

MATERIALS AND METHODS

The materials and methods adopted in the present investigation titled “**Studies on removal of brilliant green dye using marine brown macroalgal biomass *Sargassum wightii***” is presented under the following headings:

Phase -I

- ❖ Selection, collection and identification of alga
- ❖ Selection of textile dye and preparation of dye solution

Phase -II

- ❖ Batch adsorption and desorption experiments
 1. Determination of optimum biosorption conditions for decolourization
 2. Dye desorption and regeneration study
 3. Sorption Isotherm, kinetics and thermodynamics

Phase –III

- ❖ Physicochemical characterization of untreated and treated brilliant green dye
- ❖ Analytical studies
 1. UV- visible spectral analysis
 2. FTIR spectroscopic analysis
 3. SEM with EDX analysis

Phase -IV

- ❖ Commercial applications
 1. Phytotoxicity of untreated and *S. wightii* treated brilliant green dye
 2. Microbial toxicity of untreated and *S. wightii* treated brilliant green dye
 3. Cytotoxicity of untreated and *S. wightii* treated brilliant green dye
 4. Zootoxicity of untreated and *S. wightii* treated brilliant green dye

Phase –V

- ❖ Physicochemical characterization of untreated and *S. wightii* treated effluent
- ❖ *Sargassum wightii* efficiency in the removal of textile effluent- a batch experiment

4.1 Phase -I

4.1.1 Selection, collection and Identification of algal sample

Brown marine macroalga (seaweed) was collected from the coastal area of Kanyakumari, Tamil Nadu, India (Latitude- 0.0780° N and Longitude- 77.5410° S) (Fig.1). The algal species was identified as *Sargassum wightii* according to Chapman and Gellenbeck (1983); Bold and Wyne (1978). Taxonomic classification of the algal species was made according to the system developed and modified by Papenfuss (1955). The identification was based on (a) morphological, external and internal characteristics and (b) ecological distribution and habitat.

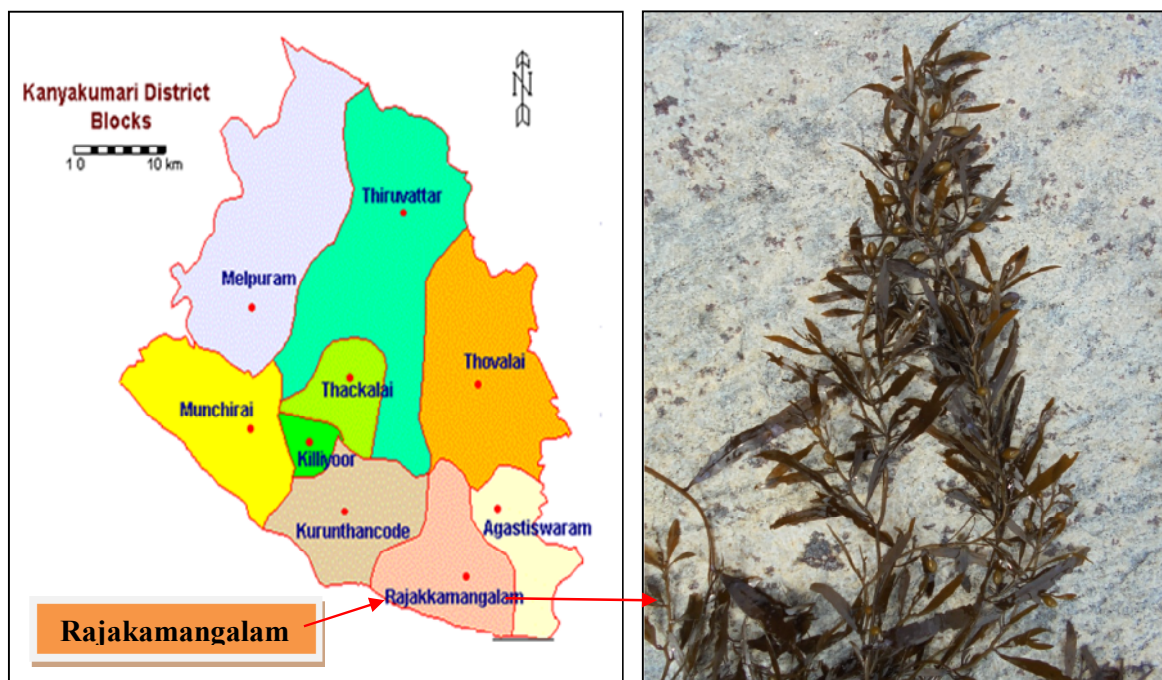


Fig. 1 Map showing the location of seaweed collection

4.1.2 Preparation of adsorbent

The seaweed was washed with sea water and fresh water thoroughly to remove the impurities. The alga was transported to the laboratory and air dried in shade (to avoid thermal degradation of the metabolites), pulverized and sieved through a mesh to get fine particles of size 150 μ m. The powdered sample (Fig. 2) was stored in an airtight container until use.



