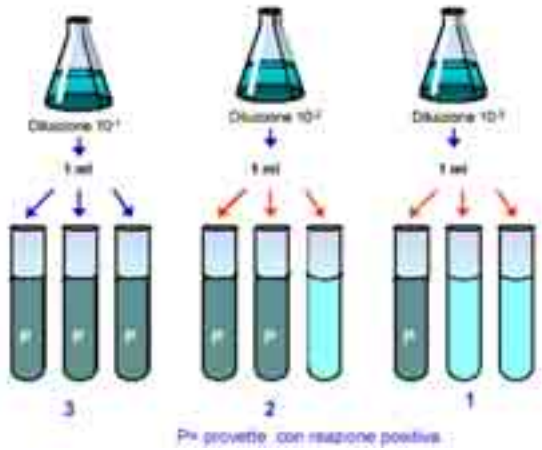


Calculation of the Most Probable Number (MPN) (4/5)

By means of statistical tables, calculation of the most probable numbers (MPN)of coliform organisms, thermotolerant coliform organisms and presumptive E.coli likely to be present in 100 mL of the sample, from the number of tubes giving positive confirmatory results.

MPN Method (5/5)

Metodo NPP/MPN (Most Probable Number)



Determination of E.coli and coliform bacteria in surface and waste water (½)

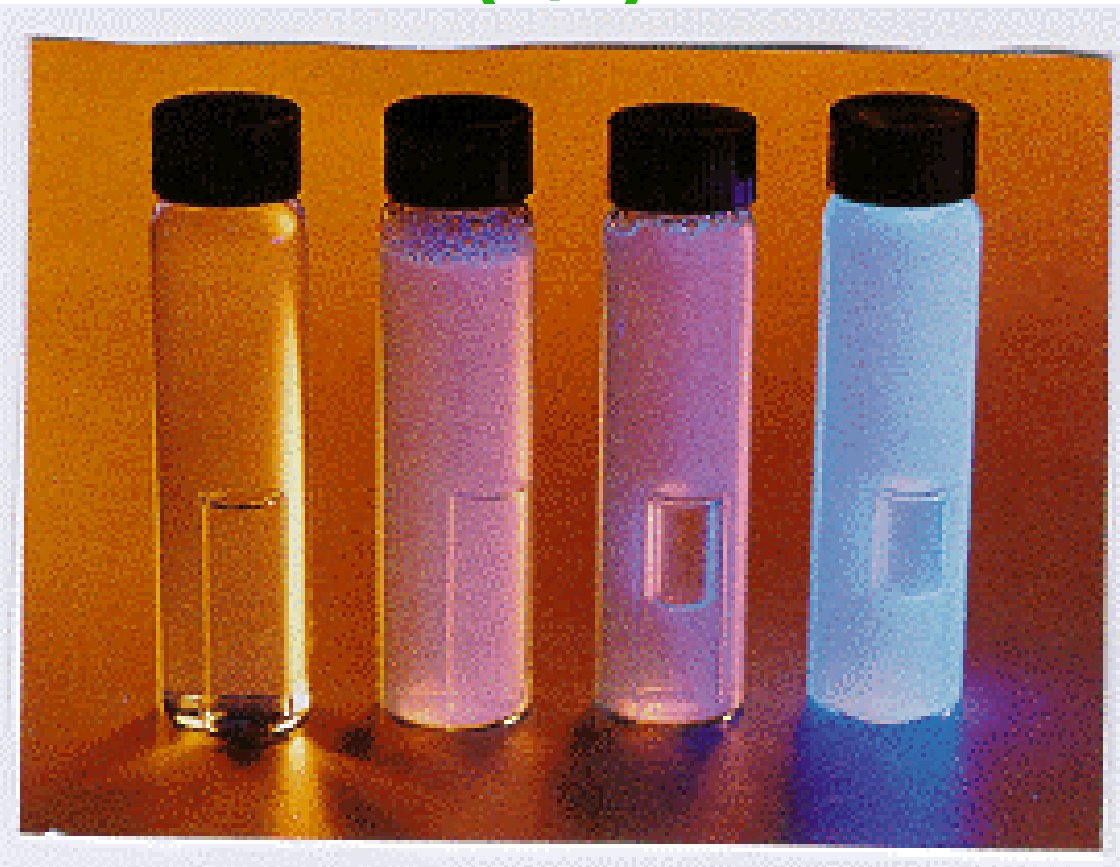
MINIATURIZED METHOD (MPN) BY INOCULATION IN LIQUID MEDIUM - PRICIPLE

The diluted sample is inoculated in a row of tubes containing dehydrated culture medium.

The tubes are examined under ultraviolet light at 366 nm in the dark after an incubation period of 36h minimum and 72h maximum at 44°C ± 0,5°C. The presence of E.coli is indicated by a blue fluorescence resulting from hydrolysis of MUG (4-methylumbelliferyl-β-D-glucoronide).

