CHAPTER III
MATERIALS AND METHODS

3.1. Area of Investigation

The present investigation on biodiversity and ecology of soil-algae was carried out in the *Oxic Dystrusteps* soils of Pathanamthitta District, Kerala State, South India. This district is situated in the south-eastern part of Kerala State, and represents mostly the midland/highland zones of the Western Ghats (9°4’-9°29’N; 76°28’-77°18’E). Therefore, the study sites (Fig. 1) selected for the collection of soil samples in the current investigations are considered to be representative areas spread over the mid-upland and high-land regions of the Western Ghats (70 to 350 m msl).

The climate of the entire area is humid with a short summer (January to April) and plentiful rainfall (average annual rain fall in the district is 2610.3 mm) available in two monsoon seasons—the southwest (May to August) and the northeast (September to December) monsoons with a short break in between. The annual average temperature of the area is 28.00°C (Soil Survey Organization 2007).

Soils of the present study area at Pathanamthitta District were mainly laterite soils. Laterite and lateritic soils are the weathering products of rock in which many weathering and mineral transformation takes place. It involves removal of bases and gradual loss of combined silica of primary minerals. The soils of the Pathanamthitta District are located at an elevation of 10 to 100 m above MSL as a strip between the coastal belt and hilly mid-upland. The area includes mounds and low hills with gentle to steep slopes. There are three different types of laterite soils found in...
Kerala. (1) Laterite soil with low amount of gravel-less than 30% with reasonable depth between 60 and 150 cm. It is present in the foot slopes of laterite mounds and laterite hills. (2) Laterite soil with 30% to 80% of gravel and high depth of 60 to 150 cm. These soils are noticed in side slopes and summits of laterite mounds and hills. (3) Shallow soils of depth less than 50 cm with indurated laterite and laterite outcrops (Soil Survey Organisation 2007).

‘Laterite’ or ‘lateritic soils’ are acid ferruginous soils of Kerala, a wet tropical zone, belonging to the southern region of the Western Ghats of South India. ‘Laterite’ is equivalent to Oxysalt in USDA soil classification system (Chandran et al. 2005). Environmental conditions influencing laterite formations in Kerala are tropical climate of prolonged rainfall with intermittent dry seasons on gneissic and granitic rocks (Narayanaswamy 2005). Stable landscape and siliceous or acidic parent material are conducive for the formation of Oxisols in Kerala (Bhattacharyya et al. 1993). Laterite soils of Pathanamthitta are basically Oxic Dystruustepts, which in Kerala are considered as a kind of inceptisols, quite juvenile soils found on laterite. This is a surface formation enriched with iron and aluminium, developed by intensive and long lasting weathering of the underlying parent rock (Soil Survey Staff 1999). In Kerala, these soils mainly occur at an elevation of 10 to 100 m above main sea level (msl) as a strip between the coastal belt and hilly mid-uplands (Soil survey Organization 2007).

Among the three types of laterite soils in the State (Soil Survey Organization 2007) the soil under the current investigations belonged to a shallow soil type of less than 50 cm depth, having indurated laterite and laterite outcrops. This soil has five different series such as the Airavon, Adoor, Ayroor, Kumaranperur and Gudarakal. The study sites of the present investigation were distributed in the Kumaranperur
and Gudarakal series in the district. Both these series are isohyperthermic, Oxic Dystrustepts. The vegetation types over the investigated soils belonged to natural forest (tropical wet or semi evergreen) and plantations such as teak over 50 years and rubber over 25 years of existence. Soils of rubber plantations examined in general receive chemical fertilizer mixture of urea, rock phosphate, and muriate of potash (10:10:10) at about 300 kg ha$^{-1}$ twice every year; the first doze during the onset of southwest monsoon and the second during the onset of the northeast monsoon season. Except the cutting of herbs and shrubs in the understoreys before the onset of monsoons, teak plantations in the region receive no other cultural or manurial support. Natural forests represent virgin vegetations without any significant anthropogenic influence.

Soil samples for each vegetation were collected from 10 different sites (each were of about 1-2 acre land) belonging to 10 different regions in the district. The first group of seven sampling sites were from the Kumaranperur series of dark reddish brown to yellowish red soils. These soils have gravelly silt loam to gravelly clay B horizon formed on granite gneiss on steep to very steep forest and rubber growing tracts in the district at an elevation of 100 to 300 m above msl. These sites extend between $9^0\ 4'-9^0\ 26'$ N and $76^0\ 38'-77^0\ 0'$ E at the central part of Pathanamthitta District in Kozhencherry and Ranni administrative zones.

