MICROBIOLOGICAL AND CHEMICAL EXAMINATION OF WATER

Water is one of the most important and abundant compounds of the ecosystem. All living organisms on the earth need water for their survival and growth. As of now only earth is the planet having about 70 % of water. But due to increased human population, industrialization, use of fertilizers in the agriculture and man-made activity it is highly polluted with different harmful contaminants.

Water provides an environment for a wide variety of microorganisms to survive and function. Microbial diversity depends on available nutrients, their varied concentrations (ranging from extremely low to very high levels).

Therefore it is necessary that the quality of drinking water should be checked at regular time interval, because availability of good quality water is an indispensable feature for preventing diseases and improving quality of life.

HOW DO YOU DO THIS----THIS LEADS US TO OUR TOPIC……..MEANING?

Microbiological and chemical examination of water simply refers to method of analysing water to estimate the numbers of microorganisms present. It is a microbiological analytical procedure which uses samples of water and from these samples determines the concentration of microorganisms. It is then possible to draw inferences about the suitability of the water for use from these concentrations. This process is used for example to routinely confirm that water is safe for human consumption.

A variety of techniques are available to monitor bacterial growth either by determining the number of cells in the population or their total mass, or by detecting their products.

The choice depends on various characteristics of the sample and the goals of the measurements.

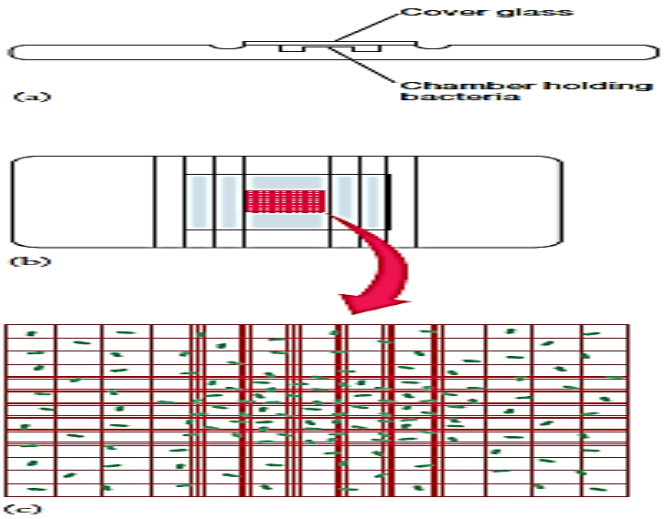
1. Direct cell counts: This method count both living and dead cells directly. It involve the use of (a) microscope and (b) cell counting instrument

A. **Direct microscopic count:** Microorganisms can be counted directly using a microscope which is one of the most rapid methods of determining the number of cells in a suspension (water). The number of bacteria in a measured volume of liquid is counted using special glass slides that hold known volume of liquid (Petroff-

Hausser counting chambers can be used for counting prokaryotes; hemocytometer can be used for both prokaryotes and eukaryotes). This can be viewed under the light microscope and the number of bacteria contained in the liquid can be counted precisely.

How can this be achieved?

A dilution of a bacterial culture is placed on a special microscope slide. Etched on the surface of the slide is a grid of precise dimension and placing a coverslip over the grid forms a space of precise volume. The number of organisms counted within that volume is used to calculate the concentration of cells in the original culture.



Note: seeing an organism under the microscope does not mean that the organism is alive. Living cells may be distinguished from dead cells by fluorescence microscopy- using fluorescent chemical dyes e.g. propidium iodide (a red dye).

Using a counting chamber is easy, inexpensive, and relatively quick; it also gives information about the size and morphology of microorganisms

B. Cell-counting instrument

(a) Coulter counter: this is an instrument that counts cells in a suspension as they pass in single file through a minute aperture (orifice). The suspending liquid must be saline or another conducting fluid, because the machine actually detects and subsequently counts brief changes in resistance that occur when non-conducting particles such as bacteria pass by. In coulter counter, the microbial suspension is forced through a small hole or orifice. Electrical current flows through the hole,

and electrodes placed on both sides of the orifice measure its electrical resistance. Every time a microbial cell passes through the orifice, electrical resistance increases (or the conductivity drops) and the cell is counted

(b) **Flow cytometer:** is similar in principle to a coulter counter except that it measures the scattering of light by cells as they pass by a laser.

