Appendix A

Aquatic Macrophyte Spirodela Polyrhiza as a Phytoremediation Tool in Polluted Wetland Water from Eloor, Ernakulam District, Kerala

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Abstract
This study involved a laboratory experiment on the efficiency of the plant duckweed (Spirodela polyrhiza) in improving the quality of two polluted wetlands of Eloor industrial area, Ernakulam, Kerala. The efficiency was tested by measuring some of the physicochemical characteristics of the control and plant treatments after each 8 days. All the parameters show considerable rate of reduction. In wetland I, the highest rate of reduction after 8 days of treatment were for heavy metals, accounting 95%, 79%, and 96% for Lead, Copper and Zinc, respectively, followed by 55% for Chromium, 45% for Mercury, 26% for Cobalt, 20% for manganese and 7% for Nickel. Other factors like pH, BOD, COD, Nitrate, Phosphate, sulphate, TDS, TSS and Turbidity decreased by 12%, 37%, 49%, 100%, 38%, 10%, 53%, 85% and 52% respectively. In wetland II also heavy metals were removed with Cd (100%), Fe (98%), Pb (91%), Cu (74%) Zn (83%) and Hg (53%) removed more efficiently. The results showed that this aquatic plant can be successfully used for wastewater pollutants removal. Other physicochemical parameters like pH, BOD, COD, Nitrate, Phosphate, sulphate, TDS, TSS and Turbidity reduced by 14%, 40%, 60%, 100%, 36%, 65%, 75%, 85%, and 51% after 8 days of treatment.

Keywords: Phytoremediation, Spirodela polyrhiza, Lemnaeaceae, Wetland, Heavy metals

Introduction
Wetlands support a wide array of flora and fauna and deliver many ecological, climatic and societal functions. Scientists often refer to wetlands as the "kidneys" of the earth. Kerala is well known for its wetlands. Eloor, an island of 11.22 sqkm, on the Periyar River is home to more than 247 chemical industries and large number of wetlands. The soil, water bodies and the wetlands in and around Eloor have been contaminated with heavy metals. Duckweed based wastewater treatment is promising to be used in efficient treatment considering organic matter, pathogen and nutrient removal [1]. Besides, duckweeds are floating plants, which reduce suspended solids by blocking light penetration. Thus, light availability causes algae die off, which settle or disintegrate. Rao et al. [2] points the advantages of using duckweeds due to its high production rate, easy manual harvest from the surface, high protein and low fiber content! The aim of the present investigation was to evaluate the effectiveness of duckweeds Spirodela polyrhiza to remove all impurities as well as heavy metals from the water samples taken from two sites of polluted wetlands in Eloor. Among macrophytes, duckweeds are very small floating aquatic macrophytes belonging to the Lemnaeaceae family which grow on the nutrient rich surface and in fresh waters and they are known for their efficiency in nutrient uptakes [3]. Likewise, Lemnaeaceae have the greatest capacity in organic matter removal and in absorbing the micro-elements such as potassium, calcium, sodium and magnesium among others. However, duckweeds plants grow only in the upper water surface layer where mainly pollutant removal takes place [4]. In India phytoremediation technique are on initial scale and detailed investigations are necessary for further research.

Materials and Methods
Sample collection
One sample (Wetland 1) was collected approximately 8 metres north of the Kulikundam Thodu creek, at a location approximately 10 m northwest of the hill site boundary (Latitude 10° 40' 51.7" N and Longitude 76° 17'32.5" E). (Table 1) The second sample (Wetland 2) was collected from the wetlands southeast of the “Amanthathu” wetland area, approximately 150 metres west of the Hill (Himalayan Insecticides Limited) site, and approximately 80 metres south of the Kulikundam Thodu creek (Latitude 10° 44' 40.31" N Longitude 76° 17'22.5" E). The samples taken from both sites were analyzed for physicochemical parameters.

Duckweed treatment system
Spirodela polyrhiza is a floating aquatic macrophyte belonging to the family Lemnaeaceae and can be found worldwide on the surface of fresh and brackish waters [5]. The Lemna and Spirodela are among the most standardized test organisms in aquatic ecotoxicology (6-9). The wetland water collected had undergone preliminary sieving step to get rid of large suspended solids. The treated water was immediately collected into the aquariums in laboratory conditions (as replicates). The treatment system with growing duckweeds in three small glass aquariums (length 18 inches) was constructed in laboratory setup. Each aquarium was 10 inches deep and 9 inches wide. These aquariums were arranged in such a way that light availability is maximum. The sides were covered to prevent light entering except at the top [10]. Duckweed (Spirodela polyrhiza) plants were collected from an unpolluted natural pond near Kochi, Kerala. The stocks were cleaned by tap water then washed by distilled water. Approximately 50 g of fresh, wet Spirodela polyrhiza plants were stacked into each of the three aquariums. Each aquarium was supplied sequentially with wetland water diluted with distilled water in 1:4 ratios. Each of the three aquariums was filled with...
same dilutions of wetland water. An aquarium is kept with same dilution but without macrophyte is considered as control. The experiment was kept under laboratory conditions of temperature (25±2) and lighting (8 light: 16 dark). Data collection time of duckweed was 8 days in the first reactor, 4 days in the second and third one. After harvesting, new and preserved duckweed was inserted. Water volume reduction by volatilization was compensated by addition of pure water.

Analytical methods

A single sample collection has been done from the study area. The water collected from the site was analysed for physio-chemical characteristics. The parameters of study are pH, BOD, COD, Nitrate, Phosphate, Sulphate, TDS, TSS and Turbidity before and after the experiment. Analysis revealed that wetland water is a cocktail of variety of metals including heavy metals. Metals like Copper, Lead, Zinc, Chromium, Cobalt, Manganese, Mercury and Nickel were present.

