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Demonstration of a plant-microbe integrated system for treatment of real-time textile industry wastewater

Mohanapriya Jayapal¹, Hema Jagadeesan¹*, Vinothkumar Krishnasamy¹, Gomathi Shanmugam¹, Vignesh Muniyappan¹, Dinesh Chidambaram², Satheesh Krishnamurthy³

Affiliations:

¹PSG College of Technology, Coimbatore, Tamil Nadu, India - 641 004.
²M/s.Dinesh Process, (Soft Flow Unit, Dyers of Knitted Fabrics), College Road, Analpalayam, Sirupuluvapatti, Tirupur, Tamil Nadu, India - 641603.
³School of Engineering and Innovation, The Open University, Milton Keynes, MK7 6AA, United Kingdom

Corresponding Author
Hema Jagadeesan,
Associate Professor,
PSG College of Technology,
Coimbatore – 641 004.
E – mail: hema.bio@psgtech.ac.in; hemajagadeesan@gmail.com
Tel (o): +91 -422 -2572177/4583

* - Corresponding author
The real-time textile dyes wastewater contains hazardous and recalcitrant chemicals that are difficult to degrade by conventional methods. Such pollutants, when released without proper treatment into the environment, impact water quality and usage. Hence, the textile dye effluent is considered a severe environmental pollutant. It contains mixed contaminants like dyes, sodium bicarbonate, acetic acid. The physico-chemical treatment of these wastewaters produces a large amount of sludge and costly. Acceptance of technology by the industry mandates that it should be efficient, cost-effective and the treated water is safe for reuse. A sequential anaerobic-aerobic plant-microbe system with acclimatized microorganisms and vetiver plants, was evaluated at a pilot-scale on-site. At the end of the sequential process, decolorization and total aromatic amine (TAA) removal were 78.8 % and 69.2 % respectively. Analysis of the treated water at various stages using Fourier Transform Infrared (FTIR), High Performance Liquid Chromatography (HPLC)) Gas Chromatography-Mass Spectrometry (GC-MS) Liquid Chromatography-Mass Spectrometry (LC-MS) indicated that the dyes were decolourized and the aromatic amine intermediates formed were degraded to give aliphatic compounds. Scanning Electron Microscope (SEM) and Atomic Force Microscopy (AFM) analysis showed interaction of microbe with the roots of vetiver plants. Toxicity analysis with zebrafish indicated the removal of toxins and teratogens.

Keywords: Textile dye wastewater; sequential anaerobic-aerobic plant-microbe treatment; total aromatic amine removal; Hydraulic Retention Time; plant-microbe association.
1. Introduction

Textile processing units are major wastewater generators and also contribute to water pollution. One of the reasons for this is the high cost of conventional treatment methods and as a result the discharge of untreated and/or partially treated wastewaters affects ecosystem’s and human health [1]. The conventional wastewater treatment methods are effective in dye removal but the overall cost, residual sludge generation, and high-energy requirements are their limitations [2,3,4,5,6,7]. The aerobic treatment requires double the energy consumed by anaerobic treatment [8].

Rhizoremediation utilizes the plants and its root-associated microorganisms for effective degradation of contaminants including dyes under laboratory conditions with synthetic wastewaters and model dyes [9,10,11]. A combination of anaerobic-aerobic reactors and rhizoremediation in constructed wetlands had treated tannery wastewater [12]. Oxygen supply in the rhizospheric niche is the major advantage [13] which acts as the primary electron acceptor [14]. Oxygen along with the root exudates that could act as nutrients and chemoattractants can enhance pollutant degradation [15].

The plant vetiver (Vetiveria zizanoides) is known for its phytoremediation abilities. A system of vetiver and associated microorganisms could effectively degrade model azo dye methyl red, and the aromatic amine intermediates formed, thereby reducing the toxicity of the treated water [11]. Several other studies have indicated the effectiveness of plant-microbe interactions in removal of dyes [16,17]. Vetiver has enormous fibrous root system that would provide microaerophilic regions in the rhizosphere [18]. Vetiver-microbe association has remediated many organic and inorganic pollutants, including dyes and heavy metals [19,20]. Cowdung was the source of inoculum for many remediation studies [21,22,23]. It includes members of Bacillus, Pseudomonas, Acinetobacter, Serratia, Staphylococcus, Flaviobacterium, Arthobacter, Enterobacter, making it suitable for treating industrial
wastewaters [24, 25]. Sequential anaerobic-aerobic reactions are used for treatment of dyes as the oxygen sensitive azo reductases are involved in the initial cleavage of the azo bonds leading to the formation of aromatic amines that are further degraded under aerobic conditions [11,26,27]. Such a system could degrade mixed pollutants in the wastewater through redox reactions in the rhizosphere by the microbiota, facilitated by the nutrient and aeration in the region as well as the adsorption of the compounds to the root surfaces [28, 29].

