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Tri-phasic engineered wetland system for effective treatment of azo dye-based wastewater

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Synthetic dyes are a major class of compounds used in the textile industry. The effluents released from these industries are carcinogenic and mutagenic in nature and pose potential threat to all life forms. Here, we designed a novel tri-phasic engineered flow through wetland system (TEWS) with three specific microenvironments (tri-phasic aquatic systems) integrated in a defined sequence to effectively treat azo dye-based textile wastewater. Tank 1 with free-floating and emergent aquatic macrophytes create anaerobic/anoxic microenvironments and facilitate reduction of the azo dye molecules. Tank 2 consists of submerged macrophytes which create an aerophilic microenvironment that direct the oxidation of carbon compounds along with nitrification. Tank 3 with free-floating plants and aquatic animals acts as filter feeder intended to remove suspended solids and colour, as well as residual carbon. The strategically designed TEWS attributes to 76%/87% of dye/chemical oxygen demand removal. The morphological toxicity test of plants and fishes illustrate the nontoxic nature of treated effluents. Thus, TEWS not only decolorizes the azo dye but also removes its toxic and mutagenic components.

npj Clean Water (2019)2:13; <https://doi.org/10.1038/s41545-019-0037-y>

INTRODUCTION

Azo dyes represent a major class of colorants used in textile industries that contain one or more azo bonds ($-N=N-$). The high color intensity is generally attributed due to the azo bond associated with chromophore.¹ These dyes are also complex due to substituted aromatic rings associated with different groups, such as chloro ($-Cl$), methyl ($-CH_3$), nitro ($-NO_2$), amino ($-NH_2$), hydroxyl ($-OH$), and carboxyl ($-COOH$) groups.² Azo dyes get decolorized by splitting the azo bonds in sequential bio-redox conditions, forming aromatic amines and further breakdown into simpler compounds.³⁻⁵ The azo bonds are environmentally electrophilic in nature and can accept the electrons from the reductive conditions to cleave the azo bond and form the aromatic amines and then breakdown into nontoxic chemicals.⁶⁻⁸ The anaerobic digestion of azo dyes form aromatic amines that are cytotoxic, mutagenic, or carcinogenic.⁹⁻¹¹ Thus, these aromatic amines can be mineralized and converted to nontoxic compounds by further subjecting to aerobic treatment with non-specific enzymes through hydroxylation and ring opening of the aromatic compounds.^{3,12} Past few years, several researchers have been working on combined, sequential, or integrated bioreactor strategies.¹³

Plants play significant role in xenobiotic degradation through their complex array of enzymes, than simply providing sites for the development of symbiotic microorganisms.¹⁴ Plant roots and their associated microbial population may perform physical removal of solids and adsorb the nutrients and dissolved constituents.^{15,16} The plants may create proper microenvironments for the treatment process. These mainly include the large rooted floating plants which allow water to become anaerobic, leading to the nitrification/denitrification. Plant metabolism of xenobiotics may lead to total or partial degradation or transformation by reductive,

oxidative or hydrolytic enzymes. Phytoremediation is easier to regulate because it is an autotrophic system that requires minimal nutrient and economic input. Moreover, plants offer protection against water and wind erosion, preventing contaminants from spreading.¹⁷ *Salsola vermiculata*, a desert plant, has been proved to be a low-cost option for the removal of large organic molecules.¹⁸ There have been few field tests to date on constructed wetlands, which have been used to treat the industrial effluents containing a wide range of chemicals.¹⁹⁻²³

The tri-phasic engineered wetland system (TEWS) was designed as an alternative process to treat the dye-based wastewaters with the aim of complete mineralization using synergistic tropical microenvironments. TEWS is an engineered wetland biosystem designed to consist of three specific microenvironments (tri-tropical aquatic systems) namely free-floating and emergent aquatic macrophytes, submerged macrophytes, and free-floating plants. The constructed wetland mimics a natural ecosystem, wherein all the trophic conditions are balanced and perform their role and in the process bring about wastewater treatment by relying entirely on the living systems.^{3,24-26} TEWS symbiotically enables ecological self-design^{25,27} with an ecosystem boundary that can self-organize.²⁵⁻³⁰ The efficiency of designed TEWS mainly depends on the root zone interactions between soil, contaminants, plant roots, and a variety of microorganisms with indigenous tropical microenvironments in each designed tanks. This primary objective is to address the current problems in the field of textile/azo dye industry by specifically providing in situ redox microenvironments with living systems for effective degradation of azo dye.

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Received: 25 October 2018 Accepted: 5 May 2019

Published online: 04 June 2019

RESULTS AND DISCUSSION

Colour removal

The efficiency of TEWS to remove color was assessed at three tropical levels with 50 mg/L of dye concentrations at a constant organic (COD) load of 250 mg/L (Fig. 1). Color removal pattern varied with the function of tank and its associated conditions. Relatively rapid and higher color removal was observed in Tank 1 outlet compared with Tank 2 and Tank 3. On individual tank basis, removal efficiency was 62%, 25%, and 17% in the outlet of Tank 1, Tank 2, and Tank 3, respectively (Fig. 1a, b). Cumulatively three tanks showed a color removal efficiency of 76%. The persistent anoxic microenvironment in Tank 1 favored denitrification that led to the azo bond breakdown of aromatic amines.

