CHAPTER 3
MATERIALS AND METHODS

The study was conducted in Kaziranga National Park (KNP) of Assam, India, for a period of two years (2020-2022). The Kaziranga National Park is recognized as the World Heritage Site by UNESCO due to its uniqueness in terms of wild life and biodiversity. It covers an area of 858.98 sq. km and has been divided in to five ranges namely Burapahar, Agaratoli, Kohora, Bagori and Biswanath ghat. Of this total area, 63.02% is occupied by grasslands, 27.95% by forestlands, 7.63% by wetlands and 0.40% by sand [1]. Our study aimed to evaluate the role of soil physicochemical, mineralogical and biological properties on carbon flux in three natural ecosystems viz. grassland, forestland and wetland of KNP. Measurements were taken during Pre-monsoon (March to May), Monsoon (June to September) and Post-monsoon (October to November) season for two consecutive years (2020-2022).

3.1. Experimental Site

The study site was selected near an Eddy flux tower situated at Burapahar range of Kaziranga National Park. The Burapahar range covers an area of approximately 100 sq. km. The area is located on the border of the Eastern Himalaya partly in Nagaon and Golaghat districts of Assam which is geographically located around 26°34′48″N, 93°6′28″E. The experimental site is presented in Figure 3.1. The floristic composition of the park can be divided into the following forest types and biomes [2]: Eastern wet alluvial grasslands, Assam alluvial plains, Semi-evergreen forests, Tropical moist mixed deciduous forests, Eastern Dillenia swamp forests, Wetlands and Sandy 'chars'. The climate of the area can be classified as humid sub-tropical according to the K¨oppen climate classification. The region experienced an average temperature of 23 ℃ to 27 ℃ during pre-monsoon, 27 ℃ to 28 ℃ during monsoon and 20 ℃ to 26 ℃ during post-monsoon. It received a total annual rainfall of 1496 mm with peak rainfall during monsoon period. Monthly average rainfall and temperature throughout the study period are presented in Figure 3.2.
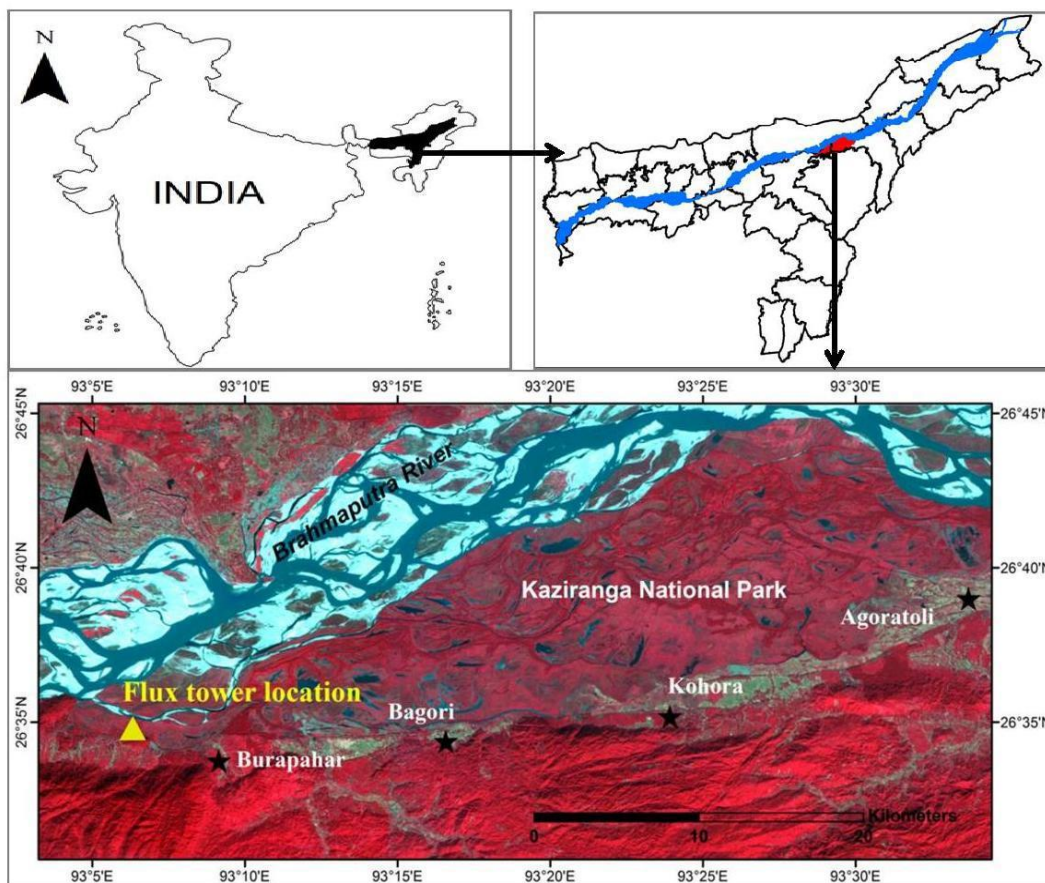
Figure 3.1. Location map of the study area at Kaziranga National Park, Assam, India.
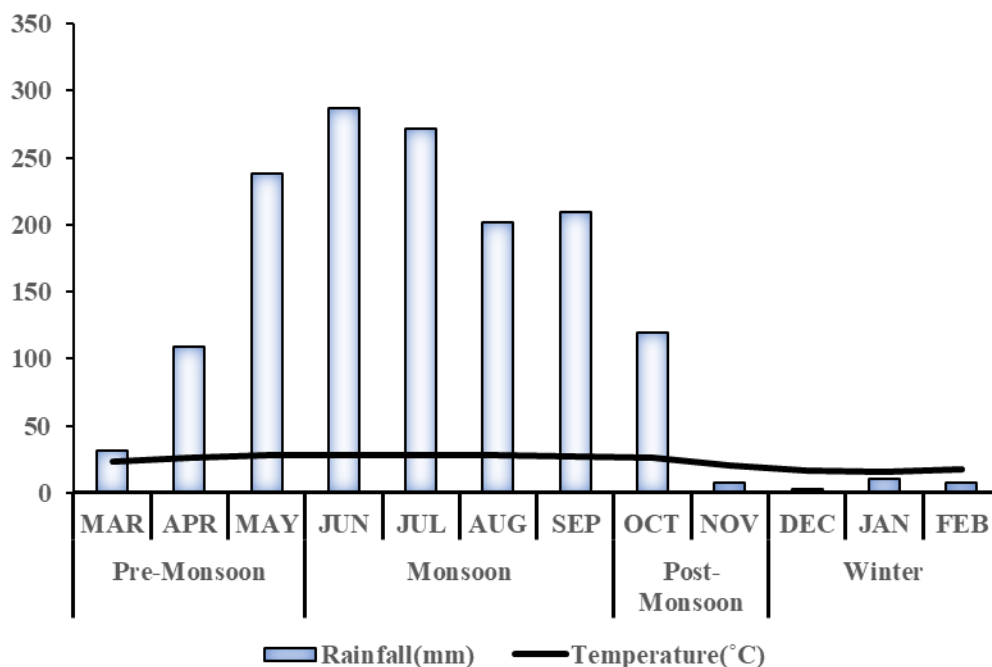