Preliminary laboratory scale study with methyl red, demonstrated decolourization of 92% and total aromatic amine removal efficiency of 89.97%. Pilot scale treatment was set up at the industry to evaluate the feasibility of the developed system to treat dyes in realtime wastewaters which contains a mixture of dyes, along with other contaminants. The setting up of the pilot plant and its evaluation is discussed.

2. Materials and Methods

2.1. Chemicals, Plants, and Microbes

HPLC grade Dichloromethane (DCM), p-dimethylaminobenzaldehyde (DMAB), citric acid, sodium hydroxide (NaOH), o-toluidine, and methanol were procured from HiMedia, Mumbai, India. All other chemicals used were analytical grade. Vetiver (Vetiveria zizanioides L.) slips were procured from the local nursery and grown in PVC pipes for 6 months to a root length of approximately 100 cm. Cowdung from a local farm was the source of microbial inoculum.

2.2. Description of the Study Site and Reactor Setup

The pilot scale study was performed on-site at a dyeing industry (M/s. Dinesh Process) at Tirupur, Tamil Nadu, India. The Industry collects its wastewater from various steps of the dyeing process and stores them in collection tanks before it is sent for treatment to a Common Effluent Treatment Plant (CETP) located in the area.
The pilot scale setup was installed adjacent to these collection tanks. The wastewater consisted of mixed dyes (mostly reactive dyes), salts such as sodium bicarbonate, hydrogen peroxide and acetic acid used for bleaching and dyeing the fabric. The temperature of the water coming to the collection tank is usually high and cannot be directly used in the experimental set-up. To avoid temperature shocks to the biological system, the wastewater from these collection tanks were diverted to a storage tank of 2 m³ capacity. In the storage tank the wastewater was diluted with treated water from the Common Effluent treatment Plant (CETP) (1:1) and all treatments were done with this water.

The treatments were done in triplicates. The treatment set-up consisted of anaerobic reactors (AN1, AN2 and AN3) each connected to the respective aerobic reactor (AE1, AE2 and AE3) through valved pipeline to control the flow. The anaerobic reactors (dissolved oxygen (DO) =0 mg/L) were completely sealed and equipped with agitators that were operated intermittently to mix the contents. The aerobic reactors contained the vetiver plants held on floats and were equipped with aerators. The DO levels in the aerobic reactors was 5-6.5 mg/L. All the reactors were 0.5 m³ capacity. A reservoir tank was used to collect the final treated water (Fig. 1). The process was operated in batch mode.

2.3. Treatment Strategy

The treatment strategy was based on the method of Desta et al., 2014. The experiments were done with pre-adapted microbial culture and vetiver plants. Microbial inoculum developed from cowdung slurry was acclimatized for a period of three months before use in the treatment. Cowdung was used as inoculum at 1% (w/v) in the anaerobic tanks and acclimatized.

The flow of wastewater was from storage tank to anaerobic reactor to the aerobic reactors. In the anaerobic reactors, 50% of the content (0.25 m³) was replaced by wastewater
from the storage tanks. The dye decolourization was regularly monitored and once it stabilized,
50% of the contents (0.25 m$^3$) were fed to the sequential aerobic reactors.

In the aerobic reactors the total aromatic amine (TAA) contents were continuously monitored. The samples were filtered and analyzed every hour using a colorimeter at 440 nm until the TAA content stabilized. Samples from both anaerobic and aerobic reactors were further collected for qualitative and quantitative analyses. All the experiments were done in triplicates project 1.mp4.

2.4. Decolorisation Assay

Decolorisation was monitored by a wavescan at 200-1100 nm using UV-Visible Spectrophotometer (UV-1601, Shimadzu, USA), as a mixture of dyes was expected in these wastewaters. The samples from anaerobic and aerobic treatments were filtered using a 0.22 µm syringe filter and absorbance was measured using colorimetry on-site at 660 nm (wavelength at which highest absorbance was observed in the wavescan). The percentage decolorization was calculated as below,

\[
\% \text{ Decolourization} = \left[ \frac{A_i - A_t}{A_i} \right] \times 100
\]

where $A_i$ is the initial absorbance and $A_t$ is the dye absorbance at sampling times.

