Aquatic Ecosystems and Environmental Frontiers, Volume 2, Issue 1, March 2024 *ISSN: 3049-186X*
https://doi.org/10.70102/AEEF/V2I1/7

Influence of Textile Dye Metabolites on Photosynthetic Efficiency in Native Aquatic Macrophytes

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Abstract: Azo dyes from textile effluents can be reduced anaerobically to form aromatic amines which can be biologically retained in freshwater systems and have a negative impact on primary producers. Photosynthesis inhibition by dyes has been reported, but the responses of aquatic macrophytes to various metabolites are still inadequately understood. A metabolite-based tool is provided that can be used to assess photosynthetic efficiency of Spirodela polyrhiza, Eichhornia crassipes and Hydrilla verticillata, along with a quantitative synthesis of existing experimental information on the toxicity of dyes. The suggested design is a completely randomised experimental design with 14 days exposure to aniline, benzidine, 1-naphthylamine and sulfanilic acid at 0–10 mg/L, plus a parent-dye comparison, and 5 replicate vessels per treatment and sampling days 0, 3, 7, 10 and 14. End points include maximum quantum yield photosystem II (Fv/Fm), chlorophyll-a, chlorophyll-b, carotenoids, malondialdehyde, and catalase and superoxide dismutase; with complimentary water quality and residual-amine analysis. Data from reports show a significant interference or disruption of the photosynthetic energy flow: 94.18% reduction in trapped energy flux and 96.10% reduction in electron transport flux in S. polyrhiza exposed to the synthetic colourants, 65.84–74.90% loss of pigments in C. vulgaris exposed to rhodamine B, and concentration-dependent and time-dependent photosystem II inhibition in E. crassipes. Water hyacinth was able to remove up to 96 % of the sulphates from the textile wastewater, but it did not grow well in highly polluted effluents. Therefore, dye removal and detoxification are not the same and chlorophyll fluorescence seems to be a good sensor to use as an early-warning system for textile wastewater monitoring. The proposed batch protocol should be performed with mass-balance closure and non-target analysis in future to quantify transformation products in different seasons and sites. Furthermore, pilot testing of the biofiltration of filter media, constructed wetlands and riverbank filtration should be conducted to control contaminant fate and minimise secondary release.

Keywords: Textile Wastewater; Azo Dye Metabolites; Aromatic Amines; Aquatic Macrophytes; Chlorophyll Fluorescence; Photosystem II; Oxidative Stress.

(Submitted: January 13, 2024; Revised: February 15, 2024; Accepted: March 13, 2024; Published: March 29, 2024)

I. Introduction

1.1. Release and Transformation of Textile Dyes

Synthetic colourants are a significant source in freshwater systems from textile dyeing. Most of the applied dye is not fixed and 15–50% of the dyes used are lost in waste water from dyeing and rinsing operations (Hashemi & Kaykhaii, 2022). Receiving waters are not only affected by colour, but also by oxygen demanding, ecotoxic and mutagenic elements of the effluent matrix (Lellis et al., 2019). Azo dyes are designed to withstand colour loss and many are not removed by conventional treatment and remain in the discharge.

Azo dyes are those dyes that contain at least one azo bond (–N=N–) between aromatic groups. In sediments, under reducing conditions, the bond is broken by microbial azoreductases and/or chemical reductants to release the aromatic amines illustrated in equation (1) (Chung, 2016):

$$R_1 - N = N - R_2 + 4[H] \rightarrow R_1 - NH_2 + R_2 - NH_2 \qquad (1)$$

Several of these are mutagenic and screening the textile azo dyes led to the identification of 40 aromatic amines that could be cleavage products of about 180 parent azo dyes (Brüschweiler & Merlot, 2017).

Decolourisation does not necessarily lead to detoxification, however, as the colour may be removed, leaving colourless amines. The metabolites produced will vary according to the structure of the dye and cannot be assumed to be present in all effluents.

Dye metabolites can affect primary producers by breaking down pigments and causing the generation of reactive oxygen species (ROS) and the disruption of photosystem II (PSII) electron transport. Chlorophyll fluorescence is considered as a quick and non-invasive tool to measure PSII functioning and is extensively used as a bio-indicator of stress responses in plants (Murchie & Lawson, 2013). An early sign of photoinhibition or reaction-centre damage is a decrease in the maximum quantum yield of PSII (Fv/Fm).