The results are given as most probable number (MPN) per 100 mL.

Fecal Coliform/E.coli
Multiple Tube Method – EC Medium + MUG (2/2)



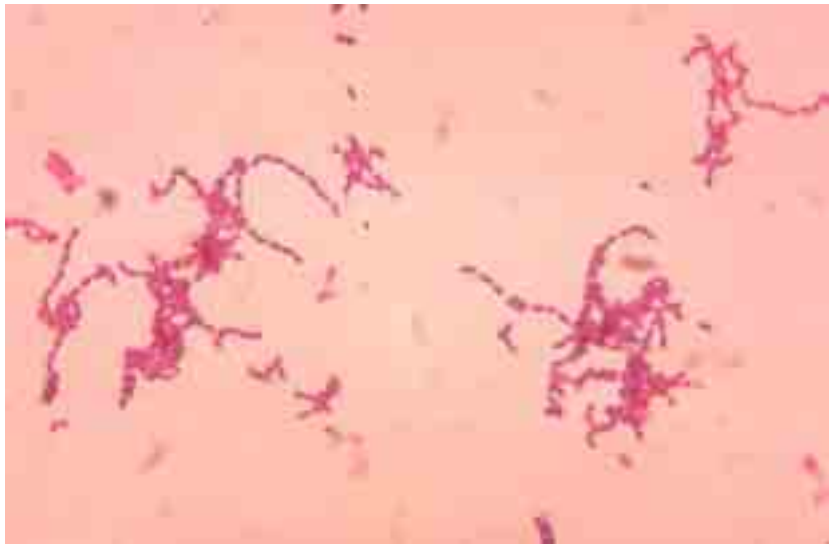
Reference Standards

- ISO 8199 Water quality – General guide to the enumeration of micro-organisms by culture
- ISO 9308-1 Water quality – Detection and enumeration of Escherichia coli and coliform bacteria Part 1: Membrane filtration method
- ISO 9308-2 Water quality – Detection and enumeration of coliform organisms, thermotolerant coliform organisms and presumptive Escherichia coli – Part 2: Multi Tube (most probable number - MPN) method
- ISO 9308-3 Water quality – Detection and enumeration of E.coli and coliform bacteria in surface and waste water Part 3: Miniaturized method (most probable number - MPN) by inoculation in liquid medium.

Reference Standards

- EN ISO 12780 Water Quality - Detection and Enumeration of Pseudomonas Aeruginosa by Membrane Filtration
- ISO 15522 Water quality – Determination of the inhibitory effect of water constituents on the growth of activated sludge microorganisms

Fecal Streptococci Method (enterococci)



Determination of intestinal enterococci in surface and waste water – Miniaturized Method (MPN) by inoculation in liquid medium (1/3)

- The major intestinal enterococci, namely E.faecalis, E.faecium, E.durans and E.hirae are frequently presents in faeces of humans and homeothermic animals.
- Other faecal Enterococcus species, namely E.avium, E.cecorum, E.columbae and E.gallinarum and Streptococcus bovis/equinus strains may occasionally be included, but they occur rarely in the environmental samples. Their recovery tends to be low. E.casseliflavus and E.mundtii are non-faecal species which, when present in water samples, are enumerated as faecal enterococci. These species and other rare non-fecal species tend to produce yellow pigment on a non-selective medium.

Determination of intestinal enterococci in surface and waste water – Miniaturized Method (MPN) by inoculation in liquid medium (2/3)

SCOPE

The miniaturized method needs detection and enumeration of major intestinal enterococci in surface and waste water by inoculation in a liquid medium.

This method is applicable to all types of surface and waste waters, particularly those rich in suspended matter

Determination of intestinal enterococci in surface and waste water – Miniaturized Method (MPN) by inoculation in liquid medium (3/3)

Micro-organisms capable of aerobic at 44 °C and of hydrolysing the 4-methylumbelliferyl-β-D-glucoside (MUD), in the presence of thallium acetate, nalidixic acid and 2,3,5-triphenyltetrazolium chloride (TTC), in the liquid medium specified.

PRINCIPLE

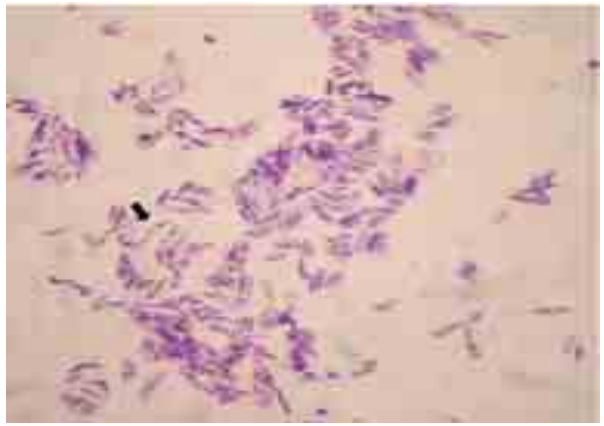
The dilute sample is inoculated in a row of tubes containing dehydrated culture medium

The tubes are examined under ultraviolet light at 366 nm in the dark after an incubation period between 36h and 72h at 44 °C ± 0,5 °C. The presence of enterococci is indicated by fluorescence resulting from the hydrolysis of MUD. The results are give as Most Probable Number (MPN) per 100 mL.