2.5. TAA Determination

TAA was measured colorimetrically on-site [30]. Briefly, sample (0.04 mL) was mixed with de-ionized water (0.16mL), Hydrochloric acid (HCl) (0.01 mL, 1M) and ethanol (0.6 mL). To this mixture, 5% p-dimethylaminobenzaldehyde (DMAB) in ethanol (0.10 mL) was added, followed by 0.1 mL of 15.7% citric acid in 6% NaOH. The mixture was incubated at room temperature for 10 minutes and de-ionized water (0.5 mL) was added. The absorbance was measured at 440 nm. TAA was quantified using o-toluidine as standard.

2.6. Quantitative Analysis of Biotransformed Products
Dyes and their degradation products were quantified using HPLC and GCMS. The changes in the functional groups was monitored by FTIR. The presence of degradation products and micropollutants in the wastewater was analyzed using LC-MS.

### 2.6.1. Sample Preparation

The samples were prepared as per the method described in Sahasrabudhe et al., 2014 [31]. The samples were concentrated five times in a water bath at 99 °C and extracted thrice with an equal volume of DCM. The DCM extracts were pooled and evaporated. The final volume of extract (5 mL) was evaporated in a fume hood (25 ± 0.5°C). The extracted powder residues were dissolved in 10 mL of 100% methanol, filtered through a 0.2 μm syringe nylon filter. The filtered samples were used for further analysis.

### 2.6.2. FTIR Analysis

The samples (control, anaerobic treated, and aerobic treated, in triplicates) were analyzed for their dye decolorization and metabolites degradation, and the corresponding changes in functional groups using FTIR analyzer (Perkin Elmer-Spectrum Two, USA) in Attenuated Total Reflection (ATR) mode. The treated samples were compared with initial samples in the mid-IR region of 400-4000 cm\(^{-1}\) with 4 cm\(^{-1}\) scan resolution. The percentage of transmittance was plotted against wavelength using OriginPro8 (version 8.0).

### 2.6.3. HPLC Analysis

The dye and its degraded products were analyzed by HPLC system (Agilent 1120 Compact LC, Germany) with a Reverse Phase (RP) C18 column (4.6 X 250 mm, 5 micron). The sample volume was 20μl and analyzed using a UV detector at 274 nm. The mobile phase was 100% methanol run in isocratic flow with a flow rate of 1 mL/min for 20 minutes to obtain chromatograms. The degradation was analyzed by comparing the peak area values.

### 2.6.4. LC-MS Analysis
The compounds present in the various samples were analyzed in Thermo LC analyzer, RP-C<sub>18</sub> column (Thermo Fisher, model LTQ XL, USA). The analysis was performed using acetonitrile as a solvent in the gradient flow and formic acid as an additive. The flow rate of the samples was 260 µL/min. The detected Mass Range and Retention Time (RT) were 0-1000 m/z and 0-26 min respectively. The chromatogram was analyzed at 254 nm to identify the aromatic compounds present.

2.6.4.1. LC-MS Data Analysis

2.6.4.1.1. Determination of Dye Degradation

The peak area for dye molecules was calculated by subtracting the background noise from the peak signal. The slope and intercept calculated for the background noise peak intensity and the values obtained were used for peak area identification for all the untreated and treated samples. The reduction percentage (peak area) for the treated samples was calculated by

\[
\text{Peak area reduction percentage} = \frac{\text{Raw dye peak area} - \text{Treated sample peak area}}{\text{Raw dye peak area}} \times 100\% 
\]

2.6.4.1.2. Determination of Other Pollutants

For all the other pollutant determination, the .RAW data files were converted to mzxml files using the proteowizard MS convert tool [32]. The formatted files were imported and baseline-corrected using mzmine version 2.53, R 4.0.3, Windows 10 Operating System. For baseline correction, filtering module (Base peak Intensity, Peak Detection baseline corrector, 1 bin width, R Caller function) was used. The baseline-corrected chromatograms were assigned to the mass detector (wavelet transform, 5 noise, 5-scale level, 30% window size, 0-1400 scans, auto RT, any polarity, and any spectrum type). Using the developed masses, ADAP Chromatogram was built (minimum group size 5, group intensity threshold 50 and minimum highest intensity 100, m/z tolerance 0.01/ 0 ppm). Peaks in the chromatogram were manually picked based on the highest peak area and compounds identified. The compounds were identified using Feature list methods online database search (PubChem, 0.01 m/z tolerance).
To compare the untreated, anaerobic and aerobic treated compounds, alignment was done using Feature list method – Alignment – RANSAC aligner (0.01 m/z, 0.5 RT tolerance, and 0.5 RT after correction, 10,000 iterations, 100% minimum no of points, and 0.5 Threshold value). The data (3D view, Scatter plot, and Peak Intensity plot) were analyzed in the Visualization module.