The second group of three sampling sites were from the Gudarakal series of dark reddish brown to strong brown soils with clay loam to sandy clay in the ‘A’ horizon and dark reddish brown to reddish yellow, gravelly sandy clay to gravelly clay in the ‘B’ horizons. Gudarakal soils are formed on granite gneiss on hilly summits of forests in the District, above an elevation of 900 m msl, which extend between $9^0\ 10'-9^0\ 27'$N and $76^0\ 54'-77^0\ 09'$E along the eastern part of Ranni and
Fig. 1: Map of Pathanamthitta District, Kerala, India
(Sites of soil and algal samples collection under different Vegetation)
Kozhencherry Taluks. In general, both the Kumaranperur soils and Gudarakal soils are gravely with good air water relationship and high water holding capacity.

3.2. Field Survey for Algae and Soil Samples

Soil samples from the three different vegetations were collected for the systematic study of algae and physico-chemical characteristics of soils in three different seasons of a year throughout the period of study (2007-10). There were ten different sites for sample collection of soils from each of the three different vegetations. These sites were labelled as F 1 to F 10 for forest, TP 1 to TP 10 for teak plantation and RP 1 to RP 10 for soils from rubber plantation. From each sampling site (1-2 acre land) soil samples were collected from ten different random plots, each of about 10 m² size.

Soil sampling and the physico-chemical analyses of soils were carried out as per Jackson (1973). In all seasons, one composite sample of soils from each of the ten different random plots was taken separately from forest, teak and rubber. In each season, altogether 30 composite samples were collected from different areas of the District, which belonged to the three different vegetations.

Soil samples (0-5 cm soil layer) were taken randomly from 10-15 different parts of each of the collection spots (10 m²). Such random samples taken from the 10 different collection spots of a site were thoroughly mixed together to a general composite sample of about 1 kg for soil chemical analysis. From each site, another 500 gm of soil was collected from the composite mixture of top soil layer for field study of algae and algal culture.

The seasons studied included summer (January to April 2007-2010), southwest monsoon (May to August 2007-2010) and northeast monsoon (September to December 2007-2010). The field samples collected were immediately put in
sterile cotton bags and brought to the laboratory. Those samples for the study of soil characteristics were kept open in the laboratory shelves for air drying till the physico-chemical analyses, which was completed with in two weeks after the collections. Samples for algal studies were processed on the same day or next day for direct field examination for algae and culture of algae as per standard methods.

3.3 **In vitro Culture Studies**

In order to explore the maximum diversity of soil-algae, initially three media were used for the culture. The three media used were Chu No.10, BG-11 and Modified Bolds Basal Medium (MBBM). Since the MBBM showed the best result for development of all three groups of soil-algae, the other two media were not used in later cultures of algae. However, the other media such as Chu No.10 and BG-11 were found useful for culture of either one or two groups of algae, but quite unsuitable for all the three groups of soil-algae.

Chu No.10 medium is a widely used one for both eukaryotic and prokaryotic algae. It was prepared as per Chu (1942). The composition of the medium was:

\[
\begin{align*}
\text{Ca(NO}_3\text{)}_2.4\text{H}_2\text{O} &- 2.0 \text{ mg}; \text{ KH}_2\text{PO}_4 &- 0.62 \text{ mg}; \text{ MgSO}_4.7\text{H}_2\text{O} &- 2.5 \text{ mg}; \\
\text{Na}_2\text{CO}_3 &- 2.0 \text{ mg}; \text{ HCl (1 mol/l)} &- 0.025 \text{ ml}; \text{ Na}_2\text{SiO}_3.9\text{H}_2\text{O; Na}_2\text{EDTA.2H}_2\text{O} &- 0.2 \text{ mg}; \\
\text{FeCl}_3.6\text{H}_2\text{O} &- 0.1 \text{ mg}; \text{ H}_3\text{BO}_3 &- 0.248 \text{ mg}; \text{ MnCl}_2.4\text{H}_2\text{O} &- 0.139 \text{ mg}; \text{ (NH}_4\text{)}_6\text{MO}_7\text{O}_{24}.4\text{H}_2\text{O} &- 0.1 \text{ mg}; \\
\text{Vit. B}_{12} &- 1 \mu\text{g}; \text{ Thiamine} &- 0.1 \mu\text{g}; \text{ Biotin} &- 0.1 \mu\text{g}; \text{ Distilled Water} &- 100 \text{ ml}; \text{ pH adjusted to 7.6}.
\end{align*}
\]