UV-Vis spectra depicted reduction of azo group corresponding to peak at 418 nm in Tank 1 compared with inlet (Fig. 1c). This infers the azo group breakdown followed by a new peak appearance in the range of 340–350 nm, which corresponds to the formation of imine groups ($R-N=C-$). The reduction of azo dye resulted in the formation of recalcitrant aromatic amine groups namely tartrazine, producing carcinogenic amines ($R-NH_2$), such as benzidine and 4-aminoaniline³¹, which are cytotoxic, mutagenic, or carcinogenic. Tank 2 due to its self-generation of aerobic microenvironment facilitates nitrification. Aromatic amines get mineralized by non-specific enzymes through hydroxylation and ring-opening structure. The UV-Vis chromatographs attribute the breaking of the aromatic ring with its respective amine, imine, and benzene-derivative groups. The intensity of the IR spectral peak significantly reduced from Tank 1 to Tank 2 at 418 nm and 350 nm, which depicts the removal of aromatic groups and their derivatives. In Tank 3, the linear UV-Vis spectra represent the total degradation of azo bond and other derived compounds.

FT-IR spectra peaks before and after the treatment of each individual tanks (Fig. 2, Table 1) illustrated the major fraction of N-H stretch at peak 3417.77 cm^{-1} and other $-OH$, $-CH$, and other non-specific functional groups at 3417 cm^{-1} , 1604 cm^{-1} , 1016.62 cm^{-1} , and 754 cm^{-1} . The major absorption band at 3414.96 cm^{-1} corresponding to the stretching vibration of $-N-H$, diminished after the degradation of dye, moreover a new absorption band at 2518.57 cm^{-1} corresponding to the $-S=O$ stretching was observed. The absorption bands at 1636.86 cm^{-1} and 1440.21 cm^{-1} can be assigned to the quinonoid ring and the benzenoid ring, respectively. Bands at 1298 cm^{-1} and 1150 cm^{-1} are due to $-C=N$ bending and in plane bending of the $-C=H$ bond. In comparison with inlet, Tank 1 followed by Tank 2 and Tank 3 treated effluents have showed disappearance of many functional groups with decreased intensity (major peaks).

Azo reductase activity

The azo reductase (AR) enzyme has the ability to degrade the azo bond in both aerobic and anaerobic conditions. AR enzyme basically functions to cleave azo bonds with the aid of an electron donor (NADH and/or NADPH). This was monitored during operation of different tropical systems with plant extract, suspending microbes, and sediment microbial consortia (Fig. 3). Tank 1 showed higher azo reductase activity in root extract (34.61 units/mL) followed by suspending microbes and sediment bacteria (34.05 and 33.18 units/mL). Whereas Tank 2 and Tank 3 showed 31.13, 32.27, and 30.53 Units/mL and 30.93, 32.61, and 30.93 Units/mL microbial azo reductase than plant root azo reductase activity, suspending microbes and sediment bacteria, respectively. In anaerobic microenvironment, aromatic nitro compounds were generated from the action of azo reductase, which later undergoes the nitro reduction reaction mediated by reduced quinone compounds resulting in quinone reductase activity.^{32–34} In aerobic conditions which were eventually degraded by other enzymes like mono, deoxygenasas, and

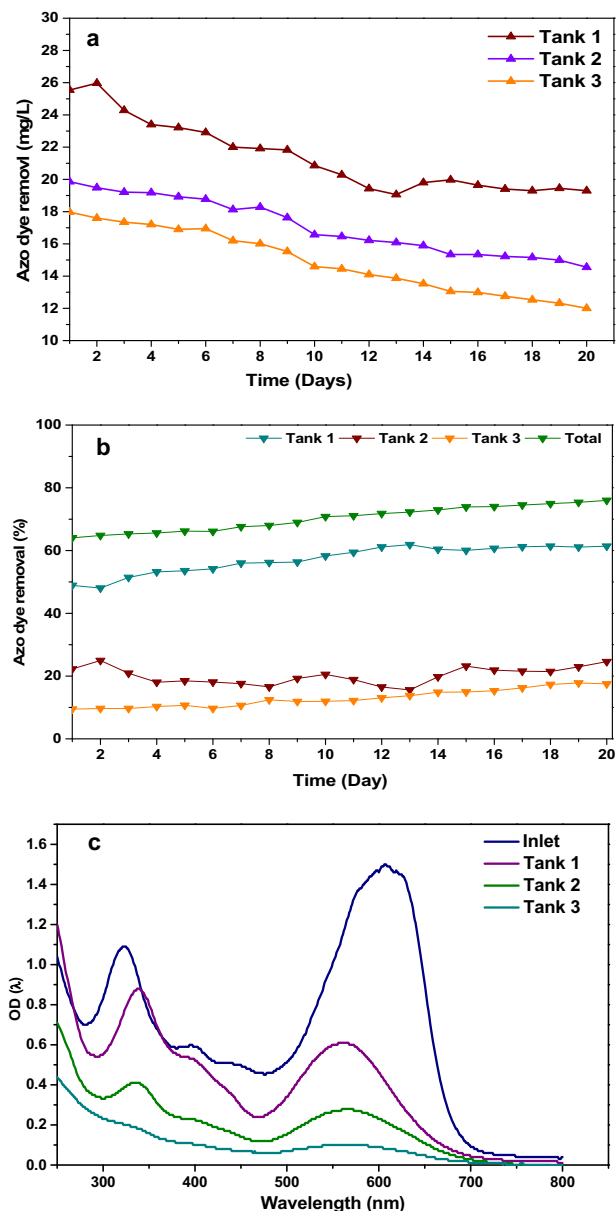


Fig. 1 a, b Variations in azo dye removal concentrations and removal efficiencies in individual tanks with respect to time, c UV-Visible spectra profile of degraded dye compounds in individual tanks

