Material and Methods
METHODOLOGY

The present study is divisible into Fields Observations and Pot Experiments. Field observations and Experiments.

The present study was carried out in 30 villages in Devli Tehsil of Tonk district of Rajasthan state. Water sample were collected from different sources viz tube wells, hand pumps, open wells and PHED supply from randomly selected village and stored in polythene bottle instead of glass bottle so that there may not be any change in the concentration of fluoride.

Water sample were periodically collected from thirty different sites of area in cleaned polyethylene bottles. These samples were brought to the research laboratory of Indira Gandhi Centre for Human Ecology, Environment and Population Studies, University of Rajasthan, Jaipur. The main focus was on the fluoride distribution in the villages but other physico-chemical parameters were also analyzed such as pH, Total Dissolved Soiled, Electrical conductivity, Alkalinity, Total Hardness, Calcium hardness, Chloride, Fluoride by using water analysis kit. Beside the soil sample and crop material were collected from fluoride rich areas and analyzed for fluoride concentration. An intensive field survey was carried out to assess the impact of high fluoride contents on human health.

*Hordeum vulgare* variety RD 2052, *cicer arietinum*, variety RSG 888 and *Triticum aestivum* variety Raj 3077 Crop plants have been selected for the study of effect of fluoride on their different parameters.

**Water Analysis**

**pH (Potential hydro genii)**

pH is negative log 10 of Hydrogen ion concentration in solution. Ph of water sample were analyzed by digital pH meter (model No.161 E).

**Regents**

Standard buffer solution of pH 4.00, 7.00 and 9.2 buffer tablets were dissolved in distilled water and diluted to 100ml.
Procedure

Standardize the instruments with electrode immersed in a buffer solution. Electrode was removed from buffer, rinsed and blotted and then immersed in a second buffer. For the sample and analysis, electrode was placed in the sample and the reading was noted on the meter.

Electrical Conductivity

Electrical conductance is the ability of a substance to conduct the electric current. Electrical conductivity of the sample was determined with the help of digital conductivity meter (Model No. 161E).

Total Dissolved Solids

Total dissolved Solids are determined as the residue left after evaporation of the filtered sample. The total dissolved solid in drinking water reveals the saline behavior of the water which indicate the organic pollution level of water. Total dissolved solid of the sample were determined with the help of water analysis kit (Model No. 161 E).

Alkalinity

Alkalinity is the measure of the capacity of the water to neutralize a strong acid.

Reagents

1. Sulfuric acid (H₂SO₄) (0.02N)
2. Methyl orange indicator
3. Phenolphthalein indicator
4. NaOH
5. Na₂CO₃

Procedure

25ml. of sample was taken in a conical flask and 1-2 drops of Phenolphthalein indicator was added. If no colour was produced, the Phenolphthalein alkalinity was absent. If the colour changes to pink after addition of Phenolphthalein, it was further titrated with 0.02N H₂SO₄ I until the colour disappeared at end point. This is
Phenolphthalein alkalinity. Total alkalinity was determined by 2-3 drops of methyl orange to the sample and titration was further continued until the yellow colour changes to pink at end point. This is alkalinity.

25ml of sample was taken in a conical flask and 1-2 drops of phenolphthalein indicator was added.

\[
\text{Alkalinity (mg/L as CaCO}_3\text{)} = \frac{\text{Reading} \times 5N \times 50 \times 1000}{\text{ML. of Sample}}
\]

Where

\(N\) = Normality of H\(_2\)SO\(_4\) used.

**Chloride**

Chloride in water samples were determined by Argento metric titration method. Silver nitrate reacts with chloride to form very slightly soluble white precipitate of AgCl. At the end point when all chloride get precipitated free silver ions react with chromate to form silver chromate of radish brown colour. High content of chloride gives salty taste to water.

**Reagents**

1. AgNO\(_3\) (0.02N) – 3.40g of dried AgNO\(_3\) (A.R.) was dissolved in distilled water to make one liter of solution and kept in a dark bottle.
2. Potassium chromate (5%) – 5g of K\(_2\)CrO\(_4\) was dissolved in 100 ml. of distilled water.