Figure 3.2. Meteorological parameters during the study period (2020-2022) at Kaziranga National Park.

3.2. Experimental Details

3.2.1. Collection of soil samples

Soils from the study area were collected for two consecutive years (during 2020-21 and 2021-22). Three major ecosystem types of the forest namely grassland, forestland and wetland were selected for the study. Number of sampling sites were fixed based on the accessibility of different land-cover (ecosystem) types across the study sites. In grassland and forestland ecosystem, the soil samples were collected from two depths viz. topsoil (0-15 cm) and sub soil (15-30 cm). A total of 120 samples were collected using grid sampling method at a distance of ≈ 2.5 km (Figure 3.3). Soils were collected in three different seasons- pre-monsoon, monsoon and post-monsoon.

After collection, soil samples were immediately stored inside the zipped plastic bags and were brought to the laboratory.

In the laboratory, collected soil samples from each ecosystem were mixed thoroughly to form composite samples. The field-moist composite sample was then divided into three sub-samples. One sub-sample was oven dried at 105°C for 24 h to determine soil physical properties. Second sub-sample was kept at 4°C in plastic bags for few days to stabilize the soil microbiological activity disturbed during soil sampling and handling after which it was analysed for biochemical and microbiological parameters. The third sub-sample was air dried at laboratory conditions, sieved through 2 mm sieve and stored in plastic zipper bags until completion of analysis for chemical parameters.
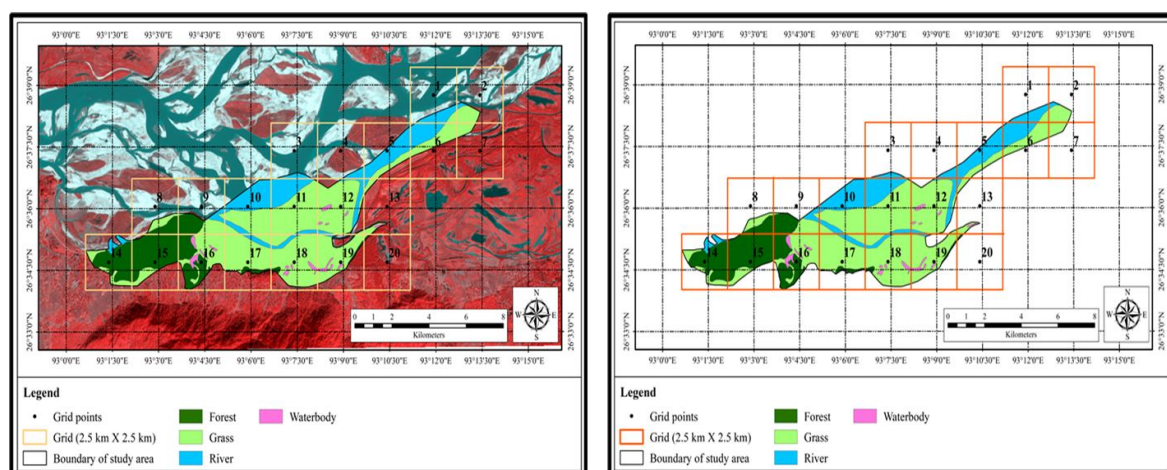

Figure 3.3. Grip Map of Burapahar range of Kaziranga National Park, Assam, India.

3.3. Analyses

3.3.1. Soil physico-chemical parameters

3.3.1.1. Soil texture

Soil texture was determined using Buoycous hydrometer method [3].

Procedure:

100 g of soil sample was treated with 30 % H_2O_2 to destroy the organic matter. Now, 200 mL of distilled water and 100 mL of sodium hexametaphosphate solution were added and mixed thoroughly. After 5 hours the solution was quantitatively transferred to the cup of a mechanical stirrer and washed 4-5 times with distilled water, and the volume was made up to 500 mL. After 10 minutes of stirring, the contents were transferred to the suspension cylinder (1000 mL), giving 4-5 washings to bring the volume up to the required level. A rubber stopper was snugly fitted, and the cylinder was carefully inverted before being vigorously shaken several times to completely disperse soil particles. The stopper was removed, and the hydrometer was placed in the suspension immediately, gently checking the up and down movement. The reading was taken exactly 40 seconds after placing the hydrometer. To assure the complete dispersion of soil particles, the cylinder was vigorously shaken and the rubber stopper was tightened. After 2 hours, the hydrometer was replaced and the reading was recorded. Another sample as blank without soil was run and the room temperature was noted with a thermometer.

Calculation:

Correction factor $(CF) = (Actual\ room\ temperature\ in\ °F - 68) \times 0.2$

Percent silt + clay $= \dfrac{(S-B)+CF}{Dry\ wt.of\ the\ sample\ (g)} \times 100$

Where, S and B stand for sample and blank readings respectively, taken at 40 seconds (first reading).

Percent clay $= \dfrac{(s-b)+CF}{Dry\ wt.of\ the\ sample\ (g)} \times 100$

Where, s and b stand for sample and blank readings respectively, taken at 2 hours (second reading).

Percent sand $= 100 - (Percent\ silt + clay)$

3.3.1.2. Soil moisture content

Moisture content was estimated using gravimetric method as described in Baruah and Borthakur [4]

Procedure:

In a pre-weighed container (C), 10 g (W_1) of fresh soil sample was taken. After recording the final weight (W), the container was kept in an oven at 105 °C for 24 hours. The final oven dry weight (W_2) was calculated by subtracting the container weight $(C - W)$, and the soil moisture content was estimated using the following equation:

Soil moisture content $(\%) = \frac{W_1 - W_2}{W_2} \times 100$

3.3.1.3. Oxidisable Soil organic carbon

Soil organic carbon was determined using Walkley and Black [5] method.

Procedure:

1 g of air-dried soil was weighed and emplaced in a conical flask where a total of 10 mL of 1 N $K_2Cr_2O_7$ and 20 mL of conc. H_2SO_4 were added. After 30 minutes, 200 mL of distilled water was added to it and mixed with 10 mL of concentrated H_3PO_4 and 1.5 mL of diphenylamine. The solution was then titrated with 0.5 N ferrous ammonium sulphate (FAS) until it was bright green. Each set included a reagent blank run without soil.