Fig. 2 Powdered *Sargassum wightii*

Taxonomical Hierarchy

Kingdom	:	Chromista
Phylum	:	Ochrophyta
Class	:	Phaeophyceae
Order	:	Fucales
Family	:	Sargassaceae
Genus	:	<i>Sargassum</i>
Species	:	<i>wightii</i>
Common name	:	Brown Alga

4.1.3 Selection of dye and preparation of dye solution

4.1.3.1 Selection of dye (Adsorbate)

All the dyes were procured from sigma- Aldrich, Mumbai, India were of 90% purity and used without further purification. All the other chemicals used in the present study were of analytical grade. De-ionised water was used for all dilutions. The preliminary pilot study was conducted with five different textile dyes viz., brilliant green, alizarin red S, bromothymol blue, malachite green, and solo chrome black T (Table 1). 100ml of different concentrations of each dye solution was taken in 100ml Erlenmeyer flask,

inoculated with 0.1-0.7g of algal powder were and placed at laboratory temperature (27° C) for a week. Later, the samples were taken and centrifuged at 4000 rpm for 5min. The cell free suspensions were collected and analysed for the percentage of colour removal. According to the pilot study, noticeable colour change was observed in brilliant green dye. Finally further study was conducted with brilliant green dye (BG) and its properties are shown in table 2.

Table. 1 Properties and structure of dyes used in pilot study

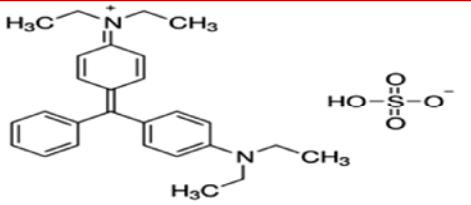
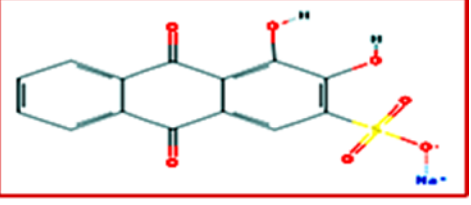
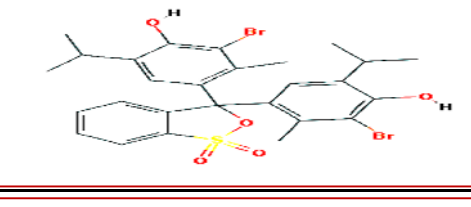
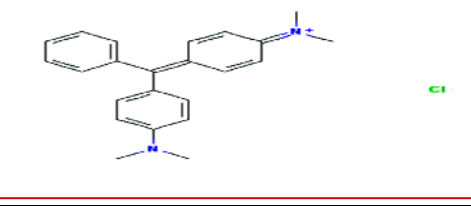
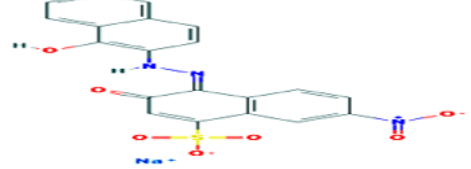
S. No	Name of the dye	Properties of the dye	Chemical structure
1	Brilliant green (C ₂₇ H ₃₄ N ₂ O ₄ S)	CAS. No :211-190-1 λ max(nm): 625 Mol. wet (g/mol) : 482.639	
2	Alizarin red S, (C ₁₄ H ₇ NaO ₇ S)	C. I. No : 58005 CAS. No :130-22-3 λ max(nm): 556 Mol. wet (g/mol) : 342.255949	
3	Bromothymol blue (C ₂₇ H ₂₈ Br ₂ O ₅ S)	CAS. No : 28631-66-5 λ max(nm) : 610 Mol. Wet (g/mol) : 624.38	
4	Malachyte green (C ₂₃ H ₂₅ ClN ₂)	C. I. No : 20470 CAS. No :569-64-2 λmax(nm): 617 Mol. Wet (g/mol) : 572.52724	
5	Solo chrome blackT, (C ₂₀ H ₁₂ N ₃ NaO ₇ S)	C. I. No : 14645 CAS. No :1787-61-7 λ max(nm) : 503 Mol. Wet (g/mol) : 461.379949	

Table. 2 Properties of BG dye

Chemical name	[4-[[4-(diethylamino)phenyl]-phenylmethylidene]cyclohexa-2,5-dien-1-ylidene]-diethylazanium;hydrogen sulfate
Synonyms	Astradamant green GX, Basic Green 1, Diamond Green, Emerald Green, Ethyl Green, Malachite Green G, Solid Green JO, Basic brilliant green
Molecular formula	C ₂₇ H ₃₄ N ₂ O ₄ S
Molecular weight	482.639 g/mol
CAS Reg. No.	633-03-4
EC Number	211-190-1
Melting point	210 deg C (lit)
Product category	Organics
Hazard code	GHS07
Industry uses	Dyes
Consumer uses	Silk and wool, spirit inks, wood stains, pigments, antiseptic

4.1.3.2 Preparation of dye solution

Before the experiment, dye stock solutions were prepared by dissolving accurately weighed dye in distilled water at a concentration of 1 g/L and left overnight to make the dye powder fully dissolved (Fig.3). The bottles were covered with aluminium foil in order to prevent the decolourization caused by light and stored in dark environment at room temperature. All the experimental solutions were prepared by diluting the stock solution to the required concentration. Absorbance values were recorded at the corresponding maximum absorbance wavelength (λ_{\max}) of 625nm.