**Procedure**

25ml of sample was taken in a conical flask and 1ml of K\(_2\)CrO\(_4\) solution was added. This solution was titrated against 0.02N silver nitrate until a persistent brick red end point appeared.

\[
\text{Chloride (mg/L)} = \frac{\text{Volume of AgNO}_3 \times \text{Normality of AgNO}_3(0.02N) \times 35.5 \times 1000}{\text{Volume of Sample}}
\]
Material and Methods

Total Hardness

Hardness is generally caused by the calcium and magnesium ions present in water. Calcium and Magnesium form a complex of a wine red colour with Eriochrome Black T at pH of 10.0 ± 0.1. The EDTA has got a stronger affinity towards Ca$^{2+}$ and Mg$^{2+}$ and therefore by addition of EDTA the former complex is broken down and a new complex of blue color is formed.

Reagents

1. EDTA Solution (0.01M)-3.723g of disodium salt of EDTA was dissolved in distilled water prepare one liter of solution and stored in polyethylene bottle.
2. Buffer solution-
   a. 16.9g Ammonium chloride (NH$_4$Cl) was dissolved in 143ml of concentration ammonium hydroxide (NH$_4$OH).
   b. 1.179g of disodium EDTA and 0.780g of MgSO$_4$.H$_2$O Were dissolved in 50 ml distilled water Both (a&b) solution were mixed and diluted to 25ml with distilled water
3. Eriochrome Black T (Solochrome Black T) Indicator – 0.04 g of Eriochrome Black T was mixed with 100g NaCl (A.R.) and grinded.
4. Sodium Sulphaide solution– 5.0g of Na$_2$S.9H$_2$O or Na$_2$S.5H$_2$O was dissolved in 100ml of distilled water, Bottle was tightly closed to prevent oxidation.

Procedure

50 ml. of water sample was taken in a conical flask is taken and 1 ml of buffer solution and a pinch of Eriochrome Black T indicator was added, the solution turned wine red colour. The solution was titrated against EDTA solution until the wine red colour changes to blue at the end point.

\[
\text{Hardness as (mg/L) } \text{CaCO}_3 = \frac{\text{ml of EDTA used x 1000}}{\text{ML of sample}}
\]

Calcium Hardness

EDTA is having affinity calcium the former complex is broken down and a new complex is formed. However EDTA has a property to combine with calcium.
Reagents
1. EDTA solution (0.01M) – 3.723g of disodium salt of EDTA was dissolved in distilled water to prepare 1 litre of solution and stored in polyethylene bottle.
2. Sodium Hydroxide, (1N)-80g of NaOH was dissolved in distilled water and diluted to 1 litre.
3. Murexide Indicator- 0.2g of Ammonium purpurate was added to 100g of NaCl (A.R.) and grinded.

Procedure

50 ml of sample water was taken in a conical flask 2 ml NaOH solution was added. Then one pinch of Murexide indicator was added. The solution turned pink colour. Solution was titration against 0.01M EDTA until the pink colour changed to purple.

\[
\text{Calcium Hardness as CaCO}_3 \text{ (mg/lit).} = \frac{\text{Volume of EDTA solution} \times 1000 \times 1.05}{\text{Volume of sample (ml.)}}
\]

Estimation of Fluoride Ion

Fluoride concentration in water, soil and plants sample were analyzed with the help of Mettle Toledo MA 235 pH/ion Analyzer was used. The fluoride ion selective electrode measures the ion activity in solution rather than concentration. Fluoride ion activity depends on the solution total ionic strength and pH and on fluoride complexing species. Adding an appropriate buffer provides a nearly uniform ionic strength back ground. Adjusts pH and break up complex so that, in effect, the electrode measures concentration.

