Investigating degradation metabolites and underlying pathway of azo dye "Reactive Black 5" in bioaugmented floating treatment wetlands

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Abstract

The direct discharge of azo dyes and/or their metabolites into the environment may exert toxic, mutagenic, and carcinogenic effects on exposed fauna and flora. In this study, we analyzed the metabolites produced during the degradation of an azo dye namely Reactive Black 5 (RB5) in the bacterial augmented-floating treatment wetlands (FTWs), followed by the investigation of their underlying toxicity. To this end, a FTWs system was developed by using a common wetland plant *Phragmites australis* in the presence of three dye-degrading bacteria (*Acinetobacter junii* strain NT-15, *Pseudomonas indoloxydans* strain NT-38, and *Rhodococcus sp.* strain NT-39). We found that the FTW system effectively degraded RB5 into at least 20 different metabolites with the successful removal of color (95.5%) from the water. The fish toxicity assay revealed the non-toxic characteristics of the metabolites produced after dye degradation. Our study suggests that bacterially aided FTWs could be a suitable option for the successful degradation of azo dyes, and the results presented in this study may help improve the overall textile effluent clean-up processes.

Introduction

Discharge of polluted water into the environment is a worldwide problem, particularly in developing countries where the discharge of untreated wastewaters directly into water bodies is a common practice (Chandanshive et al. 2016). Such a discharge of textile wastewater leads to water pollution in terms of elevated concentrations of noxious dyes. These dyes are often detrimental to living organisms and also cause depletion in the dissolved oxygen in the receiving water (Shehzadi et al. 2014; Bilal et al. 2017; Chatha et al. 2017; Hussain et al. 2018). Consequently, this discharge of textile wastewaters is a big threat to aquatic life (Bilal et al. 2016; Hussein and Scholz 2018; Hussain et al. 2019).

In the textile industry, desirable features such as bright color, simple application, and excellent color fastness make azo dyes as the most useful type of dyes (Yaseen and Scholz 2018). Reactive Black 5 (RB5) is a widely used synthetic reactive azo dye in the textile industry. Its high solubility in water and ability to form reactive groups allows strong fixation with fibers (Asad et al. 2007; Vijaykumar et al. 2007). On the other hand, a significant proportion of the dye is present in the wastewater discharge from the textile industry (Bilal et al. 2017). As previously reported, even a small quantity of azo dyes can lead to unwanted consequences in the aquatic ecosystem (Shah and Patel 2014).

As of today, several remediation approaches are developed to eliminate dyes from the textile industry wastewater. The remediation via conventional methods is hard to achieve because these dyes are highly resistant to chemicals and physical treatments (da Silva et al. 2020). Moreover, the use of nature-based solutions (NBS) is strongly encouraged for the cleanup of industrial wastewaters. To this end, the floating treatment wetlands (FTWs) system is a promising approach due to its low operational costs, simple installation structure, and low requirements for operational control (Shahid et al. 2018). In FTWs, roots of plants hang down into the water body and act as a biological filter for pollutant elimination (Benvenuti et al. 2018). Furthermore, the plant root apparatus provides a niche for the growth of microbial communities

that are essential for the nutrient and pollutant removal from wastewaters and can at the same time support plant growth (Faulwetter et al. 2009; Benvenuti et al. 2018; Riva et al. 2020). Recently, FTWs have been successfully applied to remediate different types of wastewaters, e.g., stormwater, municipal sewage as well as various types of industrial wastewater (Headley et al. 2006; Ijaz et al. 2016; Ali et al. 2017; Rehman et al. 2019). The plant-bacteria synergism in FTWs is appeared to be an effective strategy for faster treatment of real textile wastewater both at lab-scale (Tara et al. 2019a) and at pilot-scale (Tara et al. 2019b).

The degradation of azo dyes by the combined use of plants and bacteria has already been reported (Khandare et al. 2011, 2013a). Till today, little is known about the metabolites produced during the biodegradation of azo dyes in FTWs. Furthermore, the degradation of RB5 and its metabolites in FTWs inoculated with bacteria was never investigated. The identification of metabolites and underlying biodegradation pathways may contribute to future studies related to azo dyes biodegradation in FTW and/or similar systems, i.e. NBS.

