SECTION - 2

MATERIALS & METHODS
SECTION - 2

MATERIAL AND METHOD'S

Water sample's were collected from reservoir water, tap water and ground water for one year from September 1995 to August 1996. 12 sampling locations were selected in various localities covering the entire water supply system of Jhansi. The samples were collected at 15 days interval from the same points every time, in well stoppered, sterilized glass bottles for one year. Before collecting water from the tap was allowed to run at least 2 minutes before the samples were collected. The sample's thus collected were analysed in the laboratory within 6 hours of collection. 12 sampling locations so marked in different localities are given below from A to L.


Physico-chemical parameters were analysed according to the methods described by Adoni et al. (1985), I.S. : 3025 (1964) and APHA (1985), Greenberg et al. (1975), Das (1989). Temperature was determined in the field with the help of sensitive thermometer and for PH valves and conductivity analysis portable meter were used. Total Hardness, total alkalinity, chloride contents, free CO₂, dissolved Oxygen were estimated by volumetric titration methods in the lab. Details for the analysis of the physico-chemical parameter's are given below.

1. Colour: Observation for the colour of the tap water were made visually using Sacchi disc.

2. Temperature: Temperature of the water samples were recorded with the help of maximum minimum thermometer (divided from 0° to 50° C and calibrated to 0.1° C) as suggested by Welch (1948), Vyas (1968), Schowerbel (1970), Ganapati (1960), Adoni et al. (1985) and Das (1989). Reading were taken at 11 A.M. and 5.00 P.M. Necessary tables and histograms were prepared accordingly.
3. **Conductivity**. Electrical conductivity of the water samples were determined in the laboratory with the help of Systronic conductivity meter and is expressed in Micromhos. It gave an idea of soluble salts present in the samples. This method was suggested by Welch (1948), Adoni et al. (1985); Das (1989), A P H A (1985), Necessary tables and histograms were prepared accordingly.

4. **pH Value**. pH of the water samples were determined in the laboratory with the help of Systronic pH meter type 321. pH was also determined on the spot colorimetrically with the help of Lovibond comparator box using appropriate B.D.H indicator (Bromo thymol blue, Phenol red). Initially, each sample was tested by adding dropwise B.D.H indicator thereafter the alkalinity and acidity were tested respectively by way of comparing with their standard as referred by Adoni (1985) Das (1989) and APHA (1985).

5. **Chloride Contents**: Chloride was estimated according to Mohr's method titrating 50 ml. of sample by silver Nitrate using Potassium chromate as an indicator APHA (1985). For which 50 ml of water sample in a titration flask was kept over a white paper surface. Thereafter 2-3 drops of potassium chromate solution was added, this gives yellow colour to the sample and it was then titrated with 0.0141 N Silver Nitrate solution until a colour change from pure yellow to brick red end point is reached. Then blank titration was also determined by titrating distilled water in the same way. This helped to chose the end point for the titration. The formula used for calculating the chloride content’s of the water is given below.

\[
\text{Chloride Contents} \quad \text{(Ml of AgNO}_3 \text{ used for sample - for Blank) XNX35 46X1000} \\
\text{mg/L} = \frac{\text{ml of sample}}{N} \quad \text{ml of sample}
\]

\[N = \text{Normality of titrant}\]

6. **Total Hardness**: Total hardness was estimated according to the methods described by Adoni et al. (1985), I.S. 3025 (1964), APHA (1967) and Greenberg (1975) For determining the total hardness of water 50 ml of water sample in a titration flask was kept on white paper. In this solution 1 ml of Buffer
solution (dissolved 13.5 gm Ammonium chloride in 114 ml. Con. Ammonium hydroxide and added 86 ml water to make the volume up to 200 ml.) is added then 2 drops of indicator Eriochrome Black T (dissolved 0.5 gm Eriochrome Black T dye in 100 ml of 80% ethyl alcohol) is added which turned the colour of the sample wine red. Finally it was titrated with the standard EDTA titrant (0.01 m) slowly, with continuous stirring until the wine red colour disappeared from the solution and finally changes to clear blue colour. The reading is then noted and the total hardness is calculated by the following formula.