Dye solution was initially calibrated for concentration in terms of absorbance units (Fig.4). The calibration curves of BG dye solution were plotted respectively from the dye solution prepared in the concentration range from 10-110mg/L.

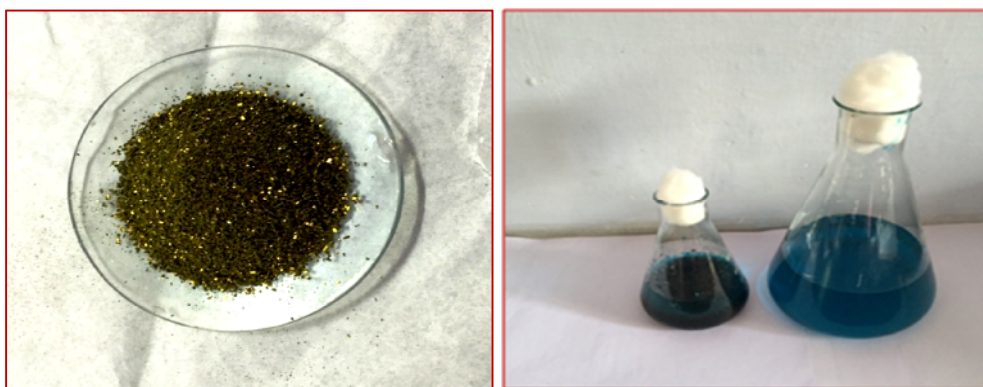


Fig. 3 A. Brilliant green dye 3B. Preparation of dye stock Solution and working solution

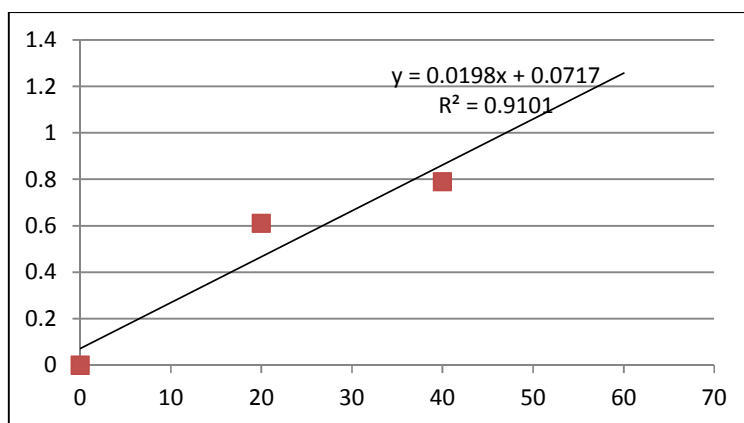


Fig. 4 Standard curve of BG solution

4.2 Phase - II

4.2.1 Biosorption optimization study (decolourization)

The effect of various parameters for the removal of BG dye from aqueous solution onto the brown marine macroalgae was studied in a batch mode (Fig. 5). The experiments were conducted in 250 ml Erlenmeyer flasks containing 100 mL dye solution with a desired quantity of adsorbent. To evaluate the effect of different environmental factors on decolourisation, the batch experiment was carried out with varying dye concentrations (10-110 mg/L), adsorbent dosages (0.05-0.6 g) and pH (2-11) at different temperatures

(20-60° C). The solution pH was adjusted by adding 0.1 N NaOH and 0.1 N HCl. Under the optimized conditions the samples (5 ml) were withdrawn at various time intervals (0, 5, 10, 15, 20, 30, 60, 120, 180, 240 min) and were evaluated for the color removal (%) and uptake capacity (q_{eq}). The absorbance of the supernatant solution was read in a colorimeter (μ P Photo Colorimeter- 1311) at 625 nm to estimate the final dye concentration. All the experiments were conducted in triplicates and conical flasks were capped in order to prevent evaporation at high temperature levels. The percentage of decolourization and dye uptake capacity were calculated using the following formulae.

$$(\%) \text{ Color removal} = \frac{\text{Initial concentration} - \text{Final concentration}}{\text{Initial concentration}} \times 100 \dots (1)$$

$$\text{Equilibrium uptake capacity (} q_e \text{)} = \frac{(\text{Initial concentration} - \text{Final concentration})V}{W} \dots (2)$$

where, V is total volume of the solution (L) and W is weight of adsorbent concentration (g).