2.7. GC-MS Analysis

GC–MS analysis of control and treated samples was performed on a GC-MS (JEOL GC MATE II GC-MS, USA), equipped with an integrated gas chromatograph with a HP5 MS column. Helium was used as the carrier gas at a flow rate of 1 mL/min. The injector temperature was 220°C, with an increase in oven temperature up to 250 °C at the rate of 10°C / min. The compounds were identified by a quadruple double-focusing mass analyzer and photon multiplier tube detector. The mass spectra were analyzed using the National Institute of Structure and Technology (NIST) library.

2.8. Study of Root Surface Characteristics

2.8.1. Scanning Electron Microscopic (SEM) analysis of root surface

The changes in the morphological structure of the roots of vetiver used in the treatment before and after the treatment were analyzed using SEM. The control and treated roots were cut into 1 cm length and lyophilized at – 60°C (0.01 Torr) [33]. The dried roots were mounted onto aluminium stubs, sputter-coated with gold, and examined with SEM (Zeiss Supra 55VP Field Emission Gun Scanning Electron Microscope (FEGSEM, Oberkochen, Germany) at 3 kV, 10K X magnification.

2.8.2. Atomic Force Microscopy (AFM) analysis of root surface

The roots were cut to 1 cm in length, dried at 40 °C, and analyzed for root surface characteristics using AFM (Asylum MFP3D, Oxford Instruments, Oxford, UK) [34]. The images were acquired at air tapping mode and processed using Gwyddion Software (version 2.53).
2.9. Teratogenicity Analysis

Teratogenicity was assessed for the wastewater and its subsequent treated waters using zebrafish (*Danio rerio*) embryos, as described earlier [11]. The embryos were incubated in control (fish water) and the sample waters, and monitored during development up to 72 hours post fertilization (hpf). Developmental morphology was recorded at 24, 48 and 72 hpf using an inverted microscope. Survival and malformation in the developing embryos were noted and embryotoxicity and malformation percentages were calculated.

2.10. Statistical Analysis

All experiments and analysis were carried out in triplicates. Data were analyzed statistically by one-way ANOVA using Microsoft Excel.

3. Results and Discussion

An integrated plant-microbe treatment system’s efficiency in completely degrading the dyes in real-time textile industry wastewater was evaluated with a focus on the efficiency of dye decolorization and removal of dye degradation by-products, namely aromatic amines. Besides, the fate of various kinds of pollutants in the mixed dye-containing real-time wastewater was also analyzed.

3.1. Decolorisation of Real-Time Textile Dyes

Dye decolorization in the storage tank (without any treatment), after anaerobic treatment and aerobic treatments were analysed. The wastewater in the storage tank showed a decolourization of $1.8 \pm 0.01 \%$, indicating the retention of dyes in the wastewater not subjected to treatment. Anaerobic treatment was performed till the dye decolourization stabilized. The maximum decolourization observed at the end of anaerobic treatment was $68.8 \pm 0.0 \%$ at the end of fourth hour of treatment. Intermittent mixing using agitators in the anaerobic reactors facilitated microbial contact with dye containing water that enhanced decolourization. Under anaerobic conditions, dye molecules act as electron acceptors, and –
N=N– azo bonds break to form the intermediate aromatic amine compounds [35, 36]. The azo bond reduction was also evident in GC-MS data (Table 1). In the subsequent aerobic reactors, decolorization of up to 78.8± 0.01% was achieved (Fig. 2a). A small increase in dye decolorization observed in the aerobic reactor might be due to the transfer of facultative microbes from the anaerobic to the aerobic reactor that could have continued the process. When natural anaerobic sludges were used, methanogens capable of withstanding high levels of oxygen and facultative bacteria when transferred to the aerobic condition [37] continued the decolorization; this could also be due to the presence of oxygen insensitive azoreductases [38,39,40]. Recalcitrant dyes could adsorb onto the plant root surfaces [11]. Adsorption occurs when molecules are highly hydrophobic. Plant roots’ have the ability to adsorb compounds with a K_{ow} (n-Octanol/Water Partition Coefficient) of greater than 2 [41]. Hence, both adsorption and microbial decolorization reduce dye content [42]. Aromatic substitutions in the dye molecules influence their biodegradability [38]. The industrial wastewater is not homogenous throughout the period of study and moreover, contained mixture of dyes. Some of them might not be completely degraded under either anaerobic or aerobic conditions. A 100% decolourization was not obtained in this study even after extended aerobic treatment (data not shown). One way ANOVA test showed the significant difference between the control and treated samples (p<0.001 and F>F_{critic}) for dye decolorization.

