Chapter 3

Materials and methods

Contents

3.1 Selection of test species
3.2 Experiments
3.3 Analysis
3.4 Statistical analysis
In the present investigation the stress impact of a synthetic anionic detergent on selected freshwater macrophytic plants was studied. Five common rooted macrophytic species in the Pallom block of the lower Kuttanad wetland was selected for the study. The selection of the plants was done after conducting a survey of the macrophytes in the area to identify the prevalent forms. The chosen plants were brought to the laboratory and grown in the field soil.

The Pallom block is a region that lies geographically between North latitudes 9° 32’ 7.8” and 9° 32’ 45” and East longitudes 76° 30’ 19” and 76° 30’ 46.2” in Kottayam district, Kerala (Fig.1). This region is densely populated and the local population uses the surrounding surface water for their daily household chores.

3.1 Selection of test species

Systematic field survey was done on the macrophytic vegetation. They were identified with the help of standard reference books (Gamble, 1935; Cook, 1996; Gosh, 2005). Details like taxonomic position, occurrence in the field, habit etc. were also noted in a data sheet (Table 1). Five common macrophytes were selected as experimental plants for the present study.
Materials and methods

Studies on the phytotoxic stress impact of a synthetic detergent on some freshwater macrophytes of lower Kuttanad wetland, Kerala

Fig. 1 Base map showing sampling sites and land use (vadakke kothari padasekharam, Pallom block)
### Table 1  Details of macrophytic vegetation in selected areas of Pallom block

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Common name (Malayalam)</th>
<th>Family</th>
<th>Occurrence</th>
<th>Habit</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alternanthera philoxeroides</em> (Mart.)</td>
<td>Kozhuppa -- Velimehembu</td>
<td>Amaranthaceae</td>
<td>Common</td>
<td>Herb</td>
<td>Shallow water pools, ditches, canals and marshes</td>
</tr>
<tr>
<td><em>Ceratophyllum demersum</em> Linn.</td>
<td>Kulavazha/Kakapola</td>
<td>Ceratophyllaceae</td>
<td>Rare</td>
<td>Aquatic Herb</td>
<td>Small ditches and Ponds at low lands</td>
</tr>
<tr>
<td><em>Colocasia esculenta</em> (Linn.) Schott</td>
<td>Chelli</td>
<td>Araceae</td>
<td>Common</td>
<td>Tuberous herb</td>
<td>Water logged ditches and stream side</td>
</tr>
<tr>
<td><em>Eichhornia crassipes</em> (Mart.) Solms.</td>
<td></td>
<td>Pontederiaceae</td>
<td>Common</td>
<td>Aquatic Herb</td>
<td>Ponds and low wetlands</td>
</tr>
<tr>
<td><em>Eleocharis dulcis</em> (Burm. f.) Trimen ex Hensch.</td>
<td></td>
<td>Cyperaceae</td>
<td>Common</td>
<td>Erect Herb</td>
<td>Marshy areas</td>
</tr>
<tr>
<td><em>Fimbristylis miliacea</em> (L.) Vahl. var. miliacea</td>
<td>Manjakora</td>
<td>Cyperaceae</td>
<td>Common</td>
<td>Erect Herb</td>
<td>Weed of cultivation, marshy areas and paddy fields</td>
</tr>
<tr>
<td><em>Hydrilla verticillata</em> (Linn. f.) Royle</td>
<td>Mullanpayal -- Karimkoovalam/ Kolachempu</td>
<td>Hydrocharitaceae</td>
<td>Common</td>
<td>Submerged herb</td>
<td>Stagnant ponds</td>
</tr>
<tr>
<td><em>Ipomoea aquatica</em> Forssk.</td>
<td>Palvally</td>
<td>Convolvulaceae</td>
<td>Rare</td>
<td>Herb</td>
<td>Ponds and ditches</td>
</tr>
<tr>
<td><em>Limnocharis flava</em> (Linn.) Buch.</td>
<td>Manjapola</td>
<td>Alismataceae</td>
<td>Common</td>
<td>Herb</td>
<td>Marshy areas</td>
</tr>
<tr>
<td><em>Ludwigia abscentans</em> (L.) Hara</td>
<td>--</td>
<td>Onagraceae</td>
<td>Rare</td>
<td>Aquatic Herb</td>
<td>Ditches and Ponds</td>
</tr>
<tr>
<td><em>Monochoria vaginalis</em> (Burm. f.) Presl.</td>
<td></td>
<td>Pontederiaceae</td>
<td>Common</td>
<td>Sub erect Herb</td>
<td>Paddy fields and wet lowlands</td>
</tr>
<tr>
<td><em>Nymphoides hydropylloides</em> (Lour.)</td>
<td>Neythel</td>
<td>Menyanthaceae</td>
<td>Rare</td>
<td>Aquatic herb</td>
<td>Ditches in grass lands and ponds</td>
</tr>
<tr>
<td><em>Nymphoides indica</em> (L.) O.Keze.</td>
<td>Neyyambal</td>
<td>Menyanthaceae</td>
<td>Common</td>
<td>Aquatic rooted floating herb</td>
<td>Ponds and ditches</td>
</tr>
<tr>
<td><em>Pistia stratiotes</em> Linn.</td>
<td>Akarathamara/ Kudapayal</td>
<td>Araceae</td>
<td>Common</td>
<td>Floating Herb</td>
<td>Aquatic</td>
</tr>
<tr>
<td><em>Persicaria barbata</em> (L.) Hara</td>
<td>Veluthamudela-mukki</td>
<td>Polygonaceae</td>
<td>Rare</td>
<td>Herb</td>
<td>Along stream sides</td>
</tr>
</tbody>
</table>
3.1.1 *Alternanthera philoxeroides* (Mart.) Grisb. [Family: Amaranthaceae]