During the treatment process subsurface (under duckweed mat) water samples for physio-chemical were collected in polyethylene bottles from all sides of each tank and then mixed. This procedure was carried out every week. Initial and final measurements after three weeks of exposure were made. The percentage of removal or removal efficiency was calculated. Physico-chemical analysis was carried out according to standard methods for examination of water and wastewater [11]. Field parameters were measured in situ. The statistical analysis was done using STATISTICA software.

Results and Discussion

The results of efficiency of Spirodela in scavenging contaminants indicate that the presence of this macrophyte was an important element for contaminant removal in wastewater. Hydroponites can supply required oxygen by oxygen leakage from the roots into the atmosphere to accelerate aerobic degradation of organic compounds in wetlands. This assumption was confirmed in the present study, since the accumulation of heavy metals was higher in plants than water. This mechanism, also referred to as phytostabilization, is based on hydroponically grown plants that have shown to be most efficient in removing heavy metals from water [12].

In physico-chemical analysis different parameters (colour, pH, BOD, COD, Nitrate, Phosphate, Sulphate, total dissolved solids, TSS and turbidity of wetland I and II) were studied. During sample collection colour of the water samples was turbid or slightly yellowish. The level of colour in the wastewater may be due to the presence of total dissolved solids.

The pH of water from wetland I was 8.2 and for wetland II was 8.4. It was found to be in the optimum range for duckweed growth [4]. After 2 days of treatment it has reduced to 7.2 and 7.9 for WT I and WT II. In the remaining two treatment chambers the pH remains 7.2 and 7.4 respectively after 4 days and 7.2 for both samples after 8 days of treatment (Tables 1 and 2). The pH level in the present experiment ranged between 2.17 and 2.35 which was within temperature tolerance limit for duckweed growth as mentioned by [13] who found that the upper temperature tolerance limit for duckweed growth was around 34°C. Duckweed tolerance allows it to be used for year-round wastewater treatment in areas where tropical macrophytes, such as water hyacinth, can only grow in summer [14].

Turbidity was reduced by 23% from 29 NTU to 22.4 NTU after 2 days for WT I. It further reduced to 19.7(32%) after 4 days of treatment. After 8 days it was 13.8 NTU which means almost half of the turbidity has been removed. In WT II turbidity was reduced by 25.1% from 382 NTU to 286 NTU after 2 days. It further reduced to 262(31.4) and to 189 NTU (50.5%) after 4 days and 8 days of treatment respectively (Figure 1). This may be attributed to decrease the concentration of suspended material because of settlement on the bottom and adsorption on aquarium glass and this was shown in statistical analysis, as it did not show significant correlation between suspended solids and turbidity (r=0.94; p<0.05).

Figure 2 shows total suspended solids (TSS) values decreased by increasing treatment periods, showing maximum concentration of 218.4 and 359 for WT I and WT II respectively before treatment. The concentration sides down to 98.66 and 181 mg/L. After 2 days (55% and 49.5% respectively) and further reduced to 65.12 and 70.1% and 125.6(60.61%) after 4 days and finally decreased by 85.4% and 85.2% (31.7 mg/L and 32.8 mg/L respectively) which corroborates the findings of Pandy [15] regarding discharging duckweed treatment system in Halsihar, Likewise Huang et al. [16] recorded a clear reduction in
resuspension of sediment in Talbo lake during 41 days which covered by floating aquatic plants, and this result agreed with the study of Al-Shehri et al. [17].

It was also revealed that total dissolved solids (TDS) of WI and WWI recorded their minimum values of 1522 mg/L (52.5%) and 158 mg/L (73.3%) after 8 days of treatment. It was 3311 mg/L (5.5% reduction) and 47.65 mg/L (96% reduction) after 2 days of treatment. It was 2928.11 mg/L (3.7% reduction) and 2063 mg/L (35.10%) after 4 days. Majority of TDS was reduced between 8 days to 8 days of treatment. This decrease was due to the plant capacity to take some organic and inorganic ions (Figure 3).

Results in (Tables 1 and 2) show that solute concentration in WI and WWI recorded 9.3% and 88.04% as reduced percentage during 2 days, 11.18% and 79.33% during 4 days and 13.8% and 63.15% after 8 days of phyto-remediation (Figure 4). The cause of reduction may be due to plant ability to absorb different types of pollutants and accumulated in their tissues [18]. The phosphate reduction percentage after 8 days of treatment was found to be unrealistic. It may be assumed that Spirodela polyrhiza is a poor tool for phyto-remediation of phosphate from waste water. The phosphate content from the wastewater was 11 mg/L and 11.74 mg/L respectively. After 2 days of treatment, it has been reduced by 16.3% and 47.9% and after 4 days has been reduced by 25.4% and 28.6% respectively. After 8 days it has been reduced by 36.36% and 70.70% for WI and reduced by 37.68% to 8.17 after 8 days of growth (Figure 8). The removal of phosphate is comparatively better than sulfate. Similarly Nitrate content was 27 mg/L for WI and 15.7 mg/L for WWI. It has been reduced to 14.7 mg/L (45.5%) and 6.4 mg/L (67.9%) respectively with 2 days of treatment. It was further down to mere 4.3 mg/L and 1.3 mg/L after 4 days (84% and 96% respectively). Eight days of treatment was enough to remove nitrate from the water completely from both samples (100%) (Figure 5).