(c) **Fluorescence activated cell sorter (FACS).** An electronic technique more suited to counting and separating cells requires an instrument called FACS. This is a machine that can rapidly separate the cells in a suspension on the basis of size and colour of their fluorescence.

In the FACS technique bacterial cells that synthesize a fluorescence protein (e.g. cyan) or that are labelled with fluorescence antibody or chemical are passed through a small orifice, and then a laser detectors measure light scatter in the forward direction-a measure of particle size and to the side, which indicates shape or granularity. In addition, the laser activates the fluorophone in the fluorescent antibody and a detector measures fluorescent intensity. Within a single culture, the FACS technique enable us to use cell size and level of fluorescence (since one sub-population may fluoresce more than or less than another) to identify and count different populations of cells.

E.g. by placing the green fluorescent protein gene (gfp) under the control of regulatory DNA sequences of a bacterial gene (making a gene fusion), researchers can count the cells expressing that gene by using FACS analysis; this analysis in turn makes it possible to determine what conditions allow expression of gene and whether all cells in the population express that gene at the same time and to the same extent.

2. **Viable cell counts.** Viable cells are cells that can replicate and form colonies on a plate. Viable cell counts are used to quantify the number of cells capable of multiplying. This method requires knowledge of appropriate growth conditions for a particular microorganisms as well as the time to allow growth to occur. They are invaluable for monitoring bacterial growth in samples such as food and water that often contain numbers too low to be seen using a direct microscopic count.

(a) Plate count (b) Membrane filter and (c) Most probable number

(i) Plate count: it measures the number of viable cells in a sample by exploiting the fact that an isolated cell on a nutrient agar plate will give

rise to one colony. A simple count of the colonies determines how many cells were in the initial sample.

i). pour plate (ii) spread plate method: these two differs in how the suspension of bacteria is applied to the agar plate.

Before plating out the cells it is usually necessary to dilute the sample- this is because sample usually contains many bacteria but the ideal number to count is 30 and 300. Dilution of sample is normally in 10-fold increments, the diluent (or sterile solution) used to make the dilution, is generally physiological saline (0.85% NaCl in H_2O). Distilled water can be used, but some bacteria may lyse in this hypotonic environment.

In pour plate method, 0.1-1.0ml of the final dilution is transferred into a sterile petri dish and overlaid with melted nutrient agar that has been cooled to 50^oC. The dish is the gently swirled to mix the bacteria with the liquid agar. When the agar hardens, the individual cells are fixed in place and after incubation form distinguishable colonies.

In spread plate method, 0.1-0.2ml of the final dilution is transferred directly onto a plate already containing a solidified nutrient agar with a sterilized rod.

In both methods the plate are incubated for specific time to allow the colonies form, which can then be counted and the original number is calculated and recorded as colony forming unit per millilitre (cfu/ml). Because it is not possible to be absolutely certain that each colony arose from an individual cell, the results are often expressed in terms of **colony forming units (CFU)** rather than the number of microorganisms.

E.g. counted no=100

No of dilution=10^{-3}

Amount/volume of water used=100ul (0.1ml)

Cfu/ml=$100x10^{-3}$ x 0.1

Advantages: Plating techniques are simple, sensitive, and widely used for viable counts of bacteria and other microorganisms in samples of food, water, and soil.

Disadvantages: The counts will be low if the agar medium employed cannot support growth of all the viable microorganisms present.

Low counts will result if clumps of cells are not broken up and the microorganisms well dispersed.

The hot agar used in the pour-plate technique may injure or kill sensitive cells; thus spread plates sometimes give higher counts than pour plates

(b). **Membrane filter**

Membrane filter is used when the numbers of organisms in a sample is relatively low, as might occur in dilute environment such as natural waters. This method concentrates the bacteria by filtration before they are plated. A known volume of liquid is passed through a sterile membrane filter, which has a pore size that retains bacteria. The filter is subsequently placed on an appropriate agar medium and the incubated. The number of colonies that grow on the filter indicates the number of bacteria that were in the volume filtered.

(c). **Most Probable Number (MPN)**

The most probable number (MPN) method is a statistical assay of cell numbers based on the theory of probability. The goal is to successively dilute a sample and determine the point at which subsequent dilution receive no cells.

To determine the MPN, three sets of three or five tubes containing the same growth medium are prepared. Each set receives a measured amount of the sample. The amount added is determined, in part, by the expected bacterial concentration in that sample. What is important is that the second set receives 10-fold less than the first and the third set 100-fold less than the previous set. After incubation, the presence or absence of growth in each tube in each set is noted; in some cases, growth along with a characteristics visible change such as gas production is noted. The results are then compared against an MPN table, which gives a statistical estimate of the cell concentration. The MPN method is mostly used to determine the approximate number of coliforms in a water sample.

