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Phytobeds with *Fimbristylis dichotoma* and *Ammannia baccifera* for treatment of real textile effluent: An *in situ* treatment, anatomical studies and toxicity evaluation

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ABSTRACT

Fimbristylis dichotoma, Ammannia baccifera and their co-plantation consortium FA independently degraded Methyl Orange, simulated dye mixture and real textile effluent. Wild plants of *F. dichotoma* and *A. baccifera* with equal biomass showed 91% and 89% decolorization of Methyl Orange within 60 h at a concentration of 50 ppm, while 95% dye removal was achieved by consortium FA within 48 h. Floating phyto-beds with co-plantation (*F. dichotoma* and *A. baccifera*) for the treatment of real textile effluent in a constructed wetland was observed to be more efficient and achieved 79%, 72%, 77%, 66% and 56% reductions in ADMI color value, COD, BOD, TDS and TSS of textile effluent, respectively. HPTLC, GC-MS, FTIR, UV–vis spectroscopy and activated oxido-reductive enzyme activities confirmed the phytotrasformation of parent dye in to new metabolites. T-RFLP analysis of rhizospheric bacteria of *F. dichotoma*, *A. baccifera* and consortium FA revealed the presence of 88, 98 and 223 genera which could have been involved in dye removal. Toxicity evaluation of products formed after phyto-transformation of Methyl Orange by consortium FA on bivalves *Lamellidens marginalis* revealed less damage of the gills architecture when analyzed histologically. Toxicity measurement by Random Amplification of Polymorphic DNA (RAPD) technique revealed bivalve DNA banding pattern in treated Methyl Orange sample suggesting less toxic nature of phytotransformed dye products.

1. Introduction

Textile industries contribute a major share to the economies of developing countries. On the other hand, dye manufacturing and processing firms of small and large scales are condemned as one of the foulest polluters of water and soil. Around 10–15% of the synthetic textile dyes having carcinogenic and other toxic effects are released during the dyeing and finishing of clothes, ultimately causing threat to all life forms (Khataee et al., 2010). Many existing chemical, physical and biological methods are available for the treatment of textile effluents. However, magnitude of pollution, secondary waste generation, leachates, cost and other technical difficulties while managing *in situ* treatments are key problems of dye treatment process. In the last decade, use of plants has appeared as a promising green and clean tool for the treatment of textile dyes (Khandare and Govindwar, 2015).

Phytoremediation, use of potential plants for environmental cleanup, is rising as a true green technology now a days (Dietz and Schnoor, 2001). Plants and their rhizospheric microbes can efficiently remove pollutants via rhizodegradation, biostimulation, biostabilization, bioaccumulation, phytoextraction and phytovolatization (Pilon-Smits, 2005). *In situ* phytoremediation is highly rational for public authorization because of being easy to run, economical, require low nutrient input and aesthetically acceptable although is still in experimental stages and needs a lot of attention (Khandare et al., 2011). Many a times, phyto-technology has been found to be less than half the price of alternative physicochemical and biological methods. Heavy metal

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removal using plants is the most successful, engineered and accepted approach when phytoremediation technology is concerned. Phytoremediation of textile dyes however has remained an unattended area of research (Khandare and Govindwar, 2015).

Although, many plants studies have reported dye removal with plants, most of them have remained lingered at laboratory scales. Pilot scale demonstrations of treatment of textile wastewater have revealed the potential of this technology. Hydroponic phyto-tunnel system has been utilized for the treatment of textile effluent (Khandare et al., 2013a, 2013b). Lab scale horizontal and vertical subsurface flow bioreactors based on plant bacterial synergistic approach were developed to treat real textile effluent (Kabra et al., 2013; Khandare et al., 2013a, 2013b). Some pilot scale operational systems using macrophytes are on record. For instance, *Phragmites australis, Typha domingensis, Alternanthera philoxeroides* were proposed in independent constructed wetlands studies on removal of textile dyes from wastewater (Ong et al., 2011; Shehzadi et al., 2014; Rane et al., 2015). Large scale treatment of textile dye effluents with combinatorial system of plants has occasionally been reported. A 200 L volume of waste water from tie and dye industry was shown to be treated with the use of cattail and cocoyam plants in independent engineered wetland systems (Mbuligwe, 2005). Co-plantation of garden ornamentals *Aster amellus-Glandularia pulchella* and *Gaillardia grandiflora–Petunia grandiflora* were explored for the efficient treatment of dye mixtures and effluents (Kabra et al., 2011; Watharkar and Jadhav, 2014). Ornamental plants because of their habitat and forms however are not suitable for the treatment of large amounts of wastewater. Water floating plants like *I. aquatica* and *S. molesta* were used to treat industrial effluent (Rane et al., 2016; Chandanshive et al., 2016). However, when complete plants are exposed to effluents, root and shoot lengths, and ultimately growth of the plants is affected. In addition, the effluent tolerance capacity and survival is also challenged. These plants are weeds and therefore their overgrowth needs to be frequently monitored. Roots of plant play a vital role in treatment of textile dyes (Khandare et al., 2014; Watharkar et al., 2015). Non-transformed adventitious roots of *I. hederifolia* have shown a potential of textile dye remediation (Patil et al., 2016a, 2016b).

In the present study, *F. dichotoma* L., *A. baccifera* L. and their co-plantation system consortium FA were tested for the treatment of Methyl Orange as the model dye, a simulated dye mixture and real textile effluent. It was however challenging to treat large amounts of effluents using a limited number of rooted plants at the edges of wetlands further. To overcome this problem, floating phyto-beds were designed and explored so that a greater surface area on the CW could be covered in such a way that plants can freely float on textile effluent. To achieve this, the use of plants from actual site of dye contamination with a potential to survive at marshy places can ideally be explored for development of floating-beds. *F. dichotoma* and *A. baccifera* were selected from actual site of contamination because of their habit and implemented for floating phyto-beds. Both the plants used for this study are annual herbs, non-edible, have massive root systems and occur naturally in consortia hence hypothesized to possess noteworthy dye removal potential. *Ammannia baccifera* L. is classified as: Kingdom – plantae, Division – Magnoliophyta, Class – Magnoliopsida, Order – Myrtales, Family – Lythraceae, Genus – *Ammannia* L., Species – *baccifera* L. and *Fimbristylis dichotoma* L. as: Kingdom – plantae, Division – Magnoliophyta, Class - Liliopsida, Order – Cyperales, Family – Cyperaceae, Genus – *Fimbristylis* L., Species – *dichotoma* L. For *in-situ* application, floating phyto-beds were developed at the common effluent treatment plant, MIDC, Kagal, India for abatement of textile effluents.

2. Materials and methods

2.1. Collection of plant material, construction and implementation of floating phyto-beds in constructed tanks for textile wastewater treatment

A. baccifera and *F. dichotoma* plants with 45–55 cm in height, 180–200 g in weight were collected from the contaminant site at Maharashtra Industrial Development Corporation, Kagal, India. Both plants were further explored individually and in consortium experiments in constructed floating phyto-bed (FPB) systems for the treatment of textile industrial wastewater.