Table 1 and 2 also reveal the gradual reduction of factor like BOD, COD, Phosphate, Nitrate etc. with time. Data revealed that Spirodela polyrhiza was effectively reduced BOD by 12.7% for WI and 14.54% for WWI reduced from 110 mg O2-L-1 at zero days reaching 46 mg O2-L-1 for WI and reduced from 341 mg O2-L-1 at zero days reaching 202,1 mg O2-L-1 (2 days treatment). After 4 days it further reduced by 20.9% (reduced to 87 mg/L) and 20.8% (reduced to 72.1 mg/L) for WI and WWI respectively. After 8 days BOD stands at 69 mg/L (reduced by 72%) for WI and WWI stands at 20.1 mg/L (reduced by 38.9%) for WI. Ziems et al. [4] found that BOD removal efficiency was higher in duckweed based ponds than in algae based ponds. Pandey [15] reported that in D-H the duckweed ponds were operated at different flow rate giving hydraulic retention time from 5.4 to 22 days, x 30 - 50% reduction in phosphate, 56 - 80% reduction in ammonium nitrogen and 66 - 80% reduction in BOD (Figure 6). In concurrence with the present findings, Oon et al. [19] mentioned that the duckweed contributed for the removal of organic materials due to their ability to direct use of simple organic compounds. The COD has been reduced by 38.75% for WI and 24.71% immediately after 2 days of phyto-remediation and reduced from the initial concentration of 320mg/L to 196 mg/L and 67.9 mg/L to 311.7 mg/L after 4 days. After 4 days it further reduced by 44.57% (reduced to 178mg/L) for WI and reduced by 34.68% (reduced to 464.14 mg/L) and finally after 8 days, reduced to mere 166 mg/L (49.37%) for WI and reduced to 206.3 mg/L (66.8%) for WWI (Figure 7). Kuarat et al. [20] mentioned that duckweed significantly enhanced COD removal in shallow batch systems. Pandey [15] reported that COD removal was in the range of 79% - 80% in the discharge of duckweed treatment systems at Halisahar. However in the present study, the COD and BOD removal by the macrophyte were not up to the capacity of Lemna minor.

Fernandes et al. [21] indicated the reliability of wastewater treatment by some aquatic plants including duckweed in absorption of the heavy metals cadmium and zinc. Vat et al. [22] reported that duckweed plants proved to be an excellent bioaccumulator of various heavy metals, which allowed it to treat a variety of wastewaters including industrial and highly polluted waters. Hammouda et al. [23] evaluated the efficiency of duckweed aquatic treatment in heavy metals removal in various water systems data obtained suggested a maximum reliability of systems with mixtures containing high ratios of wastewater. In the present study metals like Copper, Lead, Zinc, Chromium, Cobalt, Manganese, Mercury and Nickel were found in the wastewater and removed by the plant by greater extent The eight metals studied, showed Pb>Cu>Zn>Cr>Hg>Co>Mn>Ni pattern of absorbance. Hb
concentration was 26 mcg/L in the control solution. After 2 days of treatment it has been reduced by 33.4% to 17.1 mcg/L. After 4 days it further reduced by 63.2% to 9.3 mcg/L. Finally after 8 days Lead concentration is only 1.3 mcg/L, which means 99% removal (Figure 8). After 8 days of treatment Copper concentration in the water has been removed by 78.74% which means a reduction from initial concentration of 65 mcg/L to final concentration of 13.8 mcg/L. (Figure 9). Copper shows

Figure 1: This may be attributed to decrease the concentration of suspended material.

Figure 2: Total suspended solids (TSS) values decreased by increasing treatment periods.

Figure 3: Majority of TSS was removed between 4 days to 8 days of treatment.

Figure 4: Sulfate concentration in WW and WW recorded as reduction percentage during 2 days.

Figure 5: Eight days of treatment to remove nitrate from the water completely from both samples.

Figure 6: Different flow rates giving hydraulic retention time.
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Conclusion

In the current study macrophyte duckweed Spirodela polyrhiza was

was very low initially i.e. 7.2 mg/L. After 2 days it has been reduced by more 11.11%. After 4 days the concentration was measured as 8.2 mg/L i.e. 37.77%. Interestingly even after 8 days of treatment the cobalt concentration did not come down any further i.e. it remains at 27.77% removal (Figure 12). The Manganese concentration has been reduced by 20% from 8 mg/L to just 6.4 mg/L, even after 8 days of treatment. This reveals that Spirodela polyrhiza is a poor accumulator of Manganese (Figure 13). Mercury concentration remains the same even after 2 days of treatment i.e. 2.6 mg/L. The concentration reduced to 1.1 mg/L, after 8 days of treatment with the removal efficiency of 49% (Figure 14). Nickel shows the least removal after the treatment regime of 8 days. It has been reduced from 19.5 mg/L to 17.6 mg/L, with removal efficiency of just 8.80% that shows that Spirodela is a poor accumulator of Nickel (Figure 15). Iron and Cadmium were present in Wetland II but were absent in wetland I. Fe were removed 98.1% and Cd removed completely (100%) after 8 days of treatment (Figure 16 and 17).

Figure 7: After 4 days it further reduced for Wt.

Figure 8: Lead concentration removal.

Figure 9: After 8 days of treatment Copper content in the water reduced from initial concentration to final concentration.

Figure 10: Four days of treatment for removal of Zn.

Figure 11: Chromium concentration before treatment.

Figure 12: Stability after 8 days of treatment with cobalt concentration.

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Figure 13: Sporodesa polyrotata is a poor accumulator of Manganese.

Figure 14: The concentration reduced after 8 days of treatment with the removal efficiency.

Figure 15: Note the lean removal after the treatment regime of 5 days.

Figure 16: Iron and Cadmium were present in Wetland II.