3. **Measuring biomass.** Instead of measuring the numbers of cells, the cell mass can be determined. This can be done by measuring the turbidity, the total weight or the precise amount of chemical constituents such as nitrogen. These all relate to the number of cells present.

(a). Turbidity: turbidity simple means cloudiness. The cloudiness or turbidity of a broth culture is due to the scattering of light passing through the liquid by cells. The amount scattered is proportional to the concentration of cells. To measure turbidity, a spectrophotometer is used. This instrument transmits light through a specimen and measures the percentage that reaches a light detector. The number is inversely proportional to the optical density.

One limitation of assaying turbidity is that a medium must contain relatively high numbers of bacteria in order to be cloudy.one millilitre of a solution containing 1million bacteria (10^6) is

still clear, and if it contains 10 million cells (10^7), it is barely turbid. Thus, although a turbid culture indicates that bacteria are present, a clear solution does not guarantee their absence. Not recognizing these facts can have serious consequences in the laboratory as well as outside.

(b). Total weight: Determining the total weight of a culture is a tedious and time consuming method that is not used routinely. It can be invaluable, however for measuring the growth of filamentous organisms that do not readily break up into individual cells for it to be counted. To measure the wet weight, cells growing in the liquid culture are centrifuged down and the liquid removed then the weight of the resulting packed cell mass is proportional to the number of cells in the culture. The dry weight of the cell mass can be determined by drying the centrifuged cells at approximately 100^oC for 8 to 12 hours before weighing them.

(c) Chemical constituents: the quantity of a chemical constituent of a cell (typically nitrogen) can be determined and used to calculate the biomass. For example, cells can be treated with sulphuric acid, which converts cellular nitrogen to ammonia. The amount of ammonia can then be easily assayed; because cells composed of 14% nitrogen; the biomass can be mathematically derived from the amount of ammonia released.

4. **Detecting cell products**: Products of microbial growth can be used to estimate the number of microorganisms or, more commonly, to confirm their presence. These products include acids, gases such as CO_2 and ATP.

(a) Acid production: as a consequence of the breakdown of sugars which are used as energy source, microorganisms produce a variety of acids. The precise amount of acid can be measured using chemical means such as titration. Most commonly, however, acid production is used to detect growth by incorporating a pH indicator into a medium. The pH indicator changes from one colour to another as the pH of the medium changes. Several pH indicators are available and they differ in the value at which their colour changes.

(b) Gases: production of gases can be monitored in several ways. A method routinely used in the laboratory involves the use of an inverted small tube, called a Durham tube, in a broth of sugar-containing media. If the bacteria produce gas as a result of degradation of the sugar, bubbles will be trapped in the tube.

(c) ATP: the presence of ATP, the universal form of energy, can be detected by adding the firefly enzyme luciferase. The enzyme catalyses chemical reaction that uses ATP as an energy source to produce light. This method is sometimes used to

assess the effectiveness of chemical agents formulated to kill bacteria. Light is produced only if viable organisms remain.

Sanitary Analysis of Waters

Monitoring and detection of indicator and disease-causing microorganisms are a major part of sanitary microbiology.

A wide range of viral, bacterial, and protozoan diseases result from the contamination of water with human faecal wastes. Although many of these pathogens can be detected directly, environmental microbiologists have generally used **indicator organisms** as an index of possible water contamination by human pathogens. Researchers are still searching for the "ideal" indicator organism to use in sanitary microbiology.

The following are among the suggested criteria for such an indicator:

1. The indicator bacterium should be suitable for the analysis of all types of water: Tap, river, ground, impounded, recreational, estuary, sea, and waste.
2. The indicator bacterium should be present whenever enteric pathogens are present. i. e. The level of the indicator bacterium in contaminated water should have some direct relationship to the degree of faecal pollution
3. The indicator bacterium should survive longer than the hardiest enteric pathogen.
4. The indicator bacterium should not reproduce in the contaminated water and produce an inflated value.
5. The assay procedure for the indicator should have great specificity; in other words, other bacteria should not give positive results. In addition, the procedure should have high sensitivity and detect low levels of the indicator.
6. The testing method should be easy to perform.
7. The indicator should be harmless to humans.