Phragmites australis is one of the most commonly used plants for phytoremediation, because of its flexibility to grow in a wide range of freshwater environments, including the most polluted ones (Borruso et al. 2017). Furthermore, it is a helophyte plant and is extensively used in constructed wetlands due to effective plant-bacteria interactions for successful phytoremediation goals (Rehman et al. 2018; Tara et al. 2019a; Afzal et al. 2019a,b). In this study, we added three bacterial strains (*A. junii* NT-15, *P. indoloxydans* NT-38, and *Rhodococcus sp.* NT-39) to support *P. australis* functioning in the FTWs. These strains were previously isolated from activated sludge, root endosphere and rhizosphere of *Polygonum aviculare* and *Poa labillardierei* and showed successful degradation of azo dye followed by plant growth promotion activities (Tara et al. 2019a). This study particularly aims to identify metabolites produced during the biodegradation of RB5 in FTWs inoculated with bacteria, and elucidate underlying biodegradation pathways of RB5 dye. Moreover, the removal of RB5 in *Phragmites* based FTWs and the toxicity of treated water were determined.

Materials And Methods

Chemicals

RB5 was obtained from OHYOUNG Industrial Co., Ltd. (Seoul, South Korea), and was used without further purification. The solutions of RB5 were prepared according to the estimated concentrations in real textile wastewaters (El Bouraie and El Din 2009; Kumar et al. 2018). In this study, 200 and 1000 mg L^{-1} concentrations of RB5 were adapted in FTWs for metabolites investigations. The physicochemical characteristics and main functional groups of the dye are summarized in Table 1

Bacterial strains

In this study, three bacterial strains (*A. junii* NT-15, *P. indoloxydans* NT-38, and *Rhodococcus* sp. NT-39) were used (Tara et al. 2019a). They were cultivated separately at 37°C in Luria Bertani broth for 24 h.

Their cells were harvested by centrifugation at 9000 rpm and re-suspended together at the ratio of 1:1:1 in sterile 0.9% NaCl solution. The amount of each pure culture cell suspension was adjusted by a turbidimetric method (Sutton, 2011) before addition to the mixture. An inoculum (150 ml: 10^7 CFU ml^{-1}) of the mixture was augmented in the FTWs

Experimental setup

Fifteen FTW mesocosms were constructed using rectangular plastic tanks having dimensions of 52 × 38 × 30 cm (length × width × height) with a total water holding capacity of 60 liters. The floating mats were constructed by using a polystyrene role with four equidistant holes having the following dimensions: 50 × 36 × 7 cm (length × width × height) (Fig. 1). To shield the mat from sun radiation, each side of the mat was covered with aluminum foil (Rehman et al. 2018). Five seedlings of *P. australis* were put in each hole of the mat which were further supported with coconut debris. Plant growth was supported by adding soil up to 25.4 mm on the mat. The plants used were homogeneous in the length of around 600 mm and a weight of 50 to 60 g. The floating mats carrying plants were at first grown in tap water containing Hoagland solution for 30 days to establish the root system. After the acclimatization period, tap water of each mesocosm was replaced with the 50-liter dye-containing solution as per the experimental design. The dye was found water-soluble and therefore directly dissolved in water. Five different treatments, each with three replicates, were set up as follows:

Control 1 (C1) (unvegetated and uninoculated): dye solution having 200 mg RB5 L^{-1} water,

Control 2 (C2) (unvegetated and uninoculated): dye solution having 1000 mg RB5 L^{-1} water,

Control 3 (C3) (vegetated and uninoculated): FTWs having tap water,

T1

FTWs having dye (200 mg RB5 L^{-1} water) with the bacterial consortium,

T1*

FTWs having dye (1000 mg RB5 L^{-1} water) with the bacterial consortium.

An inoculum (150 ml: 10^7 CFU ml^{-1}) containing a mixture of the three bacterial strains was augmented in T1 and T1*. The amount of each pure culture cell suspension was adjusted by a turbidimetric method (Sutton, 2011) before addition to the mixture. The experiment was conducted at ambient light and temperature (April-May 2018) in the outdoor environment of the National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan (31°25'0"N73°5'28"E). The average high and low temperatures were 36.5°C and 20.5°C, respectively. Water specimens were obtained after 0, 5, 10, and 15 days of inoculum application followed by examination for color removal as per standard procedures (APHA 2005).

Decolorization studies

Aliquots of 2 ml were collected separately from three replicates of each treatment (C1, C2, T1, and T1*) containing RB5 at five days intervals. The samples were centrifuged at 6000 rpm for 12 min at 4°C to eliminate any suspended biomass. The color removal was evaluated by determining its concentration in the supernatant with Spectroquant® NOVA 60 (Merck Germany) (Tara et al. 2019). The color removal (%) was evaluated by using the following expression.

Decolorization efficiency (%) = (OD0 - OD1)/OD0 ×100

Where OD0 referred to the initial absorbance at day 0 and OD1 referred to the absorbance after 5, 10, and 15 days. The treatment showing maximum color removal was chosen for the identification of biodegraded products of RB5.