Figure 17: Fe and Cd removal competency after 8 days of treatment.

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References

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Appendix B

Phytotoxicological Assessment of Two Wetlands in Eloor, Kochi Using Aquatic Macrophyte Spirodela Polyrhiza.

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Abstract: In the current study, the duckweed aquatic macrophyte Spirodela polyrhiza was employed for assessing the toxicity of two wetlands in the Eloor industrial estate, Ernakulam district, Kerala, South India. The assessments were made according to OECD guidelines for testing (2006). The studies involve study of growth parameters, Growth Index, Biomass and changes in productivity. The water samples were collected from two different wetland sites at the same time. The spirodela plants were introduced into several dilutions of wetland water samples. The parameters were measured after 7 days of exposure. All samples except control affected all parameters. The results of this study emphasize the significance of duckweeds as standard and reliable testing material for biological parameters in polluted aquatic ecosystem.

Keywords: Growth inhibition, Spirodela polyrhiza, Growth Index, macrophyte, duckweed

I. Introduction:

Wetlands support a wide array of flora and fauna and deliver many ecological, climatic and societal functions. Scientists often refer to wetlands as the "kidneys" of the earth. Kerala is well known for its wetlands. Eloor, an island of 11.21 sq.km, on the Periyar River is home to more than 247 chemical industries and large number of wetlands. The soil, water bodies and the wetlands in and around Eloor have been contaminated with heavy metals. Standardised ecotoxicity test methods frequently uses duckweed species Spirodela polyrhiza due to their advantages such as rapid vegetative propagation, sensitivity to toxicants, easy culturing under axenic conditions (Lakatos et al. 1993).

II. Materials and Methods:

Duckweed Spirodela polyrhiza were obtained from an unpolluted natural pond near Fort Kochi, Kerala, India. It is a floating aquatic macrophyte belonging to the family Lemnaceae and can be found world wide on the surface of fresh and brackish waters (Zimno, 2003). The Lemma spp. are among the most standardized test organisms in aquatic ecotoxicology, EPA 1996,DIN2000, 2001;Eberius 2001;OECD 2002.

One sample (Wetland 1) was collected approximately 8 metres north of the Kuzhikandam Thodu creek, at a location approximately 10 m northwest of the HIL site boundary (Lat 10° 04’51.76”N and Long 76° 17’32.55”E) (see Table 1).

The second sample (Wetland 2) was collected from the wetlands southwest of the "Amanthuruthu" wetland area, approximately 150 metres west of the HIL (Hindustan Insecticides Limited) site, and approximately 80 metres south of the Kuzhikandam Thodu creek (Lat 10° 04’48.13”N Long 76° 17’22.75”E) (see Table 2).

Test solutions were prepared by diluting water samples of wetland 1 and 2 with distilled water. The solutions were prepared in 100%, 50%, 25%, 10%, 5% and 0.5% concentrations of wetland water plus a control and undergo seven days of exposure. Plants were harvested, washed with double distilled water, blotted and used for the study of various parameters. The parameters include study of vegetative characters, growth parameters and study photosynthetic pigments. All the tests were conducted in six replicates.

III. Analysis of parameters:

I. Study of growth parameters

1. A. Dry weight:

All colonies are collected from each of the test vessels and rinsed with distilled or deionised water. They are blotted to remove excess water and then dried at 60 °C to a constant weight. Any root fragments should be included. The dry weight should be expressed to an accuracy of at least 0.1 mg.
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1. B. Fresh weight:
   All colonies are transferred to pre-weighted plastic tubes with small (1 mm) holes in the rounded bottoms. The tubes are then centrifuged at 3000 rpm for 10 minutes at room temperature. Tubes, containing the now dried colonies, are re-weighed and the fresh weight is calculated by subtracting the weight of the empty tube.

1. c. Dry weight-Fresh weight
   ratio can be determined from above estimations. The plant growth index was calculated as follows:
   \[ \text{Growth Index} = \frac{\text{Biomass (t = 7 days)}}{\text{Biomass (t = 0)}} \]

1. D. Doubling time:
   To determine the doubling time (Td) of frond number and adherence to this validity criterion by the study, the following formula is used with data obtained from the control vessels:
   \[ Td = \frac{\ln 2}{\mu_1} \]
   Where \( \mu \) is the average specific growth rate.

1. e. Average specific growth rate:
   This response variable is calculated on the basis of changes in the logarithms of frond numbers, and in addition, on the basis of changes in the logarithms of another measurement parameter (total frond area, dry weight or fresh weight) over time (expressed per day) in the controls and each treatment group. It is sometimes referred to as relative growth rate. The average specific growth rate for a specific period is calculated as the logarithmic increase in the growth variables - frond numbers and one other measurement variable (total frond area, dry weight or fresh weight) - using the formula below for each replicate of control and treatments:
   \[ \mu_i = \frac{\ln (N_j) - \ln (N_i)}{t} \]
   where:
   - \( \mu_j \): 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.

1. f. Percentage of growth inhibition:
   Percent inhibition of growth rate (Ir) may then be calculated for each test concentration according to the following formula:
   \[ \%\text{Ir} = \left( \frac{\mu C_{(-i)}}{\mu C} \right) \times 100 \]
   where:
   - \( \%\text{Ir} \): percent inhibition in average specific growth rate
   - \( \mu C \): mean value for \( \mu \) in the control
   - \( \mu C_{(-i)} \): mean value for \( \mu \) in the treatment group

2. Estimation of photosynthetic pigments.
   The chlorophyll estimation is an important study parameter for the estimation of impact on pollution on photosynthetic activity. About 200mg of treated plants were weighed. This is taken in a mortar with 5ml of 90% acetone and 1ml of Magnesium carbonate. It is then ground thoroughly with pestle. This is then kept at 4°C for 4 hours for the pigments to elute. The solution is then centrifuged at 2500 rpm for 15 minutes. The extract is then decanted to a volumetric flask and the volume is made up to 50 ml with 90% acetone. The absorbance at 750, 663, 645.510 and 480 were measured in the spectrophotometric analysis using Hitachi-U-2000 spectrophotometer.

