CHAPTER 4

MATERIALS AND METHODS
Nitrogen Mineralization:

Production and consumption of $N_{\text{min}}$ occur simultaneously in the soil. The amount of $N_{\text{min}}$ present in the soil at any time shows only the balance between the two processes and does not indicate the rate of supply to the vegetation. To determine the production of $N_{\text{min}}$, it is necessary to exclude to consumption and subsequently estimation of $N_{\text{min}}$ over a definite time interval is required.

Hesselman (1917) was the first to attempt the comparison of mineral nitrogen production in different forest soils by introducing the method of storing soil material under standardized condition. This “incubation method” made it possible to carry out comparative research at several sites in a short time. Modification of this method have been introduced by Ehrhardt (1959), Eno (1960), Ellenberg (1964) and Foguelman (1966) in order to achieve a near approximation to natural condition by on-site storage of the soil samples.

In the present investigation the principle of the method introduced by Ellenberg (1964) was used, soil samples were taken periodically during different seasons of the year and stored at the site for six weeks. The samples were kept in polyethylene bags to prevent penetration of roots and leaching of mineral nitrogen. The method was adopted after Millilo (1977), Nedlhoffer et al. (1984), Gordon (1986).

Treatment of Samples:

Mineralization was investigated to a depth of 20-30 cm depending upon the site conditions. Three different horizons were recognized viz., litter layer (L), partially decomposed layer (D) and mineralized layer (M). On each sampling date
three single samples, each from an area of 20x20 cm. were taken and mixed by hand in a plastic bowl. Roots were removed as far as possible.

One part of each mixed sample was brought of the laboratory and the mineral nitrogen and water content were determined immediately. Another part was placed in a polyethylene bag (ca 20 x 32 cm) to about 1/3 of the bags were knotted tightly, to leave as little air inside as possible and subsequently replaced in the layer from where the sample was taken. The bags were placed as flat as possible so that to experience the natural condition of that horizons.

On site storage is usually effected by enclosing the soil samples in polyethylene bags, since these prevent both the absorption by roots and leaching by N_{min} while at the same time neither nitrification nor mineralization are impaired (Eno, 1960; Van schreven 1968; Runge 1970). This method was examined by Gerlach (1973, 1978) for its suitability for ecological investigations, allows approximation to natural temperature and moisture conditions. However, this implies a deviation from fully natural conditions and therefore, such determinations can only be interpreted with restrictions (Runge, 1983).

**Net nitrogen mineralization and nitrification**:

The N_{min} formed by the mineralization of organic compounds is utilized by both the microorganisms and by higher plants. Therefore by excluding the absorption by higher plants, the N_{min} which is estimated is the amount that is left behind after the consumption by microorganism. The difference between the content of mineral
nitrogen at the beginning and at the end of 6 weeks (on site incubation period) gives a measure of net mineralization. However, as the variable condition are optimally chosen, the results can be characterized as potential mineralization (Runge, 1983). In the present study, net and potential mineralization are used as synonyms.

Total mineral nitrogen has been estimated both by adding NH$_4$-N and NO$_3$-N. Nitrification is the amount of production of nitrate only. Therefore, in the present study the difference in the production of nitrate before and at the end of incubation has been considered as the net nitrification.

**Analysis:**

For the analysis, the soil was first passed through 6mm mesh sieve, 40g of the sieved soil was weighted in to a 300ml erlenmeyer flask and 80ml of a 1% KAl(SO$_4$)$_2$ was added. After shaking for half an hour the suspensions were filtered into 100ml erlenmeyer flask. The filtrate was either used immediately or stored in a refrigerator at about 6°C.

All the methods of analysis were followed after Allen *et al* (1974), Bremner (1965), Robinson (1975) and Chang (1978). Ammonium and nitrate nitrogen was measured by the distillation method, described by Bremner and Edwards (1965) and Bremner and Keeney (1966).

**Ammonium nitrogen (NH$_4$-N):**

40g of sieved soil was extracted with 80ml of 1% KAL (SO$_4$)$_2$ and filtered through whatman filter paper no. 42 after shaking for half an hour.
Kjeldhal’s distillation assembly was used in the present study, 20ml of aliquot (filtrate) was taken in 100ml kjeldhal flask and a pinch of magnesium oxide powder was added. 10ml of distillate was collected in 50ml conical flask containing 20ml of 2% boric acid indicator solution. Titration is done against N/200 H₂SO₄ till a pale colour end point. Blanks are run in similar way and substraction from sample titre.

\[
\frac{e}{a \times 0.07} = \frac{(d \times \frac{100}{b} + c) \times \frac{100}{b}}{100+e}
\]

\[
\text{NH₄-N (ppm)} = \frac{100}{d \times \frac{100}{b}} \times 1000
\]

\[
a = \text{Titrant (H₂SO₄) used ml.}
\]

\[
b = \text{Aliquot (sample used for analy ml)}
\]

\[
c = \text{Extractant (K₂ Al(SO₄)₂ solution)}
\]

\[
d = \text{Fresh wt. of soil}
\]

**Nitrate nitrogen (NO₃-N):**

Distillation procedure and other method are similar as for determination of NH₄-N. After taking out the distillate for NH₄-N, a pinch of finely powdered Devarda’s alloy was added to the same aliquot and distillate was collected in 50 ml conical flask containing boric indicator solution. Similarly it is titrated against N/200 H₂SO₄.
\[
e \quad a \times 0.07 \\
(d \times \frac{\text{---------} + c}{100+e}) \times \frac{b}{100} \\
\text{NO}_3\text{-N (ppm)} = \frac{\text{---------}}{100} \times 1000 \\
\text{d} \times \frac{\text{------}}{100+e}
\]

Total mineral nitrogen is determined by adding both NH$_4$-N and NO$_3$-N.