Fig.5 Experimental setup shows the optimization study

4.2.2 Dye desorption and regeneration study

After adsorption process, the dye loaded *Sargassum wightii* was filtered and dried overnight. Dye loaded algal biomass was placed in desorption medium (0.1N HNO₃, 0.1M HCl, 0.1N NaOH and 10% CH₃COOH) and agitated at 150 rpm at 30°C for 30 min and were repeated for 1 cycle. Samples were taken to record for its pH and maximum absorbance was read in a colorimeter. Then seaweed was filtered and rinsed with double distilled water in order to remove remaining desorbing agent and left overnight to dry. After drying, the seaweed undergoes adsorption process and the regeneration cycle continues.

% dye desorption was calculated using the formula below,

$$DE = \frac{\text{Amount of dye desorbed}}{\text{Amount of dye adsorbed}} \times 100 \dots (3)$$

4.2.3. Application of theories of adsorption

4.2.3.1 Sorption Isotherm

The efficiency of adsorption process was determined by isotherm study. Langmuir (1916) and Freundlich (1906) models have been used to describe sorption isotherm.

4.2.3.2 Sorption kinetics

The linear form of pseudo-first-order and pseudo-second-order were applied in dye adsorption study. R² values and comparison of calculated sorption capacity, q_{eq} values and experimental q_{eq} values were used to verify the adsorption kinetics of dye by *S. wightii*.

4.2.3.3 Sorption thermodynamics

Thermodynamics analysis was conducted by using different temperatures (25- 60° C). Important properties such as the Gibbs free energy change (G°), enthalpy change (H°) and entropy change (S°) were calculated from the slope and intercept of the Van't Hoff plots.

4.3 Phase -III

4.3.1 Physico-chemical characterization of untreated and treated BG dye

Treated, untreated BG dye and effluent was subjected to various physicochemical analyses. The physicochemical parameters, methods of analyses and the appendices in which the methods followed were described and the respective references are given in table 3.

Table 3 Physicochemical characterization of BG dye and effluent

S. No	Parameters	Method of analyses	References	Appendix
1.	Colour	Visual	-	1
2.	Odour	Smell	-	2
3.	Temperature (°C)	Thermometer	-	3
4.	pH	pH meter	APHA, 1998	4
5.	Electrical conductivity ($\mu\text{mho/cm}$)	Conductivity bridge	APHA, 1998	5
6.	Total suspended solids	Filtration	APHA, 1998	6
7.	Total dissolved solids	Evaporation	APHA, 1998	7
8.	Total solids	Calculation	-	8
9.	Total hardness	Titrimetry	APHA, 1998	9
10.	Total Alkalinity	Titrimetry	APHA, 1998	10
11.	Dissolved oxygen	Titrimetry	APHA, 1998	11
12.	Biological Oxygen Demand	Iodometric Method	APHA, 1989	12
13.	Chemical oxygen demand	Titrimetry	APHA, 1998	13
14.	Chlorides	Titrimetry	Vogel, 1964	14
15.	Phosphates	Titrimetry	APHA, 1998	15
16.	Sulphates	Titrimetry	APHA, 1998	16
17.	Nitrate	Colorimetry	APHA, 1998	17

All the values except pH are in mg/l

4.3.2 Analytical studies (characterization of dye solution)

4.3.2.1 Extraction of metabolites for analytical studies

The metabolites were extracted after decolourization of BG dye (100g/L) under optimized conditions. Untreated dye solution at 0 hr served as a control. 100ml of samples was filtered and centrifuged at 5000 rpm for 10 min. The cell free supernatant of control and decolorized samples were extracted with an equal volume of ethyl acetate. The extract was dried over anhydrous sodium sulphate and evaporated to dryness in a rotary evaporator. Decolorized samples were monitored using UV-vis spectroscopic analyses, characterization of functional groups was performed using FTIR spectroscopic analysis and the surface characterization of the biomass was screened by SEM with EDX.

4.3.2.2 UV-visible spectral analysis

Treated and untreated dye solutions were analyzed using double beam UV- visible spectrophotometer (Shimadzu UV- 2102 PC). Decolourization percentages were recorded from the absorption spectrum in wavelength range of 200-800 nm was recorded.

4.3.2.3 FT-IR analysis

The FT-IR spectrum of the sample before and after adsorption was obtained using KBr disk technique. The transmission of FT-IR spectra was documented between 400-4000cm⁻¹ using Shimadzu 8400S model spectrum.

4.3.2.4 Scanning electron microscopy (SEM) with EDX

Characterization of macromolecular structure of algal sample before and after adsorption was terminated by SEM. Biosorption were mounted on stubs and coated with gold palladium of 100-150 Å thickness and transferred to the sample chamber of SEM operated at 10kV to confirm the surface adsorption of the dye on the algal biomass. Chemical composition was determined by EDX. Algal samples were coated with carbon and analysis performed at 20 kV.