3.2. Removal of TAA

Aromatic amines are formed upon the breaking of the azo bond under anaerobic conditions. The treated wastewater from anaerobic and aerobic reactors were analyzed hourly on-site for TAA. The maximum TAA value was obtained at the third hour of anaerobic treatment.(Fig. 2b). The formation of aromatic amines from azo compounds under anaerobic conditions has been reported earlier [11,43]. Their subsequent degradation under aerobic conditions was observed with maximum removal in 24 h. (Fig. 2b). The effective removal of
these aromatic amines could be the combined effort of rhizosperic microorganisms that were supported and enriched by the release of root exudates and oxygen from the roots of the plants. Enhanced pollutant degradation in the rhizosphere is due to the increased number of microbes and their metabolic activity [44,45,46,47]. Aromatic amines are acted upon by oxygenases and the substitutions on them impact the rate of these reactions [48]. The sequential treatment demonstrated a significant difference between the control and treated sample (p<0.05 and F>Fcritical) for TAA degradation.

Decolorization of 68.8% (HRT – 4h) under anaerobic conditions was achieved compared to pilot scale Upflow Anaerobic Sludge Blanket (UASB) reactor (15h) and pilot-scale anaerobic reactor (90 h). Besides, the sequential aerobic treatment achieved a higher TAA removal percentage (69.2%) in 24 hours when analyzed quantitatively compared to TAA removal of 52.2% in 2.9 days reported earlier [49] and other studies [50,51].

3.3. Analysis of Biotransformed Products

The complex mixture of compounds in the realtime textile industry wastewater are degraded by anaerobic bacteria subsequently these intermediates are further degraded under aerobic condition in the reactors with vetiver plant and associated microbes.

FTIR spectrum of textile dye wastewater (S.Fig.1) showed the presence of peaks at 3401, 3195, 2878, 2841, 1659 and1401 cm⁻¹. The peaks at 3401 and 3195 cm⁻¹ indicate N-H stretching due to the presence of amines, 2878 cm⁻¹ and 2841 cm⁻¹ indicate alkane’s C-H stretching, 1659 cm⁻¹ for aromatic C=C stretching, 1401 cm⁻¹ indicates N=N (azo bond) stretching. The treated samples showed the disappearance of the azo bond at 1401 cm⁻¹ after the anaerobic treatment and reduction in N-H amine stretch at 3401 and 3195 cm⁻¹ after the aerobic treatment. This suggests the breakdown of azo bond containing dye molecules in real-time textile wastewater. A similar degradation pattern was observed when model dye methyl red and its metabolites degradation was analyzed by FTIR after the anaerobic-aerobic treatment.
Aromatic -C=C- reduction was evident in anaerobically treated samples, indicating that double bond containing aromatic compounds reduced after the 4h anaerobic treatment.

HPLC analysis with UV detector showed a major parent peak at 5.6 RT and 7.8 RT, when textile effluent was analyzed before treatment (S.Fig.2a-b). The initial peaks (at 5.6 RT and 7.8 RT) in the dye wastewater indicate the dyes and chemicals present. The reduction in peak area (70.9% for 5.6 RT and 61.3% for 7.8 RT after anaerobic treatment and 98.1% for 5.6 RT and 61.2% for 7.8 RT after aerobic treatment) correlates with degradation of compounds in the wastewater.

The parent peak reduction at 5.6 RT after anaerobic treatment was 70.9% and after sequential aerobic treatment was 98.1%. The peak area values were used for calculating percentage reduction and concentration [52]. The compound at 5.6 RT was susceptible to anaerobic conditions and breakdown occurs. In the anaerobic environment, denitrification, sulfate reduction, and organic molecules reduction (dye decolorization) occur [53,54]. Acclimatized microorganisms can degrade various kinds of dyes using them as carbon and electron sources [55] and adsorption and microbial activity in the rhizosphere [56] contributed to further decrease in the peak area at 5.6 RT. The adsorption onto the roots is correlated with the octanol-water partition coefficient (Kow) and compounds that are hydrophilic or hydrophobic character could not pass through the roots and are adsorbed [57].

The peak reduction was 61.3% for 7.8 RT after anaerobic treatment and the same after aerobic treatment, indicating the product was not amicable to aerobic degradation and neither was it adsorbed on to plant roots. The fate of the various compounds was further analyzed in detail using the LC-MS data.