Regents
1. Stock Fluoride Solution 0.221 gm anhydrous sodium fluoride ( NaF ) was dissolved in distilled water and diluted to 1000 ml. 1.00ml=100µgF
2. Standard fluoride Solution: 100 ml stock solution diluted to 1000 ml with distilled water. (1.00ml= 10 µgF). Series of standard fluoride was prepared by serially diluting this solution.
3. Fluoride Buffer : Approximately 500 ml distilled water was placed in a one liter beaker and 57 ml glacial acetic acid 0.58g sodium chloride (NaCl) and
Material and Methods

4.0g cyclohexylenediamine tetraacetic acid (CDTA) was added and stirred to dissolve. After that 6N NaOH (about 125 ml) was added with stirring, until pH is between 5.0–5.5. Solution was transferred to a one liter volumetric flask and distilled was added to the mark.

Procedure

1. 25ml of 3ppm fluoride standard solution was taken and 25 ml TISAB (Total Ionic Strength Adjustment Buffer) was added to it and instrument was calibrated.
2. Similarly instrument was calibrated with 3ppm, 6ppm, 9ppm, 12ppm, 15ppm and 18ppm standard fluoride solution.
3. Electrode slopes if the ion meter (56-60mV) for monovalent ions at 25°C was checked.
4. 25ml of water sample was taken and 25ml TISAB was added to it.
5. Rinse electrode, blot dry and place in the sample.
6. Stir thoroughly and steady reading on the meter was noted.
7. Recalibrated the instrument after every 3 hours.
8. Temperature of standard and samples should be same, preferably room temperature.
9. Electrode should be rinse with distilled water and blot dry between readings.

SOIL ANALYSIS

Soil sample was collected from agricultural fields of the study area periodically. Analysis of soil sample from field and pot experiments was done as follows:

Soil suspension was prepared by dissolving 20 gm of soil in 100 ml of distilled water. The suspension was stirred for 30 min and then the supernatant was filtered and then transferred to a beaker. Then three parameters pH, EC and chlorides was estimated from it.

pH

pH is the negative logarithm of Hydrogen ion concentration. It was determined by potentiometric measurement using a standard sensing electrode (glass electrode) and a reference electrode (Calomel electrode) (Model no.161E). Before
pH measurement, two electrodes were placed in a solution of known pH. This is called Standardization. Then, electrode was dipped into distilled water and blot dry. Then electrode was dipped into filtered soil suspension and we were note down the pH value.

**Electrical Conductivity**

Electrical Conductivity is the capacity of ions to carry electric current usually measured in (µmho/cm). It was measured by Conductivity meter (Model no.161E). Conductivity meter is calibrated by setting knob to the standard value of 1.03. Then cell was washed with distilled water. Then we were dry blot it and dip the cell into filtered soil suspension and was note down the reading for EC in µmho/cm.

**Chloride**

Chloride in the soil samples were determined by titration method 20 ml of filtrate was taken in a flask. To this of samples, 0.5 ml of 5% potassium chromate (K₂CrO₄) was added as an indicator and the solution was then titrated against silver nitrate (AgNO₃) solution of 0.0141N, change in color to brick red was considered as end point and the calculation was made accordingly using the formula given below.

\[
\text{Chloride (mg/100gm of soil)} = \frac{\text{Volume of AgNO}_3 \times \text{Normality of AgNO}_3 \times 0.02N \times 35.5 \times 1000}{\text{Volume of Sample} \times 5}
\]

**Organic Matter (Walkley - Black Method 1934)**

Organic matter in the soil samples were determined by modified Walkley-Black Method (1934) rapid dichromate oxidation technique. 5 gm oven dried soil was taken in a 500ml conical flask. To these sample 10 ml of potassium dichromate (K₂Cr₂O₇) and 20 ml. of sulphuric acid (H₂SO₄) was added. The solution was allowed to digest for 30 minutes. To the solution now, 10 ml of ortho-phosphoric acid (H₃PO₄), 200ml of distilled water and 1 ml diphenylamine as an indicator was added as a result deep blue color developed, the solution was taken titrated against ferrous ammonium sulphate till the color changed to emerald green at the end point.
Calculation was made according to the following formula,

\[
\text{Organic C\%} = \frac{(B - T) \times 0.003 \times 100}{\text{Wt. of soil}}
\]