4.4 Phase -IV

4.4.1 Phytotoxicity impact of untreated and *S. wightii* treated BG dye solution on the biometric parameters of black gram

4.4.1.1 Selection of the experimental plant

Black gram (*Vigna mungo* L.) was selected for the experiment because of the following reasons: 1. Well suited to cool and temperate growing regions with low to moderate rainfall. 2. Common food crop in Tamil Nadu with good nutritional value. 3. Germination period is very short. 4. Easy cultivation.

4.4.1.2 Site explanation

The experiment was conducted for the duration of 3 months and site was irrigated with groundwater (Control T1), untreated BG dye (T2) and treated BG dye (T3). The meteorology of the study are indicated the temperature range from 20 to 30°C with no rainfall and the moisture from 10 to 50%. The soil at the experimental site had texture class of 60% clay, 20% silt, 14% fine sand and 6% coarse sand.

4.4.1.3 Experimental design

Based on the tolerance and season, black gram was chosen and the seeds were obtained from Tamil Nadu Agricultural University (TNAU), Coimbatore. Seeds were surface sterilized with 0.1% HgCl₂ for two minutes. A field experiment was conducted; land for each treatment was prepared of 30x30x30cm of length, breadth and width respectively, seeds were soaked for 5 hours in each treatment (control (T1), untreated (T2) and treated (T3)). After soaking, the seeds were sown in the effluent irrigated field and irrigated thrice a week at respective concentrations. Each treatment was replicated thrice. Land for each treatment was prepared of 30X30X30 cm (length, width, depth).

4.4.1.3.1 Seventh day of experiment

Germination percentage, shoot length (cm), root length (cm), fresh weight (g), dry weight(g), vigour index, tolerance index and % phytotoxicity were measured on the seventh day. The fresh weight of seedlings was taken using an electrical single pan

balance. The percentage of vigour index (1973), tolerance index (1972) and % phytotoxicity (1978) were also calculated.

4.4.1.3.2 Germination percentage

The number of seeds germinated in each treatment was counted on the 7th day and the germination percentage was calculated by using the following formula,

$$\text{Germination percentage} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds sown}} \times 100$$

4.4.1.3.3 Shoot and root length (cm/seedling)

Twenty seedlings were taken from each treatment and their shoot length and root length were measured by using a cm scale and these values were recorded.

4.4.1.3.4 Fresh weight (g/seedling)

Ten seedlings were collected from each treatment and their fresh weights were measured with the help of an electrical single pan balance.

4.4.1.3.5 Dry weight (g/seedling)

The same seedlings used for fresh weight were kept in hot air oven at 80°C for 24 hours. Then, the seedlings were taken from the oven and kept in desiccators for some time. Their dry weights were taken by using an electrical single pan balance.

4.4.1.3.6 Vigour index

Vigour index of the seedlings was calculated by using the formula proposed by Abdul-Baki and Anderson (1973).

$$\text{Vigour index} = \text{Germination percentage} \times \text{Length of seedling}$$

4.4.1.3.7 Tolerance index

Tolerance index of the seedling was calculated by using formula proposed by Turner and Marshal (1972).

$$\text{Tolerance index} = \frac{\text{Mean length of longest root in treatment}}{\text{Mean length of longest root in control}}$$

4.4.1.3.8 Percentage of phytotoxicity

The percentage of phytotoxicity of dye was calculated by using the formula proposed by Chou *et al.* (1978).

Percentage of phytotoxicity =

$$\frac{\text{Radicle length of control} - \text{Radicle length of test}}{\text{Radicle length of control}} \times 100$$

4.4.1.4 Sixtieth day of experiment

Plant sampling after 90 days of transplantation was assessed to evaluate the impact of untreated and seaweed treated dye in terms of the morphological parameters such as root length (cm), shoot length (cm), total leaf area (cm²), plant dry weight (g) and number of root nodules, yield attributes like number of fruits and seed dry weight (g) were specifically analyzed on sixtieth day.

4.4.1.4.2 Shoot length and root length

The shoot length and root length of plant samples were measured by using centimeter scale and were recorded.

4.4.1.4.3 Number of leaves and total leaf area

The plant samples were collected periodically and the number of leaves were counted and recorded for each treatments. The length and breadth of the leaf samples were measured and recorded. The total leaf area was calculated by using the Kemp's constant.

Total leaf area = L × B × Kemp's constant (for Dicot-0.66)

4.4.1.4.4 Fresh weight and dry weight

After the harvest, the plants were uprooted and their dry weights were determined by keeping the plant materials in a hot air oven at 80 °C for 24 hrs and recorded.

4.4.1.4.5 Root nodules

Root nodules were detached from the roots and measured their size.

4.4.1.4.6 Fruit yield

The harvested fruits were assessed with respect to the number produced and the physical parameters as fruit weight (g).