\[
\text{Total Hardness as } \frac{\text{ml of titrant used \times 1000}}{\text{mg/L } \text{CaCO}_3} = \frac{\text{ml of water sample}}{\text{ml of water sample}}
\]

7. **Total Alkalinity**: Bicarbonate alkalinity together with carbonate alkalinity are called total alkalinity. Method used for determining total alkalinity is the one suggested by Adoni et al. (1985) and APHA (1980). For this 50 ml of sample is taken in 250 ml Erlenmeyer flask. In this two drops of phenolphthalein was added. If pink colour appear then it was titrated with 0.02 N Sulphuric Acid till the colour disappear. Then 2 drops of methyl orange indicator is added this brings the colour yellow. Titration is then continued with the same 0.02 N Sulphuric acid till the colour changes to orange. If the colour does not appear pink after adding phenolphthalein then 2 drops of methyl orange is added and then titrated as above. The reading was then noted and total alkalinity was calculated by the following formula (APHA 1967)

\[
\text{Total Alkalinity in mg/L} = \frac{\text{Total ml. of titrant used \times N \times 50 \times 1000}}{\text{ml. of water sample}}
\]

\[
N = \text{Normality of titrant}
\]

8. **Carbonate Content's**: According to Adoni et al. (1985) carbonate content's is detected by titration method. For this 50 ml of water sample to kept in a conical flask and added with 2 drops of phenolphthalein indicator. If the water sample turned pink, presence of carbonate is indicated. It is then titrated with 0.02 N Sulphuric Acid till the pink colour disappeared and the end point is noted as 'P'
According to Adoni et al. (1985) if ‘P’ is less than half ‘T’ (Total Alkalinity) then ‘P’ value will be double

Carbonate in ml of titrant ‘P’ X N X 50 X 1000
mg/L = -----------------------------------------
ml. of water sample.

if P< 1/2 T Then Carbonate value will be 2 P

9. Bicarbonate Content’s - This method was suggested by Adoni et al. (1985) For testing bicarbonate contents of water 2-3 drops of methyl orange indicator was added to 50 ml same water sample after determining the carbonate contents. Then the sample was titrated with 0.02 N Sulphuric Acid solution Untill the colour changed from yellow to orange. Bicarbonate contents was determined by using the following formula

Bicarbonate total ml. of titrant ‘T’ X N X 50 X 1000
contents in mg/L = -----------------------------------------
ml. of water sample

According to Adoni et al. (1985), when ‘P’ (phenolphthalein alkalinity) = 0. The bicarbonate = T (Total Alkalinity)

and if P<1/2T, Then bicarbonate = T - 2 P

10. Free Carbon-di-Oxide : Free CO₂ was determined by titration method (According to Adoni et al.,1985). To 50 ml of water sample 2 drops of phenolphthalein indicator were added. In case the colour changed to pink, free carbon-di-oxide were taken as absent and when the sample remained colourless its presence was indicated. The colourless solution was titrated with standard 0.02 N Sodium hydroxide titrant and free CO₂ concentration was determined by the following formula
Free CO₂ in mg/l = \[ \frac{\text{ml of titrant} \times N \times 44 \times 1000}{\text{ml of water sample}} \]

\[ N = \text{Normality of titrant.} \]

Both free CO₂ and carbonate have an end point at a common pH of 8.3

11. **Total Carbon-di-Oxide**: As suggested by Adoni *et al.* (1985) and A.P.H.A (1980), total carbon dioxide in water is the sum of free CO₂ and CO₂ existing in the form of carbonate’s and bicarbonates. Total carbon-di-oxide was determined by values of free CO₂ and total alkalinity. Formula used for the calculation of total CO₂ of water sample is as under.