Extraction of RB5 degradation products

At the end of the experiment, the water sample (200 ml) collected from three replicates of the selected treatment (T1) was centrifuged to eliminate any suspended matter. For the extraction of degradation products, an equal volume of chloroform was mixed with the supernatant. The chloroform was evaporated over anhydrous Na_2SO_4, and the extract was desiccated. The solid residue was mixed in a small amount of analytical grade CH_3OH (Sayilgan and Cakmakci 2013; Yasmin et al. 2017).

ESI-MS/MS analysis for the detection of biotransformation products

The biodegradation metabolites of RB5 were analyzed by using ESI-MS/MS as explained previously (Sayilgan and Cakmakci 2013; Yasmin et al. 2017). Tandem mass spectrometric analysis of metabolites from degradation extract was done by using LTQ-XL Linear Ion Trap Mass Spectrometer, which was equipped with Electrospray Ionization (ESI) source. The sample was dissolved in methanol, filtered through a 0.45 um syringe filter, and then injected into the mass spectrometer through the direct insertion method at a flow rate of 10 µL/min (Yasmin et al. 2017). The scanning of samples was done at a negative ion full scan mode with a mass scan range *m/z* 50-2000. Capillary voltage and capillary temperature were adjusted at 4.2 kV and 280°C, respectively. The sheath gas (N_2) and auxiliary gas (N_2) flow rates were kept at 20 and 5 arbitrary units, respectively. Helium gas was used as the collision gas. To determine the fragmentation pattern and authenticate dye degradation, a 100 µg/L standard solution of RB5 dye was also directly introduced into the mass spectrometer and analyzed accordingly. X-Calibar software compound's identification was done by correlating the obtained data with the previous studies. The structures of degradation products were determined employing the Mechanistic Chemistry approach and biodegradation pathways were deduced by adopting ChemBioDraw Ultra 14.0.

Plant growth/biomass production

To assess the impact of the RB5 dye and bacteria on plant growth and development, plants from three replicates of different treatments (C3, T1, and T1*) were harvested at the end of the experiment, i.e. after 45 days. The growth parameters such as root and shoot lengths, and root and shoot weights were measured (Tara et al. 2019a,b). The dry weights were estimated by placing plant samples in an oven at 60°C for a week and then weighed on a measuring balance.

Analysis of plant histology

Accumulation of RB5 dye in the plant tissue was also investigated by looking at the root anatomical structure under the microscope (Chandanshive et al. 2016; Kadam et al. 2018). Briefly, root tissues of plants of three replicates of different treatments (C3, T1, and T1*) were cut at a distance of 10–20 mm from the apex. At this part, root cells are well-differentiated and are generally active in the uptake of elements/compounds. The root segments were washed with 5 ml of de-ionized water and then embedded in 5% agar. Approximately 50 μm cross-sections were made with a razor blade. The cross-sections of roots were mounted in glycerin after overlying with coverslips and were observed under a Trinocular Microscope (Leica DM4000 B, Germany) with 100 × magnification.

Toxicity analysis of the water having RB5

Toxicity of untreated and treated waters was tested using *Labeo rohita* (a freshwater dwelling fish) as reported earlier (Ashraf et al. 2020). Treated water (twenty liters) by different treatments (C1, C2, T1, and T1*) were put in each clean glass aquarium (in triplicates), as per the experimental design. Ten *L. rohita* of size (~ 82 ± 10 mm) and weight (~ 4.5 ± 0.2 g) were added to each aquarium. The survival rate was assessed by counting the number of alive fish after 96 h with a 16:8 h (light: dark) photoperiod. Behavioral responses, such as liveliness, sense of balance, opercular motion, swimming velocity, sideways movement, frontward motion with the posterior side up, and motion in a circular fashion with jerks, were evaluated through visual observation (Ashraf et al. 2020).

Results And Discussion

Effect of RB5 on plant growth and its decolorization

To determine the effect of RB5 dye concentrations and bacterial augmentation on root and shoot lengths and dry weights were determined (Fig. 2). There was significantly (ANOVA, $p < 0.05$) less root (10.1% and 31.5%) and shoot (7.7% and 40%) lengths and dry weight (21.4% and 48% of root, and 12.2% and 47.3% of shoot) of the plants in T1 and T1*, respectively, than of the plants growing in tap water (C3) (without dye and bacteria). Between two inoculated treatments, *P. australis* at the dye concentration 200 mg L^{-1} exhibited more development in root and shoot length, and root and shoot dry weight than the plants grown in the water having RB5 dye concentration 1000 mg L^{-1}. Previous studies showed that azo dyes inhibited *ATPase* activity of the plant, photosynthetic oxygen evolution, and plant growth (Zhou and Xiang 2013; Kumar et al. 2018; Wei et al. 2020).