hydrolases, it was demonstrated that menadione (quinone) is a better substrate for flavin-containing NAD(P)H-dependent azo reductase compared with azo and nitro compounds.³² Therefore, azo, nitro, and quinone reductions could also be another criterion to be considered in azoreductase activity. It was also reported to play an important role of azo reductase in the detoxification of quinones apart from azo bond removal.³⁴ The quinone reduction activities of azo reductase from different microorganisms and their dependence on NAD(P)H co-factor need to be classified as NAD(P)H quinone oxidoreductases (NQOs).^{32,35–38} It was also identified that the NAD(P)H quinone oxidoreductase activity in azoreductase from *P. aeruginosa* demonstrated that the azoreductases and the NAD(P)H quinone oxidoreductases belong to the same FMN-dependent azoreductase superfamily.^{39–41}

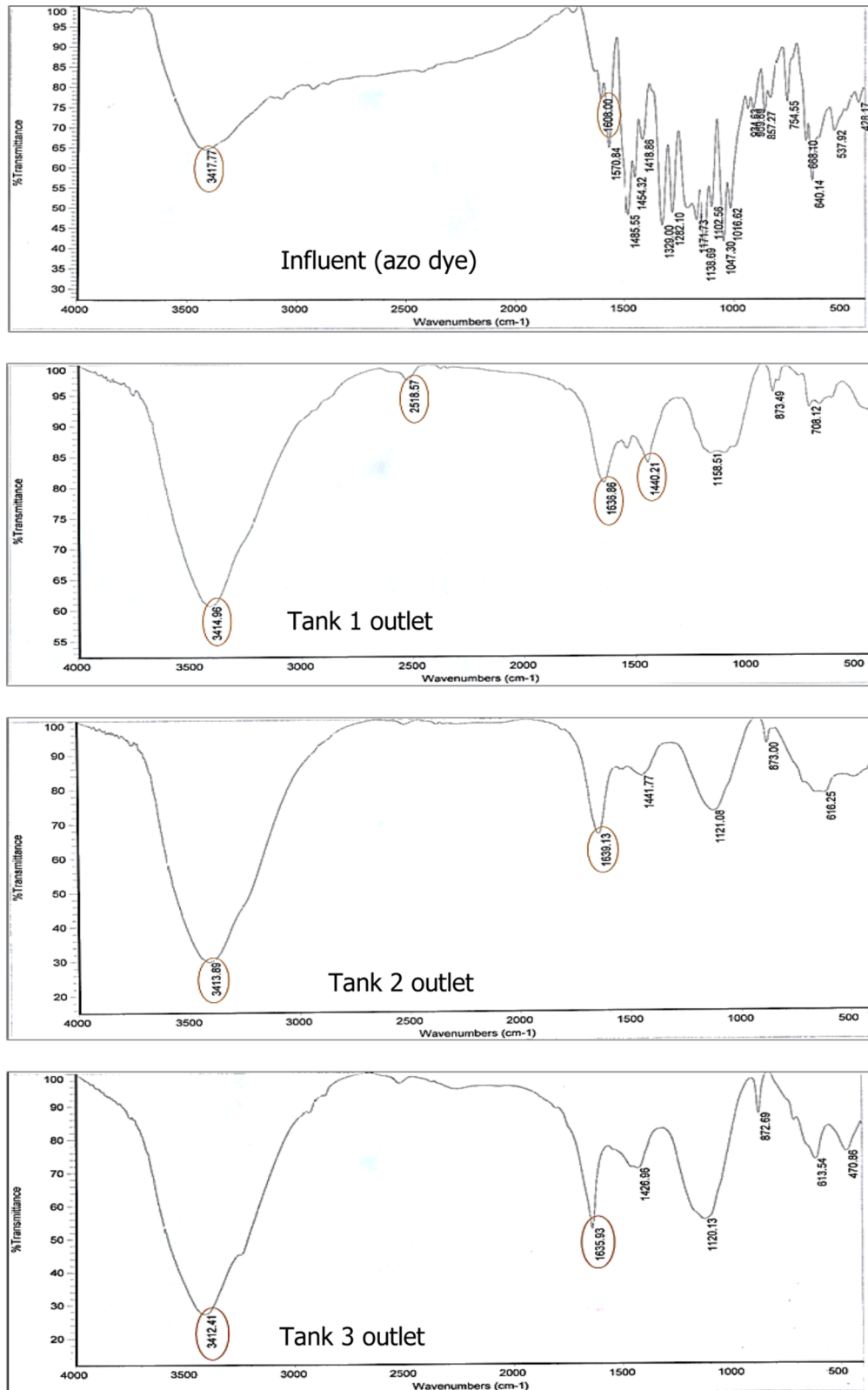


Fig. 2 Comparative FT-IR spectrum of azo dye and its degradation in inlet azo dye, Tank 1, Tank 2, and Tank 3