Treatment of textile effluent by conventional methods fails to get rid of color and high Total dissolved solids (TDS) from the effluent. The discharge of high TDS effluent onto land results in increased soil salinity and elevated TDS of ground water as well as surface water. Therefore, this work was targeted to advance earlier treatment strategy and to meet the standard treatment limitations. Phytoremediation of real textile effluent was done in cement tank with dimensions of 2.7 × 1.5 × 1.2 m (total volume 4.86 m^3, 4860 L) using floating phyto-beds. These floating phyto-beds were built using PVC pipes, aluminum metal wire gauze and the PVC plastic sheet having length 2.1 × 1.2 m. The plastic pipes were cut and joined with a plastic elbows to get the rectangular shape of 2.1 × 1.2 m body. Aluminum wire gauze was put over the body to support the plants as well as to arrest small gravel and soil present in the plastic reducers to mix in the effluent. Additionally, it easily allowed the roots to grow and pass through the mesh and get exposed to the effluent to be treated. Thirty two holes were made on the plastic sheet at every 25 cm distance. This sheet was then fixed over the aluminum gauze. A plastic reducer was placed in each hole. Each reducer was then filled with 300 g of soil for plant support. This bed was allowed to float in the effluent tank. The total weight of FPB was 7 kg and it was observed to manage to float even if the load reached 35 kg. Sixty-four plants of each selected species of *F. dichotoma* and *A. baccifera* were independent planted on FPB and 32 plants of each of *F. dichotoma* and *A. baccifera* were planted on a separate FPB (as a consortia system). They were planted in such a way that the roots could easily pass through the reducers in a downward direction towards the effluent through the soil layer (Fig. 1). Initially, these three floating phyto-beds were made to float in tap water for 2 months for root development and then exposed to treatment tanks. The treatment parameters like ADMI, COD, BOD, TDS, TSS and pH were checked after every 24 h of time interval during the treatment (APHA, 1998).

2.2. Rhizospheric soil bacterial community analysis of respective plant systems during textile effluent treatment using Terminal Restriction Length Polymorphism (T-RFLP)

The T-RFLP analysis was carried out to find the microbial community associated with rhizosphere of three different plant systems (*F. dichotoma* L., *A. baccifera* L. and consortium FA on the phyto-beds) during phytoremediation. The soil associated with roots of different plant system was collected. The whole genomic DNA of soil bacteria was isolated using bead-beating procedure (Angel, 2012). The 16s r-RNA gene was amplified by using 6 FAM (6 carboxyl fluorescein) florescent labeled forward primer F27 (5′-AGAGTTTGATCMTGGCTCMG-3′) and reverse primer R1492 (5′-TACGGYTACCTTGTTACGACT-3′) (Lane et al., 1985). The purified PCR products of a 16s rRNA gene (200–300 ng) were digested at 37 °C for 9 h with 2 U of restriction enzyme AluI (AGCT) and MspI (CCGG) separately. These digested fragments were then subjected to capillary electrophoresis with Liz 1200 (DNA size standard) in DNA sequencer (Applied Biosystem 3500). Resulted fragment size was calculated and analyzed by software GeneMapper 5. Further analysis was carried out using t-align, in which binary qualitative data matrix was constructed. The constructed binary matrix was imported into NTSYS-PC program version 2.1 (Rohlf, 1998). Jaccard similarity matrix was constructed using Jaccard coefficient. A

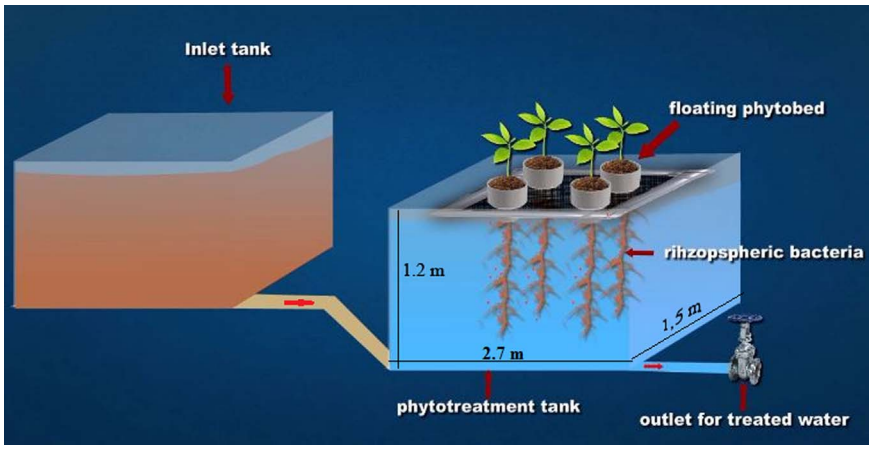

Fig. 1. Schematic representation of floating phyto-bed system.

dendrogram was constructed based on Jaccard similarity matrix using unweighted pair-group method (UPGMA) (Sneath and Sokal, 1973). Then final identification of microbial community was performed using the web-based Microbial Community Analysis III: T-RFLP Analysis interface (MiCA III - PAT$^+$) (http://mica.ibest.uidaho.edu/pat.php) (Shyu et al., 2007). PAT (phylogenetic assignment tool) uses the default database produced by MiCA online tool (Kent et al., 2003). Further Venn diagram of each set i.e. rhizospheric soil microbial count of individual plants and consortia were drawn to analyze the community survived and probably involved in dye metabolism, and transformation processes.

2.3. Initial decolorization experiments

Methyl Orange was selected as the model dye for initial experiments as it is commonly used by the local dye processors. This experiment was performed to find out the Methyl Orange degradation efficiency of selected plant systems (*F. dichotoma, A. baccifera* and their consortium). The decolorization experiments were carried out using 1000 mL glass beakers taking 400 mL Methyl Orange solution at a concentration of 50 ppm. Healthy plants having dense roots were used for decolorization experiment. Two plants of *F. dichotoma* and *A. baccifera* were independently exposed to the dye solution and single plant of the both the species were taken in another beaker with dye solution as a consortium. Abiotic control devoid of any plants and respective biotic controls with plants and dye solution were also kept throughout the experiment. One milliliter of the dye solution from each set was independently removed and centrifuged at 4561*g* for 10 min to remove any residual particles (Khandare et al., 2012). Then the absorbance of the clear solution was measured at 470 nm and percentage of decolorization was calculated using Eq. (1). All these experiments were carried out in triplicates.

$$\%\text{Decolorization} = [A_0 - A_t / A_0] \times 100 \quad (1)$$

Where, A_0 – Initial absorbance, A_t – Final absorbance

Further, a simulated dye mixture was prepared by taking Methyl Orange, Remazol Red, Blue GLL, Congo Red and Green HE 4BD to attain a final concentration 50 ppm for decolorization trials. The decolorization was monitored using simulated dye mixture and a real textile effluent. The textile effluent and dye mixture contains number of different dyes hence they do not have any particular color therefore transmittance of decolorization experiment was measured by ADMI (American dyes manufactures institutes) values. Percent ADMI color removal of dye mixture and real effluent was calculated using the Eq. (2).

$$\%\ \text{ADMI removal} = [T_0 - T_t / T_0] \times 100 \quad (2)$$

Where, T_0 – Initial ADMI removal, T_t – Final ADMI removal

2.4. Anatomical investigations for the qualitative analysis of accumulation and phytotransformation of Methyl Orange

Anatomy of root cells of *A. baccifera* and *F. dichotoma* was studied to check the dye accumulation and metabolism of Methyl Orange. Transverse sections of roots were mounted in glycerin overlaying with cover slip. The results were micro-photographed with Axio-Scope A1 Trinocular phase contrast Microscope with an attached camera at 40 X magnification.

2.5. Preparation of cell free extracts and enzyme assays after dye removal

Roots of individual plants *F. dichotoma A. baccifera* and consortium FA were collected from control as well as treatment sets of decolorization experiments. Two grams of roots were independently weighed, finely chopped and then suspended in 2 mL of 50 mM potassium phosphate buffer (pH 7.4). Suspended roots were then ground in a mortar pestle and subjected to homogenization in glass homogenizer. Homogenate was centrifuged at 8481 × *g* for 20 min. The cell free extract thus obtained was used as a source of extracellular enzyme (Rane et al., 2015).