4.4.1.5 Statistical analyses

Toxicity results obtained were subjected to one-way Analysis of Variance (ANOVA) and mean separation were accomplished by Duncan's Multiple Range Test (DMRT) for significant differences ($P \leq 0.05$) using STATISTICA SOFTWARE (1999).

4.4.1.6 Effect of soil characteristics after plant growth

To investigate the effect of untreated and treated BG dye on soil after the plant had attained full growth of 90 days. The soil sample from T1, T2 and T3 were collected for physico- chemical analyses.

4.4.1.6.1 Collection and physico- chemical analyses of soil

The soil was collected from a depth of 15 cm with a wooden spade, dried and crushed. It was sieved using a steel sieve and analyzed for its physicochemical properties (Table 4).

Table.4 Physicochemical analyses of untreated and treated soil

Parameters	Method of analyses	References	Appendices
pH ($\mu\text{mhos/cm}$)	pH meter	APHA, 1998	4
EC (%)	Conductivity bridge	APHA, 1998	5
Total Nitrogen (mg/kg)	Titrimetric	Humphrie, 1956	24
Total Phosphorus (mg/kg)	Calculation	Saxena, 1987	25
Total Potassium (mg/kg)	Calculation	Saxena, 1987	26
Sodium (mg/kg)	Calculation	Saxena, 1987	27
Calcium as Ca (mg/kg)	Calculation	Saxena, 1987	28
Iron (mg/kg)	Calculation	Saxena, 1987	29
Copper (mg/kg)	Calculation	APHA, 2005	30
Zinc (mg/kg)	Calculation	APHA, 2005	30
Chromium (mg/kg)	Calculation	APHA, 2005	30
Cadmium (mg/kg)	Calculation	APHA, 2005	30
Nickel (mg/kg)	Calculation	APHA, 2005	30
Manganese (mg/kg)	Calculation	APHA, 2005	30

4.4.2 Microbial toxicity assay of untreated and *S. wightii* treated dye

4.4.2.1 Bacterial toxicity

The bacterial toxicities of the untreated and treated dyes were determined by agar well diffusion method. Each treatment was replicated thrice. Muller Hinton agar (MH) plates were swabbed (sterile cotton swabs) with 0.1 OD cultures of gram positive (3 cultures), gram negative (7 cultures) bacteria such as *Klebsiella* sp, *Streptococcus epidermis*, *Vibrio cholera*, *Pseudomonas aeruginosa*, *Shigella* sp, *Bacillus cereus*, *Proteus vulgaris*, *Salmonella* sp, *Staphylococcus aureus* and *Escherichia coli*. Using the sterile cork borer, wells (6mm) were made into each petriplate. 10 µl of untreated dye and treated dye were introduced into separate wells and distilled water served as control. Then the plates were incubated at 37°C for 24 hours. After the incubation period, the diameter of the inhibition zones of each well was measured and the values were tabulated (Appendix 35).

4.4.2.2 Fungal toxicity

The fungal toxicities of the untreated and treated dyes were determined by agar well diffusion method. Each treatment was replicated thrice. Potato dextrose agar (PDA) plates were swabbed (sterile cotton swabs) with 0.1 OD fungal cultures including *Rhizopus* sp, *Aspergillus flavus*, *Acremonium* sp, *Trichoderma viride*, *Aspergillus fumigates* and *Aspergillus niger*. Using the sterile cork borer, wells (6mm) were made into each petriplate. 10 µl of untreated dye and treated dye were introduced into separate wells and distilled water served as the control. Then the plates were incubated at 37°C for 48-72 hours. After the incubation period, the diameter of the inhibition zones of each well was measured and the values were tabulated (Appendix 36).

4.4.2.3 Statistical analyses

Toxicity results obtained were subjected to one-way Analysis of Variance (ANOVA) and mean separation were accomplished by Duncan's Multiple Range Test (DMRT) for significant differences ($P \leq 0.05$) using STATISTICA SOFTWARE (1999).

4.4.3 Cytotoxicity study of untreated and *S. wightii* treated dye

4.4.3.1 Selection of *Allium cepa* for toxicity assay

Allium cepa is a widely used experimental plant in cytotoxicity studies because it has a suitable chromosomes and good correlation with mammalian test system. In order to test the cytotoxic effect of BG dye, the onion bulbs were exposed to control (T1), untreated (T2) and treated (T3) BG dye solution.

4.4.3.2 Collection and preparation of onion bulbs

Commercially available onion (*Allium cepa* L.) bulbs were used for cytotoxicity studies using the standard protocol (Fiskesjo, 1985). The purple variety of equal sized (width: 20-25mm, weight: 6-12mg) onion bulbs (*Allium cepa* L.) were obtained from local market of Coimbatore, Tamil Nadu, India. The bulbs were air dried for 10 days and the outer dry, brown scales were removed. The dried roots were carefully removed with a sharp razor blade and placed in distilled water to protect the primordial cells from drying up.