The presence of RB5 dye in water affects the performance of biological processes due to its toxicity or intrusion in the capability of enzymes (Iglesias et al. 2013; Glugoski et al. 2017; Lumbaque et al. 2017; Bilal et al. 2018). Therefore, the initial dye amount possesses a substantial role in the efficiency of the dye elimination process. In our experiment, there was significantly (ANOVA, $p < 0.05$) more color decolorization in the waters treated by T1 (95.5%) and T1* (45%) than in the untreated waters (C1 and C2) (Fig. 3). However, the decolorization efficiency of the FTWs decreased with the increase in the initial dye concentration. After 15 days, the decolorization efficacy of bacterial-augmented FTWs was 95.5%

(200 to 9 mg L^{-1}) at the 200 mg L^{-1} of dye. This high color decolorization might be due to the presence of RB5-degrading bacteria in the FTWs (Tara et al. 2019a). However, in the experimental design of this study, FTWs treatment with plants but without bacteria, for each dye concentration, was missed. In an earlier study, the bacterial inoculation in constructed wetlands, vegetated with *P. australis*, significantly (ANOVA, $p < 0.05$) increased the removal of RB5 compared to the non bacterized controls (Riva et al. 2019). Plants have great power to degrade pollutants by making symbiotic relations with the dye degrading bacteria (Glick 2010; Nawaz et al. 2020; Shahid et al. 2020). In such relations, the root system of the plants helps contaminant utilizing microorganisms by providing nutritious compounds and favorable environmental conditions, thus increasing bacterial actions and contaminant breakdown in the root zone (Nie et al. 2011; Khan et al. 2013a,b; Fahid et al. 2020). It was also reported earlier that the plants alone have the natural capability to remove the organic contaminants from the water (Khan et al. 2013a; Li et al. 2014; Riva et al. 2019). The decolorizing ability of bacterial-augmented FTWs was 45% at the 1000 mg L^{-1} of RB5 dye. This reduction in the decolorization potential of bacterial-augmented FTWs could be because of the poisonous effects of RB5 dye on the plant and dye-degrading bacteria (Pearce et al. 2003; Khehra et al. 2005; Sayilgan and Cakmakci 2013).

Analysis of biodegradation products

The metabolites produced due to the biodegradation of a contaminant are absorbed by plants, or they are removed in gaseous forms, e.g., CO_2 and N_2. In a worst-case scenario, the metabolites produced during the degradation of azo dyes, especially the aromatic amines, may exert even more toxicity than their parent dye compounds (Kalme et al. 2007; Ding et al. 2009; Wang et al. 2009; Aksu et al. 2015). Therefore, the comprehensive description of the biodegradation products and intermediates of synthetic dyes has a significant role in the assessment of the ecological fate of these contaminants (Ribeiro et al. 2017; Hasanin 2020).

In this study, to identify the biodegradation products of RB5 dye in bacterial-augmented FTWs, the treated dye solution (T1) with maximum (95.5%) color removal was analyzed by ESI-MS/MS. The MS/MS spectra of the RB5 and its biodegradation fragments are shown in Supplementary data (Fig. S1 and S2). The RB5 is a poly-sulfonated dye and its mass spectrum is categorized by a high extent of fragmentation and an increased level of background noise. The main functional group in the dye molecule influences its ionization response in the ESI/MS spectrum. The sodium molecular ion [M-xNa]$^{x-}$ peaks with detected charges equal to the number of sodium, were earlier categorized as precursor ions (Khandare et al. 2012). Following the previous findings, in this study, singly charged *m/z* 968 [M-Na]$^{-1}$, doubly charged *m/z* 473 [M-2Na]$^{2-}$, and triply charged *m/z* 307 [M-3Na]$^{3-}$ ion peaks were observed in the ESI-MS spectrum of RB5 (Fig. 4A). In comparison to the initial dye, no such parent dye molecular ion peaks with single, double, and triple-negative charges were observed in the ESI-MS spectrum of treated dye. Conversely, some new peaks appeared (Fig. 4B), which indicates that degradation intermediates have been formed. According to the new peaks in the ESI-MS, a total of twenty intermediary products were recognized by the mass spectrometric analysis (Fig. 5). Based on the ESI-MS/MS analysis, the dye degradation intermediates were identified and the biological degradation pathway of RB5 was suggested (Fig. 6). In the negative ion