Coliforms: Coliforms are defined as facultative anaerobic, gram-negative, non-sporing, rod-shaped bacteria that ferment lactose with gas formation within 48 hours at 35°C.

They include members of the family *Enterobacteriaceae* (e.g. *Escherichia coli, Enterobacter aerogenes,* and *Klebsiella pneumoniae*). These bacteria make up approximately 10% of the intestinal microorganisms of humans and other animals and have found widespread use as indicator organisms. When such "foreign" enteric indicator bacteria are not detectable in a

specific volume (100 ml) of water, the water is considered **potable** [Latin *potabilis,* fit to drink], or suitable for human consumption.

The original test for coliforms that was used to meet this definition involved the presumptive, confirmed, and completed tests.

The presumptive step is carried out by means of tubes inoculated with three different sample volumes to give an estimate of the **most probable number (MPN)** of coliforms in the water. The complete process, including the confirmed and completed tests, requires at least 4 days of incubations and transfers.

To test for coliforms and more effectively recover stressed coliforms, a variety of simpler and more specific tests have been developed. These include the membrane filtration technique, the **presence-absence (P-A) test** for coliforms and the related Colilert **defined substrate test** for detecting both coliforms and *E. coli.*

The **membrane filtration technique** has become a common and often preferred method of evaluating the microbiological characteristics of water. The water sample is passed through a membrane filter. The filter with its trapped bacteria is transferred to the surface of a solid medium or to an absorptive pad containing the desired liquid medium. Use of the proper medium allows the rapid detection of total coliforms, faecal coliforms, or faecal streptococci by the presence of their characteristic colonies. Samples can be placed on a less selective resuscitation medium, or incubated at a less stressful temperature, prior to growth under the final set of selective conditions.

An example of a resuscitation step is the use of 2 hour incubation on a pad soaked with lauryl sulfate broth, as is carried out in the LES Endo procedure. A resuscitation step often is needed with chlorinated samples, where the microorganisms are especially stressed.

The advantages and disadvantages of the membrane filter technique are summarized

Advantages

1. Good reproducibility
2. Single-step results often possible
3. Filters can be transferred between different media
4. Large volumes can be processed to increase assay sensitivity
5. Time savings are considerable
6. Ability to complete filtrations on site

7. Lower total cost in comparison with MPN procedure

Disadvantages

1. High-turbidity waters limit volumes sampled
2. High populations of background bacteria cause overgrowth
3. Metals and phenols can adsorb to filters and inhibit growth

More simplified tests for detecting coliforms and fecal coliforms are now available. The **presence-absence test (P-A test)** can be used for coliforms. This is a modification of the MPN procedure, in which a larger water sample (100 ml) is incubated in a single culture bottle with a triple-strength broth containing lactose broth, lauryl tryptose broth, and bromcresol purple indicator. The P-A test is based on the assumption that no coliforms should be present in 100 ml of drinking water. A positive test results in the production of acid (a yellow color) and constitutes a positive presumptive test requiring confirmation.

To test for both coliforms and *E. coli,* the related **Colilert defined substrate test** can be used. A water sample of 100 ml is added to a specialized medium containing *o*-nitrophenyl-β-D-galactopyranoside (**ONPG**) and 4-methylumbelliferyl-_β-D-glucuronide (**MUG**) as the only nutrients. If coliforms are present, the medium will turn yellow within 24 hours at 35°C due to the hydrolysis of ONPG, which releases *o*-nitrophenol**.** To check for *E. coli,* the medium is observed under long-wavelength UV light for fluorescence. When *E. coli* is present, the MUG is modified to yield a fluorescent product. If the test is negative for the presence of coliforms, the water is considered acceptable for human consumption. The main change from previous standards is the requirement to have waters free of coliforms and fecal coliforms. If coliforms are present, fecal coliforms or *E. coli* must be tested for.

Molecular techniques are now used routinely to detect coliforms in waters. 16 S rRNA gene-targeted primers for coliforms have been developed. Using these primers, it is possible to detect one colony forming unit (CFU) of *E. coli* per 100 ml of water, if an eight-hour enrichment step precedes the use of the PCR amplification. This allows the differentiation of nonpathogenic and enterotoxigenic strains, including the shiga-toxin producing *E. coli* O157:H7.

Chemical examination of water

Water is examined chemically in other to identify and quantify the chemical components and properties of the water. This includes pH, alkalinity hardness, major cations and anions, trace

elements and isotopes. Water chemistry analysis is used extensively to determine the possible uses water may have or to study the interaction it has with its environment.