The ‘alligator weed’ (*Alternanthera philoxeroides*) is a fast growing dicotyledonous species, which competes with *Salvinia molesta, Eleocharis dulcis, Ipomea aquatica* and *Eichhornia crassipes*. This species is common to oligotrophic wetlands and generally dominant in shallow waters, pools, ditches, marshes and near drainage areas. This aquatic or marshy perennial herb has a thick hollow and prostrate stem, swollen at the nodes serving as an adaptation in flotation. It is a native of South America and is now well established in India, Malaysia and Australia. In Kerala, it is found abundantly in Kottayam, Alappuzha, Ernakulam, Malappuram and Thrissur districts (Plate I -A). This plant is used as a local medicine for blood pressure. The capability of *A. philoxeroides* to provide oxygen to the underlying sediments helps the microbes in the rhizosphere region to enhance the capacity to oxidize natural organic matter (La Riviere and Bonner, 2002). In canals and rice field channels of the study area it formed sudd communities.

3.1.2 *Eleocharis dulcis* (Burm. f.) Trimen ex Hensch. [Family: Cyperaceae]

*Eleocharis dulcis*, a monocot species, generally called as ‘Chinese Water Chestnut,’ thrives well in the paleotropics. It is seen to flourish in abandoned fields, marshes and shallow waters and prefers slightly acidic soil. However, it also tolerates neutral and alkaline soils. The plant has a horizontal somewhat swollen submerged stem that roots in mud. The aerial part reaches upto 2 metres in height. They are hollow, but transversely septate at intervals. *E. dulcis*, is widely used as important forage for livestock and the corm is an ingredient in chinese cooking. This perennial emergent herb is now receiving increased attention because of its potential in aquatic weed management and pollution abatement (Socorro and Peterson, 1997). In Kerala, it spreads over Kottayam, Alappuzha, Idukki and Kasargode districts (Plate I-B).

3.1.3 *Fimbristylis miliacea* (Linn.) Vahl. var. miliacea [Family: Cyperaceae]

The ‘Manjakora’ (*Fimbristylis miliacea*), an emergent herbaceous monocot growing well in paddy fields is a major weed in our cultivated wetlands. It is also found to
grow associated with other Cyperus species in marshy areas. The plant has a tufted stem with fibrous roots (Plate I-C) and is found growing between paddy plants in the cultivated fields.

3.1.4 *Monochoria vaginalis* (Burm. f.) Presl. [Family: Pontederiaceae]

The ‘Karimkoovalam’ (*Monochoria vaginalis*) is a rooted emergent or sub-erect herb. Geographically, this monocot species is a native of tropical Asia. In Kerala, it is found in the low wetlands. The rootstock is short and creeping and the leaves long petioled and solitary at the top of the emergent stem (Plate I-D). This major weed in paddy fields is seen growing associated with *Limnophylla, Salvinia* and *Cyprus* species.