ESI-MS analysis, the sulfonic groups commonly lower the comparative numbers of the peaks of deprotonated molecules [M-H]$^-$ and cause less stability of quasi-molecular ions. Moreover, the presence of sulfate ester ($-OSO_3Na$) and sulfonate ($-SO_3Na$) groups frequently produces many fragmentations with distinctive neutral losses of SO_3, SO_2, and SO in the mass spectra. The hydrolysis of the SO_3Na groups in RB5 happened during the biological treatment producing fragments [(3, MW = 844) and (5, MW = 764)]. The detection of degradation products (3), (5), and others (18 and 20) designates that the detachment of sulfonic groups commonly happens during the biological treatment of RB5. The peaks at *m/z* 969, 873, 698, 655, 536, 473, 437, 381, 339, and 255 were identified as degradation products (1, 4, 7, 8, 10, 12, 13, 14, 16, and 19), respectively. The production of the intermediates proposes that the conversion of double bonds into single bonds, break of azo bonds, and detachment of sulfonic groups with the subsequent addition of H-atoms, respectively, in the corresponding positions during the biological treatment process. Based on these facts, a reductive degradation pathway was proposed. Although, some degradation products (e.g. 3, 5, and 15) have also been observed in the ozonation and UV/TIO$_2$-based degradation of RB5 (Castro et al. 2017; Bilal et al. 2018). But in this study, a higher number of intermediates i.e., ion peaks at *m/z* 915, 709, 607, 527, 348, and 283 were identified as products (2, 6, 9, 11, 15, and 17), respectively, which were formed by elimination and substitution reactions. The steps in the proposed pathway give clues about the route of the dye metabolism during bacterial-augmented FTWs. Moreover, these metabolites might be further completely mineralized by the vegetation and their root-associated bacteria or absorbed by the plants.

Proposed degradation mechanism and profiling of intermediates

Based on the identified degradation metabolite ion peaks in Fig. 5, the reaction pathways for the degradation of RB5 in bacterial augmented FTWs are proposed (Fig. 6). As observed, three major reactions, namely, the conversion of double into single bonds, break of azo bonds, and detachment of sulfonic groups, along with the addition of H-atoms are involved in the degradation of RB5, which indicates that RB5 degradation proceeds through a reductive pathway (Ribeiro et al. 2017; Hasanin 2020). Particularly, the detachment of sulfonic mediety from the parent compound formed product 3 (when one -SO$_3$Na group is cleaved) and product 5 (when both -SO$_3$Na groups are eliminated). Then, product (6) is formed either from the product (3) via the elimination of OH and sulfonic groups or from compound (5) via the elimination of both the OH groups with the subsequent methylation reaction. Product (5) and (6) undergo further reactions and give rise to product (8), which gives rise to the formation of product (15) via the breakage of an azo bond by undergoing several elimination and addition reactions (Castro et al. 2017; Bilal et al. 2018). Later, product (16, *m/z* = 339) is formed either from the product (15) by the elimination of amino groups with the subsequent hydroxyl addition reactions and by the addition of H-atoms at both the sulfonic groups or from the product (14) by the removal of N$_2$ and H$_2$O molecules. Product (16) can be further transformed into a product (19). The formation of products (16) and (19) is comparable to the fragments formed after the break of the azo bond in the azo dyes Remazol Black B (RBB) and Direct Red 5B (DR5B) when treated by plants in association with inoculated bacteria (Khandare et al. 2013b; El Bouraie and El Din 2016). Finally, product (15) can also be converted into a product (20) by the elimination of SO$_3$, O$_3$, and NH$_2$ groups.

Anatomical analysis of root tissues of *P. australis*

At the end of the experiment, the roots of *P. australis* were examined for histochemical studies to get knowledge about the transfer of RB5 in the tissues. The analysis of root tissues of *P. australis* grown in tap water (C3) and exposed to dye concentration (200 mg l^{-1}, T1) showed normal shape, size, and bearing no color (Fig. 7). Whereas, the root cells of *P. australis* exposed to dye concentration (1000 mg l^{-1}, T1*) exhibited the presence of residues of the dye in the outer epidermal cells, with an increase in the adjacent cortical cells. Moreover, the dye concentration (T1*) caused epidermal and cortical cells to shrink, and some of the cells were deformed. These observations revealed the uptake of the dye in the epidermal as well as in the cortical part. In an earlier study, Chandanshive and coworkers studied the accumulation of a dye, Rubine GFL, in the root tissues of *Salvinia molesta* (Chandanshive et al. 2016). They observed that the dye was stated to be compiled in the epidermal cells after 12 h of contact and subsequently transported to the cortex after 48 h. In another earlier study, methyl orange was found in the root epidermis of *Ammannia baccifera* and *Fimbristylis dichotoma* after 24 h of exposure, and was found in the cortex after 48 h and completely vanished after 72 h (Kadam et al. 2018).