They have a direct contact with contaminated water and sediments, they add oxygen to the water, they help to stabilise the sediments and provide habitat, making them appropriate pollution indicators. Three species of different growth forms are considered: free floating Spirodela polyrhiza, Eichhornia crassipes and submerged Hydrilla verticillata. There are already some studies on pollutant removal in dyeing effluent by using macrophytes such as H. verticillata in Tamil Nadu (Ahila et al., 2020). Each species must be documented as being local and E. crassipes, an introduced invasive plant in many areas is not a native indicator species but is used as a comparative test organism.

1.2. Research Questions and objectives

Research questions:

1. Do aromatic amine metabolites of azo dyes reduce photosynthetic efficiency in freshwater macrophytes?
2. Does Fv/Fm decline progressively as metabolite concentration rises?
3. Which species is most sensitive?
4. Does exposure duration intensify chlorophyll loss and oxidative stress?
5. Can chlorophyll fluorescence serve as an early-warning indicator?

The objectives of the study were to quantify the effects of selected azo dye metabolites on Fv/Fm, chlorophyll-a, chlorophyll-b and carotenoids, to analyze oxidative stress responses, to compare the tolerance of three species and to find out biological indicators for monitoring textile wastewater.

The paper starts with introduction to emerging contaminants and the role of biofilm followed by Section II which discussed about the chemical classes and mechanism and Section III which discussed about the methodology for sampling and batch experiments. It is followed by Section IV which presents contaminant uptake and substrate results, Section V, which discusses retention dynamics and treatment implications, and Section VI which concludes study limitations and findings.

II. Literature Review

2.1. Properties, Persistence and Ecological Toxicity of Dye Metabolites

Many azo dyes are highly water-soluble and are chemically and biologically resistant to breakdown and are therefore long-lived in the water column, whereas anaerobic sediments promote the reduction of the azo bond. The toxicity of the resulting amines depends on the molecular structure, substituent groups, concentration, duration and pH of the exposure, as well as on the metabolism of the organism, and cannot be extrapolated from the behaviour of the parent dye to the parent dye metabolites. Median effect concentration (MEC) exposed to methylene blue of Chlorella vulgaris and Spirulina platensis are 5.43–61.81 and 1.08–5.83 mg/L, respectively, in 24–96 h exposure, respectively, this is a strong dependence on species (Moorthy et al., 2021).

2.2. Effects on Photosynthetic Pigments and Processes

Pigments are very sensitive indicators of chemical stress. S. platensis was exposed to six textile dyes at concentrations between 5 and 40 ppm and significant reductions in growth, total chlorophyll levels (up to 50%), carotenoids (up to 50%) and phycocyanin (over 90%) were observed (Gita et al., 2021). In C. vulgaris, 96 h exposure to 50 mg/L rhodamine B led to a reduction of 71.59%, 74.90%, 65.84% and 74.20% in chlorophyll-a, chlorophyll-b, catalase and peroxidase, respectively, which was suggestive of oxidative stress as a major cause (Sudarshan et al., 2022).

There are similar responses in higher plants. Rhodamine B in H. verticillata demonstrated a dose-dependent increase in Fo/Fm and dissipation, decrease in Fm, a primary photochemistry quantum yield, a decrease in electron transport efficiency and a performance index, and initially stimulated, but then inhibited, SOD, CAT, and GPOD activity at concentrations of 5–25 mg/L for 24–72 h, respectively (Sharma et al., 2022). A reduction of the chlorophyll content in E. crassipes was observed after exposure to malachite green (50–200 mg/L) for two to six days, which resulted in the slowing of donor side electron transport, accumulation of $P680^+$ and increase in the percentage of inactive PSII reaction centres (Matpang et al., 2017).

2.3. Ecological Roles and Pollution Responses of Native Macrophytes

Floating and submerged macrophytes control oxygen, nutrient turnover and habitat structure, and a few species absorb or modify effluent components. E. crassipes has been applied as a biofilter to clean up textile effluents, and the removal of nutrients and organic pollutants is dependent on biomass weight and plant maturity (Adelodun et al., 2021). Removal capacity, however, does not reflect physiological tolerance and prolonged exposure to high concentrations of effluent can damage the plants that are doing the removal process.