Activities of the enzymes lignin peroxidase (LiP), laccase, tyrosinase, riboflavin reductase, azo reductase and DCIP reductase were determined spectrophotometrically at room temperature in the case of control and test for both the plant and consortia FA. Peroxidase activity was calculated by the previously standardized method. Propanaldehyde production was monitored at 300 nm in a reaction mixture of 2.5 mL containing 100 mM n-propanol, 250 mM tartaric acid and 10 mM H_2O_2 (Kalme et al., 2007). Tyrosinase activity was calculated in a reaction mixture of 2 mL, containing in 0.1 M phosphate buffer (pH 7.4) with 0.01% catechol at 495 nm (Zhang and Flurkey, 1997). NADH-DCIP reductase activity was measured as per an earlier report (Salokhe and Govindwar, 1999). Laccase activity was calculated in a reaction mixture of 2 mL containing 0.1 M acetate buffer (pH 4.9) with 10% ABTS and an increase in the absorbance was measured at 420 nm (Hatvani and Mécs, 2001). Riboflavin reductase activity was performed according to Russ et al. (2000).

All enzyme assays were carried out at 30 °C with enzyme blanks that contained all components present in reaction except the enzyme. The total protein content was quantified using Lowry's method (Lowry et al., 1951).

2.6. Analysis of biotransformed products

Decolorization of dye, dye mixture and textile effluent was examined with UV–Vis spectroscopic analysis (Hitachi U-2800; Hitachi, Tokyo, Japan) using supernatants, whereas the pattern of biotransformation and degradation was examined using high-performance

thin layer chromatography (HPTLC) and Fourier transform infrared (FTIR). Gas chromatography–mass spectrometry (GC-MS) was used for identification of produced metabolites. HPTLC analysis was done by using HPTLC system (CAMAG, Switzerland). Samples of dye Methyl Orange, dye mixture and its biodegraded product by *F. dichotoma* L., *A. baccifera* L. and consortium FA (dissolved in HPLC-grade methanol) were loaded on precoated HPTLC green fluorescent plate (Silica gel, Merck, Germany) by using TLC sample loading instrument (CAMAG LINOMAT 5) with help of nitrogen as spraying gas. The band length was 8 mm. Application position was from X axis 15 mm and from Y axis 8 mm. TLC plate were developed in solvent system Toluene:Methanol:Glacial acetic acid (16:3:1 v/v). After development, the plate was observed in UV chamber (CAMAG) and scanned at 470 nm with slit Dimension 4 × 0.30 mm and scanning speed 20 mm/s using TLC scanner (CAMAG). WinCATS 1.4.4.6337 software was used to generate a final result of HPTLC (Waghmode et al., 2011).

Metabolites obtained after phytoremediation and control samples of dye were examined by FTIR (FTIR-8400S Shimadzu FTIR spectrometer). FTIR analysis was carried out in the mid-IR region of 400–4000 cm^{-1}. Metabolites produced after degradation were further identified using Gas chromatography-Mass spectroscopy (GC-MS) with Shimadzu 2010 MS Engine, equipped with an integrated gas chromatograph with an HP1 column (60 m long and 0.25 mm). Helium with a flow rate of 1 mL min^{-1} was used as carrier gas. The injector temperature was maintained at 280 °C with oven conditions as follows: 80 °C kept constantly for 2 min, increased up to 200 °C with 10 °C min^{-1}, raised up to 280 °C with 20 °C min^{-1} rate. The compounds were identified on the basis of mass spectra and using the National Institute of Structure and Technology (NIST) library.

2.7. Evaluation of toxicity of products obtained after phytotransformation of Methyl Orange

The toxicity of the dye products and untreated samples were evaluated by monitoring toxic effects like histological changes and genetic mutation by Methyl Orange on freshwater bivalve *Lamellidens marginalis* gill tissue. It is known that the treated and untreated dye effluents are released in freshwater bodies therefore; *L. marginalis,* a common bivalve from freshwater was used for this toxicity assessment. The gills of bivalve are the first organ to come in contact with pollutant mixed in the water, additionally, they are very sensitive and play a role as water filter, therefore gills were the organ of choice for toxicity study. From earlier results on decolorization, enzymatic assays and analytical data, it was evident that the consortium FA was efficient in treatment than individual plants. Therefore Methyl Orange treated by consortium FA was used for toxicity study. A 50 ppm Methyl Orange and products separately were used for toxicity study since only the effects of dye and products on DNA of bivalves were to be evaluated. The experimental protocols were carried out in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, India.

The bivalves *L. marginalis* were collected from Rajaram Lake, Kolhapur India. Initially bivalves were kept in dechlorinated tap water for 5 d to adapt laboratory conditions. The toxicity assessment was carried out in three independent plastic tubs having 5 L tap water (C), 50 ppm Methyl Orange and its biotranformed dye solution by consortium FA. Equal lengths of eighteen (6 each) bivalves (60–65 mm) were distributed in three plastic tub. The solution of each tub was changed with same concentration of respective solution after every 12 h. Bivalves were dissected and gill tissues separated after 4 d of acute exposure of dye solution. The separated gill tissues were kept in bouin's aqueous fluid for tissue fixation for about 12 h. After dehydration gill tissue were embedded in wax and sectioned at five microns. The sections were stained with Hematoxelene–Eosin stain and observed under light microscope.

The remaining gills tissues were used for DNA isolation using modified C-TAB method. Measurement of concentration of DNA samples was done using Nanodrop instrument. Final concentration of each DNA sample was made to 50 ng μl^{-1}. These three DNA samples (C, T1 and T2) were subjected to RAPD molecular marker study. Initially, 10 different RAPD markers were screened and depending upon reproducibility and potential to differentiate between toxic and less toxic concentration of dye, OPA-8 primer was selected for toxicity study. The banding pattern was observed by using gel documentation system as reported by Patil et al. (2016a, 2016b).

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was performed using Tukey-Kramer multiple comparisons test to analyze the data statistically. The values obtained after taking triplicates with mean ± SD were only considered significant when P was ≤ 0.05.

3. Results and discussion

3.1. On field floating phyto-bed approach for real textile wastewater treatment

In situ treatment of the real textile wastewater with the selected plants of *F. dichotoma* and *A. baccifera* fixed on the phyto-beds were observed to root profusely when initially exposed to tap water for 2 months in cement tanks. Fully developed phyto-beds were allowed to float on real textile effluents; it revealed noteworthy reductions in essential environmental safety parameters. The plants used on these floating phyto-beds are annual herbs and possess a potential to tolerate high dye concentration. Therefore, if magnitude of such phyto-beds in terms of size and thus volume is enhanced they could even be used at larger scales and for longer terms.

Textile effluent treated using phyto-bed with *A. baccifera* gave reductions in parameters such as COD (64%), BOD (68%), ADMI (67%), TSS (48%) and TDS (56%) after 9 d. Effluent treated with *F. dichotoma* L. phyto-bed also indicated the decrease in COD (67%), BOD (70%), ADMI (70%), TSS (50%) and TDS (62%). However, the phyto-beds with co-plantation were observed to achieve superior treatment revealing noteworthy reductions in parameters such as COD, BOD, ADMI, TSS and TDS of textile effluent by 72%, 77%, 79%, 56% and 66%, respectively. Additionally, heavy metal such as cadmium, chromium, lead and arsenic reduction was also observed. Textile effluent treatment by *A. baccifera, F. dichotoma* and consortium FA phyto-beds were responsible to reduce cadmium by 33%, 44% and 55%, chromium by 48%, 61% and 72%, lead by 50%, 40% and 68% and arsenic by 60%, 54% and 72%, respectively. The pH of effluent was decreased from 9.5 to 8.4, 7.9 and 7.5 after treatment with *A. baccifera* L., *F. dichotoma* L. and consortia FA, respectively (Table 1). Hybrid CW with plant *Phragmites australis* has been shown to reduce the color by 70%, COD and TOC by 45%. Outcomes in terms of COD and TOC removal observed were independent of horizontal and vertical flow. Retention time however found to be insignificant to alter treatment process (Bulc and Ojstršek, 2008). Floating treatment of wetland using *Typha domingensis* plantation reduced the COD and BOD of sewage effluent by 87% and 87.5%, respectively (Ijaz et al., 2016). In recent reports with *A. philoxeroides* and *S. molesta* exposed to textile effluent under static condition were found to reduce the pH of different dye effluents towards normal (Rane et al., 2015; Chandanshive et al., 2016). Lowering the pH of the inflow due to acids produced by microbial actions was also noted as earlier report (Mbuligwe, 2005). The mixed cultures of *P. grandiflora* and *G. grandiflora* were also observed to show superior treatment of Remazol Orange 3R than the individual plants (Watharkar and Jadhav, 2014). Artificial neural network modeling study of the degradation of acid blue 92 by *A. filiculoides* revealed that input variables such as decolorization time, initial dye concentration, fresh weight of plant, initial pH and temperature were altering the remediation efficacy (Khataee et al.,