The equal sized bulbs were exposed to control, untreated and treated dye solution and each group consisted three replicates. For each treatment group, treatment solutions and control were changed for every 24 hours till 72 hours. After 72 hours exposure, the roots of the bulbs were removed with a forceps and their lengths were measured in centimeter with a meter scale. After sufficient root growth (1-2cm), the bulbs were removed and washed in distilled water.

After washing, the root tips were cut and fixed in fixative I [acetic acid: chloroform: alcohol (2:2:3)] for 5mins followed by fixative II [Hydrochloric acid: alcohol (1:1)] for 2mins and finally fixed in 70% alcohol. After that, the root meristems were cut and stained with 0.5% aceto-carmin for 5mins without hydrolysis. Stained root tips were squashed on microscopic slides and covered with cover slips to visualize the scorable stages under microscope.

4.4.3.3 Scoring

The slides were viewed under the binocular light microscope using the 100X objective lens with oil immersion. A total of 1000 cells were scored per slide and the most

representative abnormalities were photographed. Mitotic index and mitotic depression were calculated following the procedure of Das (1986) and Kar (1992)

$$\text{Mitotic Index (MI)} = \frac{\text{No.of dividing cells}}{\text{Total no.of cells scored}} \times 100 \quad \dots (1)$$

$$\text{Mitotic Depression (MD)} = \frac{\text{MI (control)} - \text{MI (treated)}}{\text{MI (control)}} \times 100 \quad \dots (2)$$

4.4.3.4 Statistical analyses

Toxicity results obtained were subjected to one-way Analysis of Variance (ANOVA) and mean separation were accomplished by Duncan's Multiple Range Test (DMRT) for significant differences ($P \leq 0.05$) using STATISTICA SOFTWARE (1999).

4.4.4 Toxicity evaluation of untreated and *S. wightii* treated BG dye on *Labeo rohita*

4.4.4.1 Selection of fish

An investigation was carried out in laboratory to evaluate the impact of untreated and *S. wightii* treated BG dye on the growth of *Labeo rohita* (Fig. 6). *Labeo rohita* (Hamilton 1822), commonly known as Rohu is the prime carp species and it is chosen for the present study because of the following reasons

1. Fast growth
2. Highly nutritious
3. Resistance to the disease and
4. Easily adaptable to laboratory conditions
5. Easy availability
6. Feeding flexibility
7. It is an ideal animal for toxicity studies in aquatic biology

Taxonomical hierarchy

Phylum: Chordata

Class: Actinopterygii

Order: Cypriniformes

Family: Cyprinidae

Genus: *Labeo*

Species: *rohita*



Fig. 6 Experimental fish *Labeo rohita*

4.4.4.2 Collection and maintenance of experimental fish

An Indian major carp *Labeo rohita* (length 8.0 ± 0.6 cm and weight 11.0 ± 1.5 g) were procured from Tamil Nadu Fisheries Development Corporation Limited, Aliyar Fish Farm, Tamil Nadu, India. The fishes were oxygen packed in polythene bags and brought carefully to the laboratory to avoid any injury and minimal stress during transit. Before stocking, tank was washed with 0.1% potassium permanganate to avoid fungal contamination. The fish were acclimatized to laboratory conditions (Fig. 7) for couple of weeks with continuous aeration and were fed with ad libitum with rice bran and groundnut oil cake dough once daily. Water was renewed (one – third of the water) daily to avoid accumulation and contamination of excretory materials. Feeding was withheld for 24 h before the commencement of the experiment. Prior to the investigation of experiment, healthy fishes were collected randomly and transferred into the respective treatments.



Fig. 7 Acclimatization of fish *Labeo rohita* under laboratory condition

4.4.4.3 Experimental design

Twenty fishes were introduced into a plastic (20 liter capacity) trough. The experiment was carried out in two treatments (T1 and T2) and a control (dechlorinated water). Each treatment consisted three replicates, hence each treatment and control carried 20 fishes x 9= 180 fishes were maintained under 12:12 light–dark cycle. Two treatments and tap water free from chlorine were analyzed with the following physico-chemical parameters (APHA, 2005); temperature ($25.3 \pm 1.2^{\circ}\text{C}$), pH (7.0 ± 0.05), dissolved oxygen ($5.3 \pm 0.02 \text{ mg L}^{-1}$), total hardness ($17.9 \pm 0.3 \text{ mg L}^{-1}$) and salinity ($0.3 \pm 0.05 \text{ ppt}$).

T1 – Fishes maintained in tap water (control)

T2 – Fishes maintained in untreated BG dye

T3 – Fishes maintained in treated BG dye

During the experiment, the treatment water was renewed to remove faecal matter and to maintain the desired concentration of oxygen.

4.4.4.4 Mortality rate

Mortality rate was recorded for every 6 hours during the observation period of 96 hours. Fish are considered dead if there is no visible movement (e.g. gill movements) and if touching of the caudal peduncle produces no reaction, they were removed and mortality was recorded. Observations at three and six hours after the start of the test are desirable. Records are kept of visible abnormalities (e.g. loss of equilibrium, swimming behaviour, respiratory function, and pigmentation) and percent mortality was calculated. At the end of experimental period, the fishes from each treatments and control were collected for toxicological studies.

