Methodology and planning of experiments

Chapter 3 Methodology and Planning of Experiments

3.1. Methodology and Planning of Experiments

This chapter outlines the methodologies used to conduct the experiments. The paragraphs that follow provide a brief overview of the design and technique. Further information can be found in the following chapters and the enclosed documents.

3.1.1. Climate and soil during the cropping seasons

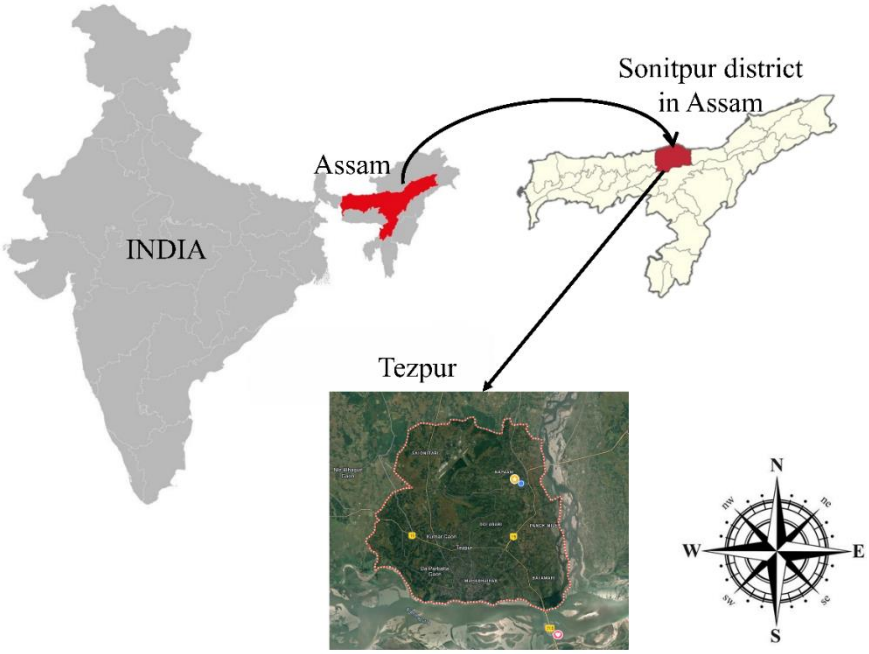

Fig. 3.1. Study area

The Krishi Vigyan Kendra (Sonitpur) provided data on precipitation and ambient temperature for the entire crop period. Climate attributes include the monthly average of ambient temperature, total rainfall, and relative humidity (RH) prevalent during the crop. The location of the study is shown in Figure 3.1. Sonitpur's soil was alluvial in character, with a black colour and a sandy-loam texture.

3.2. Phase 1: For the synthesis of Zn based nanomaterials, the plant species were carefully selected based on an extensive literature review that highlighted their remarkable antioxidant and reducing power. A thorough enquiry was conducted to review the current literature on the bioprospecting of flora native to Assam, India, with a focus on their potential for plant-mediated green synthesis of zinc-based nanomaterials. Fresh pruned green leaves of *Peltophorum inerme* Roxb, *Polygonum microcephalum* Wall. Ex D. Don., *Chrysalidocarpus lutescens* H. Wendl., *Crinum asiaticum* L., and *Aquilaria malaccensis* Lam. Were collected from Tezpur, Assam. A schematic diagram for the overall phase 1 study has been presented in Fig. 3.2. Comparing the antioxidant potentials of five different plant species for acting by way of reducing and stabilizing green synthesis agents.