Apart from the degradation of initial peaks, biotransformation occurred as evidenced from the appearance of new peaks at 5.1 RT and 19.7 RT during the treatment process of anaerobic and initial hours of aerobic treatment (S.Fig. 2c-e). Few dyes such as sulfonated
dyes could degrade optimally under aerobic conditions. For instance, *Acinetobacter baumanii YNWH 226* could break down dyes, intermediate aromatic amines formed, and their further cleavage, occurred only under aerobic conditions [58,59]. This correlates with increased intermediate peak area under the aerobic condition in this study. However, the intermediates formed at 5.1 RT and 19.7 RT were completely removed after the 24th hour of aerobic treatment, under optimum conditions, indicating their degradation under aerobic conditions. The use of adapted microbial cultures in the treatments enhanced the processes and reduced the HRT [60,61,62, 63].

Formation and further degradation of intermediates during the sequential treatment process were observed (S.Fig. 2f-g). One way ANOVA test showed p<0.001 and F>Fcritic for dye decolorization peak and hence the data is statistically significant. The intermediates formed were completely removed within 24 hours of aerobic treatment. Hence, the total hydraulic retention time for peak reduction and intermediate degradation was computed as 28 hours (4 h for anaerobic process and 24 h for aerobic process).

GC-MS analysis indicate presence of azo compounds in the samples before treatment confirmed the characteristics of textile dye and it is the treatment control (Fig. 4;Table 1). An intact -N=N- bond remained in the dye-containing effluent before the treatment process. These double bonds were broken in the anaerobic treatment and led to the formation of aromatic amine intermediates such as Benzeneamine/Aniline derivatives listed. Anaerobic azoreductases aid in reductive cleavage. The intermediates such as Benzenamine, 2,4,5-trimethyl-; Benzenamine, 4-methoxy-2-nitro formed during this process were reported to cause acute toxicity. 2-toluidine (2-Methyl-1-aminobenzene with m/z 107) formed during the treatment process was reported to be toxic and is one of the major textile dye intermediates [64].
The aromatic amine intermediates were further converted to aliphatic compounds during the aerobic process (Table 1). As the process is accompanied by aeration and oxygen supply from the rhizosphere, the aromatic amines were oxidized and aliphatic compounds formed via ring fission reaction. Oxidative enzymes aid in dye degradation by catalyzing aromatic ring cleavage [65]. Hence, the sequential anaerobic-aerobic plant-microbe integrated treatment was efficient in the complete degradation of real-time textile dye compounds (S.Fig 3a-l).

### 3.4. Dye Degradation and Fate of Other Pollutants

To understand the nature of degradation products formed during the sequential anaerobic-aerobic treatment, LC-MS analysis was performed with samples from experiments performed with real-time wastewaters under similar conditions as in pilot-scale study but with a sample size of 4 L. The degradation products formed during the treatment process were identified using freeware mzmine v2.5.3 [66].

#### 3.4.1. Dye Degradation Analysis Using LC-MS

The real-time effluent contained many different azo and anthraquinone dyes and their percentage degradation was calculated from the peak area values. Many dyes such as Acid blue 175 (786.86 m.wt and 10.4 RT), Carbanthrene Red G 2B (vat dye with 494.5 m.wt and 10.53 RT), and C.I.Direct Brown 52 trisodium salt (triazo dye with 876.76 m.wt and 10.65 RT) were found before the treatment. The mass spectrum and structure for each dye are shown in S.Fig. 4. These dyes were completely removed (100%) at the end of anaerobic treatment [(S.Fig. 5), Peak area plots (S.Fig. 6), and in Peak Intensity plot (S.Fig. 7 (a-c))]. A similar study with acid blue 204, acid yellow 79, and acid red 131 demonstrated their degradation under anaerobic condition with mixed sludge as inoculum [67].

Some dyes like Orange G free acid and Cibacron Brilliant Red 3B-A were still seen post anaerobic treatment, which could be due to unutilized dye within the HRT of 4h. Orange
G-free acid is recalcitrant at anaerobic conditions due to carbon-sulfur double bond [68], its hydrophilic nature (log P -1.09) [69], and the presence of SO₃H group. Hence, the Orange G-free acid dye molecule degradation could be only due to the microbes present in the aerobic reactor [11]. Among all the dyes analyzed, only Fast Blue B salt dye showed lesser dye decolorization (47.3%) after the treatment (S.Table 1). The lesser percentage of dye decolorization could be due to the presence of halogen group (chlorinated substituents) in the dye molecule [70].

The dye degradation was 85.61% after sequential anaerobic-aerobic treatment (S. Table. 2). The amines generated during the anaerobic process were not observed after the sequential aerobic (T7, T8, and T9) treatment (S.Fig. 8).