Table 1
Characterization of real textile effluent and treated textile effluent by on-field floating phyto-beds *A. baccifera, F. dichotoma* and consortium FA after 9 d.

Parameter	Real textile effluent	*Ammannia baccifera*	*Fimbristylis dichotoma*	Consortium FA	Discharge effluent standard into ISW
ADMI	1285 ± 1.55	427 ± 1.50	391 ± 1.53	265 ± 1.55	400
Odour	Specific	No odour	No odour	No odour	–
pH	9.5	8.4	7.9	7.5	5.5–9
COD (mg/L)	1438 ± 12.7	510 ± 9.50	475 ± 9.00	410 ± 9.30	250
BOD (mg/L)	1230 ± 10.20	390 ± 9.73	365 ± 9.20	290 ± 9.11	30
TDS (mg/L)	8230 ± 8.80	3587 ± 7.66	3173 ± 7.54	2796 ± 8.21	2100
TSS (mg/L)	5175 ± 0.7	2668 ± 0.5	2568 ± 0.5	2258 ± 0.5	100
Cadmium (ppm)	0.09	0.06	0.05	0.04	2.0
Chromium (ppm)	4.20	2.17	1.42	1.19	2.0
Lead (ppm)	0.70	0.35	0.42	0.22	0.1
Arsenic (ppm)	2.05	1.13	1.34	1.09	0.2

Values are a mean of three experiments ± SEM. ISW- Inland surface water.

2013). Similarly, amount of algal biomass of *Chara* was also previously studied to affect the performance of the reactor system using artificial neural network modeling (Khataee et al., 2010). Combinatorial phytoreactor of *I. hederifolia* and *I. aquatica* have also shown significant treatment of textile effluent than the individual plant system (Rane et al., 2016).

3.2. Microbial community analysis

T-RFLP analysis was used to examine the soil microbial community associated with all three systems of *F. dichotoma, A. baccifera* and consortium FA phyto-beds. Every peak in the profile represents a certain taxon referred as operational taxonomic unit (OTU), and the peak area corresponds to the proportion of this OTU of the microbial community. These types of peaks may be multifaceted representing more than one microorganism. However, regardless of their complexity, the T-RFLP profile is a suitable method for identification of microbial populations. Very high bacterial diversity was observed in this study using T-RFLP molecular method. The dendrogram mainly showed two clusters viz. I and II. Cluster I resembled C1 and C2 while cluster II represented C3 only (Fig. 2). To clarify this, identification of community was carried out further using the web-based Microbial Community Analysis (MiCA III) tool. The results obtained from MiCA III-PAT^{+} were supportive to earlier results, C1 and C2 possessed of 88 and 98 genera while C3 represented 223 genera (Table S1). It is evident from the venn diagram that 170 new genera were found in consortium FA phyto-bed rhizosphere which represent 56.1% of total organisms distinct from individual floating beds zone. Most of the microorganisms in community 1 (C1) and community 2 (C2) were similar. Microorganism present in community 3 (C3) differs from the both C1 and C2 communities. Thus, consortium FA with its rhizospheric community proved superior to individual plants in the treatment of textile effluent. Many species of *Pseudomonas* have been found to show dye degradation potential which could help plant system to perform with a greater potential. In addition many species of *Bacillus*, *Kocuria, Frankia*, *Comomonas*, *Nocardia*, *Burkholderia, Rhodococcus* sp. etc. have also been reported for dye degradation through their enzymatic machineries (Saratale et al., 2011).

Phytoremediation technique based on combined action of plant and microorganisms that they support within the rhizosphere participate in remediation of contaminated soil and water (Glick, 2010). Plants provide favorable condition to microbial colonization of rhizosphere for symbiotic degradation and detoxification of pollutant (Doran, 2009). Plant and microbes possess different enzymatic cascades which trigger more mineralization of textile waste than individual system. Microorganisms growing in the root vicinity also play a role in the dye treatment and support the overall remediation process. *In vitro* grown *Zinnia angustifolia-Exiguobacterium aestuarii* and *Portulaca grandiflora-Pseudomonas putida* plant-bacteria co-systems were reported to achieve superior treatment of Remazol Black B and Direct Red 5B, respectively than the individual organisms (Khandare et al., 2012, 2013a, 2013b). Some growth promoting substances secreted by plants helps to better growth of microbes. Further the mixed plantation of *F. dichotoma* and *A. baccifera* in this study involves additional substances secreted by both plants that supported growth of more number of bacteria and achieved an enhanced treatment of dye wastewater. This also enables phytoremediation processes to comply with the fluctuation pollutant load present at the actual dye disposal site.

3.3. Decolorization of Methyl Orange, dye mixture and textile effluent

Initially decolorization of Methyl Orange was carried out using *F. dichotoma A. baccifera* and consortium FA. Methyl Orange was

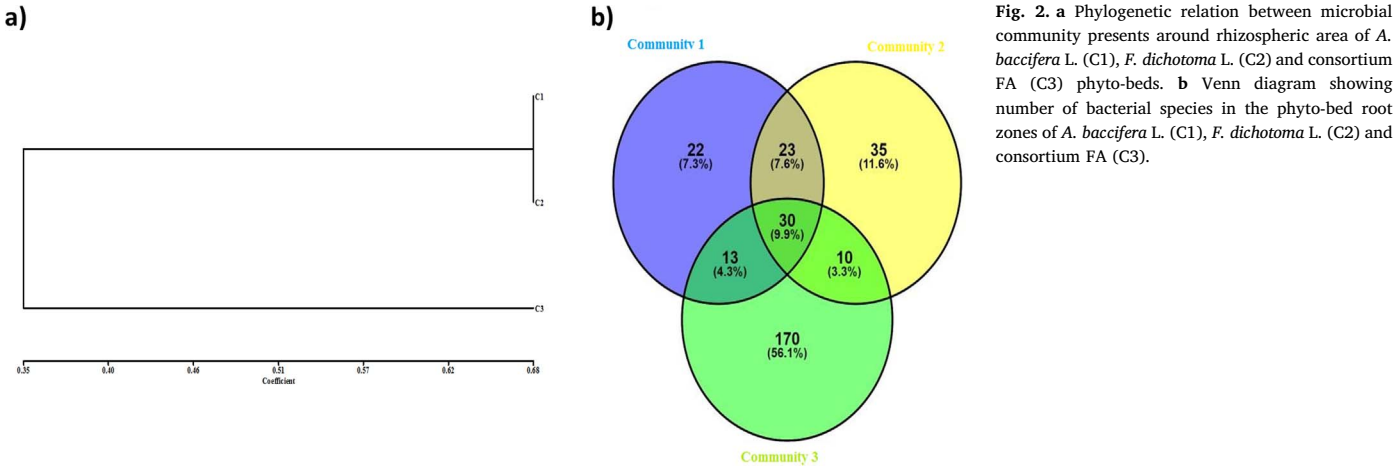

Fig. 2. a Phylogenetic relation between microbial community presents around rhizospheric area of *A. baccifera* L. (C1), *F. dichotoma* L. (C2) and consortium FA (C3) phyto-beds. **b** Venn diagram showing number of bacterial species in the phyto-bed root zones of *A. baccifera* L. (C1), *F. dichotoma* L. (C2) and consortium FA (C3).