Where,

- \(B\) = Vol. of ferrous ammonium sulphate solution required titration of blank.
- \(T\) = Vol. of ferrous ammonium sulphate solution required titration of soil sample
- \(W\) = Weight of soil sample
- Actual C\% = Organic C\% \times 1.3
- Organic matter\% = Actual C\% \times 1.724
  (here 1.724 = Van Bommden Factor).

**Fluoride Estimation**

**Digestion:** Soil samples were separately packed and oven dried for 24 hrs at 80°C. Then the samples were powdered and digested with perchloric acid, followed by neutralization with aqueous KOH and analysis for fluoride by the potentiometric method with a fluoride ion selective electrode.

**PLANT ANALYSIS**

Plant material was collected from agricultural fields of the study was periodically. Analysis of plant material from field and pot experimental was done as follows.

**Plant Length**

All the five plants from each field and pot of five replicates were taken out length of root, shoot, ears and pods of these plants were measured by meter scale from the soil surface to the base of the fully expanded top leaf at each harvest. Average was calculated on the basis of number of plants replicates.

**Biomass Estimation**

For dry weight a determination individual plants were carefully removed from pots and field keeping the root, shoot, Grains and seeds system intact. Plant roots were thoroughly washed in running water to remove soil particles. Roots, shoots,
Grains and seeds were separated and dried in the oven at 80oC for 48 hours then their dry eight were reduced. These weights were expressed in gram.

**Chlorophyll Estimation**

Samples of known weight (1 gm) of fresh leaves of individual plant species was macerated thoroughly in pestle mortar with a little and 80% acetone (Acetone: Distilled water :: 80: 20). The suspension of macerated material in acetone was centrifuged 2000 rpm for 30 minutes. Supernatant solution was transferred to a volumetric flask and made up to 25 ml. The optical density of the solution was measured by spectrophotometer at 645nm and 663 nm.

Following formulae was used to calculate the amount of chlorophyll a and b (Arnon 1949):

\[
\text{Chlorophyll a mg/l} = 12.7 \times A_{663} - 2.69 \times A_{645}
\]

\[
\text{Chlorophyll b mg/l} = 22.9 \times A_{645} - 4.68 \times A_{663}
\]

\[
\text{Chlorophyll (a+b) mg/l} = 8.02 \times A_{663} + 20.20 \times A_{645}
\]

Where

\[A_{645}\] = Absorbance at 645nm

\[A_{663}\] = Absorbance at 663nm

The amount of chlorophyll was calculated in mg/l fresh leaves material.

**Plant Nitrogen and Protein Content**

Microkjeldahl method (Kjeldahal, 1883) was used for the estimation of plant nitrogen. 0.5 gm of the plant sample (coarsely ground) was digested for about 90 min. in 3.5 ml of conc. H$_2$SO$_4$ and 2.0 gm of catalytic mixture (K$_2$SO$_4$ + CuSO$_4$·5H$_2$O + Se). The digested sample upon cooling was distilled in the Kjeldahl flask, along with 100 ml of NaOH (40%) and few granules of Zn. The distillate was collected in 2.5 ml of boric acid cum indicator solution, which in turn was titrated against 0.01N HCl, till colour changes to pink indicating the end point of titration.
The amount of nitrogen was calculated as follows:

\[
\% \text{ Nitrogen} = \frac{(T-B) \times N \times 1.4}{S}
\]

Where:
- \(T\) = Sample titration, ml.
- \(B\) = Blank titration, ml.
- \(N\) = Normality of titrant (0.01N HCl).
- \(S\) = Weight of plant material, gm.