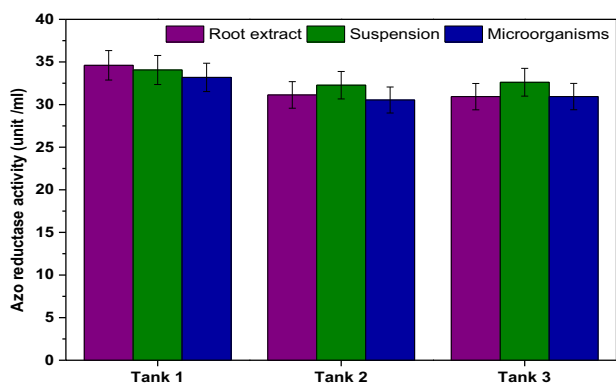
Morphoanatomical and phytotoxicity

The movement and biotransformation of azo dye in macrophyte's vegetative parts was evaluated in *E. crassipes*, *H. verticillata*, and *P. stratiotes* (Fig. 4). The dye-treated and non-dye-treated young

roots, stems, petioles, and offsets were selected from macrophytes. Cross-sections were made for each vegetative part and observed under microscope with $\times 10$ magnification. The azo dye transportation, bioaccumulation, and morphological changes

Table 1. The IR spectral data of the azo dyes and their complexes (KBr, cm^{-1})

Samples	Wavenumber cm^{-1}
Influent dye solution	-N-H 3417.77 cm^{-1}
	-OH stretching 3417
	-CH stretching 1608
Tank 1 Outlet	-OH stretching 3414
	-CH stretching 2914
	-C=O stretching 1637
	-C-C Symmetric stretching 1429
	-CH bending 1371
Tank 2 Outlet	-OH stretching 3413
	-CH stretching 2901
	-C=O stretching 1639
	-C-C Symmetric stretching 1431
	-CH bending 1371
Tank 3 Outlet	-OH stretching 3412
	-C=O stretching 1635
	-C-C Symmetric stretching 1426

**Fig. 3** Azo reductase enzyme activity of the root extract, microorganisms in suspension, and rhizosphere, each value is represented as mean of three replicates and statistical differences between values at a 5% level of significance

were not found in the epidermis, parenchyma cells, phloem, xylem, and other vascular bundles of dye treated when compared with non-dye-treated macrophytes. Morphological observations of dye-treated and control non-treated plant sections revealed no cellular morphological changes. It indicates that the metabolites formed during dye degradation were nontoxic in nature as the growth of the plant was not inhibited.

Morphological toxicity analysis

The aquatic toxicity was studied with 10 *Gambusia affinis* (length = 2.2 ± 0.06 cm; width = 2.5 ± 0.014 cm) selected randomly from Tank 3. These fishes were killed for analysis. Autopsy of fishes was done upon chronic exposure for RBC counts and blood smear. The RBC measurements made by a hemocytometer standardized with a micrometer scale as parallel magnification ($\times 10$ and $\times 100$). The percentage of morphological RBCs was calculated by observing 100 RBCs in 20 microscopic fields ($\times 10$ and $\times 100$) using an oil immersion and found that no morphological abnormalities were found in the RBC cells. Fish population was assessed for a physiological variation where there was no visible colour change observed on the skin layers.

COD removal

Co-substrate degradation based on COD removal was comparatively evaluated in the three tanks. The inlet was fed with a

constant organic load of 250 mg of COD/L. The co-substrate provided the energy required for the growth and survival of the microorganisms. Higher degradation was observed in Tank 1 with COD reduction from 250 to 110 mg of COD/L (56%) followed by Tank 2 (44 mg of COD/L; 60%) and Tank 3 (34 mg of COD/L; 34%) (Fig. 5a, b). TEWS in total contributed for 87% of COD removal efficiency. During the initial phase of operation, outlet COD from three Tanks varied between 190 and 120 mg of COD/L, due to requirement of adaptability. During the initial stage of operation, plants showed stress and then with time adapted to the chemotrophic nature. The substrate degradation can be attributed to the co-existence of tri-phasic microenvironments viz., anoxic, aerobic, and anoxic in Tank 1, Tank 2, and Tank 3, respectively. The anoxic microenvironment in Tank 1 facilitates the mineralization/reduction of the substrate by facultative bacteria. The floating aquatic plants grown as a mat on the surface of water, usually cause the water to be isolated from the atmosphere and result in the quiescence and hinderance of the gas transfer, creating anoxic conditions, particularly in Tank 1. The quiescent condition also supports the system microenvironment for effective dye removal. Aerobic condition in the Tank 2 due to the submerged macrophytes facilitate the growth of aerobic microflora, which metabolize the reduced intermediates and are capable of absorbing nutrients and some organic compounds. As compared with floating macrophytes, it may be considered that the main removal mechanism is by bacteria rather than plant uptake particularly in Tank 2. Apart from oxygen release, the photosynthetic mechanism of the submerged plants provide symbiotic association, which positively influenced the overall treatment efficiency.