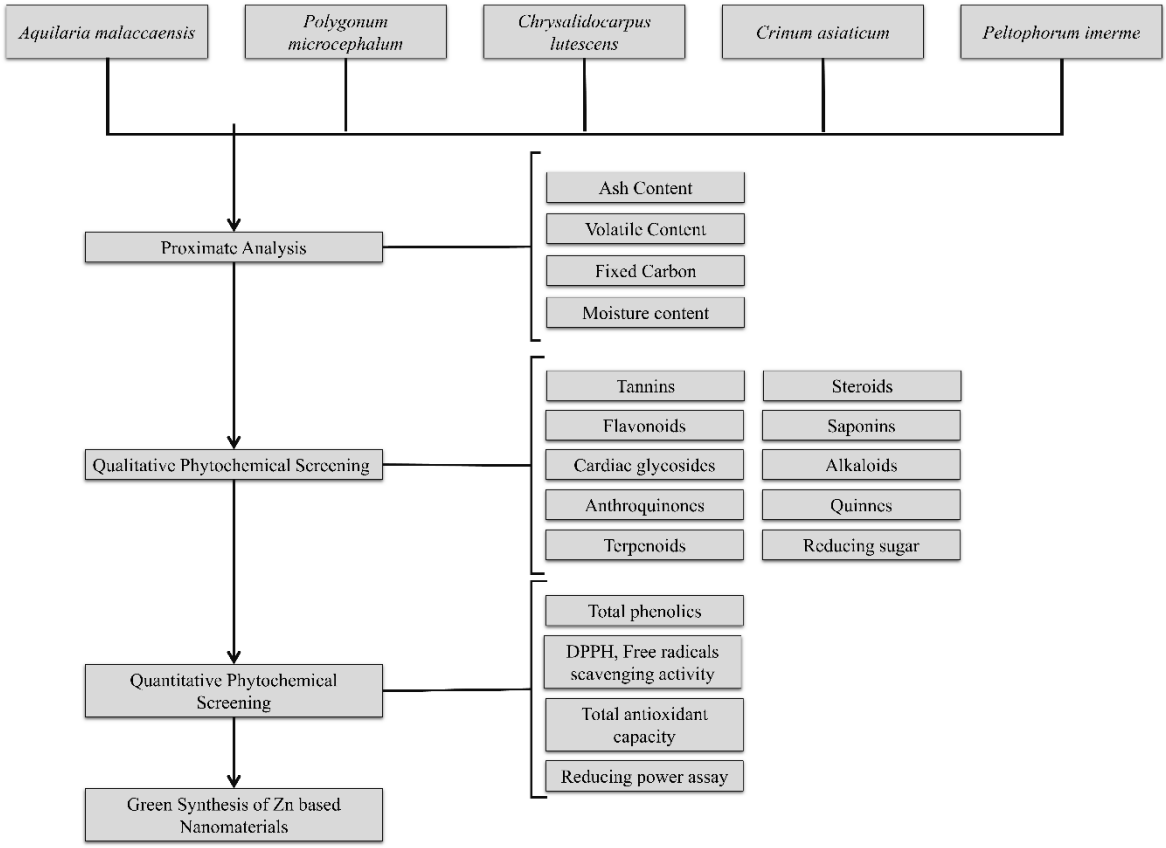

Fig. 3.2. Overview of Phase 1

3.3. Phase 2: Optimization and characterization of the synthesized Zn-based nanomaterials

Our subsequent goal was to entail the freshly synthesized nanoparticle solutions to rigorous study over a duration of 30 days to discern any alterations in coloration or stability, monitored via UV-Vis spectroscopy and Dynamic Light Scattering (DLS) analysis to characterize their hydrodynamic diameter and ensure optimal performance. A schematic diagram for the overall phase 1 study has been presented in Fig. 3.3.

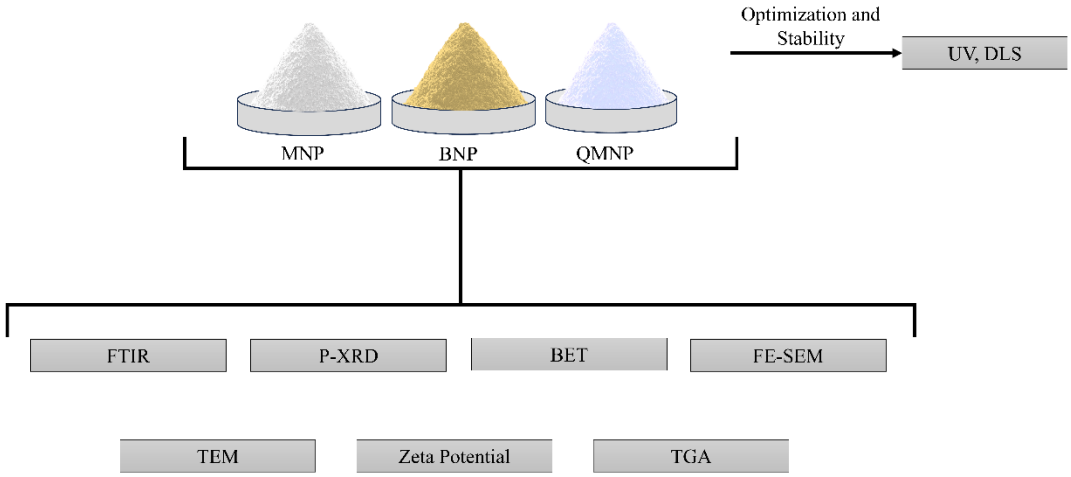

Fig. 3.3. Overview of Phase 2

3.4. Phase 3: Dose establishment study of Zn-based nanoparticles in plant-microbe-soil interface.

The next step was to carefully determine an ideal dose (EC_{50}) for the complex plant-microbe-soil interaction, which was a critical step towards realizing the full potential of this symbiotic relationship. Bengal gram or chickpea, revered as the essential source of lean protein across the Indian subcontinent, was selected as the focal point of our investigation. A schematic diagram for the overall phase 3 study has been presented in Fig. 3.4.