Calculation:

Soil organic carbon $(\%)$

$= 10 \times \frac{Titrant\ in\ blank\ (mL) - Titrant\ in\ sample\ (mL)}{Titrant\ in\ blank\ (mL)} \times 0.003 \times \frac{100}{wt.of\ sample}$

3.3.1.4. Soil pH

Procedure:

Initially, 10 g of soil sample was placed in a conical flask and shaken for 30 minutes with 25 mL of distilled water. pH of the solution was measured with pH meter (Eutech digital pH meter).

3.3.1.5. Soil EC

Procedure:

In a conical flask, 10 g of soil sample was mixed with 25 mL of distilled water and the mixture was shaken for 30 minutes. The conductivity of the supernatant was measured with conductivity meter (Eutech digital EC meter).

3.3.1.6. Total Nitrogen

Total nitrogen in powdered samples was analyzed using CHN analyzer (Perkin Elmer 2400 Series 2 Elemental Analyzer, USA).

3.3.1.7.Available Phosphorus

Available phosphorous was estimated following the methods of Bray and Kurtz [6].

Procedure:

In a conical flask, 2.5 g of soil was mixed with 25 mL of Bray's extractant (0.025 N HCl and 0.03 N NH_4F). The solution was shaken for 5 minutes before being filtered through Whatman filter paper. In a 50mL volumetric flask, 10 mL of extractant was placed and 10 mL of ammonium molybdate reagent was added to it. The volume of the mixture was made up to 40 mL by adding distilled water. A 2 mL solution of stannous chloride ($SnCl_2$) was added to the volumetric flask, and the volume was made up to 50 mL with distilled water. The mixture was kept for 10 minutes and subsequently absorbance at 660 nm was measured. One blank without soil sample was prepared. Calibration curve was prepared using standard KH_2PO_4 (2 ppm) solutions (1 mL, 2 mL, 3 mL, 5 mL, and 10 mL) following the same procedure without soil. The slope of the linear calibration curve was determined after obtaining the linear calibration curve, and the concentration of the unknown solution was calculated using the equation y = mx + c.

Available P (kg ha^{-1}) = Sample concentration (ppm) $\times$ 2.24

3.1.1.8. Available Potassium

Available K was estimated following the method of Jackson [7].

Procedure:

In a conical flask, 5 g of soil was placed with 25mL of neutral normal ammonium acetate. The solution was shaken for 30 minutes and being filtered through Whatman No.1 filter paper. The potassium in the extractant was measured in a flame photometer with a K filter.

Available K (kg ha^{-1}) $= \dfrac{R \times volume\ of\ extractant \times 2.24}{Dry\ wt.of\ soil\ (g)}$

Where, R is the ppm of K in the extract (photometer reading).

3.1.1.9. Carbon-mineralization

C-mineralization (as CO_2 evolved) was studied in a set of sealed polyethylene bottles with soil collected at each season from all the three ecosystems for a period of 90 days following the method of Angers and Recous [8]

Procedure:

Initially, 50 g field moist soil was taken in each polyethylene bottle and kept at 27±3 ℃ for incubation. Set of 28 polyethylene bottles was used to include five replications from each ecosystem (two depths for grassland and forestland ecosystems). A set of three bottles were kept without soil (control). In each bottle, glass vials of 5 mL capacity containing 1 M NaOH were hung to trap CO_2-C respired. Samplings were taken at 5 days interval till 90 days after the start of incubation. In each sampling, the vials were replaced with a new set containing fresh NaOH and the bottles were placed back in the incubator. To determine the CO_2 production, a back titration of NaOH solution with 0.5 M HCl in excess of 0.5 N $BaCl_2$ (5 mL) with phenolphthalein (2-3 drops) as an indicator was used. Amount of CO_2 evolved were expressed as mg CO_2-C g^{-1} soil after being divided by the mass of the soil samples.

The CO_2 evolved is calculated as follows:

CO_2 (mg g^{-1} soil)$= \dfrac{(mL\ of\ NaOH \times N\ of\ NaOH)in\ blank - (mL\ of\ NaOH \times N\ of\ NaOH)in\ sample \times 22}{Initial\ soil\ wt.(g)}$

..(a)

CO_2 (mg kg^{-1} soil) $= a \times 1000$

3.3.1.10. Kinetics of C-mineralization

CO_2 evolution data was used to calculate the cumulative CO_2 evolved during the incubation period (90 days) and the obtained values were plotted in graph.