3.1.5 *Nymphoides indica* (L) O.Ktze. [Family: Menyanthaceae]

The ‘Water Snow Flake’ (*Nymphoides indica*), locally known as ‘Chinnambal’ is an aquatic rhizomatous dicotyledonous herb found growing in all districts of Kerala. This perennial floating-leaved aquatic plant is seen to thrive well in shallow ponds, pools, ditches and flooded lowlands especially during monsoons (Plate I-E). In the study area it was seen growing along the sides of the channels.

3.2 Experiments

3.2.1 Experimental Design

Laboratory experimental studies were carried out to assess the effect of the synthetic anionic detergent (*Surf excel blue*) on selected macrophyte species. Tests to evaluate phytotoxicity were done using static experiments in 35 litre polypropylene tubs as per the method followed by Mohan and Hosetti (1997). Aged tap water was used for the experiment and care was taken to use only one type of container throughout the study.
Plate I – A *Alternanthera philoxeroides*

A₁ A part of the plant showing leaves

Plate I – B *Eleocharis dulcis*

B₁ Inflorescence
Plate I – C *Fimbristylis miliacea*

C₁ A plant

Plate I – D *Monochoria vaginalis*

D₁ A leaf and inflorescence

Plate I – E *Nymphoides indica*

E₁ Nodal part of the plant
3.2.2 Soil type

The soil in the study area is clay loam. Topsoil was taken after removing the uppermost layer of leaf litter and rocks. Soil was air dried and sieved to < 2mm and then it was thoroughly mixed. 5kg of this prepared soil was transferred into the tubs for the pot culture experiment. The soil used had a pH 3.83, alkalinity 0.2 mg l\(^{-1}\), acidity 4.0 mg l\(^{-1}\), chloride 59.99 mg l\(^{-1}\) and electrical conductivity 0.483 mS cm\(^{-1}\). The procedures given by APHA (1998) were followed for analysis of soil parameters.

3.2.3 Transplantation and acclimation

Healthy young plants of the selected macrophytes were collected from the Pallom region of lower Kuttanad. They were maintained in the prepared containers (tubs) for two weeks for acclimation so as to stabilize the increase in enzymatic activity due to the stress caused by handling and transplantation of plants (Singh et al., 2004). The experimental plants were maintained under greenhouse conditions.

3.2.4 Detergent application and period of study

*Surf excel blue*, a commonly used anionic synthetic detergent was selected as the contaminant. The dose to be administered was determined by a pilot study done considering the general survival capacity of the plants under different concentrations of the detergent. The study was conducted for a period of 30 days exposing the experimental plants to detergent concentrations of 0.1 g l\(^{-1}\), 0.25 g l\(^{-1}\), 0.5 g l\(^{-1}\) and 1.0 g l\(^{-1}\). **Five sets of experiments were carried out for each concentration of the detergent and the mean values were taken.** For analysis, the plant parts (leaf and root) were washed in distilled water, blotted on paper, weighed and processed. Analysis was carried out on days 1, 3, 5, 10, 15, 20, 25 and 30. Periodic analysis was also done for the water quality. The data obtained were tabulated, statistically analyzed and graphically represented.
3.3 Analysis

3.3.1 Analysis of plant characteristics

3.3.1.1 Growth analysis

The specific leaf area and relative growth rate were determined as they are significant growth criteria for analyzing vegetative growth (Beadle, 1993).

(i) Specific leaf area (SLA)

The specific leaf area was determined as per method suggested by Zhou and Qiu (2005). Leaves of the control and stressed samples were collected in replicates, rinsed with distilled water and blotted dry. The outline of the leaf was traced out on a paper that had a uniform distribution with area. The leaf shape was cut out and the copy was weighed. The leaf dry weight was determined as per method of Weatherly (1970). Specific leaf area (SLA) was calculated and expressed in cm$^2$ gm$^{-1}$ (dry wt.).