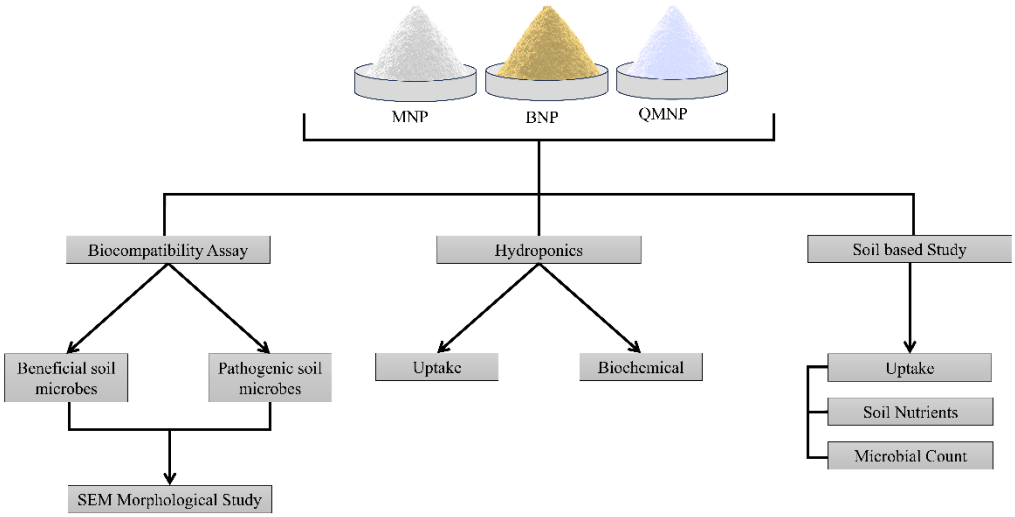

Figure 3.4. Overview of Phase 3

Table 3.1. Treatments combinations for microbial compatibility assay:

Treatments	Denotes
250	250 µg ml^{-1}
500	500 µg ml^{-1}
1000	1000 µg ml^{-1}
1500	1500 µg ml^{-1}
2000	2000 µg ml^{-1}
G	Gentamicin 1405-41-0
Zn Salt	Zinc Sulphate 250 µg ml^{-1}
Zncu salt	Mixture of Zinc Sulphate and Copper Sulphate 250 µg ml^{-1}
QM salt	Mixture of Zinc Sulphate, Copper Sulphate, Ferric Chloride and Manganese Oxide 250 µg ml^{-1}
PE	Plant Extract of *C.lutescens*

3.5. Crop Selection

Chickpeas are leguminous crops that can fix atmospheric nitrogen using nitrogen fixing bacteria in the root nodules. This process of nitrogen fixation increases soil fertility, strengthens

soil structure, and decreases the demand for synthetic nitrogen fertilisers, encouraging sustainable agriculture methods[1–3]. Chickpea cultivation offers economic opportunities to farmers in Assam and across the Indian subcontinent. It provides an income and a way of life for millions of smallholder farmers, helping to improve rural development and alleviate poverty. Chickpeas are a staple in many parts of India, including Assam. It is high in protein, carbs, vitamins, and minerals, making it an important part of any diet, particularly for vegetarians[4,5]. It is grown as both a kharif (rainy season) and rabi (winter season) crop, contributing to food security and rural livelihoods.

3.6. Lab-Scale Hydroponic farming

Hydroponic farming in a laboratory setting provides researchers with a controlled environment in which to conduct experiments and studies on plant physiology, nutrient intake, growth patterns, and environmental responses. It provides exact control over variables, making it excellent for scientific study and agricultural innovation. To achieve optimal plant development, lab-scale hydroponic systems must be observed and maintained on a consistent basis. This includes monitoring nutrient levels, ph levels, and water quality, as well as adjusting environmental conditions as necessary. Pests and fungal infections may also need to be addressed using proper techniques.