Statistical Analysis
   Analysis of variance for each test were conducted using STATISTICA software (One way Anova).
   The significant difference between treatments were determined by Duncan’s multiple range test (P<0.05).
   Each test was conducted in six replicates.

IV. Results and conclusions:
   The concept of average specific growth rate is based on the general exponential growth pattern of duckweed in non-limited cultures, where toxicity is estimated on the basis of the effects on the growth rate, without being dependent on the absolute level of the specific growth rate of the control; slope of the
concentration-response curve or on test duration. The use of average specific growth rate for estimating toxicity is scientifically preferred. In the current study ASQR and frond doubling time (Td) of the control and treatment with 0.5% concentration of the plant yield the same result. The inhibition of growth in this concentration is negligible. As the concentration of effluent increases, all the parameters vary. When the frond doubling time exceeds 2.5, the test solution is considered toxic. In the study 50% and 100% concentration treatments in wetland I and 2 shows Td values more than 2.5, thus found to be toxic. The values are given in table 3.

Plant growth index were measured after 7 days of exposure with different dilutions of water from both wetlands. At 0.5 % and 5% dilution GI is greater than control values in both waters. At 10% W2 treatment shows less GI than control but W1 still has values above control which shows the water quality of W1 is better than W2. From 25% GI values shows sharp decline in both treatments. The finding is given in table 4.

The growth indexes of both wetland water are similar in lowest concentration (0.5%). From 5% dilution onwards it is quite clear that growth of Spirodela is affected more in wetland II. But at 25% dilution GI is surprisingly similar. At extreme a concentration again GI varies. The differences in GI under different concentrations are illustrated in Table 3.

Changes in Dry weight fresh weight ratio indicate that in exposed Spiroula plants, growth retardation takes place in comparison to the control. At 0.5 % concentration, the biomass yield is same as that in control. In 5% concentration of wetland I and 2 water, slight increase in DW/FW ratio recorded in wetland 2 effluents than control. At 10% and 25% solutions, the DW/FW ratio is higher in wetland water 1 in comparison with Wet 2. But in 50% and 100% the ratio shows sharp decline. It was noted that at 50% concentration, spiroula growing in highly polluted wetland 2 water yield higher FW/DW ratio than those growing in wetland 1 ( Table 5). The decline in biomass ratio may be due the presence of excess heavy metals present in wetland. It has been shown that accumulation of heavy metals disturb the plant water status which eventually results in osmotic stress and growth reduction (Perlas-Barbecho et al. 2012; Poschenreider and Barcelo 2010). Water especially in wetland 1. In wetland 1 the biomass (DW/FW ratio) percentage were 100, 100.7, 103.9, 100, 78.5, 64.27 and 57.1 for dilutions 0.5, 5, 10, 25, 50 and 100 respectively. Similarly for wetland 2 it was 100, 99.9, 100.07, 78.4, 74.9, 64.2, and 57.04 for dilutions 0.5, 5, 10, 25, 50 and 100 respectively.

Phytoplankton and carotenoids are the central part of the energy manifestation of every green plant system and therefore; any significant alteration in their levels is likely to cause a marked effect on the entire metabolism of the plant. The productivity of plants is directly related with changes in the content of photosynthetic pigments chlorophyll a and b, carotenoids. Industrial wastewater not only affects the chlorophyll content but also the chlorophyll activity also (Song and Huang 2001; Baron et al., 1995; Lewis, 1995).

In the study photosynthetic pigments were inhibited due to metal toxicity. Duckweed leaves started to show signs of chlorosis (pigment loss) following 7d exposure to surface water samples. At 50% and 100% dilutions necrosis could also seen. The Carotenoids contents are found to be less affected. At the end of 7 days of exposure 0.5% dilution of wetland 1 and 2 shows slight increase in chlorophyll a content (Hormess) while at the same dilution and Carotenoids content remain unchanged. From 5% to 100% all pigments shows gradual decrease in concentration. Chl b degraded at a much slower rate than chl a which indicates that greater damage of pollutants present in water samples on chl a. The loss of photosynthetic pigment content has been reported in duckweed plants following exposure to Cu, Pb and Ni (Astelli et al. 2003; Hou et al. 2007; Kanou- boute et al. 2009). The destruction of photosynthetic pigments by heavy metals could be due to impairment of ETC, replacement of Magnesium ions associated with chlorophyll ring, inhibition of important enzymes (Van Asche and Clijsters 1990) associated with chlorophyll synthesis or peroxidation processes in chloroplast membrane lipids by reactive oxygen species (Sandalio et al. 2001).

Acknowledgement:
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V. Conclusion:
The study revealed that both wetlands are highly polluted. Wetland 2 has more pollutants compared with wetland 1 which is evident from the assessment of vegetation growth and photosynthetic pigment parameters. The study also points towards the importance of conservation of wetlands in the area.