(ii) Relative Growth Rate

Relative growth rate (RGR) is the rate of growth relative to the original size. It was determined and expressed in mg g$^{-1}$ day$^{-1}$ (dry wt.) following the method of Beadle, (1993).

$$RGR= \frac{\ln W_1 - \ln W_2}{T_1 - T_2}$$

where, $W_1$ and $W_2$ are the dry weights at times $T_1$ and $T_2$ (days) respectively.

3.3.1.2 Biochemical analysis

Biochemical analysis for the parameters was done for control and experimental samples of the selected plants.

(i) Chlorophyll

The chlorophyll content in the leaves was analyzed following the Arnon (1949), method. Absorbance of the sample was recorded at 645nm, 663nm and 652nm using UV visible spectrophotometer (Genesys, Thermospectronic make). The total
chlorophyll content in the sample was calculated from the absorbance and expressed in \( \text{mg g}^{-1} \) (fresh weight).

**Chlorophyll Stability Index**

Chlorophyll stability index (CSI) was calculated from the total chlorophyll content as per method suggested by Praderm *et al.* (2003).

\[
\text{CSI} = \frac{\text{Total chlorophyll content in experimental leaf sample as on day 8}}{\text{Total chlorophyll content in control leaf sample}}
\]

(ii) **Soluble Sugar**

The soluble sugar content in leaves was analysed by the phenol sulphuric acid method (Dubois *et al.*, 1956). In hot acidic medium, glucose was dehydrated to hydroxymethyl furfural. This formed a green coloured product with phenol and the absorbance was read at 490nm. The soluble sugar content was calculated and expressed in \( \text{mg g}^{-1} \) (fresh weight).

(iii) **Total Protein**

The method developed by Lowry *et al.* (1951), was followed for the estimation of total protein content in the leaf samples. The color developed was due to the presence of (1) biuret reaction of the protein with copper ion in alkali and (2) reduction of phosphomolybdic phosphotungstic reagent in the Folin-Ciocalteau reagent by tyrosine and tryptophan present in the treated protein. This colour developed was read at 660nm using spectrophotometer (Genesys) and the protein concentration of the test samples was calculated and expressed in \( \text{mg g}^{-1} \) (fresh weight).

(iv) **Electrolyte leakage and Membrane Injury**

For the study of electrolyte leakage from tissues, the method adopted by Bhattacharjee *et al.* (1996), was followed. Leaf and root tissues (200mg) of representative samples from each treatment were taken in vials containing 15 ml of deionised water and incubated at 25°C for 24 hours and the electrical conductivity of
the bathing medium was measured using conductivity meter (Systronics). The tissue and the leachate were then autoclaved and the electrical conductivity of total leachate was again measured. The electrolyte leakage was calculated and expressed in $mS \, g^{-1}$ (fresh weight).

The percentage of Membrane Injury (MI %) was calculated using the formula of Sullivan (1972).

$$MI \% = 1 - \frac{(1-T_1/T_2)}{(1-C_1/C_2)}$$

where, $C_1$ and $C_2$ are the electrical conductance of the control sample before and after autoclaving and $T_1$ and $T_2$, that of the of the test sample before and after autoclaving.

(v) Lipid peroxidation

Membrane lipid peroxidation was estimated in terms of malondialdehyde (MDA) accumulation. MDA level is routinely used as an index of lipid peroxidation. To estimate MDA accumulation, thiobarbeturic acid (TBA) test was performed by the procedure of Heath and Packer (1968). The concentration of MDA was calculated using an extinction coefficient of $155 \, mM^{-1} \, cm^{-1}$. Absorbance of the extract was read at 532nm and the measurements were corrected for unspecified turbidity by subtracting absorbance at 600 nm and expressed as $\text{nmoles MDA g}^{-1} \, (\text{fresh weight})$.

(vi) Total amino acid

Total free amino acids in leaf tissue were estimated by the method of Moore and Stein (1948) using ninhydrin, a powerful oxidizing agent that decarboxylates the alpha-amino acids and yields a bluish purple product. The colour developed was spectrophotometrically measured at 570nm. Amino acid content was expressed in $mg \, g^{-1} \, (\text{fresh weight})$.

