3.1 Area of the study

Two wetland stations in Ernakulam district namely Eloor and Kannamaly were selected for the study (Fig: 1). Eloor, an island with 11.21 sq/km, on the Periyar River is land of more than 247 chemical industries and large number of wetlands. Most of these units have been here for the last fifty years and use extremely obsolete and polluting technologies. Toxic pollution from heavy metals to chemicals and radioactivity is found in air, soil and in the water bodies, which spreads the contamination to the Vembanad Lake, Cochin and to the Arabian Sea. This leads to a large-scale devastation of aquatic life in the area, the agricultural land and it is also affecting the health of the population.
Fig. 1: Drainage map of Ernakulam prepared by Central Pollution Control Board. Two wetlands stations selected for present study are marked by star symbol. The upper one marks Eloor and lower mark denotes position of Kannamally.
The soil, water bodies and the wetlands in and around Eloor have been contaminated with heavy metals like zinc, lead, cadmium, chromium and persistent organic pollutants like DDT. Since aquatic plants are present in these waters in large quantities, they are constantly exposed to these pollutants all the time.

Fig.2: Eloor - Edayar Industrial belt in Ernakulam marked by Central Pollution Control Board.
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The chemical industries mainly fertilizers require large quantities of fresh water for their processing. To have access to large quantities of fresh water these factories are established along the sides of rivers. In Kerala there are 44 small rivers with plenty of freshwater. Periyar river open to cochin backwaters. This backwater is one of the most productive estuarine systems with an estimated annual gross production of nearly 300g C/m² (Qasim et al. 1969). Unfortunately a large number of small and large industries comprising industrial estate established on the banks of Periyar and creating enormous environmental problems.

3.2. Sampling stations in the district

The Eloor-Edayar region on the banks of River Periyar, near the river-estuary confluence region, houses Kerala's largest industrial cluster including Fertilizers and Chemicals Travancore Ltd. (FACT), Hindustan Insecticides Ltd (HIL), Indian Rare Earths Ltd., Travancore Cochin Chemicals etc. Some of the major industries, their products, pollutants and permitted effluent discharge to the Kochi estuary. In Eloor one sample (W1) was collected from a location approximately 10 m northwest (W1- Lat.10°04′51.76″N, Long. 76°17′32.55″E) of the HIL site boundary (Fig.3). The second sample (W 2) was collected from the wetlands approximately 40 meters south (Lat.10°04′48.13″N Long. 76°17′22.75″E ) of the Kuzhikandam creek (Fig. 4).
Fig. 3: Eloor Wetland 1 sampling location

Fig. 4: Eloor Wetland 2 sampling location

From Kannamaly, one sample (W1) was collected approximately 100 metres south of the Kannamaly St. Joseph pilgrim centre and close to India Seafood Factory, at a location approximately at Lat. 9.8704°N and Long.
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76.2665° E. The second sample (W2) was collected from the wetlands south to the wetland I area, approximately 1.8 km away and located at Lat. 9.8612° N Long. 76.2642° E (Fig. 5 and 6).

**Fig. 5:** Kannamaly Wetland 1 sampling location

**Fig. 6:** Kannamaly Wetland 2 sampling location
3.3. Collection of surface water samples from sampling stations

Depending upon the location of Kuzhikkandam creek which carries waste discharges from industries mentioned above, two sampling stations each (W1 and W2) from Eloor. In Kannamaly two sampling stations (W1 and W2) were selected on the basis of proximity to India seafood Company. For a sample of water to be the true representative of water quality, water must be well mixed. Therefore a due care was taken in selecting the distances between each sampling station so that the maximum mixing of the waste discharge with the wetland water ensured the true water quality of the river.

Water collected from both wetlands (W1 and W2) located in Eloor and Kannamaly during pre-monsoon, monsoon and post monsoon period as parameters vary during different seasons. Wetland water samples were transferred to the laboratory and carried out preliminary sieving step to get rid of large suspended solids. The transferred water was immediately collected into 3 opaque tanks. (For treatment for 2 days, 4 days and 8 days). The opaque tanks were used to prevent light entering except at the top. These aquariums were arranged in such a way that light availability is maximum.