The CO_2 emission data was fitted using one- and two-pool models of C mineralization [9, 10]. The first order kinetic equation was used to determine potentially mineralizable C (C_0) and rate constant of C mineralization k.

$$C_t = C_0(1 - e^{-kt})$$

$$C_t = C_1(1 - e^{-kt}) + C_2(1 - e^{-ht})$$

Where, C_t is the cumulative C at any specific time t (day), C_0, C_1 and C_2 are potentially, easily and slowly mineralizable C, k and h are the first and second order rate constant (day^{-1})

3.3.1.11. Half-life of C

Half-life of soil C under various treatments was calculated using the formulae

$$Halflife = 0.693/k$$

Where, k is first order rate constant

3.3.2. Soil Biochemical Parameters

3.3.2.1. *β- 1,4, D Glucosidase*

β- 1,4, D Glucosidase activity was determined following the method of Tabatabai [11]

Procedure:

Soil sample (1g) was added with 0.5 M MUB buffer (4 mL) in a conical flask. Following this, 1 mL of *p*-nitro phenyl glucopyranoside was added to the mixture and were kept at 37±1°C in a BOD incubator. The reaction was stopped after one hour by adding 4 mL of 0.1 M THAM and 1 mL of 0.5 M $CaCl_2$. The mixture was then centrifuged for 10 minutes at 400 rpm, and the absorbance was measured at 400 nm in a spectrophotometer. Calibration graphs were prepared using standards containing 10, 20, 30, 40, and 50 g PNP, and enzyme activity was expressed as µg PNP g^{-1}h^{-1}.

Calculation:

$$\beta\text{- 1,4, D Glucosidase activity} = \frac{Amount\ of\ PNP\ from\ standard\ curve\ (\mu g)}{Soil\ weight\ (g)}$$

3.3.2.2. N acetyl- 1, 4 Glucoaminidase

N acetyl-Glucoaminidase activity was determined following the method given by Tabatabai [11]

Procedure:

Soil sample (1g) was added with 0.5 M acetate buffer (4 mL) in a conical flask. Following this, 1 mL of N acetyl-Glucoaminidase as substrate was added to the mixture and kept at $37\pm1°C$ in a BOD incubator. Reaction was stopped after one hour by adding 4 mL of 0.1 M NaOH and 1 mL of 0.5 M $CaCl_2$. The mixture was then centrifuged for 10 minutes at 400 rpm and the absorbance was measured at 400 nm in a spectrophotometer. Calibration graph was prepared using standards containing 10, 20, 30, 40, and 50 g PNP, and enzyme activity was expressed as µg PNP g^{-1} h^{-1}.

Calculation:

$$\text{N acetyl- glucoaminidase activity} = \frac{Amount\ of\ PNP\ from\ standard\ curve\ (\mu g)}{Soil\ weight\ (g)}$$

3.3.2.3. Acid Phosphatase

Phosphatase activity was determined following the method given by Tabatabai and Bremner [12]

Procedure:

In a conical flask, 1 g of soil was sampled. 0.5 M acetate buffer (4 mL) was added to the soil samples. Following this, 1 mL of *p*-nitro phenyl phosphate was added to the mixture. For one hour, samples were kept at $37\pm1°C$ in a BOD incubator. The reaction was stopped after one hour by adding 4 mL of 0.1 M NaOH and 1 mL of 0.5 M $CaCl_2$. The mixture was then centrifuged for 10 minutes at 400 rpm, and the absorbance at 400 nm was measured using spectrophotometer. Calibration graph was prepared using standards containing 10, 20, 30, 40, and 50 g PNP, and enzyme activity was expressed as µg PNP g^{-1} h^{-1}.

Calculation:

$$\text{Phosphatase activity} = \frac{Amount\ of\ PNP\ from\ standard\ curve\ (\mu g)}{Soil\ weight\ (g)}$$

3.3.3. Soil Microbiological Parameters

3.3.3.1. Microbial biomass carbon (MBC)

Soil MBC was determined using $CHCl_3$ fumigation-extraction method [13].

Procedure:

Three sets of 10 g soil were weighed. One set was kept in an oven at 105 °C to determine the moisture content. The second set was fumigated and the third set was refrigerated until the analysis.

A. Fumigation

In a beaker, 10 g of field moist soil samples were weighed and placed in a vacuum desiccator with $CHCl_3$ in another beaker. The desiccator was sealed with vaseline to prevent leaks and incubated for 48 hours at 25±2 °C.

B. Extraction

Both the fumigated and non-fumigated samples were shaken for 10 minutes with 10 mL of 0.5 M K_2SO_4 (extractant). Whatman No. 42 filter paper was used to filter the content.