Reference Standards

- ISO 7899-1 Water quality – Detection and enumeration of intestinal enterococci in surface and waste water Part 1: Miniaturized method (most probable number) by inoculation in liquid medium
- ISO 7899-2 Water quality – Detection and enumeration of intestinal enterococci Part 2: Membrane filtration method

Clostridia



Introduction

The spores of sulfite-reducing anaerobes (clostridia) are widespread in the environment. They are present in human and animal faecal matter, in waste water and soil. Unlike E.coli and other coliform organisms, the spores survive in water for long periods as they are more resistant than vegetative forms to the action of chemical and physical factors.

They may thus give an indication of remote or intermittent pollution. They may even be resistant of chlorination at levels which are normally used for the treatment of water, and they are thus useful for control purposes.

Definition “Clostridia”

Sulfite-reducing, spore-forming, anaerobic micro-organisms which belong to the Bacillaceae family and the genus Clostridium.

Method by enrichment in a liquid medium (1/2)

PRINCIPLE

The detection of spores and sulfite-reducing anaerobes (clostridia) in a specified volume of a water sample requires the following steps.

SELECTION OF SPORES

Selection of spores in the sample by applying heat for a period of time sufficient to destroy vegetative bacteria.

ENRICHMENT CULTURE

Detection and enumeration of spores of sulfite-reducing anaerobes by inoculating volumes of the sample into liquid enrichment media, followed by incubation at 37°C ± 1°C for 44±4h in anaerobic conditions.

Method by enrichment in a liquid medium (2/2)

CULTURE MEDIA AND REAGENTS: BASIC MATERIALS

In order to improve the reproducibility of the results, it is recommended that, for the preparation of the diluents and culture media, dehydrated basic components or complete dehydrated media be used. Similarly, commercially prepared reagents may also be used. The manufacturer's instructions shall be rigorously followed.

The chemical products used for the preparation of the culture media and the reagents shall be of recognized analytical quality.

Membrane filtration and culture (1/1)

Filtration of the water sample through a membrane filter having a pore size such that bacterial spores (0,2 μm) are retained in or on the membrane filter.

Placing of the filter on a specialized selective culture medium (sulfite-iron-agar), followed by incubation at 37°C ± 1°C for 20 ± 4h and 44 ± 4h, and counting of any black colonies.

Reference standards

- ISO 6461-1 Water quality Detection and enumeration of the spores of sulfite-reducing anaerobes (clostridia) Part 1: Method by enrichment in a liquid medium.
- ISO 6461-2 Water quality Detection and enumeration of the spores of sulfite-reducing anaerobes (clostridia) Part 2: Method by membrane filtration.

Analytical Data Quality

- At the end of the presentation we are able, if also shortly, to give a look to a very important part of the microbiological analysis: Accuracy and Precision.
- Accuracy is defined as the difference between the true value and the measured value.
- False negative occur, for example, when the micro-organisms counted belong to the group sought, but result of the final enumeration underestimates the true number.
- False positive occur, for example when a number of micro-organisms not belonging to the group sought are considered and counted as such, so that the final value is greater than the true value. Both types of error can occur simultaneously.

Precision

This is defined as the closeness of agreement between results obtained by a particular method from the same sample either in the same laboratory (repeatability) or in different laboratories (reproducibility).

Precision is often expressed in terms of confidence limits within which the true value of the result generally lies for a given degree of probability (usually 95%).

Precision of MPN procedure and of colony-count procedures

PRECISION OF MPN PROCEDURE

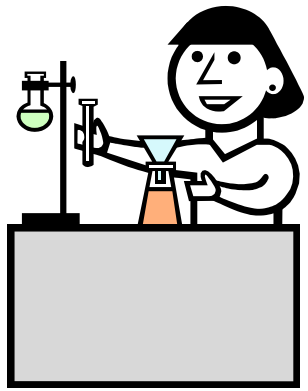
The total precision for a given system varies according to the number the tubes inoculated with each dilution.

PRECISION OF COLONY-COUNT PROCEDURES

With colony-count procedures, precision increases as the number of colonies increases.