Fish toxicity assay

Discharge of colored textile wastewaters directly into lakes and rivers in most of the developing countries has a toxic impact on aquatic life (Liu et al. 2004; Carmen and Daniela 2012). Therefore, it is important to evaluate the toxic effects of dyes and their metabolites (degradation products). In this study, the toxicity analysis revealed that the untreated water having 1000 mg RB5 L^{-1} (C1) was significantly ($p < 0.05$) more toxic (as all fish died) than the water having 200 mg RB5 L^{-1} (C2) (as 8 out of 10 fish died) (Table 2). However, treated waters by bacterial-augmented FTWs (T1 and T1*) were significantly ($p < 0.05$) less toxic than the untreated water (C1 and C2). The treated water (T1) initially having a concentration of 200 mg L^{-1} of RB5 was significantly ($p < 0.05$) less toxic (no fish died even after 96 h) than the untreated waters (C1 and C2) and the treated water (T1*) initially having a concentration of 1000 mg L^{-1} of RB5. The observations thus showed that the metabolites were less toxic in the treatment T1 than the parent compound (untreated dye solution), where eight fish died. The results highlight the possible implication of FTWs in association with bacteria for the detoxification and mineralization of azo dyes.

The investigation of behavioral changes of *L. rohita* is an important indicator in the assessment of the toxicity of a pollutant. Fish showed more behavior changes, such as liveliness, sense of balance, opercular motion, swimming velocity, sideways movement, frontward motion with the posterior side up, and motion in a circular fashion with jerks when exposed to untreated waters (C1 and C2) as compared to the treated waters (T1 and T1*) (Table 3). Earlier studies also reported that biological treatments, including phytoremediation, reduced the toxic effects of the pollutants present in the water (Ashraf et al. 2020; Shehzadi et al. 2014, Tara et al. 2019b). Moreover, fish showed more changed behavior in the treated water (T1*) initially having RB5 concentration of 1000 mg L^{-1} as compared to the treated water (T1) initially having a concentration of 200 mg L^{-1} of RB5. The abnormal behavioral acts include lying

laterally at the bottom, loss of balance, swimming forward in circular form with jerks, rapid opercular movements with opened mouth, and forward movement with the posterior side up.

Conclusions

Floating treatment wetlands, vegetated with *P. australis* and inoculated with the dye-degrading bacteria (*A. junii* NT-15, *P. indoloxydans* NT-38, and *Rhodococcus sp.* NT-39), efficiently decolorized (95.5%) the RB5 dye in T1 having 200 mg L^{-1} of RB5. While, dye concentration (1000 mg L^{-1}) in T1* was found more toxic to the plants, and the dye decolorization was decreased up to 45%. Although in the experimental design of this study, FTWs treatment with plants but without bacteria was missed, bacterial augmented FTWs degraded and transformed RB5 dye into different metabolites predominantly following the reductive pathway. ESI-MS/MS analysis revealed the identification of 20 metabolite products. The conversion of double bonds into single bonds, the break of azo bonds, and the detachments of sulfonic groups with the subsequent addition of H-atoms are proposed as the major reductive reaction in the proposed pathway. The metabolites formed after RB5 dye degradation were found to be non-toxic when checked by fish toxicity assays. Thus, it is proposed that bacterially augmented FTWs could be a wise strategy for the degradation of RB5 dye and could lead to a low cost and environment-friendly method for future textile wastewater clean-up programs.

Declarations

Competing interests: It is declared that all the authors have no competing interests.

Declarations

All manuscripts must contain the following sections under the heading 'Declarations':

Ethics approval and consent to participate (not applicable)

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- Authors' information (optional)

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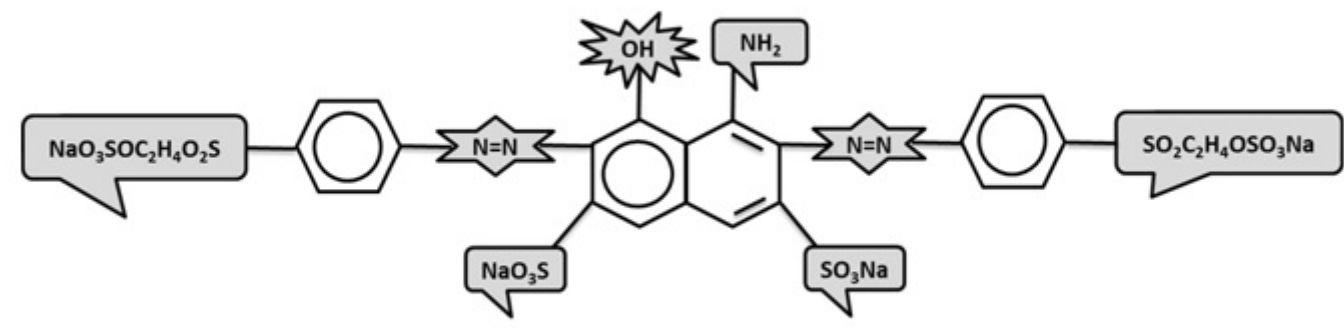
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Tables