2.4. Research Gaps

Very few studies look at individual degradation metabolites, instead focusing on the whole dye. There are few examples of direct comparisons among different aromatic amines in macrophytes. The effects of metabolites on chlorophyll fluorescence are still not quantified. Data on floating and submerged native species are scanty and limited. Relationships between metabolite accumulation, ROS production and PSII inhibition are not well understood.

III. Materials and Methods

3.1. Study Design and Plant Material

A fully randomized factorial laboratory experiment design is suggested involving species, metabolite type, concentration and exposure time as factors. S. polyrhiza is chosen for rapid clonal growth, appropriate for processing in fluorescence assays, E. crassipes has extensive roots, and H. verticillata has submerged tissue in direct contact with the water column, which is established wastewater research. The plants are gathered from areas with known permit and species identification, put into a nutrient media for 7-10 days of acclimatization.

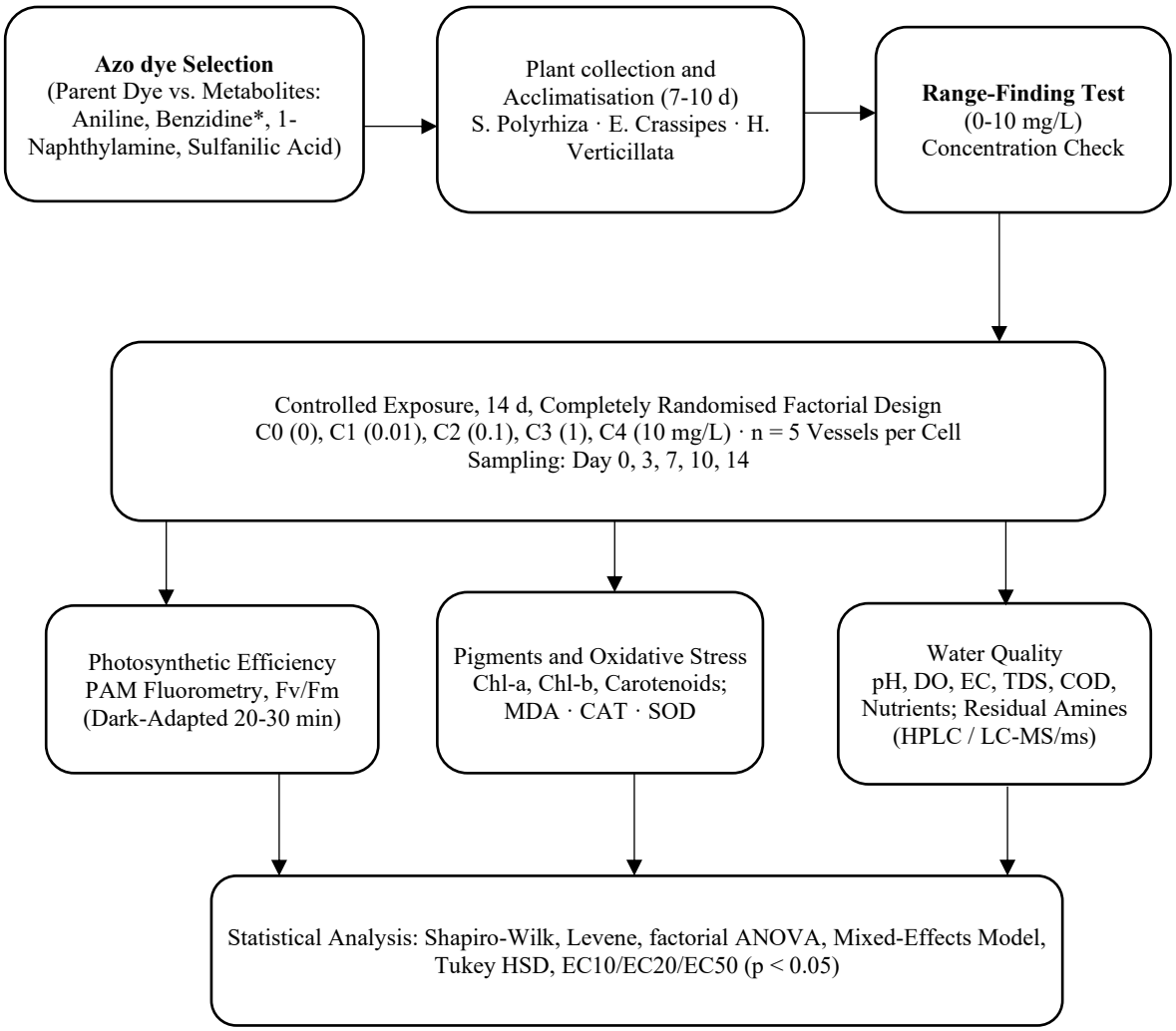


Figure 1: Experimental Workflow

Figure 1 outlines the sequence from dye selection and plant acclimatisation through range-finding, the 14-day factorial exposure, parallel measurement of photosynthetic efficiency, pigments, oxidative stress and water quality, and final statistical analysis, with all branches converging on a single analytical framework. The asterisk marks benzidine, which requires institutional hazard approval, containment and certified waste disposal before inclusion; exclusion from the first screening phase remains an option for a lower-hazard first phase.

3.2. Metabolites, Concentrations and Controls

Aniline, benzidine, 1-naphthylamine and sulfanilic acid are suggested for preliminary testing following confirmation of their formation from the parent dyes under the planned reduction conditions. The treatments are: 0 (control), 0.01, 0.1, 1 and 10 mg/L these are laboratory screening doses and not concentrations found in the environment. When there is no response or total mortality, this series is adjusted by range-finding. Metabolite effects are separated from the original dye effects by a parent-dye treatment, procedural controls and analytical blanks. The experiment is carried out for 14 days and measures are taken on days 0, 3, 7, 10 and 14.