C. Oxidation

In a 50 mL conical flask, 8 mL of the extract was taken and this was treated with 2 mL of 0.2 N $K_2Cr_2O_7$. Following this, 15 mL of digestion mixture (conc. H_2SO_4 and Orthophosphoric acid in a 3:1 ratio) was added. The flask was then left for 30 minutes to complete the oxidation process. After 30 minutes, 25 mL of distilled water was added. The contents were mixed after addition of 2-3 drops of ferroin indicator. The solution was then titrated with 0.005 N ferrous ammonium sulphate (FAS) until the appearance of the desired brick red colour. With each set, a reagent blank was run without soil.

Calculation:

1. Soil water content (WS, %)

$$WS = \frac{Weight\ of\ wet\ soil\ (g) - Weight\ of\ oven\ dried\ soil\ (g)}{Weight\ of\ oven\ dried\ soil\ (g)} \times 100$$

2. Weight of soil sample (oven-dry weight equivalent) (MS, g)

$$MS = \frac{Weight\ of\ wet\ soil\ (g)}{\{100 + WS\ (\%)\}} \times 100$$

3. Total volume of solution in the extracted soil (VS, mL)

$$VS = Wet\ soil\ weight - Oven\ dry\ soil\ weight + Extractant\ volume$$

4. Determination of extractable C (Ext C in µg ml^{-1})

   a) Standardization of FAS solution

   Normality of FAS Say x $N =$

   $$\frac{Volume\ of\ K_2Cr_2O_7\ (2\ mL) \times Strenght\ of\ K_2Cr_2O_7\ (0.2N)}{Average\ titre\ value\ for\ the\ blank\ (mL)}$$

   b) Volume of $K_2Cr_2O_7$ consumed (Y, mL)

   $$Y = \frac{Normality\ of\ FAS \times Titrant\ volume}{Normality\ of\ K_2Cr_2O_7} \times 100$$

   c) Volume of $K_2Cr_2O_7$ consumed for oxidizing easily mineralizable C in 10 mL of extractant $= (2 - Y)$ mL

   d) Amount of extractable C (Ext C in µg mL^{-1}) $= \frac{600 \times (2-Y)}{10}$

5. Total weight of extractable C (µg g^{-1} soil) in fumigated (CF) and non-fumigated (CNF) samples

   $$CF\ or\ CNF = Ext\ C \times \frac{VS}{MS}$$

6. Microbial biomass carbon (µg g^{-1} soil or mg kg^{-1} soil) $= \frac{CF - CNF}{K}$

   where, $K = 0.25$ and represents the efficiency of extraction of microbial biomass carbon.

3.3.3.2. Soil Bacterial Metagenomics

The soil metagenomic data was outsourced from AgriGenome Labs Pvt Ltd, Kochi, Kerala. The metagenomic workflow at the Agrigenome lab is given in Figure 3.4.

a. DNA isolation and 16s rRNA amplicon-based Illumina Library preparation

The DNA from the soil samples was isolated and the concentration was estimated using a Qubit Fluorimeter (V.3.0). The 16S rRNA V3-V4 region was amplified using the V3 Forward primer CCTACGGGNBGCASCAG and the V4 Reverse primer GACTACNVGGGTATCTAATCC. To remove non-specific amplifications, the amplified product was tested on a 2% agarose gel and gel purified. The NEBNext Ultra DNA library preparation kit was used to prepare a library from 5ng of amplified product (Steps followed for library preparation are illustrated in Figure 3.5). The Agilent 2200 TapeStation was used to quantify and estimate the quality of the prepared library. The library was sequenced on the Illumina HiSeq 2500 platform.
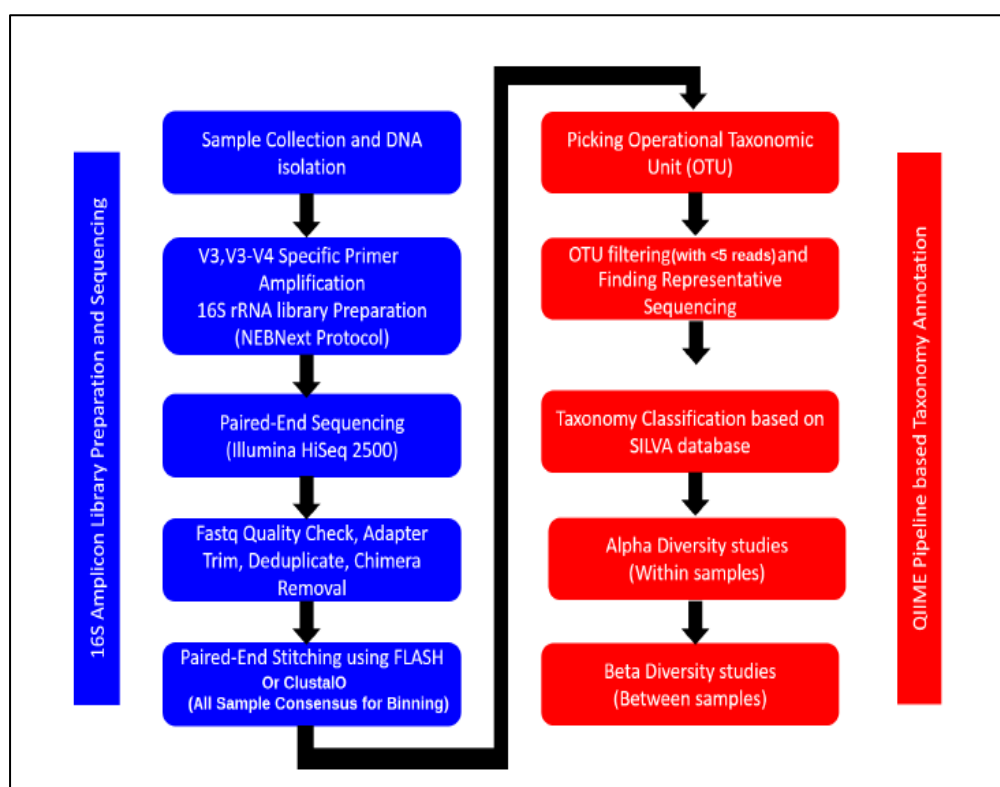