\[ \text{Total CO}_2 \text{ in mg/L} = \text{Mg/L Free CO}_2 + 0.88 (A+B) \]

Where as, \( A = \) mg/L of Bicarbonate alkalinity.
\[ A = \text{mg/L of Carbonate alkalinity} \]

\[ B = \frac{2}{2} \]

12. **Dissolved Oxygen**: The occurrence of dissolved Oxygen in drinking water may be mainly attributed to 2 distinct phenomenon

  1. Direct diffusion from the air.
  2. Photosynthetic evolution by aquatic autotrophs.

Dissolved Oxygen was determined by modified Winkler’s method as given by Adoni *et al.* (1985). Samples were collected from different localities of Jhansi city, were taken to laboratory in plastic bottles for analysis. Through delivery tube connected to the outlet tap of Ruttner’s water sampler, sample were collected in 250 ml BOD glass bottles. Delivery tube was inserted down to the bottom of the bottle and was taken out when the overflow of sample started. The samples were immediately ‘fixed’ for determination of dissolved oxygen by addition of a succession of the three regents: Manganese sulphate, alkaline iodide azide solution, and con. Sulphuric Acid. After carefully removing the stopper of 250 ml sample bottle, then 2 ml of manganous sulphate reagent and 2 ml of alkaline iodide azide reagent were added one after the other to it by means of 2 ml pipette.
dipped to the bottom of the bottle and slowly drawing out as the reagent are added. The stopper was replaced and bottle was inverted 14 or 15 times for a thorough mixing of reagents. Brown precipitate was formed which gradually settles to the bottom.

Now 2 ml of concentrated sulphuric acid was added and shaken to dissolved the brown precipitate. 50 ml of this solution was transferred to a conical flask placed on a white background and titrated with 0.025 N sodium thio sulphate (Na₂S₂O₃) solution. Hypo solution was added drop by drop until the colour turned pale yellow. Then, 2 drops of starch solution was added to impart blue colour and the titration was continued till it becomes colourless. Blank titration was also done to eliminate the error. The dissolved oxygen was determined with the help of the following formula.

\[
8 \times 1000 \times N = \frac{\text{Dissolved Oxygen in mg/L}}{V} \times v
\]

\[
N = \text{Normality of the titrant}
\]

\[
V = \text{Volume of sample}
\]

\[
V = \text{Volume of titrant used}
\]

13. **Biochemical Oxygen Demand (B.O.D.)** - Biochemical Oxygen demand is the amount of dissolved oxygen required in milligrams per litre for stabilizing the biodegradable organic matter by microorganisms of the sample under aerobic condition’s. B.O.D was determined by standard method APHA (1985). It was measured by incubating the sample at 20 °C for 5 days. Samples were collected in six 250 ml. B.O.D. glass bottles without bubbling. Then added with 1 ml of 0.05% allyl thio urea solution (Dissolved 500 mg. of allyl thio urea in a litre of B.O.D. free water) to each bottle. Dissolved oxygen was determined of three bottles by modified Winkler’s method. In this method 2 ml manganous sulphate solution (Dissolved 182 gm. MnSO₄. H₂O in distilled water and diluted upto 500 ml) and 2 ml of alkaline iodide azide solution (Dissolved seperately 300 gms potassium hydroxide and 75 gm. potassium iodide in distilled water and diluted.
upto 500 ml and dissolved seperately 5 gms sodium azide (NaN₃) in 20 ml. distilled water then both solution were added to the alkaline iodide reagent) were added one after the other, then stopper was replaced and mixed thoroughly, when brown precipitate was formed, then 2 ml of con. H₂SO₄ was added to dissolve the precipitate. Then 50 ml of solution was transferred in stoppered flask and titrated with 0.025 N sodium thio sulphate solution (Dissolved 6.205 gm sodium thio sulphate in freshly boiled and cooled distilled water and diluted upto 3 litre then added one pellet of NaOH for preservation). Blank titration was performed for accurate readings. This was “D₁” initial dissolved oxygen, the remaining three bottles were incubated in B.O.D. incubator at 27 °C for 3 days. After 3 days incubation oxygen concentration of these samples were also estimated as described above. This was “D₂”. B.O.D. were estimated with the help of the following formula.