Table 1: Summary of the Proposed Experimental Design

Factor	Specification
Species	*S. Polyrhiza*, *E. Crassipes* (Free-Floating); *H. Verticillata* (Submerged)
Metabolites	Aniline, Benzidine, 1-Naphthylamine, Sulfanilic Acid; Parent-Dye Comparison
Concentrations	0, 0.01, 0.1, 1, 10 mg/L (Confirmed by Range-Finding)
Duration	14 d; Sampling on Days 0, 3, 7, 10, 14
Replication	5 Independent Vessels Per Cell; N = 3 × 5 × 5 × 4 = 300 Units
Primary Endpoints	Fv/Fm; Chlorophyll-a, Chlorophyll-b, Carotenoids
Secondary Endpoints	MDA, CAT, SOD; Water Quality; Residual Metabolite Concentration

Table 1 consolidates the factorial structure. The number of experimental units used is 300 minus the number of parent-dye treatments (parent-dye blanks) and procedural controls (parent-dye blanks) and microbiological blanks. The "replicate unit" is the independent vessel to prevent pseudoreplication. Primary endpoints refer to photosynthetic function and secondary endpoints refer to oxidative status and exposure chemistry, thus permitting separation of physiological changes from changes in medium composition. Treatment allocation to ships is random and concentrations are provisional until range-finding has been completed.

3.3. Photosynthetic Efficiency, Pigments and Oxidative Stress

F_v/F_m is measured with a pulse-amplitude-modulated fluorometer after 20–30 min of dark adaptation under constant instrument settings defined in equation (2) and (3):

$$F_v/F_m = \frac{F_m - F_o}{F_m} \tag{2}$$

$$I(\%) = \frac{F_c - F_t}{F_c} \times 100 \tag{3}$$

Where F_o and F_m are minimum and maximum fluorescence, F_v is variable fluorescence, and F_c and F_t are control and treated F_v/F_m.

Pigment Extractions

Pigments are extracted in 80% acetone and read at 663, 646, and 470 nm shown in equation (4) – (6):

$$\text{Chl-a} = 12.25A_{663} - 2.79A_{646} \tag{4}$$

$$\text{Chl-b} = 21.50A_{646} - 5.10A_{663} \tag{5}$$

$$\text{Carotenoids} = \frac{1000A_{470} - 1.82(\text{Chl-a}) - 85.02(\text{Chl-b})}{198} \tag{6}$$

Concentration Conversion

Concentrations (μg/mL) convert to mg/g fresh weight as in equation (7):

$$\frac{C \times V}{1000 \times W} \tag{7}$$

Biochemical Assays

Lipid Peroxidation: Assessed by MDA through a TBARS assay.

Catalase Activity: Measured by the decline in H_2O_2 absorbance at 240 nm.

SOD Activity: Determined by its conversion of superoxide to hydrogen peroxide.