decolorized by *F. dichotoma, A. baccifera* up to 91% and 89% after 60 h exposure, respectively. While 95% decolorization of the dye was achieved when treated with consortium FA just within 48 h of exposure. Consortium FA reduced the ADMI of dye mixture (Methyl Orange, Remazol Red, Blue GLL, Congo Red and Green HE 4BD) up to 96%, while individual plants of *A. baccifera* and *F. dichotoma* reduced the ADMI values up to 83% and 86%, respectively within 60 h. In case of the textile effluent treatment, ADMI values were reduced up to 89% by the *F. dichotoma* L. and 87% by *A. baccifera* L. While consortium FA was found to reduce the ADMI value up to 97%. Efficient treatment of dye wastewater with plant consortia systems relies on the synergistic metabolism of the participating plants (Khandare and Govindwar, 2015).

Laboratory developed plant-plant consortium of *A. amellus* and *G. pulchella* was found to show efficient removal of 20 mg L^{-1} Remazol Orange 3R up to 100% within 36 h, on the other hand, the individual plant could only remove it after 72 and 96 h, respectively (Kabra et al., 2011). In another experiment, an *in vitro* grown consortium of *G. grandiflora* and *Petunia grandiflora* gave 94% color removal after 36 h while their individual plants could only achieve only 62% and 76% decolorization at 20 mg L^{-1} concentration, respectively (Watharkar and Jadhav, 2014).

3.4. Anatomical studies of roots during dye removal

It is important to understand the dye removal mechanisms of plants to have a better insight about phytoremediation. Anatomical study of root cells of *A. baccifera* and *F. dichotoma* for understanding the histology, movement and metabolism of Methyl Orange was carried out up to 72 h exposure to Methyl Orange. The decolorization of Methyl Orange was completed in 60 h; plants were further continued for exposure to this decolorized water. The root section of *A. baccifera* and *F. dichotoma* plants before exposure to Methyl Orange (Fig. 3a and e) showed no coloration of any cell and they appeared to be normal and undisturbed. After 24 h of Methyl Orange exposure to *A. baccifera* and *F. dichotoma* (Fig. 3b and f), dye was defused through the root and found to be accumulated in the outer epidermal cells with some amount towards the inner epidermis. This accumulation was increased up to the cortical layer after 48 h in both plants (Fig. 3c and g). However, the cells at 72 h exposure showed no dye in the epidermal and cortical cells confirming the complete degradation of Methyl Orange (Fig. 3d and h). Dye molecules accumulation take place up to 48 h (Fig. 3a to g) during this period plant sense the abiotic stress on cells because of inability to utilize these molecules. Further they trigger the enzyme cascade to mineralize these complex compounds in to simpler form. As a result, concentration of dye start decreasing and at the time of 72 h it becomes significantly less. Accumulation and subsequent degradation are the reported mechanisms of plants while treating textile dyes. *Typha angustifolia* was shown to accumulate Reactive Red 41 in the epidermal and cortical tissue and achieved 60% decolorization (Nilratnisakorn et al., 2007). Anatomy of *I. hederifolia* after exposure to Scarlet RR revealed the presence of dye molecule in the epidermis at 6 h which was further extended to cortical cell and started to disappear after 48 h (Rane et al., 2014). Similarly *A. philoxeroides* after 8 h exposure accumulated Remazol Red in the epidermal cells which was later found to move in the cortex at 32 h and completely disappeared at 48 h (Rane et al., 2015). In another experiment, *Salvinia molesta* stem epidermis showed the presence of Rubin GFL after 12 h of exposure which was found in cortex at 24 h and subsequently degraded after 48 h (Chandanshive et al., 2016). *Eichornia crassipes* root and shoot cells also showed accumulation of Methylene Blue and Methyl Orange after a 20 d exposure while showing 98% and 67% dye removal, respectively (Tan et al., 2016).

3.5. Enzymatic analysis of root cells of A. baccifera, F. dichotoma and consortium FA after phytoremediation of Methyl Orange at 48 h

Root cells of *A. baccifera, F. dichotoma* and consortium FA were analyzed for changes in various oxido-reductive enzymes before and after dye decolorization. The *A. baccifera* root cell after 60 h of dye exposure showed enhancement in the activities of lignin peroxidase (LiP), tyrosinase, NADH-DCIP reductase, azo reductase, riboflavin reductase and laccase by 4.08%, 2.80%, 17.72%, 18.19%, 38.74% and 53.33%, respectively. However *F. dichotoma* root cells showed different induction pattern in activities viz. lignin peroxidase (3.89), NADH DCIP reductase (136.19), azo reductase (19.63), riboflavin reductase (315.95) and laccase (167.64%). While assay of the roots of consortium FA revealed 29.45%, 9.51%, 145.03%, 86.44%, 206.87% and 189.00%

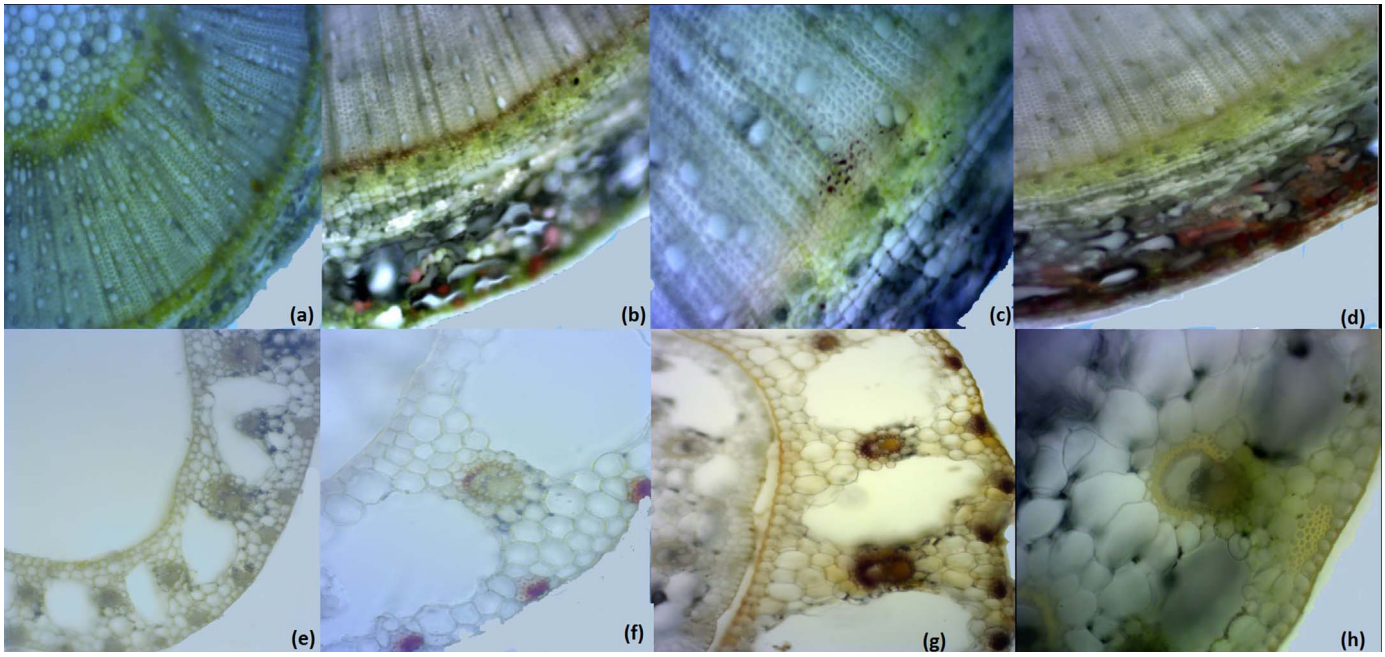

Fig. 3. Anatomical analysis of roots of *A. baccifera* L. and *F. dichotoma* L. after Methyl Orange exposure at, a and e) 0 h, b & f) 24 h, c & g) 48 h and d & h) 72 h, respectively.