3.2. Treatment combinations for hydroponics study

Treatments	Denotes
MNP-C1	Zinc Oxide nanoparticles @ 50 mg L^{-1}
MNP-C2	Zinc Oxide nanoparticles @ 100 mg L^{-1}
MNP-C3	Zinc Oxide nanoparticles @ 200 mg L^{-1}
MNP-C4	Zinc Oxide nanoparticles @ 250 mg L^{-1}
MNP-C5	Zinc Oxide nanoparticles @ 500 mg L^{-1}
Nn-Zn(C1)	Non-nanoscale Zinc @ 50 mg L^{-1}
Nn-Zn(C2)	Non-nanoscale Zinc @ 100 mg L^{-1}
Nn-Zn(C3)	Non-nanoscale Zinc @ 200 mg L^{-1}
Nn-Zn(C4)	Non-nanoscale Zinc @ 250 mg L^{-1}
Nn-Zn(C5)	Non-nanoscale Zinc @ 500 mg L^{-1}

BNP-C1	Zinc Copper Bimetallic nanoparticles @ 50 mg L^{-1}
BNP-C2	Zinc Copper Bimetallic nanoparticles @ 100 mg L^{-1}
BNP-C3	Zinc Copper Bimetallic nanoparticles @ 200 mg L^{-1}
BNP-C4	Zinc Copper Bimetallic nanoparticles @ 250 mg L^{-1}
BNP-C5	Zinc Copper Bimetallic nanoparticles @ 500 mg L^{-1}
Nn-zncu(C1)	Non-nanoscale Zinc Copper @ 50 mg L^{-1}
Nn-zncu(C2)	Non-nanoscale Zinc Copper @ 100 mg L^{-1}
Nn-zncu(C3)	Non-nanoscale Zinc Copper @ 200 mg L^{-1}
Nn-zncu(C4)	Non-nanoscale Zinc Copper @ 250 mg L^{-1}
Nn-zncu(C5)	Non-nanoscale Zinc Copper @ 500 mg L^{-1}
QNP-C1	Zinc Copper Iron Manganese Quadrimetallic Nanoparticles @ 5 mg L^{-1}
QNP-C2	Zinc Copper Iron Manganese Quadrimetallic Nanoparticles @ 15 mg L^{-1}
QNP-C3	Zinc Copper Iron Manganese Quadrimetallic Nanoparticles @ 25 mg L^{-1}
QNP-C4	Zinc Copper Iron Manganese Quadrimetallic Nanoparticles @ 40 mg L^{-1}
QNP-C5	Zinc Copper Iron Manganese Quadrimetallic Nanoparticles @ 45 mg L^{-1}
Nn-QMC1	Non-nanoscale Zinc Copper Iron Manganese @ 5 mg L^{-1}
Nn-QMC2	Non-nanoscale Zinc Copper Iron Manganese @15 mg L^{-1}
Nn-QMC3	Non-nanoscale Zinc Copper Iron Manganese @15 mg L^{-1}
Nn-QMC4	Non-nanoscale Zinc Copper Iron Manganese @ 15 mg L^{-1}
Nn-QMC5	Non-nanoscale Zinc Copper Iron Manganese @ 15 mg L^{-1}

3.7. Soil experiments

The geography of Assam, particularly its diverse terrain and waterways, influences soil formation via erosion, deposition, and drainage. Assam's environment, which is characterised by significant rainfall and temperature, has an impact on soil fertility by seeping nutrients and altering weathering processes. Ph of the soil was obtained in freshly collected soil samples using Eutech instruments ph 700 and thermofisher probe. The soil was thoroughly agitated with water n the ratio 1:2.5 following standard protocol by Blakemore et al.[6]and Kalra et al. [7] Bulk density was estimated using the method given by Grossman [8] where weight (W1) and volume (V) of the empty bulk density bottle were taken. The bottle was then filled with the organics up to the rim and repeatedly tapped for about 15-20 times. The final weight (W2) was then

recorded after capping the bottle. Bulk density (g CC^{-1}) was measured using the following formula:

$$Bulk\ density = \frac{W2 - W1}{V}$$

Moisture content was estimated using gravimetric method as described in Baruah and Borthakur.