Biochemical Oxygen demand in mg/L = D₁ - D₂

Where, D₁ = Initial D. O in the sample

D₂ = D O after 3 days incubation.

**Phytoplankton Collection & Analysis**

Phytoplankton are chlorophyll bearing suspended microscopic organisms. Plankton sample’s were collected at fort-night intervals from 11 sampling locations of Jhansi City. The time of collection varied between 9 A.M. to 12 Noon.

For quantitative and qualitative studies, 50 litres of water was passed through the plankton net. Plankton sample of one litre water was taken in a glass bottle and 10 ml. Lugols iodine (dissolved 10 gm. potassium iodide in 20 ml distilled water then 5 gm. iodine was added to it after then 50 ml. of water and 5 gm Sodium acetate were added and mixed it) was added to it and allowed to stand for 24 hours. Clean liquid was taken out with the help of pipette and
remaining concentrated 10 to 100 ml. sample was kept for the estimation of phytoplankton

During the preliminary investigations counting was done by Drop Count Method as described by A.P H A 1980 and Adoni et al (1985)

One drop of thoroughly shaken plankton concentration was put on the microslide with the help of standard dropper held vertically and a suitable size coverglass was applied. The microslide was then fixed under the microscope on moving stage focused one edge of the coverglass. Species-wise counting of the phytoplankton was done, then this process was repeated again for other edges and observed the whole coverglass. Organisms per liter of water sample were calculated by the following formula

\[
\text{Organisms/Litre} = \frac{A \times X1}{L \times n \times V}
\]

Where,  
\(A\) = Number of organisms per drop
\(V\) = Volume of one drop (0.05 ml).
\(n\) = Total volume of the concentrated sample (ml.)
\(L\) = Volume of original sample.

Estimation of the plankton were also made with the help of a "haemocytometer" and the number's were expressed in per litre of the sample.

The planktonic net used was cone - shaped with a mouth of a diameter of 26 cm and length of 50 cm. Planktonic net made of silk No. 25, mesh size 64, micron i.e 200 thread per inch and lower end was fitted into a detachable glass tube of 50 ml. capacity. Sample's were preserved in 5% formaline to which a little of glycerins was also added.
The systematic identification of the phytoplankton was done with the help of standard works ie - Adoni et. al. (1985), Deshikachari(1965)

**Quantitative Estimation of Bacteria**

Total viable microorganisms and detection of coliform organisms specially *E. coli* was estimated by the standard plate count (SPC) method. In this method water samples were collected in pre-sterilized bottles 1 ml. each of undiluted and diluted (1·10, 1·100, 1·1000) samples were transferred in separate petri plates in triplicate then 10 ml of Lukewarm Agar medium was added in each petridish and contents of the petriplate, was mixed by gentle circular movements. After some time medium gets solidified then each petriplate was incubate at 37 o c. for 2 days in an incubator. Colonies were counted using “Qubec Colony Counter”. SPC per millilitre of sample was calculated with the help of the following formula.

Standard Plate Count per ml = Average no.of Colonies per plate X Dilution.

**Standard Bacteriological test of Water**

Too much reliance can not be made upon a single bacteriological analysis One should be aware of the condition of the area from which the sample originates. Some pathogenic forms may enter in water from sewage outlets thus attempt should be made to isolate the pathogenic forms. The pathogenic forms are relatively difficult to be isolated specially from over loaded waters. There procedure for cultivation and identification are time consuming and difficult as to isolate one cell of typhoid organism would be difficult from a mixed population. At a very low concentration of such organism in water supply might cause serious Typhoid out break Thus if an index organism *Escherichia coli* is sought out, which are found in high number in the intestinal wastes, one can say that water is polluted by enteric organisms. The standard technique to determine the presence of pollution specially to sewage is to look for *Escherichia coli*.