(vii) Proline

Proline was determined by the method of Bates et al. (1973). The selective extraction with sulphosalysilic acid precipitated proteins as a complex. The other interfering
Materials and methods

Studies on the phytotoxic stress impact of a synthetic detergent on some freshwater macrophytes of lower Kuttanad wetland, Kerala

3.3.1.2 Proline

Proline materials were removed by absorption to the protein-sulphosalysilic acid complex. The extracted proline was made to react with ninhydrin in acidic conditions to form the chromophore and the absorbance read at 520nm and expressed in $\mu$ moles g$^{-1}$ (fresh weight).

(viii) Ascorbic acid

Ascorbic acid in the leaves was determined by the method of Roe (1954), using 2, 4 dinitro phenylhydrazine as a color-developing compound. Ascorbic acid was first dehydrogenated by bromination. Dehydro ascorbic acid was then reacted with 2, 4 dinitro phenylhydrazine to form osazone which was dissolved in sulphuric acid to give an orange red color solution and the absorbance was measured at 540nm using spectrophotometer. The ascorbic acid /ml in the test sample was calculated and expressed in mg g$^{-1}$ (fresh weight).

(ix) Total Phenolics

Total phenolic content in the leaves was determined adopting the method followed by Folin and Denis (1915) with Folin-Ciocalteau reagent. Phenols react with phosphomolybdic acid in the Folin-Ciocalteau reagent in alkaline medium and form a blue colored complex. The color developed was spectrophotometrically read at 650nm. The total phenol concentration of the test sample was calculated and expressed in mg g$^{-1}$ (fresh weight).

3.3.1.3 Histological studies

Histological studies were done to observe the effect of the detergent in the leaves of the control and experimental macrophytes. Free hand sections of 10-20$\mu$ thickness were taken, washed in distilled water and stained with 1% aqueous solution of safranin. The excess stain was washed off with distilled water and the material was mounted in 50% glycerin. Sections of control and tests were observed under a magnification of 1000X and photographs taken using Nikon H-III microscope with photo micrographic equipment.
3.3.2 Analysis of water quality

Periodic physico-chemical analysis of the water in the tub containing the experimental plants was done to evaluate the possible effect of the detergent on water quality. A similar set without plants was also maintained simultaneously to note the variations in the water quality with the presence of the detergent only.

3.3.2.1 Physico-chemical analysis

Analysis of water samples was carried out as per standard procedures (APHA, 1998) for pH, electrical conductivity, TDS, turbidity, dissolved oxygen (DO), biochemical oxygen demand (BOD), chemical oxygen demand (COD) and phosphate.

(i) pH

pH was measured using a digital pH meter (Eutech).

(ii) Electrical conductivity (EC)

Electrical conductivity is the measure of the ability of an aqueous solution to conduct an electric current. The EC was measured by a conductivity meter (Systronics) and expressed in mS cm⁻¹.

(iii) Turbidity

Turbidity (NTU) of the water sample was measured by a nephelometric turbidity meter (Systronics).

(iv) Total dissolved solids (TDS)

TDS was determined using conductivity meter (Systronics) that was calibrated using standard KCl solution and expressed as ppt.

(v) Dissolved oxygen (DO)

The azide modification of Winkler’s method was used for determination of DO and was expressed as mg O₂ l⁻¹.
(vi) Biochemical oxygen demand (BOD)

The biochemical oxygen demand (BOD) is a measure of the biodegradable organic matter present in a water sample and can be defined as the amount of oxygen required by microorganisms in stabilizing the biologically degradable organic matter under aerobic conditions. The sample was incubated at $20^\circ$C for five days in a BOD incubator and BOD was calculated.

$$\text{BOD (mg O}_2 \text{ l}^{-1}) = \text{Initial DO} - \text{Final DO}. $$

(vii) Chemical oxygen demand (COD)

COD was estimated by open reflux method. The organic and inorganic matter present in the sample was oxidized completely by potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) in the presence of sulphuric acid ($\text{H}_2\text{SO}_4$) to produce CO$_2$ and H$_2$O. Mercuric sulphate was added to neutralize the effect of chlorides and silver sulphate used as catalyst. The excess of $\text{K}_2\text{Cr}_2\text{O}_7$ was titrated against ferrous ammonium sulphate {$\text{Fe} (\text{NH}_3)_2(\text{SO}_4)_2$} using ferroin as indicator. The amount of $\text{K}_2\text{Cr}_2\text{O}_7$ used was proportional to the oxidisable organic matter present in the sample. COD was expressed as $\text{mg O}_2 \text{l}^{-1}$.