3.4. Physico chemical analysis of surface water samples

Various physico-chemical parameters studied for water quality assessment of Eloor and Kannamaly wetland water samples. For assessing physico-chemical characteristics, samples were collected from all the sampling stations every season in triplicates. During the pre-monsoon and post-monsoon period samples were collected in the first week of every month. To avoid floating material, samples were collected at about 5 cm depth from three points of the
site using the dip and grab sampling method and stored in clean polythene bottles. Samples were transferred to the laboratory and carried out preliminary sieving step to get rid of large suspended solids and later analyzed for various parameters using CPCB standard methods (CPCB, 2008). Temperature, pH, DO (Dissolved oxygen) and conductivity were measured in the field at the time of collection of samples by using portable star series Orion (USA) meter. The parameters of study were temperature, pH, BOD (Biological oxygen demand), COD (Chemical oxygen demand), EC (Electrical conductivity), Total alkalinity, TDS (Total dissolved solids), TSS (Total suspended solids), Nitrate, Phosphate, Ammonia, and Turbidity along with analysis for heavy metals before and after the experiment. Heavy metals were analyzed using AAS (Atomic absorption spectroscopy). Each parameter was determined in triplicate and the average of three values was recorded. All the measured data are presented as average values for premonsoon, monsoon and post-monsoon seasons.

Physico-chemical analysis of water was carried out as per CPCB guidelines (2008). Temperature, pH and TDS were measured in situ. BOD was measured respirometric method provides direct measurement of O₂ consumed by microorganisms from air in a closed vessel under conditions of constant temperature and agitation. Alkalinity of sample was estimated by titrating with standard sulphuric acid (0.02N) at room temperature using phenolphthalein and methyl orange as indicator. Conductivity meter was used to measure the conductance (EC) generated by various ions in the solution/water. The open reflux method is suitable to find out COD for a wide range of wastes with a large sample size. The dichromate reflux method was used. Turbidity is measured by its effect on the scattering light, which is termed as Nephelometry.
Phosphorous occurs in natural waters and in wastewater almost solely in the form of various types of phosphates. Stannous chloride method was used to determine phosphate content. Ammonia is produced by the microbiological degradation of organic nitrogenous matter or by leakage of ammonia. Nesslerisation method was used for the determination of ammonia (CPCB, 2008). UV spectrophotometer method was useful for the measurement of nitrates (CPCB, 2008). The ultraviolet absorption at 220 nm enables rapid determination of nitrate (CPCB, 2008). Turbidimetric method used for the determination of sulphate ions (CPCB, 2008).

3.5. **Plant material for bioassay and remediation studies**

Test organism is an aquatic macrophyte *Spirodela polyrhiza*. It is found worldwide in many types of freshwater and backwater habitat. It is a perennial aquatic plant usually growing in dense colonies, forming a mat on the water surface (*Fig. 7*). The plant is smooth, round with flat disc shaped leaves called fronds. It produces several minute roots. *Spirodela* species has a free-floating thallus; 2-5 plants may remain connected to each other. Plants are green, but may have a red or brown underside. Due to anthocyanin pigmentation (*Fig. 8*). Multiple roots (7 - 12) emerge from each frond (*Fig.9*).
Fig. 7: The plant forming dense colonies in control

Fig. 8: The abaxial side with anthocyanin pigmentation
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Fig. 9: Multiple roots from the fronds

Fig. 10: The plants are easy to remove from water
Spirodela polyrhiza is a species of duckweed known by the common names greater duckweed, giant duckweed, and duck meat. It is found to be worldwide in distribution in freshwater habitat. It is a perennial aquatic plant usually growing in dense colonies, forming a mat on the water surface. Each plant is a smooth, round, flat disc one half to one centimeter wide. It produces several minute roots. It also produces a pouch containing male and female flowers. The top part dies in the fall and the plant often overwinters as a turion. Spirodela is largest among duckweeds. Its fronds are measuring as much as 20 mm across. An individual frond may produce as many as 20 daughter fronds during its lifetime, which lasts for a period of 10 days. The bulk of the frond is composed of chlorenchymatous cells separated by large intracellular spaces that are filled with air and provide buoyancy. The plant can be easily removed from the water surface (Fig. 10).