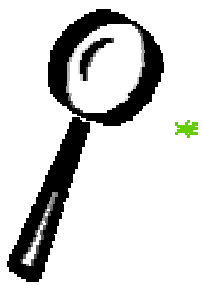
Limit of detection

The limit of detection of a procedure can be considered either as the volume in which a single micro-organism sought can be detected, or the smallest detectable number of micro-organisms contained in a given volume of water, generally 1 mL or 100 mL.



Limit of detection with the MPN procedure

The limit of detection depends on the volume of the sample actually examined. Increasing the total volume examined will therefore improve the limit of detection. With the MPN procedure this may be achieved either by increasing the test portion volumes for each tube.



Biological analysis

Ecotoxicological analysis

Ecotoxicity tests

Ecotoxicity tests are biological experiments performed to examine if either a potentially toxic compound, or an environmental sample (e.g. effluent, sediment or soil sample) causes a biologically important response in tests organisms, and if so to determine the concentration that produces a given level of effects or produces an effect that cannot be distinguished from background variation.

Some criteria of test

In a test, organisms are exposed to different concentrations or doses of a test substance or a test substrate (e.g. waste water, sludge, or a contaminated soil or sediment), sometimes diluted in a test medium. Typically, at least one group of test organisms (the control group)is not exposed to the test substance or substrate, but is otherwise treated in the same way as the exposed organisms.

The endpoint's relationship with the concentration of the tested chemical or substrate is examined.

Endpoints

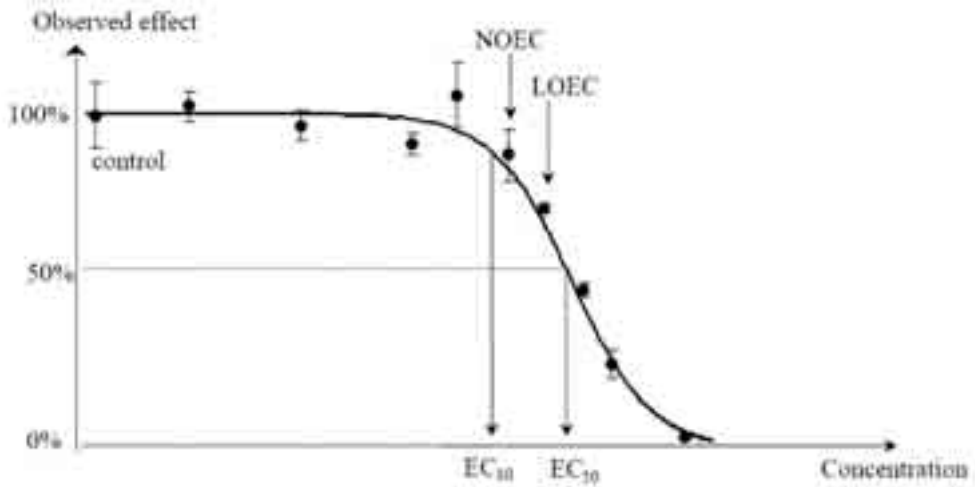
An ecotoxicological study can have one or many endpoints. The endpoint is the biological parameter observed, e.g. survival, number of eggs, size or growth, enzyme level.

- Some endpoints
- ECX Effective concentration
- LCX Lethal concentration
- Etx is the time at which an effect of x% is expected at a specified test concentration when the test organisms are exposed to a given concentration of material (in water or sediment or soil).
- LCx is estimated when the response of interest is mortality, etc.

LOEC - NOEC

The Lowest Observed Effect Concentration is the lowest concentration out of the tested concentrations at which a statistically significant difference from the control group is observed.

The No Observed Effect Concentration is the tested concentration just below the LOEC.



Relevant OECD Ecotoxicity Test Guidelines

OECD 201 Alga, growth inhibition test
OECD 202 Daphnia sp. acute immobilisation test
OECD 203 Fish acute toxicity test
OECD 204 Fish prolonged toxicity test
OECD 210 Fish early life-stage toxicity test
OECD 211 Daphnia magna reproduction test
OECD 212 Fish, short-term toxicity test on embryo and sac-fry stages
OECD 215 Fish juvenile growth test
OECD 219 Sediment-Water Chironomid Toxicity Test Using Spiked Water

Reference Standards

- ISO 6341:98 Water quality – Determination of the inhibition of the mobility of Daphnia magna Straus (Cladocera, Crustacea) – Acute toxicity test
- ISO 8692:99 Water quality - Fresh water algal growth inhibition test with Scenedesmus subspicatus and Selenastrum capricornutum (Pseudokirchneriella subcapitata)
- ISO 11348-1 Water quality – Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test) – Part 1: Method using freshly prepared bacteria

Reference Standards

- ISO 11348-2 Water quality – Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test) – Part 2: Method using liquid-dried bacteri
- ISO 11348-3 Water quality – Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test) – Part 3: Method using freeze-dried bacteria