Where, 10 g (W1) of fresh soil was taken in a pre-weighed container (C). The samples were kept in a hot air oven at 105 °C for 24 hours and the final weight (W) was recorded. The final oven dry weight (W2) was obtained by subtracting the weight of the container (C-W), and the soil moisture content was determined using the following equation:

$$Soil\ moisture\ content = \frac{W1 - W2}{W1} \times 100$$

Nitrogen (N), phosphorus (P), and potassium (K), the three macronutrients that plants require for optimal growth and development. These nutrients, essential in many physiological activities, including photosynthesis, cell division, and enzyme activation[9]. NPK fertilisers are designed to supply appropriate amounts of these nutrients to plants, supporting optimal growth and production. The conventional NPK fertilizer application requirements in Assam soils may differ depending on type of soil, crop variety, and nutrient requirements[10]. Nutrient recommendations for *Cicer arietinum* 20–30 kg N and 40–60 kg P and 17 to 30 kg K_2O per ha. Farmyard manure, or FYM, is organic matter formed from animal waste like cow dung, horse manure, or poultry droppings, as well as bedding materials and other organic leftovers. FYM is high in organic carbon, nitrogen, phosphorus, potassium, and other vital nutrients, making it an effective soil conditioner and fertiliser[11]. It improves soil structure, increases water retention and aeration, encourages microbial activity, and gives plants a slow-release source of nutrients. The normal FYM application rate in Assam soils varies depending on soil fertility levels, crop type, and management approaches. FYM is typically sprayed to soils at rates ranging from 2 t/ha[12], depending on soil nutrient needs and crop nutrient uptake. Vermicompost is a nutrient-rich organic fertiliser made from the breakdown of organic matter by earthworms (vermi) and microorganisms. It is high in organic matter, humus, nitrogen, phosphorus, potassium, and other micronutrients that plants require for growth[13,14]. Vermicompost improves soil structure and fertility, boosts microbial activity, and promotes nutrient cycling in the soil-plant system. It also helps to control soil-borne illnesses and boosts

plant resistance to environmental challenges[15]. The normal vermicompost application rate in Assam soils varies based on soil type, crop variety, and nutritional requirements. Vermicompost is typically applied to soils at rates ranging from 0.8 t/ha, depending on soil fertility and crop nutrient requirements (ICAR, 1995)[12].

Table 3.3. Treatment combinations for soil based study

Sl.no.	Treatments	Denotes
1.	Control	No treatment with plantation
2.	NPK	Urea, DAP, Potash dose recommended by ICAR
3.	VC	Vermicompost
4.	FYM	Farmyard Manure
5.	MNP25	Zinc monometallic nanoparticles @ 25 mg kg^{-1}
6.	MNP50	Zinc monometallic nanoparticles @ 50 mg kg^{-1}
7.	MNP100	Zinc monometallic nanoparticles @ 100 mg kg^{-1}
8.	MNP200	Zinc monometallic nanoparticles @ 200 mg kg^{-1}
9.	MNP250	Zinc monometallic nanoparticles @ 250 mg kg^{-1}
10.	MNP500	Zinc monometallic nanoparticles @ 25 mg kg^{-1}
11.	MNP1000	Zinc monometallic nanoparticles @ 1000 mg kg^{-1}
12.	BNP25	Zinc copper bimetallic nanoparticles @ 25 mg kg^{-1}
13.	BNP50	Zinc copper bimetallic nanoparticles @ 50 mg kg^{-1}
14.	BNP100	Zinc copper bimetallic nanoparticles @ 100 mg kg^{-1}
15.	BNP200	Zinc copper bimetallic nanoparticles @ 200 mg kg^{-1}
16.	BNP250	Zinc copper bimetallic nanoparticles @ 250 mg kg^{-1}
17.	BNP500	Zinc copper bimetallic nanoparticles @ 500 mg kg^{-1}
18.	BNP1000	Zinc copper bimetallic nanoparticles @ 1000 mg kg^{-1}
19.	QM10	Zinc copper iron manganese quadrimetallic nanoparticles @ 10 mg kg^{-1}
20.	QM25	Zinc copper iron manganese quadrimetallic nanoparticles @ 25 mg kg^{-1}
21.	QM35	Zinc copper iron manganese quadrimetallic nanoparticles @ 35 mg kg^{-1}
22.	QM40	Zinc copper iron manganese quadrimetallic nanoparticles @ 40 mg kg^{-1}
23.	QM45	Zinc copper iron manganese quadrimetallic nanoparticles @ 45 mg kg^{-1}
24.	Base soil	No Treatments without plantation

3.8. Quality Assurance and Quality Control

Tezpur University's QC requirements were followed during the experimental studies. Quality assurance can be defined as "a set of coordinated actions such as plans, specifications, and policies used to assure that a measurement programme can be quantifiable and produce data of known quality". Quality control refers to the repetitive use of methods to attain and maintain a specific degree of quality for measuring systems.