Table 1. Physiochemical characteristics of the Reactive Black 5

| Molecular formula: $C_{26}H_{21}N_5Na_4O_{19}S_6$ | Molecular wt. (g mol^{-1}): 990.87 | Absorbance-λ max. [nm]: 598 |

Table 2. Fish toxicity assay of Reactive Black 5 dye solution treated by floating treatment wetlands augmented with the bacteria

Treatment	Total fish	Death over time										Total deaths	Death (%)
		0h	1h	2h	4h	8h	12h	24h	48h	72h	96h		
C1	10	0	0	0	0	2	1	2	1	1	1	8[b] (0.47)	80
C2	10	0	2	3	4	1	0	0	0	0	0	10[a] (0.0)	100
T1	10	0	0	0	0	0	0	0	0	0	0	0 (0.0)	0
T1*	10	0	0	0	0	1	0	2	1	1	1	6[c] (0.47)	60

Control 1 (C1) = RB5 at 200 mg L^{-1} without *P. australis* and bacterial augmentation, Control 2 (C2) = RB5 at 1000 mg L^{-1} without vegetation and bacterial inoculation, T1 = FTWs planted with *P. australis* and augmented with bacteria for the remediation RB5 (200 mg L^{-1}) solution, and T1* = FTWs vegetated with *P. australis* and inoculated with bacteria for the remediation RB5 (1000 mg L^{-1}) solution. All values are the means of triplicates, whereas values in the parenthesis represent the standard error, and means followed by the same letter are not significantly different at a 5% level of significance.