System redox variation

During decolourization, the pH of influent and effluent varied between 7 and 8 in each tanks. A marginal decrease in pH was observed in the outlet of Tank 1 compared with influent. There was a slight higher and consistency of pH observed in Tank 2 and Tank 3, respectively (pH 7.65 and 7.1) (Fig. 6a). In anaerobic microenvironment (Tank 1), azo dye reduction occurred which might have resulted in the breakdown of the azo bond subsequently forming respective aromatic amine intermediates. Due to these, amine compounds stability utilizes the protons, it reduces proton concentration and increases the OH^- concentration in the aqueous solutions. This is attributed to the rising of pH which was maintained at near-neutral conditions, as well as anoxic microenvironment. The fluctuation of the pH will depend on the buffering capability of the water. However, Tank 2 and 3 showed marginally higher pH and values than the Tank 1, which might have influenced by the DO concentration. Most of the studies showed that the submerged macrophytes (*Hydrilla*) could decrease the anaerobiosis due to producing the oxygen gas. The higher DO (>1 mg/L) facilitates the biodegradation and improves the effluents redox status. In general, nitrification process occurs at higher oxidative conditions in the Tank 2.

Dissolved oxygen

The DO concentration varied in the system during operation, based on the day (12:00 am) and night (6:30 pm) conditions (Fig. 6b). The inlet DO remained more or less constant near 2.1 ± 0.1 mg/L during the operation. Tank 2 showed higher DO concentrations [morning, 5.8 mg/l; night, 3.88 mg/L] followed by Tank 3 (4.04 mg/L and 3.08 mg/L), and Tank 1 showed least DO concentration (<0.2 mg/L), when measured during day and night time of operation. During night DO, values are relatively low due to the low photosynthetic activity. Tropical behavior based on DO concentration was engineered by the indigenous specific macrophytes induction in tanks. The microenvironments in Tank 1 lead to enrichment of specific anoxic consortia showed lowest DO

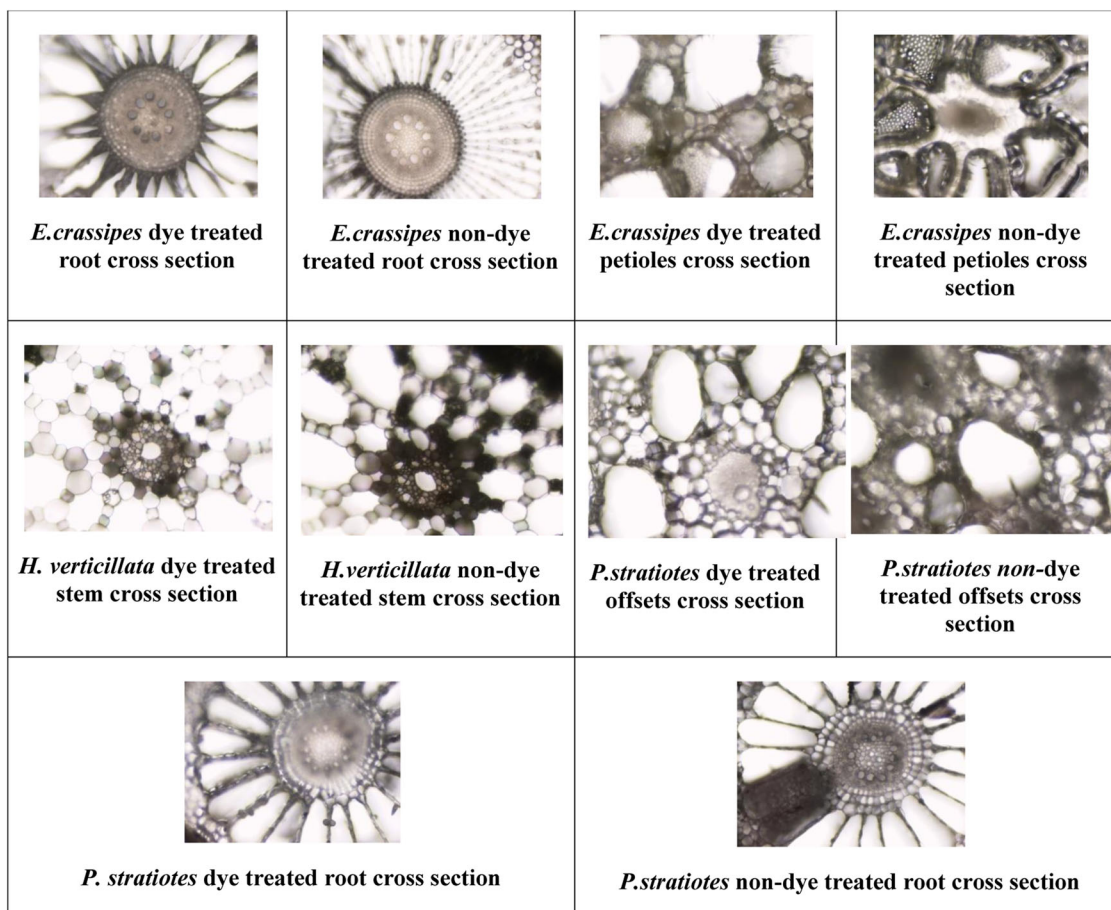


Fig. 4 Morphoanatomical and phytotoxicity analysis of the root, stem, petioles, and offsets of plants cross-sections in each tank when exposed and not exposed to dye