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Table 1: Wetland I

Table 2: Wetland II

Table 3: ASGR, Td and Ir% of S. polyyrrhiza after 7 days of treatment with various dilutions of wetland I and 2. Mean (N)=5, Mean (T)=7 and (T)=0. Standard deviations were presented by error bars. Each values are means of triplicates. The significant difference between treatments P<0.05.
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<table>
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<td>63.64</td>
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<td>3.484</td>
<td>1.555</td>
<td>18.177</td>
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<tr>
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<td>1.548</td>
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<td>63.64</td>
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</tbody>
</table>

Table 4: Growth Index of Spirodea plant in different dilutions of Wetland 1 and 2 after 7 days of exposure. Standard deviations were presented by error bars. Each values are means of six replicates. The significant difference between treatments is *P*<0.05.

![Growth Index Graph](image)

Table 5: Biomass DW/FW ratio of *S. palustris* after 7 days of exposure in water from Wetland 1 and 2. Standard deviations were presented by error bars. Each value is means of six replicates. The significant difference between treatments is *P*<0.05.

<table>
<thead>
<tr>
<th></th>
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<th>0.5</th>
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<th>10</th>
<th>25</th>
<th>50</th>
<th>100</th>
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<td>3.2</td>
<td>3.4</td>
<td>3.3</td>
<td>2.9</td>
<td>2.6</td>
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<td>1.8</td>
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<tr>
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<td>3.2</td>
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<td>3.5</td>
<td>2.6</td>
<td>2.1</td>
<td>2.0</td>
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</table>

Table 6: Relative phycocyanin pigment concentration after 7 days of exposure in different dilutions of wetland 1. Standard deviations were presented by error bars. Each values are means of six replicates. The significant difference between treatments is *P*<0.05.
Phytotoxicological Essment of Two Wetlands In Eloor, Kochi Using Aquatic Macrophyte Spirodela

Table 7: Relative photosynthetic pigment concentrations after 7 days of exposure in different dilutions of wetland 2. Standard deviations were presented by error bars. Each values are means of triplicates. The significant difference between treatment is *P*<0.05.
Appendix C

Environmental & Analytical Toxicology

Research Article

Phytotoxicological Assessment of Two Backwater Wetlands in Kannamaly, Ernakulam Using Aquatic Macrophyte - Spirodela Polyrhiza.

Anil Lovesson and Rajathy Sivalingam
School of Environmental Studies, Cochin University of Science and Technology, Kerala, India

Abstract

In the current study, the duckweeds Aquatic macrophyte Spirodela polyrhiza was employed for assessing the toxicity of two backwater wetlands in the Kannamaly, Ernakulam district, Kerala, South India. The assessment was made according to OECD guidelines for testing (2006). The studies involve a study of growth parameters, Growth Index, biomass, and changes in productivity. The water samples were collected from two different wetland sites at the same time. Spirodela plants were introduced into several dilutions of wetland water samples. The parameters were measured after 7 days of exposure. All samples except control affected all parameters. The results of this study emphasize the significance of duckweeds as standard and reliable testing material for biological parameters in polluted aquatic ecosystem.

Keywords: Growth inhibition, Spirodela polyrhiza; Growth Index; macrophyte; Duckweed.

Introduction

Wetlands support a wide array of flora and fauna and deliver many ecological, climatic, and societal functions. Scientists often refer to wetlands as "the lungs" of the earth. Kerala is well known for its wetlands. The Kerala coast is bordered by 29-backwaters running parallel to the coastline. The water quality of these backwaters is deteriorating due to population explosion, rapid industrialization, siltation, tourism, and agricultural activities. Effluents from industries are major cause of pollution in coastal area. The waste water/effluents from seafood processing plants located at Kannamaly, Ernakulam panchayath, Ernakulam district directly discharge the waste water to the neighbouring water bodies. Apart from raising the BOD at immediate vicinity, limited effluents do not cause any severe damage to the system. But at high level, cause severe pollution and adversely affect the aquatic flora and fauna. Standardized ecotoxicity test methods frequently uses duckweeds Spirodela polyrhiza due to their advantages such as rapid vegetative propagation, sensitivity to toxicants, easy culturing under aerobic conditions [1]. Spirodela polyrhiza has tolerance to moderate saline conditions. Duckweeds are salinity tolerant, adapt with time to high salinity, remove salinity, and have a potential for desalinization in agricultural detention ponds [2].

Objectives

The objective of current study to assess the toxicity of water from two wetlands by standard testing procedure that includes growth analysis and photosynthetic pigment analysis.

Materials and Methods

Duckweed Spirodela polyrhiza were obtained from an unpoluted natural pond near Fort Kochi, Kerala, India. It is a floating aquatic macrophyte belonging to the family Lemnaceae and can be found worldwide on the surface of fresh and brackish waters [3]. The duckweeds are among the most standardised test organisms in aquatic ecotoxicology [4]. One sample (Wetland 1) was collected approximately 50 meters south of the Kannamaly pilgrim centre and close to India Seafoods Factory, at a location approximately 9.8794’N and 76.2665’E (Figure 1).

The second sample (Wetland 2) was collected from the wetlands south to the ‘wetland I area, approximately 1.8 km away and located at 9.8612’N (Long 76.2642’E) (Figure 2).

Wetland water samples collected from two sites were analysed according to APHA standards [9]. Metals were analysed using AAS. Test solutions were prepared by diluting water samples of wetland 1 and 2 with distilled water. The solutions were prepared in 16%, 32%, 25%, 10%, 5%, and 0% concentrations of wetland water plus a control (water taken from an unpoluted site near Kumballang), 5 km away from Kannamaly. After seven days of exposure, plants were harvested, washed with double distilled water, blotted and used for the study of various parameters. The parameters include study of vegetative characters, growth parameters and study photosynthetic pigment. All the tests were conducted in six replicates.

Figure 1: One sample (Wetland 1) was collected approximately 50 metres south of the Kannamaly pilgrim centre and close to India Seafoods Factory.