Table 2
Enzyme analysis of *A. baccifera* and *F. dichotoma* plants tissue at 0 h and after 60 h of 50 mg L^{-1} Methyl Orange dye exposure.

Enzymes	*Ammannia beccefera*		*Fimbristylis dichotoma*		Consortium FA	
	Control	Test	Control	Test	Control	Test
Lignin peroxidase	54.05 ± 0.63	56.26 ± 1.21	73.53 ± 0.41	76.39 ± 0.86*	71.43 ± 1.21	92.47 ± 0.21***
Tyrosinase	0.43 ± 0.09	0.44 ± 0.15	0.83 ± 0.09	0.78 ± 0.13	0.70 ± 0.06	0.77 ± 0.70
NADH-DCIP reductase	37.16 ± 0.89	43.75 ± 0.55*	22.06 ± 0.64	52.08 ± 1.11**	32.14 ± 0.39	78.77 ± 0.91***
Azoreductase	41.13 ± 0.83	48.91 ± 0.74*	47.95 ± 0.95	57.37 ± 0.73**	55.90 ± 1.06	104.22 ± 0.73***
Riboflavin reductase	4.29 ± 0.20	5.95 ± 0.18*	5.84 ± 0.14	24.25 ± 0.91***	11.34 ± 0.27	34.79 ± 1.04***
Laccase	0.03 ± 0.001	0.05 ± 0.001*	0.02 ± 0.001	0.03 ± 0.001**	0.02 ± 0.001	0.06 ± 0.003***

Values are a mean of three experiments ± SEM. Significantly different from control (0 h) at *P < 0.05, **P < 0.01 and ***P < 0.001 by one-way ANOVA with Tukey–Kramer comparison test. All enzyme assays were carried out in triplicate. The enzyme activities were determined in mg mL^{-1} min^{-1}.

induction in the activities of lignin peroxidase, tyrosinase, NADH-DCIP reductase, azo reductase, riboflavin reductase and laccase, respectively (Table 2). Because of the synergistic involvement of enzyme from both the plants in co-culture, it was found to be efficient than the individual plants. Mixed plantation and consortia enzymatic involvement of *A. amellus* and *G. pulchella* had also shown such kind of enhancement in the activity of plant enzyme while treating Remazol R (Kabra et al., 2011). *P. grandiflora* with *P. crinitum* was also reported to show increased activities of dye degrading enzymes when used for treatment of synthetic dye effluent (Watharkar and Jadhav, 2014). Recently, mixed bed plantation of *I. hederifolia* and *I. aquatica* could also achieve superior treatment of dye wastewater by virtue of their synergistic enzyme involvement (Rane et al., 2016).

3.6. Analysis of metabolites before and after phytodegradation

The differential FTIR spectra of the control and treated samples with individual as well as plant-plant consortia of Methyl Orange, simulated dyes mixture and textile effluent confirmed their changes in functional group of newly formed metabolites after phytoremediation treatment (Fig. S1 and Table S2).

HPTLC analysis (Fig. S2) of the untreated Methyl Orange (Lane a) showed four peaks at R_f values of 0.12, 0.14, 0.39 and 0.53 with absorbance of 68.6, 46.4, 535.1 and 90.7 AU, respectively. The products of Methyl Orange after treatment by *A. baccifera L.* showed three peaks at R_f of 0.11, 0.13 and 0.56 with absorbance of 45.6, 43.7 and 539.0 AU, respectively (Lane b). *F. dichotoma* L. treated dye showed three peaks at R_f 0.11, 0.13, 0.53 and 0.61 with absorbance 65.4, 52.6, 56.8 and 33.4 AU, respectively (Lane c). Methyl Orange degraded by consortium FA showed three peaks at R_f of 0.12, 0.15 and 0.63 with absorbance 57.8, 53.4 and 32.0 AU, respectively. Untreated dye mixture (Lane e) represent five major peaks at R_f value of 0.12, 0.23, 0.34, 0.46 and 0.60 with absorbance 633.7, 218.5, 41.2, 176.1 and 50.1, respectively. *A. baccifera* L. treated dye mixture showed three peaks at R_f of 0.11, 0.15 and 0.63 with absorbance 61.6, 57.2 and 410.1, respectively (Lane f). Lane g showed the dye mixture degraded by *F. dichotoma* L. with three peaks at R_f of 0.12, 0.15 and 0.61 with absorbance 93.0, 55.5 and 52.5, respectively. The consortium FA treated dye mixture showed three peaks at R_f 0.12, 0.16 and 0.64 with different absorbance 42.3, 35.2 and 159.3 AU, respectively (Lane h). This confirmed that the parent dyes transformation to different products after treatment by plant species.

GC-MS analysis results were used to find out chemical nature of extracted metabolites as well as to propose degradation pathways of Methyl Orange. The probable degradation pathway of Methyl Orange was predicted considering induction in the enzyme activity and metabolite produced during degradation (Table S3). When dye solution treated with *A. baccifera* L. and *F. dichotoma* L. Methyl Orange undergoes asymmetric cleavage by lignin peroxidase and laccase to form sodium 4–(phenyldiazenyl)benzenesulfonate. Further in case of *A. baccifera L.* 4–(phenyldiazenyl)benzenesulfonate (m/z = 282, mw = 284) underwent reduction to yield 4–(phenyldiazenyl)benzenesulfenate (m/z = 251, mw = 252) (Fig. 4a). The FTIR bond vibrational frequencies only at 2430.39 and 2337.80 cm^{-1} representing NH^+ stretching and at 2195.07 and 2058.11 cm^{-1} showing NH^+ vibrations support the removal of N containing species after cleavage of the dye structure. Further, the only peak at 3400.62 cm^{-1} shows removal of oxygen viz. reduction explaining the formation of the second metabolite shown in the degradation pathway by *A. baccifera* (Table S2). While in case of Methyl Orange treated with *F. dichotoma* 4–(phenyldiazenyl) benzenesulfonate (m/z = 281, mw = 284) further degraded followed by benzene removal, sodium 4–diazenylbenzenesulfonate (m/z = 206, mw = 208) were formed (Fig. 4b). The only C≡N Stretching as shown in the FTIR spectrum at 2339.73 cm^{-1} shows deamination along with a peak for supporting removal of N along with aromatic structure. In addition, the only peak at 3412.19 cm^{-1} represents removal of aliphatic N in the form of NH^+ vibrations (Table S2). In consortium FA treated Methyl Orange, laccase split model dye in to 4-(dimethylamino) phenol (m/z = 135, mw = 137) and 4–diazenylbenzenesulfonate (m/z = 207, mw = 208). Further 4–diazenylbenzenesulfonate were oxidized then subsequently reduced to yield sodium 4–hydroxybenzenesulfinate (m/z = 179, mw = 180) and 4 – sulfanylphenol (m/z = 129, mw = 126), respectively (Fig. 4c). The FTIR peak at 3410.26 cm^{-1} showing O-H stretching reveals oxidative cleavage of the dye structure. The NH^+ stretching represented as peaks at 2480.54 and 2333.94 cm^{-1} further show oxidative deamination of the intermediate. Loss of peaks at 1386.86 and 1174.69 cm^{-1} reveals the S=O bond breakdown which was not seen in the products (Table S2). Lignin peroxidase is specifically reported for asymmetric cleavage of dye molecule (Kabra et al., 2011; Khandare et al., 2013a, 2013b; Rane et al., 2015). The laccase is well known for oxidative cleavage and desulphonation of dyes (Kagalkar et al., 2015).