3.9. Sample Storage and Preservation

After collecting, the leaves of plant species were air dried, crushed, and sieved in accordance with AOAC guidelines[16]. Air-dried samples were stored in vacuum sealed zipper bags and dated accordingly. Most samples were analysed within 30 days after collection. Samples were kept at dark and dry place to maintain sample properties. Similarly, freshly synthesized green nanoparticles solution were stored in 4°C for up to 28±2 days while continuing the stability study of nanoparticles. Moreover, powdered nanoparticles which were oven dried in clean environment right after the successful UV-vis studies were transferred to amber bottles to ensure quality of the material. Before measuring and analysing, ensured that the apparatus, materials, and reagents are free of contamination.

3.10. Chemical, reagent, and labware purity

All the chemicals used were GR grade (guaranteed reagent) and confirmed to be 90-99% pure. To prepare reagents, ultrapure water was typically obtained from a water purification system (Sartorius Stedim, Germany). All reagents were made fresh on the day of each analysis. The glassware and plasticware were washed with reagent water, rinsed with double distilled water, then oven dried before each use.

3.11. Calibration procedures

Analytical instruments were required to be calibrated and adjusted before use. Calibration was checked a day before analysing new samples. The technique would involve at least one blank and multiple standards. Several Standard measurements were conducted regularly for instruments such as ph metres that do not necessitate blanks. Standard Reference Materials, which provide certified values for analytes, were employed through the trials to assure accurate analysis. All the standards were maintained with the highest purity, including ph metres, electrical conductivity metres, UV-VIS spectrophotometers, CHNS, and AAS.

3.12. The initial performance demonstration

It characterises instrument and laboratory performance, including determining linear calibration ranges and technique detection limits, before conducting analyses.

3.13. Linear calibration range (LCR)

The LCR for critical instruments such as UV-Vis spectrophotometers, Kjeltec N analyzers, and Thermo Scientific Flash 2000 CHNS was first determined and validated every 6 months or whenever a significant change in instrument response was noticed. Linearity was validated using one blank and three standards. To ensure the accuracy of the CHNS results, the standard was injected after every 5 samples. Linearity was reestablished when verification data surpassed original values by ±10%.

3.14. Method detection limit (MDL)

For each analyte, mdls were generated using either reagent water (blank) or standard solutions. To calculate MDL values for separate studies, seven duplicate aliquots of working standard solutions were analyzed using the same analytical techniques. To calculate concentration levels in the relevant units, all methods were used as defined. The mdls were determined using the formula below.

$MDL = t \times S$

Where t is the student's t-test value for a 99% confidence level standard deviation estimate with n-1 degrees of freedom (dof) [t= 3.14 for seven replicates]. Here, S is the standard deviation of the replicates. Mdls should be calculated twice every six months. The appendix includes a list of mdls for different analytes.

3.14.1 Instruments and equipment

a. Operations and maintenance

   All instruments and equipment were kept in top condition, with detailed records of proper functioning, calibration, and troubleshooting. The guidelines for the daily functioning of the following laboratory equipment are as follows:

b. Equipment

   CHNS instrument, UV-visible spectrophotometers, ph Meter & probes, Weighing Balances, and Automated Pipettes. Calibrations was done after every 10 samples

analysed, following the instrument's initial warm-up and de-gassed for best optimization.

i. CHNS instrument

The reagent blank was performed with empty tin cans provider for sample injection and the standard thus used for the study was BBOT (2,5-Bis(5-tert-butyl-2-benzo-oxazol-2-yl) thiophene).

Maintenance: Maintenance done once yearly by professionals.

ii. UV-VIS Spectrophotometers

Calibration was verified after every 40 samples analysed, following the instrument's initial warm-up.

Maintenance: Maintenance done once yearly by professionals.

iii. Ph Meter & probes: calibrating the instrument at ph 4, 7 and 9 with standard solutions, every 90 samples,

Maintenance: Checking of probes regularly and ensuring that electrodes are filled.

iv. Balances: The balances were checked daily before use. The balances were kept in airtight cabinets to nullify the errors in measurement. The precision of the balances was routinely checked by weighing authentic weighs.

Maintenance: After every use necessary cleaning was performed with ethanol. The dust and fallen chemicals during weighing was vacuum cleared. The instrument was serviced and calibrated by certified company engineers once in a year.

v. Automated Pipettes: Calibration done every 15 days.

Maintenance: Suitable cleaning after every use.

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