4.4.4.5 Collection of blood, determination of haematology, biochemical composition and enzymes

Blood was drawn from the cardiac region by cardiac puncture using a plastic disposable syringe fitted with 26 gauge needle which was coated with an anticoagulant heparin. The collected blood was expelled into the separate heparinised plastic vials and immediately used for haematological analysis. The remaining sample was centrifuged for

20min at 10000rpm to separate the plasma, which was used for estimation of biochemical composition (plasma glucose and protein) and enzymes (GPT, GOT, LDH and GSH).

4.4.4.5.1 Haematological analyses (Appendix, 31)

RBC and WBC were counted by haemocytometer method of Davidson and Henry (1969). Haemoglobin content of the blood was estimated by Acid - haematin method (Sahli, 1962). Haematocrit was estimated by microhaematocrit (capillary) method (Nelson and Morris, 1989). Erythrocyte indices (Stoskopf, 1993) like mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) were also calculated according to standard formulas

$$\text{MCV (cubic micra)} = \frac{\text{Hct (\%)}}{\text{RBC (millions X cu mm X } 10^6)} \times 100$$

$$\text{MCH (picograms)} = \frac{\text{Hb (g/dl)}}{\text{RBC (millions X cu mm X } 10^6)} \times 100$$

$$\text{MCHC (g/dl)} = \frac{\text{Hb (g/dl)}}{\text{Hct (\%)}} \times 100$$

4.4.4.5.2 Biochemical analysis

Plasma glucose was estimated by O- Toluidine method developed by Cooper and McDaniel *et al.* (1970). Plasma protein content was estimated (Lowry *et al.*, 1951) using bovine serum albumin as a standard (Appendix, 32).

4.4.4.5.3 Enzymological analyses

Plasma Glutamic oxaloacetate transaminase (GOT) and Glutamic pyruvate transaminase (GPT) activities were estimated according to the colorimetric method of Reitman and Frankel (1957). The enzyme Lactate dehydrogenase (LDH) activity was estimated by the method of King (1959). Reduced glutathione (GSH) was estimated using the Ellman's method (1959) (Appendix, 33).

4.4.4.6 Histological examination

The tissues of gill, kidney and liver were dissected from control, untreated (T1) and treated (T2) exposed fishes and for histological studies the methods of Pearse (1968), Roberts (1978) and Humason (1979) were followed. The histological changes in the tissues were examined under light microscope and photomicrography. The changes in the

tissues of the treated fishes were observed and compared with the control fish (Appendix, 34).

4.4.4.7 Statistical analyses

Toxicity results obtained were subjected to one-way Analysis of Variance (ANOVA) and mean separation were accomplished by Duncan's Multiple Range Test (DMRT) for significant differences ($P \leq 0.05$) using STATISTICA SOFTWARE (1999).

4.5 Phase -V

4.5.1 Collection of textile effluent

The textile effluent used in this study was obtained from Perundhurai Common Effluent Treatment Plant (PCETP) Private Limited, Perundhurai, Tamil Nadu, India. All the chemicals used in this study were of analytical grade and purchased from M/s Hi-Media, Mumbai, India.

4.5.1.1 Physicochemical characterization of untreated and treated effluent

Treated and untreated effluent was subjected to various physicochemical analyses. The physicochemical properties studied, methods of analyses, and the appendices in which the methods followed were described and the respective references are given in table 3.

4.5.1.2 *Sargassum wightii* efficiency in the removal of textile effluent- a batch experiment

The batch adsorption experiment was conducted to optimize the various operational parameters such as biosorbent dose, pH, temperature and incubation time to obtain optimum colour removal from the effluent. Batch studies were conducted in 250ml Erlenmeyer flasks containing 100ml of the diluted effluent(50%) with fixed amounts (100-500g/L) of adsorbents and pH (3-10) at different temperatures (25-60 °C) which were shaken separately in a rotary orbital shaker at 150rpm for definite time periods (1-8days). The pH was adjusted using diluted NaOH and HCl solutions. At the end of the predetermined time intervals, samples were withdrawn from the shaker and centrifuged for 5 minutes. The absorbance of the supernatant solution was read in a colorimeter at 340nm

to estimate the final dye concentration. All experiments were carried out three replicates with respect to each condition and the average values were obtained. The control experiments were also conducted under the same conditions without adsorbent.

Colour removal efficiency was calculated using the following formula:

$$\text{Removal efficiency (\%)} = \frac{C_0 - C_i}{C_0} \times 100$$

where, C_0 is the initial absorbance and C_i is the final absorbance of the solution



Fig. 8 Map showing the location of Perundurai Common Effluent Treatment Plant (PCETP) Private Limited, Perundurai, Tamil Nadu, India.