3.4. Water Quality, Replication and Statistics

All throughout: pH, temperature, dissolved oxygen, conductivity, total dissolved solids, COD (dichromate method), nitrate and phosphate (molybdenum blue) are measured. Because of the ability of plants and microorganisms to remove or transform test compounds, residual concentration of metabolites is determined at the beginning and the end of the exposure by HPLC or LC-MS/MS. Data were presented as mean ± SD and statistically analyzed using Shapiro–Wilk and Levene tests, factorial ANOVA, repeated-measures or mixed-effects model, Tukey's HSD, Pearson correlation and dose–response regression analyses ($p < 0.05$).

IV. Results

All quantitative results reported below are combined results from experiments on photoautotrophs exposed to dye in water and should not be interpreted as results of the proposed 300-unit experiment.

4.1. Photosynthetic Efficiency Across Species, Concentrations and Exposure Periods

Photosynthetic energy flow is well inhibited according to datasets. In S. polyrhiza, 2.5 g/L synthetic colourants led to a decrease of 94.18% in trapped energy flux (TR_0/CSm) a decrease of 96.10% in electron transport flux (ET_0/CSm) and further decreases in primary photochemistry yield, electron transport efficiency, and performance index. For E. crassipes, the inhibition increased with the concentration and duration of treatment with malachite green (50–200 mg/L for 2, 4 and 6 days). A 5–25 mg/L concentration of rhodamine B reduced Fm and performance index in H. verticillata within 72 h.

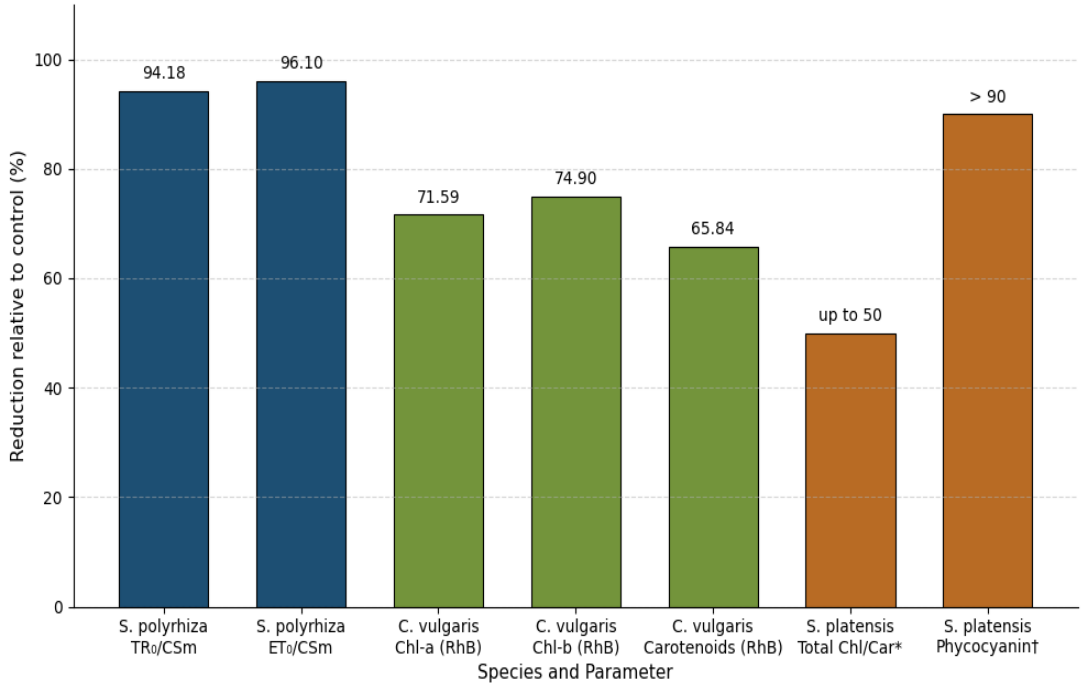


Figure 2: Reductions in Photosynthetic and Pigment Parameters

Figure 2 compares reported percentage reductions relative to controls across three photoautotrophs. The energy flux parameters of S. polyrhiza had the greatest decrease, over 94%, despite the exposure amount being measured in g/L. Chlorella pigments were reduced by approximately two thirds to three quarters and Spirulina phycocyanin decreased more than 90% under exposure of 50 mg/L of Rhodamine B. The values represent magnitude and are from different species, chemicals and protocols. Values with a star or dagger indicate bounds and error bars are not available for summaries.