3.7. Toxicity evaluation on bivalve Lamellidens marginalis

Because of the known threats possessed by dyes their toxicity assessment becomes inevitable to check the harmfulness after remediation process. Freshwater animals are most affected species as the dye effluent many a times are released in to water bodies. Textile wastewater was reported to alter gill structures in *Salmo trutta* freshwater fish nonspecifically (Bernet et al., 2004). Various toxic effects such as hyperplasia, hypertrophy inflammation, desquamation and abnormal cell

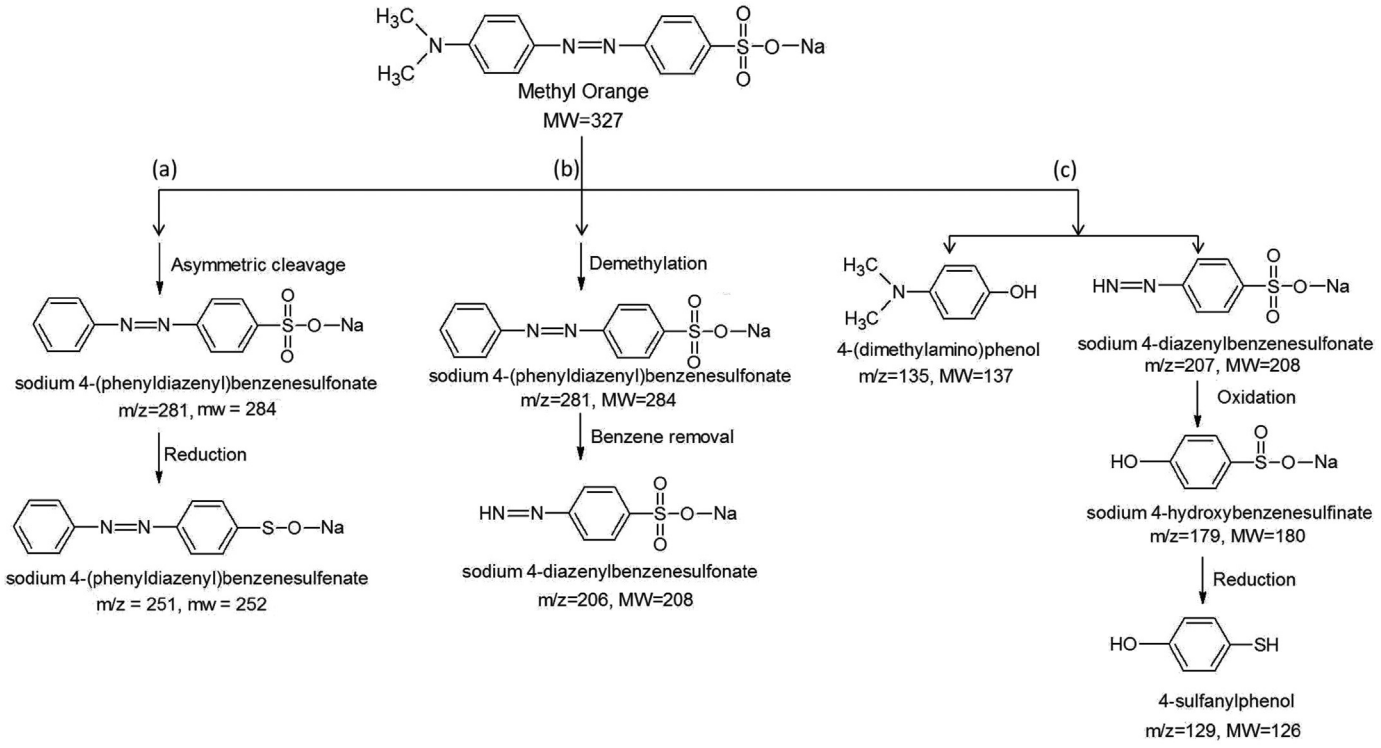

Fig. 4. Proposed pathways for metabolites of Methyl Orange transformed by a) *A. baccifera* L. b) *F. dichotoma* L. and c) consortium FA.