Water chemistry analysis is often the groundwork of studies of water quality, pollution, hydrology and geothermal waters.

Components commonly analysed are pH, the cations Na, K, Mg, the anions Cl, F, SO_4, NO_3, trace metals and metalloids Fe, Mn etc unstable volatile (dissolves gas) such as CO_2, H_2S and O_2

Measuring pH of a liquid using a pH meter and probe:

1. Turn on the pH meter and calibrate the probe using two standard solutions. Calibration procedures vary by instrument, so following the manufacturer's instructions is highly recommended. BE SURE TO RINSE THE PROBE THOROUGHLY BETWEEN BUFFERS USING DEIONIZED WATER AND CAREFULLY BLOT THE PROBE DRY USING A KIM WIPE. pH meters should be calibrated before each use (before each series of samples, not between each sample itself) or when measuring a large range of pH.
2. Collect sample water in a glass or plastic container. Collect enough so the probe tip can be submerged in sample; either rinse the probe with deionized water (and blot dry) or with sample before inserting the probe into the collection vessel.
3. Submerge the probe into the sample and wait until the pH reading on the meter stabilizes. Record the measurement when the pH reading is stable.

Alkalinity

Alkalinity is an aggregate property of the water sample which measures the acid-neutralizing capacity of a water sample. The alkalinity of surface water is due to the carbonate, bicarbonate and hydroxide content and is often interpreted in terms of the concentrations of these constituents. Higher the alkalinity, greater is the capacity of water to neutralize acids. Conversely, the lower the alkalinity, the lesser will be the neutralizing capacity.

Alkalinity of sample can be estimated by titration with standard H_2SO_4 or HCl solution. Titration to pH 8.3 or decolourisation of phenolphthalein indicator will indicate complete

neutralization of OH^- and 1/2 of CO_3^{2-}, while to pH 4.5 or sharp change from yellow to orange of methyl orange indicator will indicate total alkalinity.

Procedure

1. Mix 100 ml of the sample with two or three drops of phenolphthalein indicator in a conical flask over a white surface. If no colour is produced, the phenolphthalein alkalinity is zero. If the sample turns pink or red, determine the alkalinity by titrating with standard acid until the pink colour just disappears. In either case, continue the determination using the sample to which phenolphthalein has been added.
2. Add a few drops of methyl orange indicator. If the sample is orange without the addition of acid, the total alkalinity is zero. If the sample turns yellow, titrate with standard acid until the first perceptible colour change towards orange is observed.
3. The determination by means of mixed indicator is done in the same way as with methyl orange. The mixed indicator yields the following colour responses: above pH 5.2, greenish blue; pH 5.0, light blue with lavender grey; pH 4.8, light pink-grey with a bluish cast; pH 4.6, light pink.

Any difficulty experienced in detecting the end-point may be reduced by placing a second 100-ml sample with the same amount of indicator (phenolphthalein, methyl orange or mixed indicator) in a similar container alongside that in which the titration is being carried out. Another way to provide a standard end-point is to prepare buffer solutions to which are added indicators in the same amount as in an alkalinity titration.

To detect the different types of alkalinity, the water is tested for phenolphthalein and total alkalinity, using Equations:

$$Phenolphthalein\ alkalinity\left(\frac{mg}{L}\right)\ as\ CaCO_3 = \frac{A \times Normality\ of\ acid \times 50{,}000}{mL\ of\ sample}$$

$$Total\ alkalinity\ \left(\frac{mg}{L}\right)\ as\ CaCO_3 = \frac{B \times Normality\ of\ acid \times 50{,}000}{mL\ of\ sample}$$

Where: A = titrant (mL) used to titrate to pH 8.3

B = titrant (mL) used to titrate to pH 4.5

N = normality of the acid (0.02N H_2SO_4 for this alkalinity test)
50,000 = a conversion factor to change the normality into units of $CaCO_3$

Total hardness

Hardness in water is that characteristic, which "prevents the lathering of soap". This is due to presence in water of certain salts of calcium, magnesium and other heavy metals dissolved in it. A sample of hard water, when treated with soap does not produce lather, but on other hand forms a white scum or precipitate. This precipitate is formed, due to the formation of insoluble soaps of calcium and magnesium. Thus, water which does not produce lather with soap solution readily, but forms a white curd, is called hard water. On the other hand, water which lathers easily on shaking with soap solution, is called soft water. Such water consequently does not contain dissolved calcium and magnesium salts in it.