Table 2: Quantitative Evidence of Dye-Induced Photosynthetic Impairment

Organism	Stressor	Exposure	Reported Response
S. Polyrhiza	Synthetic Colourants	2.5 g/L	TR_0/CSm −94.18%; ET_0/CSm −96.10%
E. Crassipes	Malachite Green	50–200 mg/L; 2–6 d	Lower Chlorophyll; More Inactive PSII Centres; Concentration- And Time-Dependent
H. Verticillata	Rhodamine B	5–25 mg/L; 24–72 h	Lower Fm, φPo, φEo, PIabs; Higher Fo/Fm; SOD, CAT Rise then Fall
C. Vulgaris	Rhodamine B	50 mg/L; 96 h	Chl-a −71.59%; Chl-b −74.90%; Carotenoids −65.84%
S. Platensis	Six Textile Dyes	5–40 ppm	Chlorophyll and Carotenoids up to −50%; Phycocyanin > −90%

Table 2 shows the evidence base utilized for interpretation. Organism, stressor, exposure and principal response are listed in each row. The responses are from losses of energy flux due to fluorescence in the macrophytes to loss of pigment and protein in the microalgae. Since there are no isolated aromatic amines in any of the rows, the table serves for mechanistic inference only, and specifies the data gap that the proposed metabolite-specific experiment is intended to close, which is the quantified responses to individual amines.

4.2. Chlorophyll Content and Oxidative Indicators

A permanent reduction in chlorophyll level was a frequent response. In S. platensis, the total chlorophyll and carotenoids decreased by as much as 50%, in C. vulgaris, the pigments decreased by 65.84-74.90% after 96 h at 50 mg/L of rhodamine B and the chlorophyll in E. crassipes decreased gradually with the dose of malachite green and the duration of exposure. The antioxidant enzymes followed a biphasic response where SOD, CAT and GPOD increased at lower concentrations and exposure times, but decreased with higher concentration and exposure time (which represents defence overload).

4.3. Comparison Among Species and Phytoremediation Performance

Exposure units, stressors and endpoints are not the same for Direct sensitivity ranking. However, measurable damage to PSII was observed in all three macrophytes. Sulphate (96%), ammonia-nitrogen (91%) and phosphate (84%) were removed from textile wastewater but removal of the other parameters i.e. total kjeldahl nitrogen, hardness, chloride and organic nitrogen were comparatively less (55% each). However, the plants did not grow well in very polluted effluent after 8 weeks.

Figure 3 ranks removal efficiencies of water hyacinth for seven wastewater parameters. Highest removals were achieved for sulphates (more than 84%), ammonia-nitrogen and phosphate (more than 84%), while the removals of hardness, chloride, organic nitrogen and total Kjeldahl nitrogen were between 53% and 55%. The high removal rate was associated with low survival rate in effluent treatment performance after eight weeks, indicating that treatment performance is not equivalent to physiological tolerance or long-term survival and tolerance testing prior to field deployment is warranted.