levels. The rapid growth nature of *Eichhornia* indicates the development of the anaerobic/anoxic conditions, which mediate the rapid decolorization of azo dye under an aerobic microenvironment, involving the reductive transformation of the azo chromophoric system and forms the aromatic amine. These aromatic groups are substituted with the amino group, which does not have adsorption in UV region of the spectrum and results in the decolorization of wastewater. Higher DO present in the Tank 2 can detoxify the toxic aromatic amino compounds by the aerobic microenvironment. Sustainable O_2 released by submerged *Hydrilla* in Tank 2 may help in enrichment of the aerobic bacteria, which facilitated the conversion of amines to nitrates by nitrification process. Tank 3 maintained the DO concentration for the growth of the fishes and aquatic plants. The outlet of the effluents have higher DO levels than the inlet to Tank 1. The presence of oxygen in the system facilitates the growth and provides habitat for flora and fauna of aquatic nature.

The results obtained from the TEWS system substantiate sustainable solution for effective azo dye-based wastewater treatment employing synergism of anaerobic and aerobic microenvironment simulated with near-natural conditions. Essential part of the study contributes significantly to color and COD removal, which is supported by FT-IR and UV-Vis multi-scan spectra. An overall efficiency of 76% of dye removal was achieved, with individual tanks removal efficiency accounting to 62%, 25%, and 17% in Tank 1, Tank 2, and Tank 3, respectively. TEWS has the advantage of the tri-phasic microenvironment and flow connections that helps to mineralize azo dye by sequential redox reactions with anoxic, aerobic, and filtering mechanisms.

METHODS

Tri-phasic engineered free-floating wetland system

TEWS was designed with three rectangular tanks having dimensions of $55 \times 30 \times 30$ -cm length, width, and height, using perplex sheets with the total/working volume of 50/35 L (free board of 5 cm) as described elsewhere.²⁵ The three tanks were placed on a customized stand with natural gradient of 15 cm allowing the flow with gravity (Fig. 7). The feed tank was kept at the highest point followed by the series of other tanks. The tanks were labeled from the top to bottom as Tank 1, Tank 2, and Tank 3, respectively (Table 2). The tanks were provided with baffles to prevent cross discharge and to equalize the flow. Vents were provided at a distance of 5 cm from the top of the tank. The system was operated in continuous mode with a constant flow rate of 2 L/h. Tank 1 and Tank 2 were filled with sediment obtained from Nacharam lake, Hyderabad upto a height of 5 cm from the bottom, and Tank 3 was filled with fine sand.

Macrophytes

The microenvironment in three tanks were designed to simulate natural ecological conditions based on different hydroponic macrophytes, and Tank 1 was designed to house free-floating macrophytes (*Eichhornia crassipes*). It covers the water surface of the tank and helps to maintain anoxic microenvironment. Moreover, *Eichhornia crassipes*, owing to its strong tolerance to pollutants, develops a good fibrous root system, which is favorable for the growth of different microbial communities in the rhizosphere with rapid growth and reproduction. Tank 2 contains slender, submerged, perennial aquatic herbs (*Hydrilla verticillata*; Water thyme), which helps in maintaining aerobic condition in the tank by respiring underwater. The growth of these plants is very rapid and they contain a tuberous root system anchored to the sediment. Tank 3 was designed to have *Pistia stratiotes* (water cabbage) along with fish (*Gambusia affinis*) and snails (*Cornu aspersum*), which enables final water polishing by the filter-feeding action of snails and fish and consume the bacteria and planktons.

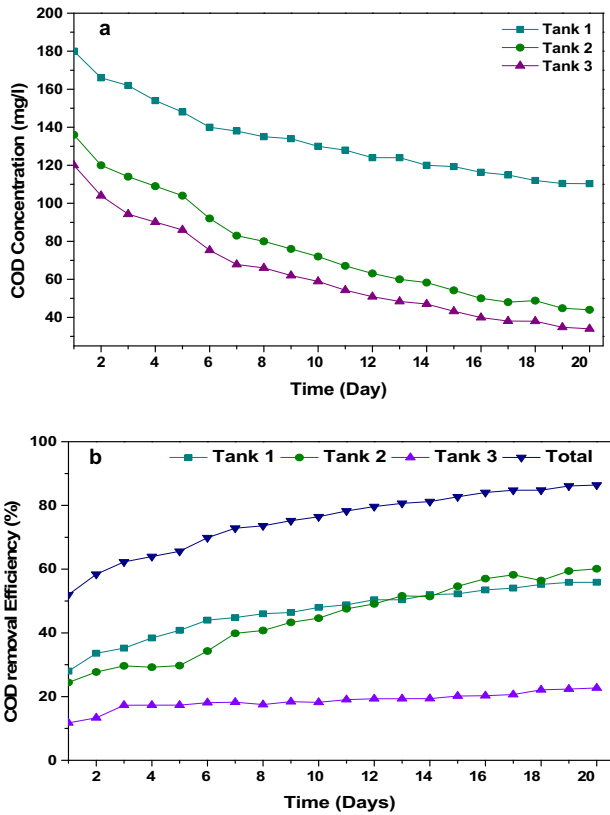


Fig. 5 Variations in the substrate removal monitored for Tank 1, Tank 2, and Tank 3 with respect to time, **a** chemical oxygen demand (COD) removal in concentration, **b** COD removal efficiency