### 3.4.2. Fate of Other Pollutants

The effect of treatment on molecules other than dyes and their byproducts was observed. The aromatic compounds present before treatment and after anaerobic treatment were mostly halogenated (S.Table.3&4). Halogenated aromatic compounds are known to be recalcitrant. The halogenated aromatics in textile dye effluent also act as a precursor in dye manufacturing [71, 72]. The halogenated aromatic compounds are toxic and are major environmental pollutants of concern [73]. The chlorinated aromatic compounds are resistant to degradation due to their highly stable C-Cl bonding in the molecule [74]. The presence of halogenated compounds and toxic aromatic amines, render the effluent more toxic.

In the present study, few halogenated compounds were not degraded under anaerobic conditions and new halogenated compounds were formed after anaerobic degradation. Those halogenated compounds were degraded only under the sequential aerobic condition (S.Table. 5). This could be due to the action of microbes that utilize chlorinated compounds in the dehalorespiration process under anaerobic conditions and by the dechlorination process under aerobic process [75,76]. Dioxygenases are implicated in these degradations that finally result
in various metabolites of the tricarboxylic cycle (TCA) like acetyl CoA, pyruvate, succinate, oxaloacetate, and acetaldehyde [72].

Textile effluents contain a wide variety of compounds both organic and inorganic many of which are known toxic compounds (S.Table. 3), and these mixed pollutants impact the biological degradation and cannot be achieved by a single process. The additives used in the dyeing process such as sodium sulfate, sodium carbonate and acetic acid influence the pH of the effluent. The sodium carbonate contributes to the alkalinity of the released textile effluent and influences the growth of the microbial community and thus impact treatment [77].

The use of integrated plant and microbial system in the degradation of multi-pollutants are reported [78]. The end products of this sequential anaerobic-aerobic treatment were found to be non-toxic, as most of them are endowed possible therapeutic properties. The compounds are non-toxic nature while some are known to have medicinal property (S.Fig. 9 & S.Fig. 10)

After treatment all aromatic compounds were reduced, and aliphatic compounds and microbial metabolites showed an increase (S.Table. 6; S.Fig. 11 (a-g) & S.Table 6). Major decrease in peaks was observed during the sequential aerobic treatment (seen as peaks in the chromatogram ((S.Fig. 12 a-i), Histogram data (S.Fig. 13 a-c), and 3D peaks(S.Fig. 14 a-c)). This indicates the importance of the aerobic stage of treatment as well as the presence of plants. which results in treated water that contained aliphatic or non-toxic aromatic compound.

3.5. Root analysis for dye and microbial attachment

To assess plant-microbe association during the treatment process, the treated and untreated roots were analyzed for the attachment of dyes/microbes using SEM and AFM techniques.

3.5.1. SEM analysis

The control and treated roots were analyzed for morphological changes before (Fig. 3a) and after treatment. The bacterial attachment onto vetiver roots was seen in colonies after the
treatment (Fig. 3b). Plant roots acted as a matrix for bacterial attachment. The aerobes are associated with roots on rhizoplane and in the rhizosphere due to the release of oxygen from the rhizospheric root region. Rhizodegradation of aromatic compounds was previously reported [11,84].

3.5.2. AFM analysis

The roughness values depend on the adsorbent and, in general, bacterial attachment increases the roughness [11]. Ra and Rq are measures of surface roughness in AFM. Ra is the arithmetic mean of absolute values of surface profile to the height; while Rq is the root mean square average of the distribution of surface height. It is the statistical quantity calculated by the standard deviation of surface heights to minimize the outliers. Ra and Rq are calculated using the formulae 2 and 3 respectively, as below

\[ R_a = \left[ \frac{1}{L} \int_{0}^{L} |z(x)| \, dx \right] \]

\[ R_q = \left[ \left( \frac{1}{L} \right) \int_{0}^{L} |z(x)|^2 \, dx \right]^{\frac{1}{2}} \]

The greater the Ra, the greater is the bacterial attachment. Similarly, the greater the Rq values, the greater the surface roughness [85].

The control bare vetiver root has roughness parameters (Ra=76.3 & Rq=97.7) shown in Fig. 5c. For the roots taken from the treatment reactor, the surface roughness parameters Ra and Rq significantly increased (Ra=117.3 & Rq=142.2). The increased roughness is due to the bacterial attachment onto the vetiver roots (Fig 5d).

3.6. Analysis of Teratogenicity

Zebrafish (Danio rerio) have 87% concurrence with human disease genes and it has toxicity levels similar to rodents [80]. Hence, it is used as a model for teratogenic studies. Fish water from the breeding tank (positive control), untreated dye wastewater (negative control), and treated samples from the sequential anaerobic-aerobic reactor were analyzed. The
development of zebrafish embryos was monitored from the gastrula (24 hpf) to the hatching stage (72 hpf).