**Systematic position**

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
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<tbody>
<tr>
<td>Phylum</td>
<td>Angiosperms</td>
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<td>Class</td>
<td>Monocotyledons</td>
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<tr>
<td>Order</td>
<td>Alismatales</td>
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<tr>
<td>Family</td>
<td>Araceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Spirodela</td>
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<tr>
<td>Species</td>
<td>polyrhiza</td>
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</table>
Some cells of have needle like raphides which are presumably composed of calcium oxalate. The upper epidermis is highly cutinized and is unwettable. Stomata are on the upper side. Anthocyanin pigments found on abaxial side of the leaf. *Spirodea polyrhiza* has greatly reduced vascular bundles. Roots are adventitious type. They are usually short but this depends on species and environmental conditions and vary from a few millimetres up to 14cm.

Duckweed (*Spirodea polyrhiza* L.) is used in water quality studies to monitor heavy metals and other aquatic pollutants, because duckweed, like other water plants, may selectively accumulate certain chemicals. *Spirodea* plant is the smallest available representative of angiosperms. The plants possess same physiological and biochemical properties of terrestrial macrophytes. By assessing the plant, we can assess the toxicity of surrounding media. They shows rapid growth between pH 5 - 9, and vegetatively propagated, which make them an ideal test system. The *Lemna* and *Spirodea* are among the most standardized test organisms in aquatic ecotoxicology studies (EPA, 1996; DIN, 2000 & 2001; Eberius, 2001; OECD, 2002).

The plant duckweed has several other advantages such as

1) It is the world’s “greenest” feedstock. Fast growing, high in protein and dietary minerals, and easily harvested, the plant is cultivated as a feed supplement for chicken, livestock, and farmed fish, especially in developing countries. The growth rate of duckweed under ideal light, temperature and pH would be exponential if there were no limitation in terms of mineral deficiencies or excesses. In ideal conditions it may reach about 1.2kg/m² duckweed (fresh).
2) An inexpensive, earth-friendly source of the biofuel ethanol. Unlike corn, potato etc duckweed requires minimal human-made energy to grow and it doesn’t deplete the world’s food supply. Spirodela polyrhiza is an ideal system for biofuels since it has more starch content than potato.

3) Bioremediation efficiency coupled with other aspects of fast-growth, direct contact with media enable duckweed-based wastewater treatment systems provide genuine solutions to the problems of urban and rural human waste management with simple infrastructure at low cost.

4) A natural wastewater treatment option. The plant feeds on organic pollutants like nitrogen, phosphate and other metallic pollutants the very stuff treatment plants aim to remove from wastewater. The recycling of water through waste water treatment works or purification of water for human use from presently polluted surface water. Duckweed will remain an underutilized resource unless governments accepts that polluted water cannot be released into water bodies without removal of minerals .There is a vast need for research support for this little plant with such a great potential.

Duckweed (*Spirodela polyrhiza*) plants were collected from JNTBG, Trivandrum and maintained in the local outdoor conditions for 3 months before the experiment for acclimatization. The stocks were cleaned by tap water then washed by distilled water. Five healthy and fresh, wet *Spirodela polyrhiza* plants were stocked into each of the three aquariums. Each aquarium was supplied sequentially with 5 liters of wetland water. Each of the three aquariums was filled with same amount of wetland water. An aquarium is kept with distilled water with nutrients and macrophyte is considered as control.
The experiment was kept under outdoor local environmental conditions for 2, 4 and 8 days retention time.

3.6. Exposure to surface water samples

In the present study, the test plant was exposed to the water samples for phytotoxicological assessment and phytoremediation studies. In phytotoxicological assessment, the sample water collected from all sampling sites during different seasons were immediately transferred to the lab aquarium (as replicates) and treated with Spirodela polyrhiza plants for 2 days, 4 days and 8 days. In the second part of current research, toxicity and bioaccumulation of Copper and Lead were studied. In this part, Spirodela polyrhiza plants were exposed with different concentrations of Copper and Lead mixed with Hoagland’s 10% nutrient media as per OECD guidelines (2002, 2006). The concentration of exposure were 1 mcg/L, 10 mcg/L, 20 mcg/L, 40 mcg/L and 80 mcg/L for a duration of 8 days. The concentration ranges were selected on the basis of its concentration in the wetland water samples. During phytoremediation studies, water samples collected over 3 seasons from both wetlands of Eloor and Kannamaly were brought to the laboratory and treated with Spirodela polyrhiza plants for 2 days, 4 days and 8 days of intervals and monitor the changes in the water parameters after each interval.