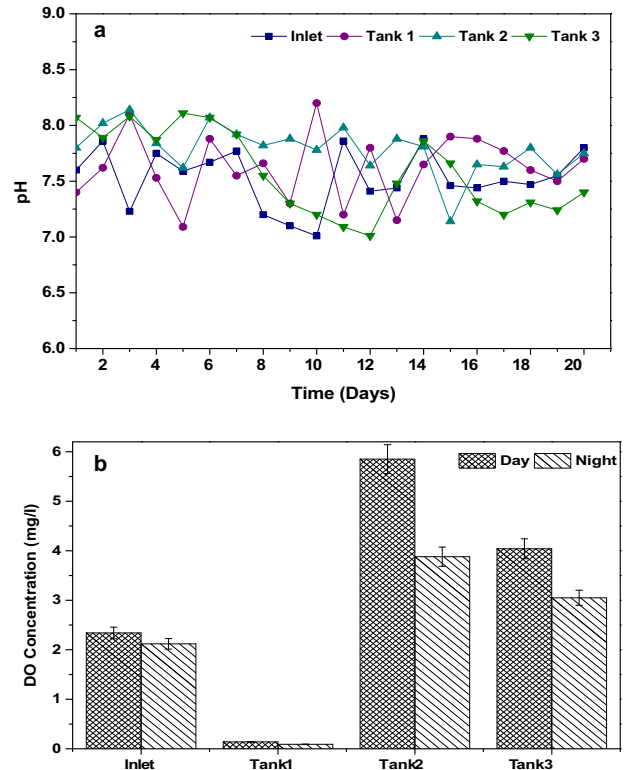


Fig. 6 Bioprocess monitoring in TEWS **a** pH in individual tanks, **b** dissolved oxygen profile in individual tanks, each value is mean of three replicates and statistically significant differences between values at a 5% level of significance