The degree of hardness of drinking water has been classified in terms of the equivalent $CaCO_3$ concentration as follows:

Soft	0-60mg/L
Medium	60-120mg/L
Hard	120-180mg/L
Very Hard	>180mg/L

In a hard water sample, the total hardness can be determined by titrating the Ca^{2+} and Mg^{2+} present in an aliquot of the sample with Na_2EDTA solution, using NH_4Cl-NH_4OH buffer solution of pH 10 and Eriochrome Black-T as the metal indicator.

$$Na_2H_2Y \text{ (Disodium EDTA solution)} \rightarrow 2Na^+ + H_2Y^-$$

$$Mg^{2+} + HD^{2-} \text{ (blue)} \rightarrow MgD \text{ (wine red)} + H^+$$

D (metal-indicator complex, wine red colour) + $H_2Y^- \rightarrow Y^-$ (metal EDTA complex colourless) + HD^{-N} (blue colour) + H^+

Ethylenediamine tetra-acetic acid (EDTA) and its sodium salts form a chelated soluble complex when added to a solution of certain metal cations. If a small amount of a dye such as Eriochrome black T is added to an aqueous solution containing calcium and magnesium ions at a pH of 10 ± 0.1, the solution will become wine red. If EDTA is then added as a titrant, the calcium and magnesium will be complexed. After sufficient EDTA has been added to complex all the magnesium and calcium, the solution will turn from wine red to blue. This is the end point of the titration.

$$Total\ hardness\ as\ CaCO_3\ (ppm) = \frac{Vol.\ of\ EDTA(mL) \times 0.1 \times molarity\ of\ EDTA \times 10^6}{Vol.\ of\ the\ sample\ (mL)}$$

Total dissolve solid

A well-mixed sample is filtered through a standard filter and the filtrate is evaporated to dryness in a weighed dish and dried to constant weight at 180^{o}C. The increase in dish weight represents the total dissolved solids.

Calculation

Total dissolved solid (TDS) = $\frac{\mathbf{B-A \times 1000}}{\mathbf{Volume\ of\ sample}}$

Chemical Oxygen Demand (COD):

Theory:

COD is used as a measure of oxygen equivalent to organic matter content of a sample that is susceptible to oxidation by a strong chemical oxidant. For samples from a specific source, COD can be related empirically to BOD. COD determination has advantage over BOD determination in that the result can be obtained in about 5 hours as compared to 5 days required for BOD test.

The organic matter gets oxidized completely by $K_2Cr_2O_7$ in the presence of H_2SO_4 to produce CO_2 and H_2O. The excess of $K_2Cr_2O_7$ remained after the reaction is titrated with ferrous ammonium sulphate. The dichromate consumed gives the O_2 required for oxidation of organic matter.

DRINKING WATER QUALITY STANDARDS:

S/N	Characteristic/Parameter	BIS	ICMR	WHO
1	Colour	5	2.5	-
2	Odour	Agreeable	Unobjectionable	Unobjectionable
3	Turbidity	10 NTU	5 NTU	2.5 NTU
4	Ph	6.5-8.5	7.0-85	7.0-8.5

5	TDS	500 mg/l	500 mgl	500 mgl
6	Hardness	300 mg/l	300 mgl	200 mgl
7	Ca	75 mgl	75 mgl	75 mgl
8	Mg	30 mgl	50 mgl	30 mgl
9	CL	250 mgl	200 mgl	200 mgl
10	Sulphate	200 mgl	200 mgl	200 mgl
11	Fe	0.3 mgl	0.1 mgl	0.1 mgl
12	Nitrate	45 mgl	20 mgl	45 mgl
13	Phenolic compounds	0.001 mgl	0.001 mgl	0.001 mgl
14	Cd, Sc	0.01 mgl	-	0.01 mgl
15	Cu, As	0.05 mgl	0.05 mgl	0.01 mgl
16	Cyanides	0.05 mgl	-	0.01 mgl
17	Pb	0.1 mgl	-	0.01 mgl
18	Anionic detergents	0.2 mgl	-	-
19	PAH	-	-	-
20	Residual Chlorine	0.2 mgl	-	-
21	Pesticides	Absent	-	-

BIS- Beaurau of Indian Standards
ICMR- Indian Council for Medical Research.
WHO- World Health Organization.