The treatment system for growing duckweed in small glass aquarium tanks (Fig. 11) were constructed in laboratory set up. After preliminary work, the entire set up was shifted to outside the laboratory for exposure to natural conditions. Each aquarium tank was 18 inches long, 10 inches deep and 9 inches wide. The stocks were cleaned by tap water and distilled water. Five
healthy fronds of Spirodela polyrhiza were stocked into the aquariums initially. All parameters were measured after 2, 4 and 8 days of exposure. The sides of each exposure chamber were covered with black chart paper to avoid light entry through the sides consequently preventing algal growth.

Fig. 11: Spirodela polyrhiza treatment tanks for the exposure

3.7. Phytotoxicity assessment end points

The phytotoxicity assessment was carried out by measuring the changes in morphological parameters, average specific growth rate, frond doubling time, estimation of biomass, estimation of photosynthetic pigments and estimation of protein and carbohydrates.

3.7.1. Changes in morphological parameters of the plant

At the start of the test, frond and colony numbers in the test vessels are counted and recorded, taking care to ensure that overlapping but distinctly visible fronds are accounted for. Frond and colony numbers (normal and abnormal) and their appearance were determined at the beginning and end of
the test when effects are assessed in terms of the average specific growth rate over the full duration of the test. Counts of frond numbers after intermediate exposure periods were taken since average specific growth rate need to be determined at intervals during the period of the test. Changes in plant development (e.g. frond size, appearance, necrosis or chlorosis, colony break-up or loss of buoyancy, root length, morphology or breakdown) were noted. Other morphological parameters observed were root number, root length and leaf size measurements. Total frond area was measured in cm² by image analysis with adobe Photoshop software. Root lengths were measured with a simple millimeter scale.

3.7.2. Changes in growth and biomass

Duckweed growth was determined measuring ASGR (Average Specific Growth Rate), Frond doubling time (Td) fresh weight (FW, biomass) and dry weight (DW) and DW/FW ratio (as per OECD, 2006 test protocol). The frond number was scored at the start of the experiments (t₀) and 2, 4 and 8 days after (t₀). All visible fronds were counted. ASGR is determined by the following formula.

\[ \mu_{i-j} = \frac{\ln(N_j) - \ln(N_i)}{t} \]

where - \( \mu_{i-j} \) : average specific growth rate from time \( i \) to \( j \), \( N_i \) : measurement variable in the test or control vessel at time \( i \), \( N_j \) : measurement variable in the test or control vessel at time \( j \), \( t \) : time period from \( i \) to \( j \).

\( T(d) \) can be calculated by equation \( Td = \ln 2 /\mu \) where \( \mu \) is the average specific growth rate.
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Dry weight to Fresh weight ratio can be calculated by measuring frond weight by calculating dry weight (gms)/ Fresh weight (gms).

$$\text{Growth Index can be calculated by} \frac{\text{Biomass (t - 8 days)}}{\text{Biomass (t - 0)}}$$

Plants were surface-dried between layers of paper towels, and the fresh weight was determined. To measure dry weight, plants were dried at 80°C overnight. The growth parameters were measured according to OECD guidelines (2006).

3.7.3. Estimation of photosynthetic pigment concentration

Chlorophyll content was determined by the acetone method by Arnon, (1949). For estimation of photosynthetic pigments plant material (100 mg) was ground in chilled 80% acetone in dark. After centrifugation at 10,000 × g for 10 min at 4°C, absorbance of supernatant was taken at 750, 663, 645,510 and 480 nm. Chlorophylls and carotenoid content was calculated using the formula given by Arnon (1949). Photosynthetic pigments Chlorophyll-a, Chlorophyll-b and Carotenoids were estimated using spectrophotometer (Hitachi-U-2000 spectrophotometer).