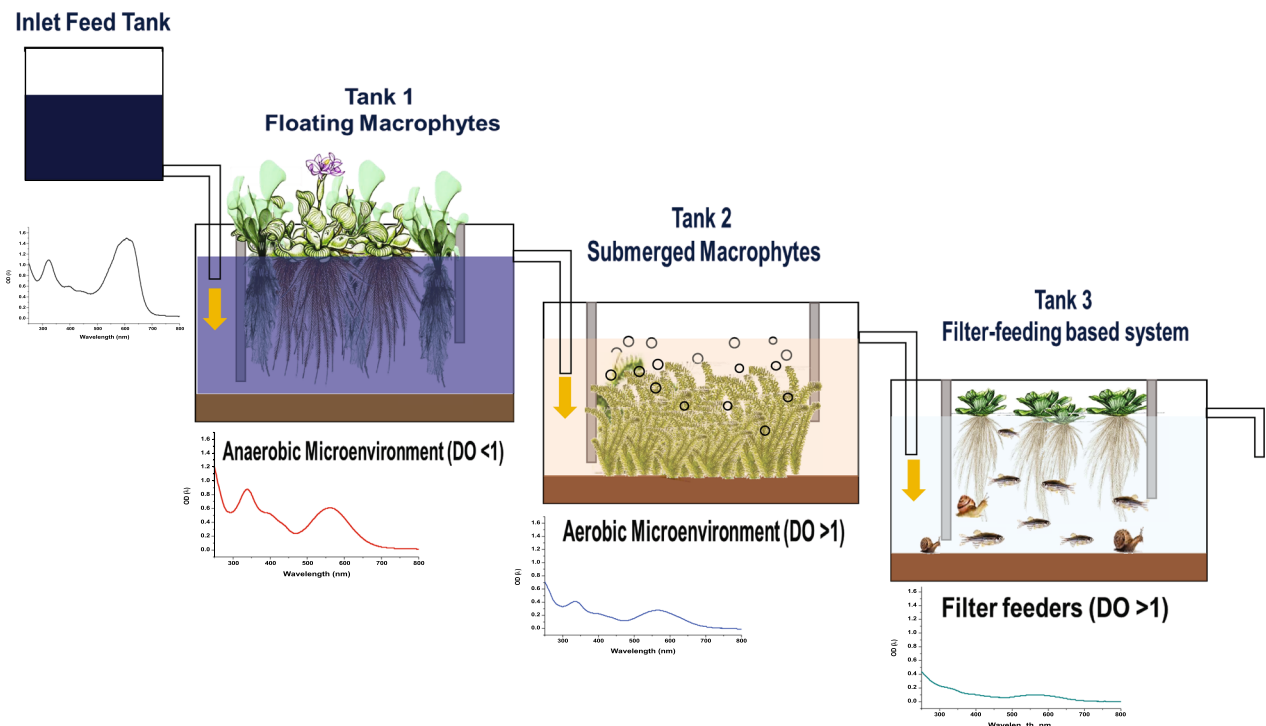


Fig. 7 Schematic representation of the TEWS system; Tank 1 grown with free-floating macrophytes (*Eichhornia crassipes*) helps to maintain the anoxic microenvironment. Tank 2 contains slender, submerged, perennial aquatic herbs (*Hydrilla verticillata*), which helps in maintaining aerobic conditions in the tank by respiring underwater. Tank 3 was designed to have *Pistia stratiotes* (water cabbage) along with fish (*Gambusia affinis*) and snails (*Cornu aspersum*), which enables final water polishing by the filter-feeding action of snails and fish and consume the bacteria and planktons. The spectra graphs represent the degradation of the dye from inlet in the tanks

Table 2. Tri-phasic engineered wetland system with various types of biota along with the individual function of tanks

Treatment system	Type of system and components	Functions	Advantages
Tank 1	Floating macrophyte based system with <i>Eichhornia crassipes</i>	To favors anoxic microenvironment and enrich the anaerobic microbial consortia	Fast growing, high nutrient uptake, the sponge-type root system, creates low oxygen conditions beneath the mat
Tank 2	Submerged macrophyte-based system with <i>Hydrilla verticillata</i>	To increase DO concentration in water and enhances the aerobic microbes	High photosynthetic O ₂ producer, a fast-growing plant, provides a good habitat for aerobic bacteria
Tank 3	Filter-feeding-based system with <i>Pistia stratiotes</i> and fish	To enables final water polishing	Removes the suspended particles, reduce pathogenic microorganisms, insects larva

Wastewater

C.I. Acid Black 10B [4-amino-5-hydroxy-3-[(4-nitrophenyl) azo]-6-(phenyl azo)-2, 7-naphthalene disulfonic acid disodium salt, C₂₂H₁₄N₆O₉S₂Na₂] related to the azo group was used to prepare simulated dye wastewater (SDW) by dissolving 50 mg/L of dye in designed synthetic water (g/L): glucose 0.25, NH₄Cl 0.5, KH₂PO₄ 0.25, K₂HPO₄ 0.25, MgCl₂ 0.03, CoCl₂ 0.025, FeCl₃ 0.025, ZnCl₂ 0.0115, NiSO₄ 0.050, CuCl₂ 0.0105, CaCl₂ 0.005, and MnCl₂ 0.015). Prior to feeding, pH of SDW was adjusted to 7.0 ± 0.1 using 1 N HCl/NaOH.

Operation

TEWS was continuously operated with 50 mg/L of simulated azo dye-based wastewater with dissolved organic carbon load of 250 mg/L as a co-substrate for a period of 90 days in a continuous mode with a constant flow rate of 2 L/h. The wastewater allowed to flow in series through Tanks 1, 2, and 3 by gravity, while the treated effluent from Tank 3 was used for gardening. After stabilization of the system performance (growth of the plant vegetation was stabilized), the last 20 days of operation data were reported in the communication. Harvesting of the aquatic plant biomass was done once in 20–25 days, depending on the growth. The degradation toxicity of treated dye-based wastewater in TEWS was evaluated by subjecting to fishes in Tank 3 throughout the experiment.

Process monitoring

To determine the decolorization efficiency, samples were withdrawn from TEWS systems at periodic time intervals during reactor operation. Samples were centrifuged (10,000 rpm; 5 min; 28 °C) to remove suspended biomass with appropriate dilutions, and the absorbance was measured at λ_{max} of 618 nm⁴² using a spectrophotometer (Gene Quant 1300). Azo dye concentration was quantified from a standard curve, and the samples were analyzed for the changes in COD as per standard procedures.⁴³ Azo reductase activity was determined to understand the reductive breakdown of azo bond in the dye molecule using a colorimetric method by estimating the extracellular azo reductase activity with NADH as co-substrate.^{42,44} Reaction mixture constituting 600 μl of sample, 600 μl of test dye, 1.2 ml of potassium phosphate buffer, and 7 mg/mL of NADH was incubated for 12 h at 37 °C. The absorbance of the overnight incubated reaction mixture was monitored at λ_{max} of 618 nm. One unit of azo reductase accounts for the amount of enzyme required to decolorize 1 mol of dye per min. Process monitoring was observed with pH and DO as per APHA (4500-O) methods. The azo dye bonds were analyzed based on FT-IR spectra data from each tank inlet and outlet. Toxicity test was carried out through fish survivability analysis in treated dye wastewater with respective to each tank.

DATA AVAILABILITY

All data generated or analyzed during this study are included in this article.

ACKNOWLEDGEMENTS

The authors greatly acknowledge financial support from Department of Biotechnology (DBT), Government of India in the form of project (BT/PR18965/BCE/8/1401/2016). DKY duly acknowledge CSIR, New Delhi, for providing research fellowship. IICT Manuscript No. IICT/Pubs./2019/116.

AUTHOR CONTRIBUTIONS

D.K.Y., P.R. and A.K.K. contributed executing the experiments and writing the paper equally. S.V.M. designed and supervised the experiments and associated in writing the paper.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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