Figure 3.4. Metagenomic workflow at Agrigenome Laboratory, Kerala, India.
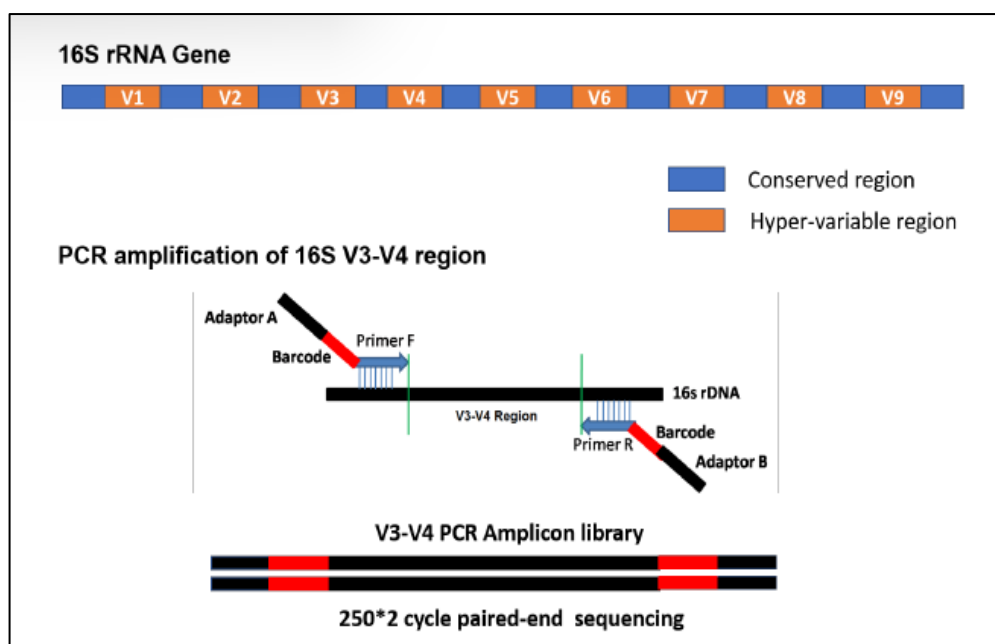
Figure 3.5. Graphical representation of 16S rRNA V3-V4 library preparation and sequencing.

b. Identification of V3-V4 region from paired-end reads

Following steps were performed to extract V3-V4 region from Illumina paired-end sequences.

i. Trimming of sequencing primers

The forward V3 specific primer and reverse V4 specific primers were trimmed using In-house PERL script. The properly paired end reads with Phred score quality (Q>20) were considered for V3-V4 consensus generation.

ii. Building consensus V3-V4 region from trimmed paired-end reads

Trimmed primers with high-quality paired-end reads were carefully paired and allowed to merge/stitch to produce the V3-V4 amplicon consensus FASTA sequences. The FLASH programme (version 1.2.11) was used to merge the reads, with a minimum and maximum overlap of 10bp and 240bp, respectively, and no mismatches. All consensus reads were formed with an average contig length of 350 to 450bp while creating the consensus V3-V4 sequence. Chimeras were removed using the de-novo method UCHIME (version 11) implemented in the tool VSEARCH.