**Total Nitrogen:**

500mg of air dried sample was digested with 5ml of con H$_2$SO$_4$ in presence of copper sulphate- potassium sulphate-selenium dioxide. The digested aliquot is diluted with distilled water to 100ml.

10ml of aliquot was run with 10ml of 40% sodium hydroxide for distillation and 30ml of distillate was collected in 5ml of 2% boric acid indicator solution. Distillate was titrated against N/28 HCl from green to a pale end point. The total nitrogen was calculated as follows.

\[
T \text{ (ml)} \times \text{Solution volume (ml)} \\
N \text{ (%) } = \frac{10^2 \times \text{aliquot (ml)} \times \text{Sample wt (g)}}{1} \\
T = \text{Titrant volume}
\]

**Moisture content:**

1 g of sieved and dried sample was taken in a weighed crucible and dried at 105°C for 6 hours, cooled and reweighed.

\[
\text{Loss of wt. on drying (g) } \times 100 \\
\text{Moisture (%) } = \frac{\text{---------------}}{\text{Initial sample wt. (g)}}
\]
Temperature treatment:

Apart from the on site incubation methods, experiments were designed to see the effect of temperature by incubating soil samples of all the three horizons ie L, D and M layers in the laboratory at 25\(^0\)C and 35\(^0\)C in incubators. Since average temperature of soils vary mostly in this range the difference was to be seen between on-site and laboratory incubations.

Statistical Analysis:

The data was analyzed for standard deviation and standard error. Analysis of variance was carried out among different factors i.e., sites, seasons and to rate of mineralization and nitrification, statistical methods were followed after Mather (1966) and Snedecor and Cocharan (1980).

Microbial population (Nitrifiers)

Nitrification is the microbial transformation of nitrite or nitrate from ammonium compounds. Two separate steps are involved in this chemoautotrophic nitrification process. In the first, oxidation of ammonium to nitrite by ammonium oxidizing bacteria i.e. *Nitrosomonas* group and the second is oxidation of nitrite to nitrate by nitrite oxidizing bacteria i.e. the *Nitrobacter* group. All the energy required for growth and maintenance of nitrifiers population is obtained from the oxidation of inorganic nitrogenous compounds.

Counting of nitrifiers gives additional information in studies on soil microbial nitrification.
The study of population ecology of nitrifiers has suffered from the lack of a quick, reliable method for counting population. For counting the nitrifier population, the most probable number (MPN) technique has been described by Halvorson and Ziegler (1933) and Cochran (1950). The MPN technique is based on a determination of presence or absence of microorganism in individual aliquots of several dilution of soil materials.

In the case of Nitrosomonas, dilutions, of soil inoculated into inorganic medium containing ammonium as an energy source of nitrogen, and for Nitrobacter soil inoculated into medium free organic material containing nitrite as an energy source, are required.

In the present investigation, estimation of nitrifiers population, was carried out of following the method of most probable number (MPN) count technique (Alexander and Clark, 1965).

Soil samples were taken periodically during different seasons and at an interval of six weeks, the duration of incubation of samples for determination of N-mineralization. Effect of temperature were also seen on the population of nitrifiers by storing the soil samples at different temperatures.

Sample collection:

Soil sample collection and storing method is similar as for determination of nitrogen mineralization. The fresh samples were kept in polyethylene bags and stored at 4°C for one or two weeks without drying, in refrigerator.
Preparation of Dilutions:

For preparation of dilutions, the soil samples were first passed through 6mm. mesh sieves. 10g. of the sieved soil was taken into a 250ml conical flask containing 95ml sterilized water. The capped flasks were shaken for 10 minutes and 10ml suspension was immediately transformed by 10ml sterile pipette into a 90 ml sterilized water containing conical flask.

This suspension established a $10^2$ dilution. Tenfold dilutions of the suspension in sterilized distilled water were made by using 90ml dilution blanks.

Composition of media:

1. Ammonium-calcium-carbonate medium for *Nitrosomonas*.

   Distilled water, 1000ml, (NH$_4$)$_2$SO$_4$, 0.5g; K$_2$HPO$_4$, 1.0g; FeSO$_4$. 7H$_2$O, 0.03g;

   NaCl, 0.3g; MgSO$_4$. 7H$_2$O, 0.3g; CaCO$_3$. 7.5g

2. Nitrite-calcium-carbonate medium for *Nitrobacter*.

   Distilled water, 1000ml; KNO$_2$ 0.006g; K$_2$HPO$_4$, 1.0g; NaCl 0.3g; MgSO$_4$. 7H$_2$O, 0.1g; FeSO$_4$.7H$_2$O, 0.03g; CaCO$_3$, 1.0g; CaCl$_2$, 0.3g.