In positive control, embryos survived and have shown their proper developmental stages at the 24th, 48th and 72nd hour of post-fertilization (hpf). In the negative control, most of the embryos coagulated and severe malformation was observed due to toxicity. Anaerobically treated water had shown a better survival rate than untreated wastewater but severe malformations such as lack of eye and ear formation, a kink in the tail, retarded growth during their developmental stages were observed. However, the aerobically treated water have shown a better survival percentage and lesser malformation ratio (>65% survival and <10% malformation percentage in all stages of development) than the control untreated wastewater (40% survival and 50% malformation) and anaerobic treated water (>45% survival and >25% malformation percentage in all developmental stages) (Fig. 4 & 5; S.Table 7). Teratogenic effects decreased tremendously after sequential aerobic treatment at 72 hpf. The endpoints were compared with the observations of previous researchers who have tested the teratogens ethanol [81], silica nanoparticles [82], and PAH [83]. The survival percentage of zebrafish embryos and their malformation for treated water showed a significant difference after treatment (p<0.05, F>Fcritic). The treated water has proven to be less toxic on zebrafish embryos and hence the treated water might be reused.

4. Conclusion

The efficacy of a plant-microbe integrated system operated in a sequential anaerobic-aerobic mode to treat a large volume of real-time textile industry wastewater was evaluated at a pilot scale. The pre-adapted microbial culture and plant-microbe system efficiently degraded dyes and their intermediates within 1.2 days of HRT. The degradation of dye molecules and the formation of aliphatic compounds were also confirmed in GC-MS and LC-MS analyses. Vetiver-microbe association during dye degradation was evident from SEM and AFM images.
The better survival and decreased malformation of the embryos in the treated water proved the reduced toxicity of the treated water. The use of plants that release oxygen in the rhizosphere would reduce the aeration cost as well as providing the microorganisms with nutrients through root exudates. Thus, the treatment system developed with an attempt to achieve cost-effective, and sustainable rhizoremediation technology to treat real-time textile effluent and to overcome sludge production was found to be efficient. The microbial population would be an evolving population in real-time scenario. Further studies are required to develop a functional signature(s) for effective treatment of different industrial wastewaters by performing metagenomics analyses.

5. ACKNOWLEDGEMENTS

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References:


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30
doi: 10.1098/rstb.2012.0377


Tables:
Table 1: GC-MS data of textile dye effluent (a) before treatment, (b) anaerobic and (c) subsequent aerobic treatment during HRT determination

<table>
<thead>
<tr>
<th>Sample(s)</th>
<th>m/z</th>
<th>RT (min)</th>
<th>Dye and its metabolites</th>
<th>Type of compounds present</th>
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<tr>
<td>168</td>
<td>17.12</td>
<td>Benzenamine, 4-methoxy-2-nitro</td>
<td>Aromatic amines</td>
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<td>198</td>
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<td>Azoxybenzene</td>
<td>Azo compound</td>
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<td>212</td>
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Figures:

Fig 1: Schematic diagram of the pilot-scale sequential anaerobic-aerobic treatment setup
Fig 2: (a) Decolorisation of real time textile effluent and (b) Estimation of Total aromatic amines after sequential anaerobic and aerobic treatment
Fig 3. SEM micrographs and AFM images of roots taken before and after the dye degradation experiment. SEM image of (a) bare vetiver root surface (b) root surface after use in the integrated plant and microbe system. AFM image depicts c) root incubated in water - control (Ra = 76.29 and RMS = 97.73 nm) (d) dye and bacterial attachment onto root surface (Ra = 117.34 nm and RMS = 142.20 nm)
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Fig 4. Embryonic development of zebrafish embryos incubated in Positive Control (Fishtank water), Negative Control (Untreated Wastewater, anaerobic (AN1, AN2, AN3) and aerobic (A1, A2, A3) treated wastewater at 72 hpf.

Fig 5. Zebrafish Embryotoxicity and Malformation analysis at 24 hpf, 48 hpf and 72 hpf.
Author contributions:

**Mohanapriya Jayapal**: Investigation, Formal analysis, Data Curation, Methodology, Software, Writing – Original Draft, review and editing.

**Hema Jagadeesan**: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – Review and editing.

**Vinothkumar Krishnasamy**: Investigation.

**Gomathi Shanmugam**: Investigation.

**Vignesh Muniyappan**: Investigation.

**Dinesh Chidambaram**: Industry collaborator.

**Satheesh Krishnamurthy** – Project Collaborator, Project discussion
Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

One of the authors is the Managing Director of the industry where the study was carried out and is actively trying to identify processes to treat the dyeing industry wastewaters.

The other authors do not have any competing interests.