(viii) Phosphate-P

Phosphate was determined by the stannous chloride method. An aliquot of the sample was made alkaline by adding phenolphthalein followed by NaOH. The solution was decolourised with H$_2$SO$_4$ and then the sample was subjected to persulphate digestion for converting all the forms of phosphates into one form. After 30 minutes of digestion, the sample was cooled to room temperature and one drop of phenolphthalein indicator was added and it was again neutralized using NaOH or H$_2$SO$_4$. Ammonium molybdate and stannous chloride were then made to react with the digested solution and the intensity of colour developed was measured at 690nm using spectrophotometer. The concentration of phosphate was expressed in $\text{mg l}^{-1}$. 
Materials and methods

3.3.2.2 Anionic surfactants as MBAS

Determination of anionic surfactants in the water samples was done as per APHA, (1998). The surfactant was isolated from dilute aqueous solution by the process of sublation to yield a dry residue relatively free of non-surfactant substances. The anionic surfactant in the water samples was estimated by the methylene blue active substance (MBAS) method. MBAS brings about the transfer of methylene blue, a cationic dye, from aqueous solution into an immiscible organic liquid upon equilibration. This occurs through ion pairing between MBAS anion and methylene blue cation. The intensity of the resulting blue color in the organic phase is a measure of MBAS.

The method comprised of three successive extractions (i) from acid aqueous medium containing excess methylene blue to chloroform (CHCl₃) (ii) an aqueous back wash (iii) measurement of blue color in CHCl₃. The absorbance of the developed color was determined at 652 nm against a blank of CHCl₃ using spectrophotometer. The concentration of anionic surfactant in the test samples was calculated and expressed as mg MBAS l⁻¹.

3.3.3 Analysis of anionic surfactants (mg MBAS l⁻¹) in the natural system

Seasonal analysis of LAS (anionic component in the synthetic detergent) in the water samples collected from the natural site was done to determine the levels of detergent contamination in the natural system from where the experimental macrophytes were collected. A survey was conducted in the Pallom block in Kuttanad wetland ecosystem and six sites (S₁ – S₆) were identified (Fig.1) for collection of water samples to study seasonal variations for LAS during the year 2006 – 2007.

The sites selected included an abandoned paddy field (Site 1), that had been permanently fallow since last 8 years and had a water course fully covered with both floating and rooted species of macrophytes and other weeds. A high rate of decay of macrophytes was noticed in this site located at the northern side of the study area.
Materials and methods

Site 2 was an irrigation canal close to an excavated ditch, an open place in Vadakke Kothakari padashkaram. The channel extends through the cultivated field. Growth of macrophytes was seen to be restricted more towards the sides, rather than in the water retained area.

A part of the Kodur River, where the water flow is comparatively high was chosen as Site 3. It was noticed that bathing, washing and other human activities were common in this part of the river.

Site 4 was a point where the Kottayam – Neelamperoor canal (KN canal) drains into the Kodur River. The canal water seemed to be polluted. Effluent discharge from the nearby factories and wastes from households drained into this part of the system but a dilution effect due to the flow of the river could occur. Growth of emergent species was seen at the sides.

An area located in the beginning of the canal near a factory was chosen as Site 5. Human activities like bathing; washing of clothes and utensils etc. were common there. This canal received domestic sewage and agricultural runoff from the surrounding paddy fields. Two factories were found working near Site 6. Human dwellings and an ayurveda resort are there on the sides of this canal. Growth of both floating and rooted species of macrophytes was found in this area. A high rate of decay of these plants was also noticed.

3.4 Statistical Analysis

Datas (mean values) were tabulated and statistically analyzed as per the method followed by Gomez and Gomez (1984). SPSS was employed for the analysis. ANOVA with 1-factor CRD (completely randomized design) and 2-factor CRD were applied for growth and biochemical changes to test the level of significance between the control and experimental samples. Variations between species, concentration, duration and their interaction effects for studies on phytoremediation potential of the selected macrophytes were tested by 3-factor CRD. Correlation studies were done for comparison of biochemical constituents and for changes in water quality.