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Appendices

Analysis of Parameters

Phytochemical analysis of wetland water

Study of growth parameters:

1. a. Dry weight: All colonies are collected from each of the test vessels and mixed with distilled deionised water. They are homogenised to ensure mass water and then dried at 80°C to a constant weight. Any root fragments should be included. The dry weight should be expressed to an accuracy of at least 0.1 mg.

b. Fresh weight: All colonies are transferred to pre-weighed plastic cups with small (1 mm) holes in the bottom numerous. The cups are then centrifuged at 3000 rpm for 10 minutes at room temperature. Then, the centrifuged, de-watered colonies, are re-weighed and the fresh weight is calculated by subtracting the weight of the empty cup.

c. Dry weight/fresh weight ratio can be determined from above estimations. The plant growth index was calculated as follows:

\[ \text{Growth Index} = \frac{\text{Fdcm}}{\text{Idcm}} \]  

Where: F = fresh weight, I = initial weight

1. b. Troubling time: To determine the doubling time (TD) of fresh weight and substances to this validation solution by the study the following formula is used with data obtained from the control vessels:

\[ \text{TD} = \ln 2 / \text{r} \]  

Where: \( \text{r} \) is the average specific growth rate

2. c. Average specific growth rate: This response variable is calculated by the time of changes in the logarithms of (fresh weight), and in addition, on the basis of changes in the logarithms of another measurement parameter (total fresh area, dry weight or fresh weight), over time (expressed per day) in the controls and each treatment group. It is sometimes referred to as an absolute growth rate. The average specific growth rate for a specific period is calculated as follows the logarithmic increase in the growth variables (x) measured at time 1 (t = 1) and at time 2 (t = 2) is:

\[ \text{r} = \ln(N_2) - \ln(N_1) \]  

Where:  
- \( N_1 \) = average specific growth rate from time 1 to 2
- \( N_2 \) = measurement variable in the test or control (initial) at time 1
- \( N_2 \) = measurement variable in the test or control (initial) at time 2

1. c. Time period time (t)

1. d. Percentage of growth inhibition: Percent inhibition of growth rate is generally calculated as follows:

\[ \% \text{Inhibition} = \left( \frac{\text{Test} - \text{Control}}{\text{Control}} \right) \times 100 \]  

Where:
- \( \% \) = percent inhibition in average specific growth rate
- \( \text{Control} \) = mean value for \( \mu \) in the control group
- \( \text{Test} \) = mean value for \( \mu \) in the treatment group

Estimation of photosynthetic pigments:

The chlorophyll estimation is an important study parameter for the evaluation of impact of pollution on photosynthetic activity. About 200mg of treated plants were weighed. This is taken in a mortar with 20% ethanol and 1% of Magnesium carbonate. It is then ground thoroughly with pestle. This is then kept at 4°C for 2 hours for the pigments to diffuse. The solution is then centrifuged at 3000 rpm for 15 minutes. The extract is then dispersed with a volumetric flask and the volume is made up to 30 ml with 95% alcohol. The absorbance at 750, 645, 665, 490 and 480 nm were measured in the spectrophotometric analysis using Hitachi U-1000 spectrophotometer.

Statistical analyses:

Analysis of variance for each test was conducted using STATISTICA software (One way ANOVA). The significant differences between treatments were determined by Dunnett's multiple range test. Each test was conducted on six replicates.

Results and Discussions:

Physical and chemical parameters of water from local wetlands are given in Table 1. The values of BOD, Nitrate, Nitrite, Ammonia, TKN, TSS, Turbidity and heavy metals Ca, Pb, Zn and Cd were significantly high in wetland 1 in comparison to the control.

<table>
<thead>
<tr>
<th>No</th>
<th>Parameter (mg/L)</th>
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<th>Wetland 2</th>
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<td>1</td>
<td>Temp (°C)</td>
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<td>20.1</td>
<td>20.1</td>
</tr>
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<td>pH</td>
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<td>7.8</td>
</tr>
<tr>
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<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
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<td>DO (mg/L)</td>
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<td>2.6</td>
<td>2.6</td>
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<tr>
<td>6</td>
<td>PO₄-(P) (mg/L)</td>
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<td>0.09</td>
<td>0.11</td>
</tr>
<tr>
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<td>Nitrate (mg/L)</td>
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<td>1.5</td>
<td>1.5</td>
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<td>Ammonia (mg/L)</td>
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<td>0.2</td>
</tr>
<tr>
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<td>TAN (mg/L)</td>
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<tr>
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<td>Turbidity</td>
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<tr>
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<td>Calcium (mg/L)</td>
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<td>Zinc (mg/L)</td>
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<td>Cadmium (mg/L)</td>
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<td>&lt;0.005</td>
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</table>

Table 1. Results of physico-chemical analyses of Wetland 1 and 2 water before treatment. Each value is mean ± standard error. The significant differences between treatments are (P<0.05).
Appendices


Duckweeds show great tolerance to changes in physicochemical parameters of water. The growth rate of duckweed is favoured by organic pollutants as well as inorganic nutrients. Gupta and Chamanal [9] reported the maximum biomass of L. polyrrhiza and S. polyrhiza from road-side pools and ditches in India within an electrolyte conductivity range of 630-1,400 µS/cm.