c. **Picking Operational Taxonomic Units (OTUs) and Taxonomy classification**

For OTU selection and taxonomy classification, pre-processed consensus V3-V4 sequences were used. Pre-processed reads from all samples were pooled and clustered into OTUs based on sequence similarity using the QIIME Uclust programme (similarity cuto. = 0.97). For the entire downstream analysis, the QIIME1 programme [Version: 1.9.1] was used [14]. Using the PyNAST programme, representative sequences from each clustered OTU were chosen and aligned against the SILVA core set of sequences. Furthermore, RDP classifier was used to perform taxonomic classification by mapping each representative sequence against the SILVA OTUs database.

d. **Alpha diversity with samples and rarefaction curves**

Shannon, Chao1, and observed species metrics were used to assess the microbial diversity in the samples. QIIME software was used to perform the metric calculation. KRONA plot is also presented which is a graphical representation commonly used in metagenomics and microbiome research to visualize and explore taxonomic and functional abundance data. KRONA stands for "Key Role in Navigational Analysis," and it provides an interactive and hierarchical view of the data.

3.3.4. Soil mineralogical Parameters

3.3.4.1. Fourier transmission infrared analysis

The soils samples for XRD analysis were prepared by vigorous grinding in a mortar pastel. Powder X-ray diffraction of all soil samples were recorded on Bruker D8 Advance diffractometer using Cu Kα X- radiation (λ = 1.54056 Å) at 40 kV and 30 mA. Diffraction patterns were collected over a 2θ range of 5−80° at a scan rate of 1° min^{-1}. PDXL Ver.2-series was used for Rietveld refinement.

3.3.4.2. X-ray diffraction analysis

Fourier transmission infrared (FT-IR) spectroscopy analysis was carried out using a Perkin Elmer Frontier MIRFIR spectrometer. Soil sample (1 mg) was mixed with 150 mg of KBR to make the pellet. The sample chamber was purged with purified compressed air to remove water vapour and CO_2 prior to scanning the pellet. The transmission of infrared spectra bands was analysed in the range between 400 and 4000

cm^{-1} at room temperature (25±1°C). The spectra were collected at a certain resolution by performing a series of interleaved sample and background scans.

3.3.4.3. Thermogravimetric analysis (TGA) and differential thermogravimetric analysis (DTGA).

The TGA and DTGA were performed by heating the soil samples (≈ 13 mg) from room temperature to 800°C using a NETZSCH thermogravimetric analyser (NETZSCH-Gerätebau GmbH, Germany) at a heating rate of 10˚C min^{-1}. Samples were placed in Pt crucibles and purged by a stream of nitrogen flowing at 80 mL min^{-1}. Experiments were performed in platinum crucibles under a nitrogen atmosphere (50 mL min^{-1}). The TGA and DTGA were performed to assess the thermal stability of the organic compounds, which could indicate the extent of humification of SOC [15, 16].

3.3.4.4. Micromorphological analysis

Finely grounded soil samples were dispersed on the carbon conductive tape which was placed on the standard brass stub of 10mm diameter. Samples were kept at 40°C in the vacuum oven for 15-30 mins to remove the moisture. After sufficient drying, the samples were coated with a conductive material Platinum (Pt) using sputter coater unit of Model no. Jeol JSM 1600 to prevent charge build-up on the surface of the samples. The metal (Pt) was applied in a controlled manner in the sputter coater. It is critical because the coating should be thick enough to prevent charging ~ 10 nm but not thick enough to obscure specimen surface details. After coating, the stubs were mounted in the holder of Field Emission Scanning Electron Microscope (FESEM) for analysis.

To identify the elemental composition, the soil samples were prepared following the aforementioned procedure. SEM-EDX was calibrated with company provided Cu sample with OXFORD INCA software. After proper calibration of the instrument, the prepared samples were mounted in the holder for analysis.

3.4. Statistical Analysis

Statistical analyses were performed using SPSS 26 software package (SPSS Inc., Chicago, IL, USA). Analysis of variance (one-way ANOVA) and Duncan's multiple range test (DMRT) was executed at $p \leq 0.05$ to process the data and to determine statistical difference between treatment means. Standard deviation ($p \leq 0.05$) was used to represent variations in collected data. In addition, mineralogical spectra were developed using OriginPro 8.5

(OriginLab®, MA, USA). Correlation matrices and Principal Component Analysis (PCA) were performed to estimate their relationships between the soil variables using R statistical software (R studio). The figures were created with GraphPad Prism 8.3.0.

Photo 3.1. Three representative ecosystems (A) Grassland (B) Forestland (C) Wetland of Burapahar range of Kaziranga National Park, Assam, India.

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