The Second medium used for the culturing of algae was BG-11 medium. It was found more effective for the culturing of blue green-algae and was prepared as per Stanier et al. (1971). The composition of this medium was: \(\text{NaNO}_3\) 1.5 gm; \(\text{K}_2\text{HPO}_4.3\text{H}_2\text{O}\) 0.040 gm; \(\text{MgSO}_4.7\text{H}_2\text{O}\) 0.075 gm; \(\text{CaCl}_2.2\text{H}_2\text{O}\) 0.036 gm; Citric acid 0.006 gm; Ferric ammonium citrate 0.006 gm; EDTA (disodium salt)-0.001
gm; Na₂CO₃- 0.02 gm; Trace Metal Mix**- 1.0 ml; De ionised water- 1 L; Trace Metal Mix** H₃BO₃- 2.86 gm; MnCl₂.4H₂O- 1.81 gm; ZnSO₄.7H₂O- 0.22 gm; Na₂MoO₄.2H₂O- 0.39 gm; CuSO₄.5H₂O- 0.079 gm; Co (NO₃)₂.6H₂O- 49.4 mg; Distilled water-1L; Finally pH adjusted to 7.1.

The third medium used for the culture was MBBM, which was prepared as per Stein (1973). It was found always suitable for the growth of all the type of algae including green-algae, blue-green algae and diatoms. It was prepared from stock solutions of the following compounds: (1) NaNO₃- 25 gm/l distilled water (dw) (2) CaCl₂.2H₂O- 2.5 gm/l dw (3) MgSO₄.7H₂O-7.5 gm/l dw (4) K₂HPO₄- 7.5 gm/l dw (5) KH₂PO₄- 17.5 gm/l dw (6) NaCl- 2.5 gm/l dw (7) Di Sodium EDTA- 50.0 gm/l dw (8) KOH- 31.0 gm/l dw (9) FeSO₄.7 H₂O- 4.98 gm/l acidified water (10) H₃BO₃-11.42 gm, ZnSO₄.7H₂O- 8.82 gm, MnCl₂.4H₂O- 1.44 gm, MoO₃ - 0.71gm, CuSO₄.5H₂O- 1.57 gm and CoNO₃.6H₂O- 0.49 gm/l dw.

The liquid MBBM medium for algal culture was prepared by dissolving 10.0 ml of solutions from 1ˢᵗ to 6ᵗʰ stock; 1.0 ml of solution from 7ᵗʰ to 9ᵗʰ stocks; 2ml of solutions from 10ᵗʰ stock; 1gm of bacto-peptone and 5gm of sucrose were added to 950 ml of distilled water. Solidified MBBM was prepared by adding 1.5% of agar to the stock before autoclaving and the pH was adjusted to 6.8. These solid and liquid medium were taken in 250 ml conical flask and 10 x 75 mm culture tubes. These were incubated at 25⁰± 2⁰ C under continuous light intensity of 4000 lux conditions. Cultures were allowed to grow for 20-40 days prior to counting and identification process.

Isolation and culture of soil-algae were carried out as per Mansour and Shaaban (2010) and Zancan et al. (2006). 10 gm of composite soil samples taken randomly from the upper 0.5-1.0 cm soil layer of collection spots for algal studies.
were used for soil culturing. Fresh soil samples were shaken with sterile water (90ml) for 2 hours on a rotary shaker. One ml of the soil suspension obtained was spread on solidified and liquid Modified Bold’s Basal medium (MBBM). For the culturing of blue-green algae, MBBM without nitrogen was used. Standard plating or streaking techniques were also used for isolation and pure culture of algae (Stainer et al. 1971). Green and blue-green algae were developed after 20 days and 30 days of culturing, respectively, in MBBM. But development of diatoms was noted only after 40 days of culturing.

3.4. Analyses of Climatic and Physico-Chemical Soil Parameters

Rain fall data was the only climatic factor examined in this study by using rain gauge. Electrical Conductivity (EC), temperature and soil moisture content were the physical parameters determined whereas soil pH, organic carbon, total N, available P and K were the chemical parameters analysed. Soil pH and EC were measured from 1:2 (neutral distilled water) soil pastes of air dried sample. Soil pH was measured using a pH meter (Systronics 324) and EC using an electrical conductivity bridge (Systronics Conductivity Bridge 303). Soil temperature was directly measured from the field using a thermometer. Soil moisture was estimated on the same day of collection as per Clarson (2002); Organic carbon, total nitrogen (N), available phosphorous (P) and available K were estimated as per Jackson (1973).