The protein content was calculated as follows:

\[
\% \text{ Protein} = \% \text{ nitrogen} \times 6.25
\]

**Carbohydrate Estimation**

Standard Anthrone method was used for the estimation of carbohydrates. Weigh 0.1 gm of dried leaf sample was hydrolyzed by keeping it in a water bath with temperature 80 – 100°C for three hours with 5 ml of 2.5N HCl and cools it to room temperature. Neutralized with solid sodium carbonate until the effervescence cease. The extract was centrifuged and then volume was made up to 100 ml. 1ml aliquots were taken for analysis. 4 ml of Anthrone reagent was added to each sample and heated for eight minutes. Aliquot was cooled rapidly to room temperature. Green colour was developed. Absorbance absorbance (O.D.) was observed at 630nm using UV-VIS Spectrophometer SHIMADZUV mini-1240.

Standard glucose solution was used for standard graph, stock glucose solution was prepared by adding 0.1 g of glucose in 100 ml of distilled water. Taking 10 ml of the stock solution and making it to 100 ml was prepare working standards. From this working standard, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the solution was poured into a series of test tubes and the volume was made up to 1.0 ml in each by adding distilled water. Absorbance was recorded at 630 nm and a standard graph was drawn by plotting concentration of the standards on the x-axis versus absorbance on y-axis was prepared to calculate the amount of Carbohydrate.

Following formulae was used to calculate the amount of carbohydrate (mg/gm):

\[
\text{Absorbance of Carbohydrate (mg/gm)} = \frac{\text{Absorbance at 630nm X 100}}{\text{Volume of test sample}}
\]
Plant Phosphorus

Phosphorous content of plant was determined by stannous chloride ammonium molybdate method.

Reagents
1. Stock phosphate solution - Dissolve 0.711gm anhydrous KH$_2$PO$_4$ and dilute to 1 liter; 1 ml= 0.5mg PO$_4$/L.
2. Phosphate working solution- Solution dilutes 100ml of stock solution to 1 liter; 1ml=0.05 PO$_4$/L.
3. Ammonium molybdate solution= Dissolve 2.5gm ammonium molibdate in 17.5 ml of double distilled H$_2$O cautiously add 28ml of cons.H$_2$SO$_4$ to 40ml of double distilled H$_2$O Cool add molybdate solution and dilute to 100ml.
4. Phenolphthalein indicator solution = Dissolve 1gm of phenolphthalein in 100ml of 95% ethyl alcohol and add 100ml of double distilled H$_2$O.
5. Stannous chloride solution I = Dissolve 2.5gm of fresh SnCl$_2$.H$_2$O in 10ml glycerol. Heat in a bath and Stir with a glass preservation non special storage.
6. Dilute stannous chloride reagent II = Mix 8ml stannous chloride reagent I with 50ml glycerol. This reagent stable for at least 6 months.

Procedure
Stannous chloride ammonium molybdate method was used for the estimation of plant Phosphorus Estimation. 0.25gm of the plant sample(Coarsely ground) was digested for about 30 minute in 9ml of concentrated HNO$_3$ and 3ml of concentrated HCL. Digestion temperature was 100°C after one hour we make up the solution up to 100ml by distilled water. Then we add 4ml ammonium molybdate and 5-10 drops of SnCl$_2$ to it at a last we analyzed by Colorimetric 690nm.

Fluoride Estimation

Digestion: Plant samples were separately packed and oven dried for 24 hrs at 80°C. Then the samples were powdered and digested with perchloric acid, followed by neutralization with aqueous KOH and analysis for fluoride by the potentiometric method with a fluoride ion selective electrode.
Genotype of the Species

1. *Hordeum vulgare* variety RD 2052

   Common Name is Barley
   
   Kingdom - Plantae
   Order - Poales
   Family - Poaceae
   Sub-Family - Pooidae
   Tribe - Triticeae
   Genus - *Hordeum*
   Species - *Hordeum vulgare*
   Edible Part - Ear
   Sowing - November
   Maturity - March

Experimental design for the effect of Fluoride of *Hordeum vulgare* variety RD 2052 test species, through pot experiment.