*Escherichia coli* is a short gram negative, non-spore forming rod that ferments lactose, with both acid and gas production. Bacteriological water analysis envolves three major steps.
A. **Presumptive Test**: When tubes of Lactose Broth are inoculated with water and incubated the production of acid and gas through the break down of lactose leads to the presumption that coliform bacteria are present in water. Coliform include all species of the genera *Escherichia* and *Aerobacter*. Aerobacter is generally of soil or plant origin. Faecal sample may harbour them in about 10% of the samples. *Escherichia coli* is mainly of faecal origin. Two or more species of bacteria by interaction may also yeild acid and gas from lactose due to synergism. For this reason, it is important that the above presumptive test is subjected to further confirmation.

B. **Confirmed Test**: Selective media containing substances bacteriostatic for gram-positive bacteria are used so that the gram-negative species present in the presumptive lactose broth tube will have a better opportunity to develop. Endo Agar, Eosin methylene blue agar, may be used to confirm the presence of coliform as the cause of positive presumptive test. Coliform appear as coloured colonies with or without a metallic like lustre on the surface, when examined in reflected light.

C. **Complete Test**: Having isolated the suspicious looking colonies on the selective medium the next step is to select a colony and inoculate in a fresh tube of lactose broth and on standard nutrient agar. If this pure culture ferments the sugar with acid and gas production, and if a gram stain of the agar colony reveals short gram negative rods it is confirmed that water contained coliforms.

Additional test were done to further pin point and acertain the organism. These test are for convinience reffered as IMViC reactions. l-reffers to Indole test, M-methyl red test; V-refers to Voges Proskauer test and C-citrate test. The small letter 'i' is inserted purely to make the word more pronounceable and does not refer to any particular test. A typical stain of *Escherichia coli* is therefore IMViC + + - - while a typical *Aerobacter aerogenes* is IMViC - - + +. There are 14 other
combination for these 4 reactions and the organism enhabiting in between reactions are called 'Intermediates'.

M.P.N. Valuation in Water:

MPN denotes the probable number of coliform organisms present in 100 ml of the water sample. For this, sample were inoculated in, 5 tubes of 1:10 dilution in double strength of MacConkey Broth, 5 tubes of 1:100 and 5 tubes of 1 1000 dilution in single strength MacConkey Broth. Then inoculated tubes were incubated at 37 °c. in an incubator for 2 days and examined each tube at the end of 24 and 48 hours for the formation of acid and gas production.

Statistical Processing Of The Data

Data collected was statistically analysed with the help of Standard formula given in 'Standard Statistical Work' of Schefler (1969).

**Mean** - Mean is 'the sum of all the members of a distribution divided by the number of members in that distribution' and was calculated with the help of the following formula:

\[
\bar{X} = \frac{\sum x}{N}
\]

**Standard Deviation** - Standard deviation is the 'square root of the sum of squared deviation's from the mean' and was calculated by the following formula.

\[
SD = \sqrt{\frac{\sum x^2 - (\sum x/N)^2}{N-1}}
\]

**Standard Score** - Standard score is 'the number of standard deviation's above or below the mean of a distribution' and was calculated by substituting the obtained values in the following formula:

\[
Z = \frac{x - \bar{X}}{Sd}
\]
**Standard Error** - Standard error is the relation of standard deviation of a distribution with respect to square root of number's in the distribution and hence can also be called as standard error of the mean and was calculated by the following formula

\[
SX^- = \frac{SD}{\sqrt{N}}
\]

**Variability** - Variability or range of mean in the population often called as estimation is the mean range on a particular confidence limit in the given distribution and was calculated with the following formula

\[
Va = X^- \pm SD(Z).
\]