3.7.4. Biochemistry

1. Estimation of protein content

Proteins were estimated by the method of Bradford, (1976). Fresh leaves (0.5 g) were homogenized in 1 ml phosphate buffer (pH 7.0). The crude homogenate was centrifuged at 5000 × g for 10 min. 0.5 ml of freshly prepared trichloroacetic acid (TCA) was added and centrifuged at 8000 × g for 15 min. The debris was dissolved in 1 ml of 0.1N NaOH
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and 5 ml Bradford reagent was added. Absorbance was recorded photometrically at 595 nm (Beckman 640 D, USA) using bovine serum albumin as a standard.

2) Estimation of soluble carbohydrates

Total soluble carbohydrate was estimated as per Dey, (1990). Leaves (0.5g) were extracted twice with 90% ethanol. The extracts were combined. The final volume of the pooled extract was made to 25 ml with double distilled water. A suitable aliquot was taken from the extract and 1 ml 5% phenol and 5 ml concentrated sulphuric acid were added. Final volume of this solution was made to 10 ml by addition of double distilled water. Absorbance was measured at 485 nm using UV-Vis spectrophotometer.

3.7.5. Metal analysis by Atomic Absorption Spectroscopy (AAS)

All sample containers and glass wares used were washed with detergent, rinsed with water and immersed in concentrated nitric acid (AR grade) and kept for 12 hours. Then it was taken out and washed with distilled water in order to remove the unwanted traces of metallic and non metallic contents.

For determining the accumulation of metals in the samples, the samples were taken at the end of the exposure period and thoroughly washed with double distilled water to remove metal content smeared on the root and leaf surfaces. Four ml of conc. nitric acid and 1 ml of perchloric acid in the ratio of 4:1 was added into it. The digestion was carried out in small 100 ml beaker covered with small glass funnels kept in sand bath. The samples were evaporated to dryness and it was washed with double distilled water and made
up to 10 ml. Care was taken to prevent the solution from boiling. The samples were subjected to spectroscopy using Atomic Absorption Spectrophotometer of the model Hitachi Z. 8000 polarized Zeeman AAS and expressed as µg/100 mg of dry weight. AAS method was helpful in water analysis of wetland samples from Eloor and Kannamaly during three different seasons (Pre monsoon, monsoon and post monsoon). It was also helpful in determining phytoremediation capacity of the plant after different periods of exposure (8 days) to water samples in different seasons. The experiments on the accumulation of metals in Spirodea plant helped in determining the uptake of metals (Copper and Lead) with reference to the increasing concentration (1 mcg/L, 10 mcg/L, 20 mcg/L, 40 mcg/L and 80 mcg/L) of the metal in the medium and time duration of 8 days.

### 3.7.6. Bioaccumulation, Elimination and BCF

During the bio accumulation studies, the test material is collected from the treatment chamber after 8 days of exposure and analysed using AAS to find out percentage of removal or removal efficiency. BCF is the Bio concentration factor, determined to find out the efficiency of an organism to take up a metal from the surroundings in which it is living.

\[
BCF = \frac{\text{Metal concentration in the plant ( mg/KgDW)}}{\text{Metal concentration in the solution( mg/L)}}
\]

During phytoremediation studies, the metallic and non metallic parameters were measured according to the guidelines issued by Central Pollution Control Board (CPCB). Some of the parameters were measured in situ. All others were estimated in the laboratory before the exposure and after...
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2, 4 and 8 days of exposures. The percentage of elimination of metallic and non metallic pollutants were estimated by following equation (Kellaf and Zerdaoui, 2009).

\[
\text{Elimination (\%)} = \frac{C_0 - C_f}{C_0} \times 100
\]

in which \( C_0 \) and \( C_f \) are initial and remaining concentration.

3.7.7. Tolerance: Determination of NOEC and EC\textsubscript{50}

Tolerance was calculated by determining NOEC and EC\textsubscript{50} based on biomass after exposing the plants for 8 days in various concentrations of Copper and Lead. NOEC and EC\textsubscript{50} were determined Dunnet method.

3.8. Statistical analysis

The statistical analysis was done using ‘R’ software. All the experiments were conducted in triplicates and average values were taken. The relative standard deviations of means of triplicate measurement were less than 5%. Analysis of variance (ANOVA) for each test was conducted using “R” software, Zigmaplot and Microsoft Excel.