Analysis:

3ml of prepared medium of each for *Nitrosomonas* and *Nitrobacter* was transferred to each tube and the tubes were tightly capped with cotton plug. Tubes were sterilized by autoclaving at 15 pound pressure for 15 minutes.

Five tubes of each dilution were prepared for the two oxidizers. 1ml from each of the inoculated dilution was transferred to the every tube containing 3ml of medium.
Tubes were then incubated for 3 week at 28° C±2° C temperature along with blanks that were considered as control.

After incubation each tube was tested for the presence of nitrite and nitrate by using the Griess-Ilosvay reagent and zine-copper-manganese oxide mixture.

For the development of colour, few drops of Griess-Ilosvay reagent mixture (sulfanilic acid, α-Nepthylamine and Sodium acetate) were added. Development of a purpl red colour indicates the presence of nitrite and raddish colour indicates the nitrate.

Most probable number (MPN) of organisms in the sample was calculated by selecting the positive tubes in the least concentrated dilution in which all tubes were positive or the greatest number of the tubes were positive (P₁). Numbers of tubes in most higher dilution were considered to be as P₂ and P₃. By selecting the number of tubes in P₃ as positive, the MPN value was obtained from the standard table (Cochran, 1950). By muntiplying this with appropriate dilution factor, MPN for original sample was obtained. The 95% confidence limit for MPN value was calculated from prepared tubes (Appendix-I).

Enzyme activity (Dehydrogenase) :

The 2, 3, 5 triphenyl tetrazolium chloride (TTC) technique is used to estimate the dehydrogenase enzyme activity of microflora in the soil. The reduction of the tetrazolium salt to a stable coloured formazan in soil was shown to be influenced by substrate concentration as well as soil type.
For the estimation of enzymatic activity, the TTC- dehydrogenase technique as described by Lenhard (1956) and Stevenson (1956,1959) was followed. These enzymatic tests are suitable for qualitative studies of various soil processes. However they are not applicable for the quantitative estimation of total enzyme.

In the present investigation Lenhard’s (1956) technique is used for dehydrogenase enzyme activity in soil.

Soil samples were take periodically during different seasons. Soil samples collection and storing method are similar as for determination of nitrogen mineralization.

Analysis:

20g fresh soil was taken in a 100ml conical flask and 200 mg CaCO₃ and 2 ml 1% 2,3,5 triphenyl tetrazolium chloride were added. Samples were throughly mixed and incubated at 30°C in incubator (Relative Humidity 70%) for 24 hours. After incubation, 25ml methanol was added to the flask and shaked well for 5 minutes. Suspension was immediately filtered by whatman filter paper-5 and the methanol through the soils until the extractant appeared colourless. Volume of extractant used for individual sample was recorded.

The density of coloured extractant was determined by spectrophotometer at a wavelenght of 485 mμ with methanol used as the reference blank, concentrations were calculated by comparing with a standarded curve of Triphenyl formazan in methanol.
Reducing sugars:

Carbohydrates are universally found in living tissue and a very important substrate for metabolism. Several colorimetric and titrimetric methods have been given to estimate sugars in solution and other samples.

The Hagedorn-Jensen method (Hodgy and Davis, 1952) based on the quantitative oxidation by potassium ferricyanide, was followed for estimation of reducing sugars in the present study. Soil sample collection and storing method are similar as for determination of nitrogen mineralization.

Extraction:

50 mg of air dried soil was taken in to 100 ml conical flask containing 30 ml water and covered with glass bubble. Flask is kept on a hot plate for 2 hours then cooled slightly and suspension was filtered through whatman filter paper No.44 into as 50 ml volumetric flask.

Analysis:

5 ml of extract is taken into 100 ml conical flask, 5 ml glucose standard and 5 ml water are taken in a separate 100 ml conical flask, 5 ml potassium -ferricyanide-sodium carbonate reagent was added to it, and glass bubble covered flask were kept in boiling water for 15 minutes. Flasks were then cooled for 3 minutes under running water. 5 ml potassium - iodide - zinc sulphate-sodium chloride reagent was added. This resulted in the formation of white precipitate and liberation of iodine. Thereafter, 3 ml of 5% acetic acid was added in to these solutions followed by a few drops of
starch indicator to develop blue colour. These were titrated with 0.01m thiosulphate solution until the blue colour was disappeared.

Calculation:

$$\text{Reducing sugar as glucose (\%)} = \frac{A-C (\text{ml}) \times \text{extract volume (ml)}}{A-B (\text{ml}) \times \text{aliquot (ml)} \times \text{Sample wt (g)} \times 2}$$

A = ml of the water blank
B = ml of the glucose standard
C = ml of the sample extract.