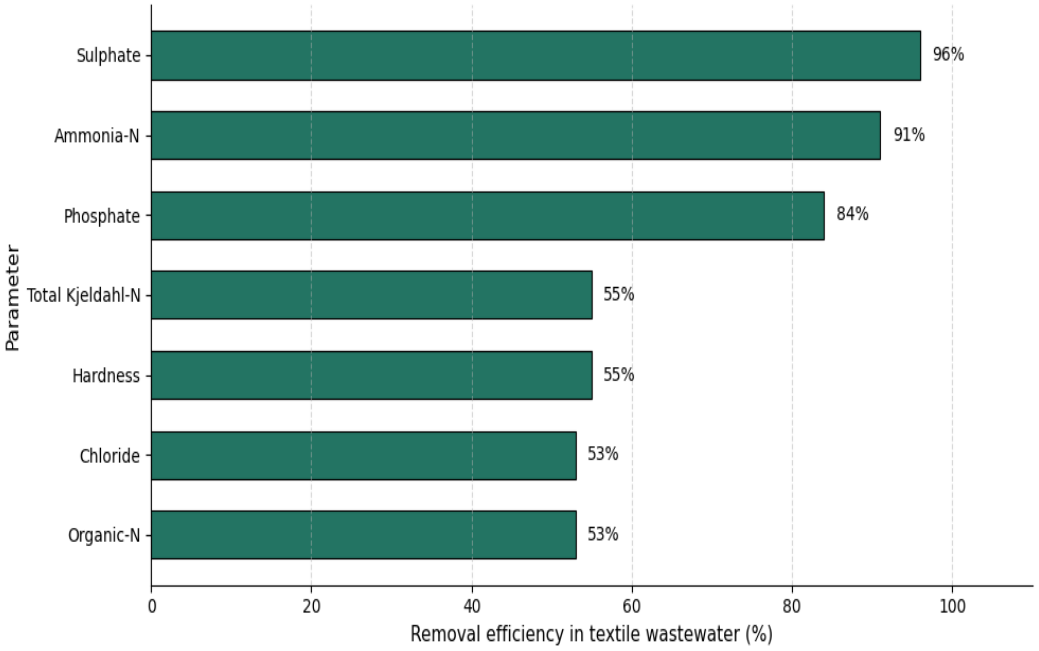


Figure 3: Pollutant Removal by E. Crassipes in Textile Wastewater

4.4. Statistical Significance and Dose–Response Patterns

In all systems, inhibition was seen to increase with increasing concentration and length of exposure, and was monotonic for photosynthetic impairment. The values in percentages of different studies cannot be added together in one statistical comparison and no EC10, EC20 and EC50 values exist for aromatic amines in macrophytes. In the reporting of the proposed experiment, Fv/Fm (mean ± SD) and chlorophyll-a (mg/g FW) should be presented by species, treatment and day, based on actual observations only.

V. Discussion

For parent dyes and synthetic colourants and photosynthetic efficiency, the content of pigments and PSII performance decreases with increasing concentration and exposure time. Qualitative support H3 is provided with contrasting responses of floating, submerged and microalgal systems. The four hypotheses are speculative for metabolites; none of these studies tested isolated aromatic amines.

The responses observed may have a number of causes. Excessive ROS can result in oxidative damage to lipids in the membrane, to proteins and degradation of pigments; an increase in MDA would reflect this damage. PSII disruption (donor- or acceptor-side blockage, loss of active reaction centres) reduces electron transport and Fv/Fm. The reduction of chlorophyll decreases the ability to harvest light, and increases in antioxidant enzymes may be a stress response, which will only further decrease in ability to harvest light under chronic stress, so enzyme activity should always be considered in context with damage markers.

Tolerance may vary according to form of growth. The small body size and fast clonal growth of S. polyrhiza favour the early detection of stress. E. crassipes has a large root mass and biomass for adsorption and accumulation, but can be inhibited by high concentrations of effluent for a long period. H. verticillata has submerged leaves that are in direct contact with the medium, providing an informative contrast. This is a testable expectation and ranking can only be based on comparative data.

If the photosynthesis of the macrophytes is impaired, it can result in decreased primary productivity and oxygen production, changed nutrient cycles, habitat degradation and changed food-web relationships, and increased susceptibility to other stressors. Colour removal is not enough to consider wastewater

management a success. Residual dye and aromatic amine concentrations, COD, BOD, acute and chronic toxicity, and photosynthetic stress should all be monitored.

Differences between single amines and complex effluents, sensitivity of fluorescence readings to plant age, nutrient levels, light and temperature, change of metabolites during exposure, lack of sediment and microbial interactions in the laboratory, and dependence on parent-dye studies as analogues. There is a need for future work to include the integration of chemical, fluorescence, biochemical assays and field monitoring.

VI. Conclusion

The threat of textile dye metabolites to the photosynthetic performance in freshwater macrophytes is a sound one. It has been consistently shown in evidence that there is a decrease in PSII efficiency, electron transport and pigment content as dye stress increases with dye concentration and time, respectively. *S. polyrhiza* was the most severely impaired with losses of trapped and transported energy flux of 94.18% and 96.10%, respectively, while E. crassipes had high pollutant removal and limited tolerance to strongly polluted effluent. Based on this sensitivity, it seems to be species-dependent, growth form-dependent, stressor-dependent, and requires metabolite-specific testing to determine a definitive ranking. Decolourisation alone is not sufficient as a treatment endpoint as colourless aromatic amines can remain after visible colour is removed, hence the need to monitor in addition to amine quantification with biological indicators of photosynthetic stress. Fv/Fm and electron transport parameters are likely to be useful as early indicators of damage and are recommended to be used in conjunction with visible chlorosis and growth loss. Long-term and field-based studies should be given special attention in future work and the following actions are recommended to assess pollution and control the management of textile wastewater.

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