<table>
<thead>
<tr>
<th>Treatment Levels</th>
<th>Distilled water (Control), 3ppm, 6ppm, 9ppm, 12ppm, 15ppm, 18ppm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency of fluoride treatment levels</td>
<td>Daily</td>
</tr>
<tr>
<td>applied</td>
<td></td>
</tr>
<tr>
<td>Number of fluoride treatment events</td>
<td>105</td>
</tr>
<tr>
<td>Site of application of fluoride treatment levels</td>
<td>Soil surface in the pot</td>
</tr>
<tr>
<td>Mode of application</td>
<td>Poured in the pot</td>
</tr>
<tr>
<td>Duration of the experimental treatment</td>
<td>105 days</td>
</tr>
<tr>
<td>Number of harvest</td>
<td>Total three harvest - Pre-flowering, Peak-flowering and Post-flowering</td>
</tr>
<tr>
<td>Individual pot size</td>
<td>12 inches diameter</td>
</tr>
<tr>
<td>Population of plants maintained per pot</td>
<td>5</td>
</tr>
<tr>
<td>Plant harvest per harvest per level</td>
<td>5</td>
</tr>
</tbody>
</table>
### Experimental design for the effect of Fluoride of *Cicer arietinum* variety C888 test species, through pot experiment.

<table>
<thead>
<tr>
<th>Treatment Levels</th>
<th>Distilled water (Control), 3ppm, 6ppm, 9ppm, 12ppm, 15ppm, 18ppm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency of fluoride treatment levels applied</td>
<td>Daily</td>
</tr>
<tr>
<td>Number of fluoride treatment events</td>
<td>120</td>
</tr>
<tr>
<td>Site of application of fluoride treatment levels</td>
<td>Soil surface in the pot</td>
</tr>
<tr>
<td>Mode of application</td>
<td>Poured in the pot</td>
</tr>
<tr>
<td>Duration of the experimental treatment</td>
<td>120 days</td>
</tr>
<tr>
<td>Number of harvest</td>
<td>Total three harvest- Pre-flowering, Peak-flowering and Post-flowering</td>
</tr>
<tr>
<td>Individual pot size</td>
<td>12 inches diameter</td>
</tr>
<tr>
<td>Population of plants maintained per pot</td>
<td>5</td>
</tr>
<tr>
<td>Plant harvest per harvest per level</td>
<td>5</td>
</tr>
</tbody>
</table>
3. *Triticum aestivum variety Raj 3077*

Common Name is Wheat

Kingdom - Plantae
Order - Poales
Family - Poaceae
Sub-Family - Pooideae
Tribe - Triticeae
Genus - Triticum
Species - Triticum aestivum
Edible part - Ear
Sowing - November
Maturity - March

**Experimental design for the effect of Fluoride of *Triticum aestivum variety Raj 3077* test species, through pot experiment.**

<table>
<thead>
<tr>
<th>Treatment Levels</th>
<th>Distilled water (Control), 3ppm, 6ppm, 9ppm, 12ppm, 15ppm, 18ppm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency of fluoride treatment levels applied</td>
<td>Daily</td>
</tr>
<tr>
<td>Number of fluoride treatment events</td>
<td>125</td>
</tr>
<tr>
<td>Site of application of fluoride treatment levels</td>
<td>Soil surface in the pot</td>
</tr>
<tr>
<td>Mode of application</td>
<td>Poured in the pot</td>
</tr>
<tr>
<td>Duration of the experimental treatment</td>
<td>125 days</td>
</tr>
<tr>
<td>Number of harvest</td>
<td>Total three harvest- Pre-flowering, Peak-flowering and Post-flowering</td>
</tr>
<tr>
<td>Individual pot size</td>
<td>12 inches diameter</td>
</tr>
<tr>
<td>Population of plants maintained per pot</td>
<td>5</td>
</tr>
<tr>
<td>Plant harvest per harvest per level</td>
<td>5</td>
</tr>
</tbody>
</table>