The concept of average specific growth rate is based on the general exponential growth pattern of duckweed in non-limiting cultures, where toxicity is estimated on the basis of the effect on the growth rate, which is dependent on the absolute level of the specific growth rate of the control-slope of the concentration-response curve or on test duration. The use of average specific growth rate for estimating toxicity is scientifically preferred. In the current study, ASGR and Td doubling time (Td) of control and treatment with 6% and 5% concentration of T1W yield the same result: T1W water shows different result ASGR and Td remains same as control up to 25% concentration. The inhibition of growth in this concentration is negligible. As the concentration of effluent increases, all the parameters vary. For the test to be valid, the doubling time of fluoride number in the control must be less than 2.5 days (60 h) (OECD guideline) [7]. When the found doubling time exceeds 2.5, the test solution is considered toxic. In the study only six dilutes 100% concentration of water from wetland 2 shows Td values more than 2.5, found to be toxic. The values are given in Table 2.

Plants growth index were measured after 7 days of exposure with different dilutions of water from both wetlands. At 0.9% and 5% dilution GI is greater than control values in both waters. At 10% W2 treatment shows less GI than control but W1 still has values above control which shows the water quality of W1 is better than W2. From 25% GI values show sharp decline in both treatments. The finding is given in Table 3.

The growth index of both wetland water is similar in lowest concentration (0.9%). From 5% dilution onwards it is quite clear that growth of Spirodela is enhanced more in wetland 1. This may be due to the increase in crude protein content of duckweed however, seems to increase to a maximum of 40-50% GI over the range from trace ammonia concentrations to 7-12 mg N/L. [10]. Khondker, Islam and

<table>
<thead>
<tr>
<th>Site</th>
<th>Median</th>
<th>Mean</th>
<th>Mean ASG (µL)</th>
<th>Td</th>
<th>%r</th>
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<td>4.253</td>
<td>1.772</td>
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<td>5%</td>
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<td>4.253</td>
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<td>10%</td>
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<td>3.871</td>
<td>1.4</td>
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</tr>
<tr>
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<td>4.253</td>
<td>1.4</td>
<td>9.03</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 2: ASGR, Td and %r of S. polyrhiza after 7 days of treatment with different dilutions of wetland 1 and 2. Mean ± SD. Average of 4 replicates. Standard deviations were presented by error bars. Each value is means of six replicates. The significant difference between treatments is P<0.05.

Mukhtar [11] observed that both phosphate and silicate concentrations had significant positive correlation with the biomass of L. perpusilla in Bangladesh. But at 25% dilution GI is surprisingly similar. At extreme concentrations again GI rate, the differences in GI under different concentrations are illustrated in Table 4.

Changes in dry weight fresh weight ratio indicate that in exposed Spirodela plants, growth retardation takes place in comparison to the control. At 9.5% concentration, the biomass is similar as in the control. In 5% concentration of wetland 1 and 2 water, slight increase in DW/FW ratio recorded in wetland 1 effluents than control. At 10% and 25% solutions, the DW/FW ratio is higher in wetland 1 than in comparison with W1 and W2, but in 50% and 100%, the ratio shows sharp decline. It was noted that at 50% concentration, Spirodela growing in highly polluted wetland 2 water yield higher DW/FW ratio than those growing in wetland 1. (Table 5).
The decline in biomass was due to the presence of excess heavy metals present in soil. It has been shown that the accumulation of heavy metals disturbs the plant water status; which eventually results in complete stress and growth reduction [17.18]. Water content in soil 1 is about 1% lower than the mean water content in soil 2. Chlorophyll a and carotenoids have the same trend in both soils. Industrial wastewater does not affect the chlorophyll content but the chlorophyll a is lower than that in soil 2. In the study, photosynthetic pigments were not inhibited due to metal toxicity. Industrial wastewater not only affects the chlorophyll content but also the photosynthetic activity [14-16].

Unaffected species started to show signs of chlorosis (pigment loss) following 50 µmol exposure to heavy metal ions. All 30% and 100% solutions are also seen. The Carotenoids are more sensitive to be less affected. At the end of 7 days of exposure 0.5% diluted chlorella 1 and 2 show slight increase in chlorophyll a content (Fig. 8) while at the same time and carotenoids content remain unchanged. From 50% to 100%, all pigments show gradual decrease in concentration. Chlorophyll degradation at a much slower rate than chlorophyll a which indicates that the degree of damage to pigments present at water samples in A.1. The loss of photosynthetic pigment content has been reported in aquatic plants following exposure to Cu, Pb and Ni [17-19]. The destruction of photosynthetic pigments by heavy metals could be due to impertinence of ETC replacement of Magnesium ions associated with chlorophyll a ring, inhibition of important enzymes [20] associated with chlorophyll synthesis or precipitation processes in chloroplast membranes [21] (Table 6).

### Table 6 Relative photosynthetic pigments concentrations after 7 days of exposure in different dilutions of metal 1. Standard deviations were presented by error bars. Each value is mean of 4 replicates. The significant difference between treatments is P<0.05.

<table>
<thead>
<tr>
<th>Pigment</th>
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</thead>
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<td>0.36</td>
<td>0.36</td>
<td>0.43</td>
<td>0.36</td>
<td>0.29</td>
<td>0.28</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>0.28</td>
<td>0.25</td>
<td>0.25</td>
<td>0.24</td>
<td>0.22</td>
<td>0.20</td>
<td>0.18</td>
</tr>
<tr>
<td>Carotenoid</td>
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<td>0.24</td>
<td>0.18</td>
<td>0.15</td>
<td>0.13</td>
<td>0.11</td>
<td>0.11</td>
</tr>
</tbody>
</table>

### Conclusion

The study revealed that both methods are highly polluted. Wastewater 2 has more pollutants compared with wastewater 1 which is evident from the assessment of vegetation growth and photosynthetic pigments parameters. The study also points towards the importance of conservation of wetlands in the area.

### Acknowledgement

The authors are grateful to the School of Environment Studies, CUBAR, for providing technical assistance to carry out the work.

### References
Appendices