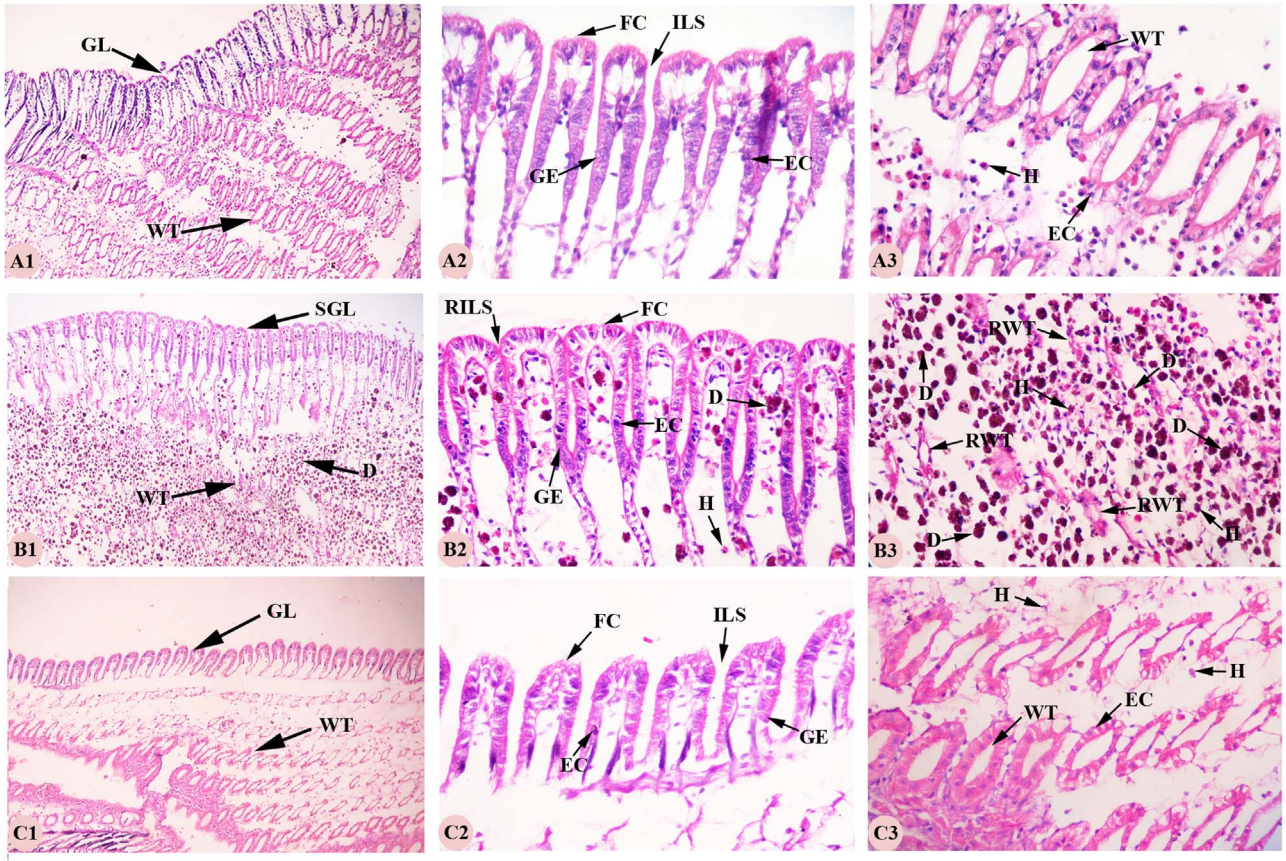

Fig. 5. Histology of bivalve gill tissues exposed to A1) fresh water showing undisturbed and normal gill lamellae (GL), water tubes (WT), gill lamellae with (A2) frontal cell (FC), inter lamellae space (ILS), endothelial cells (EC), (A3) water tubes with normal size, hemocyte (H); L.S. of gills exposed to (B1) untreated Methyl Orange showing dye accumulation and disturbed cells, (B2) gill lamellae with aberrations such as swelling of gill lamellae (SGL), reduced inter lamellae space(RILS), (B3) water tubes with distortions such as dye accumulation (D), reduced size of water tube (RWT) and L.S. of gill showing (C1) unaltered gill architecture of bivalve exposed with treated Methyl Orange showing normal gill/less disturbed structure, (C2) gill lamellae in normal shape with very little bifurcation and (C3) water tubes with normal shape and size, and devoid of dye.

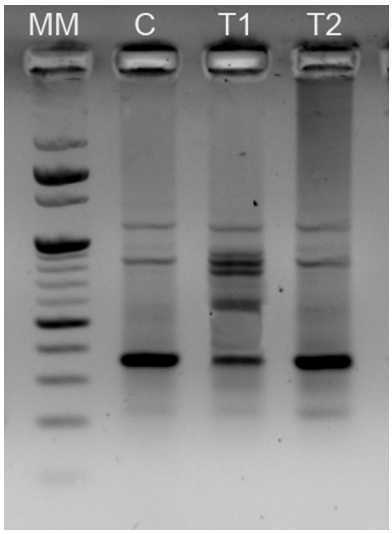

Fig. 6. RAPD banding pattern of bivalve DNA shown as Lane i) molecular marker, ii) normal bivalve, iii) bivalves exposed with untreated 50 ppm of Methyl Orange and iv) bivalves exposed with treated dye.

sizes of lamellae and hemorrhage was observed in the gills of *Etheostoma olmstedi* when exposed to a textile effluent for 12 d. however the phyto-remediated effluent with *P. crinitum* showed no such toxic effects (Watharkar et al., 2015). A similar distortion in the gill structures like blood congestion, missing of secondary lamellae and curling was observed in the gills of *Devario aequipinnatus* fish upon exposure to Remazol Red solution at 70 mg L^{-1} for 14 d. The phyto-remediated samples treated in a constructed wetland with *A. philoxeroides* revealed normal gill morphology in the fishes exposed to dye (Rane et al., 2015).

Fifty ppm of Methyl Orange dye and its metabolites obtain after consortium FA treatment independently exposed to *L. marginalis* revealed reduced toxicity of dye metabolites. The gill of control bivalve showed uniformly arranged lamellae with inter-lamellar space (Fig. 5A1 & A2). The surface of each lamella was covered with a monolayer of epithelium. Several normal structures of water tubes (WT) were present below the gill lamellae (Fig. 5A3). The gill architecture however was observed to be seriously damaged and dye particles were observed throughout the gill lamellae with significant swelling and distortion as evident from reduced inter-lamellar space. Swelling of gill lamellae (SGL) and reduction of inter lamellar space (RILS) (Fig. 5B1 & B2). Excess dye deposited in haemolymph vessels (H) and around the water tube (WT) showed reduction in size of water tubes (Fig. 5B3) when compared to control (Fig. 5B1 and B2). The presence of Methyl Orange was also observed in the water tubes (Fig. 5B1 and B3). The metabolite exposed bivalve gills on the other hand revealed normal morphology with intact water tubes and lamellar structure with normal inter lamellar space (Fig. 5C1 and C2). The water tubes were found normal in shape and no dye or product stresses accumulation visualized (Fig. 5C1 and C3).

The toxicity analysis performed on bivalves using molecular marker RAPD revealed reduced toxicity of the treated dye. RAPD toxicity assay can help to detect a sudden change in at least 2% of the DNA (Jones and Kortenkamp, 2000). Changes in nucleotide sequence of DNA can give different RAPD pattern as new binding sites become available to RAPD primers for further amplification. Chemical pollutants such as benzene and hydrogen peroxide were exposed to *Dicentrarchus labrax* embryonic cells and toxicity assessment was carried out using RAPD-PCR (Rocco et al., 2013). In this work, the DNA of exposed organisms to Methyl Orange (T2) was subjected to RAPD using single primer OPA-8 (5′-G-TGACGTAGG-3′). The DNA banding pattern of water, Methyl Orange and phyto-degraded dye exposed bivalves gave a clear impression about toxic nature of Methyl Orange over phytotransformed dye (Fig. 6). Control bivalve DNA (Lane 2) showed three RAPD bands of molecular weight 1.1, 0.9 and 0.3 kb. The exact banding pattern was observed in DNA of phyto-treated dye exposed to bivalve (Lane 4). While the DNA of dye exposed bivalve showed six RAPD bands having molecular weight 1.1, 0.9, 0.88, 0.81, 0.6 and 0.3 kb (Lane 3), respectively. The bands with molecular weights of 1.1, 0.9 and 0.3 kb were common in all samples hence they were monomorphic bands. Dye exposed bivalve DNA showed some polymorphic band with molecular weight 0.88, 0.81 and 0.60 kb. It seems that model dye induced some changes in DNA of *L. marginalis* after acute exposure. This toxic effect was however not observed in treated sample because of reduced toxicity after biotransformation of Methyl Orange.

Methyl Orange (50 ppm) was able distort the gill structure of freshwater bivalve *L. marginalis* also responsible for changes at genetic level within 96 h. Both the toxicity assessment studies i.e. histology and molecular markers revealed reduction of Methyl Orange toxicity due to phytoremediation.

4. Conclusions

Consortia of plants (*F. dichotoma* L and *A. baccifera* L.) efficiently treated Methyl Orange and textile effluents as compared to individual plants. Floating phyto-beds developed using these plants treated the effluents to the remarkable extents and significantly reduced COD, BOD, TDS and color of effluents. The toxicity assessment of bivalves and RAPD revealed reduction in toxicity of phyto-remediated Methyl Orange. Heavy metals were also found to be reduced note-worthily. Rich microbial diversity developed during the treatment of phyto-bed of consortium FA might have significant role in efficient phytoremediation. The outcomes suggest that use of phytobeds can be a wise approach for textile waste management.

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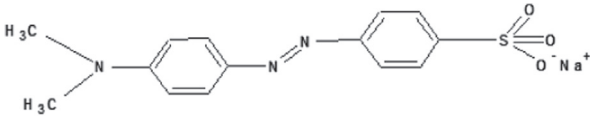
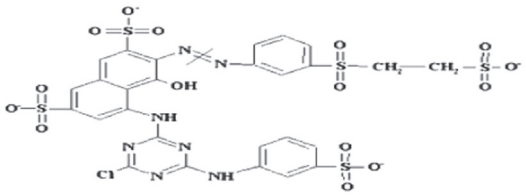
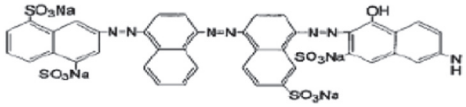
Appendix A

The chemical information of textile dyes used in this study.

Name of the dyes	C. I. name	λ_{max} nm	Chemical nature	CAS No.	Chemical structure of dyes
Methyl Orange	Acid orange 52	470	monoazo	447-58-0	
Remazol Red	NA	530	monoazo	NA	
Blue GLL	Direct blue 71	620	direct	NA	

Appendix B. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2017.09.009.

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