3.5. Biodiversity Characterization

In addition to direct observation of field samples in wet seasons, two additional procedures were used to identify the biodiversity of soil-algae. Colonies of the different strains of algae grown on agar plates not identifiable by direct observation were isolated into uni-algal cultures (Neustupa 2001). Isolates were
obtained by transferring a small amount of the cells from a colony to sterile test tube with liquid Modified Bolds Basal medium (MBBM) and leaving them to grow for 2-4 weeks in the incubation chamber before identification. The second method used was that of ‘growth slides’ (Zancan et al. 2006). Sterile glass-slides were placed over soil samples moistened with sterile water and in the incubation chamber for 1-2 days, and afterwards covered with sterile incubated cover-slips for examination using LX400 Trinocular Microscope. The actively growing algae adhering to the slides were examined under the microscope and the photographs were taken using SONY Digital Camera W 310 attached to the microscope.

All the algae were identified up to the species/variety. Since a monograph on tropical soil-algae is unavailable, identification of them on the basis of the records on aquatic algae was carried out. Identification of micro-algal species was carried out on the basis of morphological characteristics, such as cell structure and size, cell organisation and number, and position of flagella. Some biochemical properties such as pigments and food storage were also utilized. Characterization and classification of green-algae were carried out in accordance with on-line databases of Guiry and Guiry (2012). In addition to that, systematic keys of Ralfs (1848), Randhawa (1958, 1959), Prescott (1962), Ramanathan (1964), Philipose (1967), Iyengar and Desikachary (1981) and Pham et al. (2011) were also employed.

The nature of filaments, shape and size of vegetative cells, width and length of intercalary cells, presence or absence of constriction at the cross wall, sheath nature, presence or absence of heterocysts and akinetes were taken into consideration during the identification of the blue-green algae. Identifications were made using the floras of Desikachary (1959), Prescott (1962), on-line databases by Guiry and Guiry (2012) as well as descriptions in the Komarek (2007).
Diatom samples were prepared by the method of Van de Vijver and Beyens (1998). Small parts of the samples were cleaned by adding $\text{H}_2\text{O}_2$ and heating to $80^0\text{c}$ for about 1 hr. The reaction was completed by addition of $\text{KMnO}_4$. The resulting cleaned materials were mounted in Naphrax. Taxonomical identifications were followed by Gandhi (1956), Sarode and Kamat (1984) and Taylor et al. (2007).

The descriptions of all the isolated green, blue-green algae and certain diatoms are supported with light microscopic images (LM). But the identification of majority of diatoms is carried out by using SEM images.

3.6. **SEM Analysis of Field Soil**

Collected soil samples were placed in air tight plastic bags to prevent moisture loss from the algae and repeat samples were sprayed with iodine to preserve them. The samples were then sieved through a 75 micron nylon mesh.

SEM studies were carried out with cleaned specimens and examined in high vacuum mode with a Jeol make model-JSM-6390 LA. The investigated material was fixed to carbon tapes (1x15 cm) placed on aluminium stubs, coated with gold and underwent magnetron sputtering by autofine coater-Jeol JFC-1600. The accelerating voltage was 20 kv. SEM analyses were carried out in the laboratory of the Sophisticated Test and Instrumentation Centre (STIC), University of Science and Technology, Cochin.

3.7. **Environment Relationships and Population Dynamics of Algae**

Environment relationships of soil-algae such as their relations to vegetation, season and physico-chemical characters of soil were found out. In addition to this, some other ecological parameters such as relative abundance of each algae, species richness, species evenness and diversity index (Shannon-Wiener Index) of algae in each season, and correlation of diversity to the soil parameters were also described.
Relative abundance of a species was calculated by the formula: \( \frac{Y}{X} \times 100 \), where \( X \) = total number of samples collected, \( Y \) = number of samples from which soil-algae was isolated (Dey et al. 2010).

The Shannon-Wiener Index for algal diversity has been studied as per Dey et al. (2010), using the formula

\[
H_s = -\sum_{i=1}^{S} \left( P_i \ln P_i \right),
\]

where, \( H_s \) = diversity in a sample of \( S \) species or kinds, \( S \) = the number of species in the sample, \( P_i \) = the relative abundance of \( i^{th} \) species or kinds, \( \text{measures} = n/N \), \( N \) = total number of individuals of all kinds, \( n_i \) = number of individuals of \( i^{th} \) species, \( \ln \) = log base 2.

Species Richness was calculated with the following formula:

\[
D = \frac{n}{\sqrt{N}},
\]

where, \( n \) = the number of different species in the sample and \( N \) = the total number of individual organisms in the sample. Species Evenness was calculated by the following formula \( E = \frac{H}{\ln(S)} \); Where \( H \)=diversity index value and \( S \)= the number of species in the sample.

3.8. Statistical Methods

Physico-chemical parameters and algal abundance are found out on the basis of the mean of ten independent samples. The data were statistically performed using ANOVA by SPSS package. Species richness, species evenness and diversity index were also statistically analysed and compared by PAST package.