Figures

Figure 1

Experimental set up of FTWs for the degradation of Reactive Black 5 (RB5). Control 1 (C1) (unvegetated and uninoculated): dye solution having 200 mg RB5 L-1 water, Control 2 (C2) (unvegetated and uninoculated): dye solution having1000 mg RB5 L-1water, Control 3 (C3) (vegetated and unioculated): tap water having FTWs, T1: FTWs having dye (200 mg RB5 L-1 water) with the bacterial consortium, T1*: FTWs having dye (1000 mg RB5 L-1 water) with the bacterial consortium.
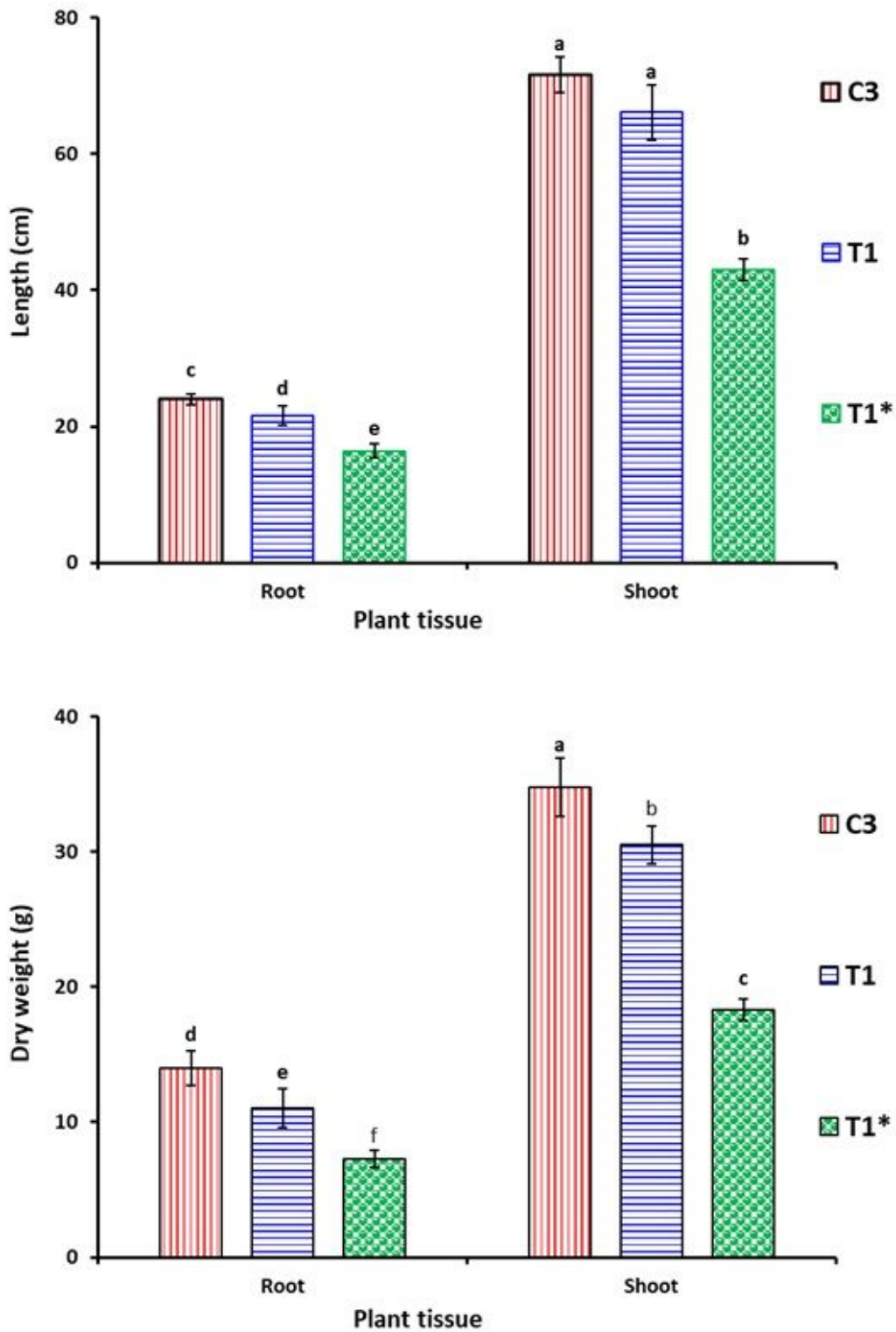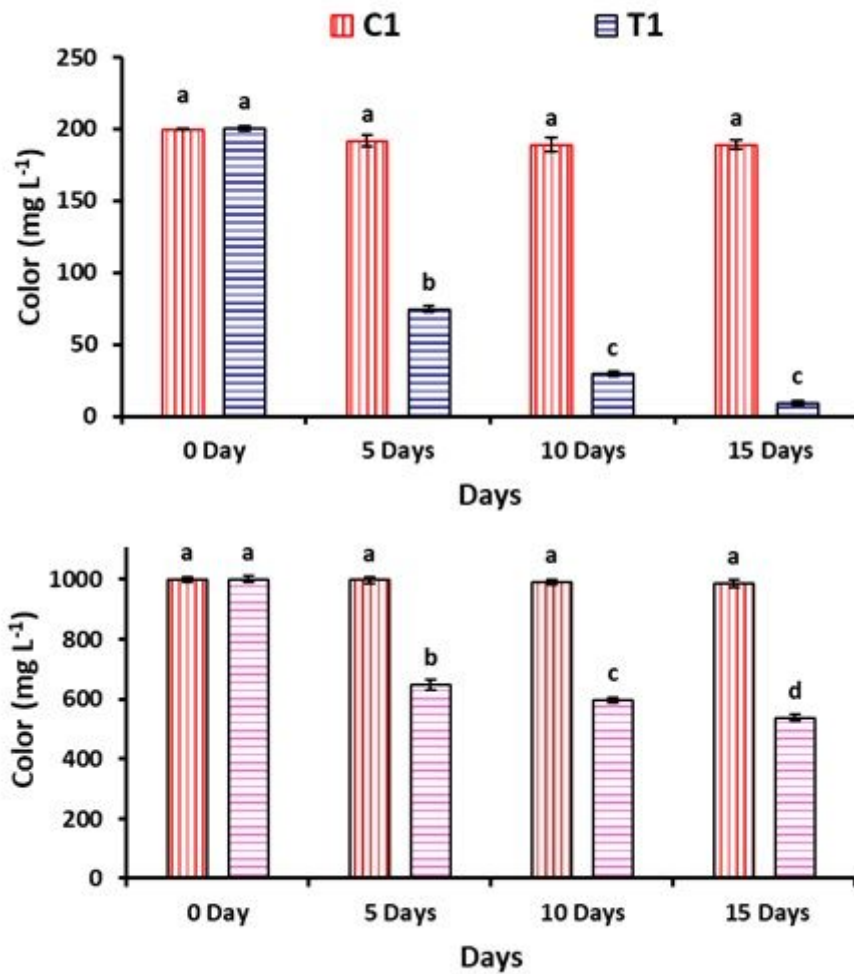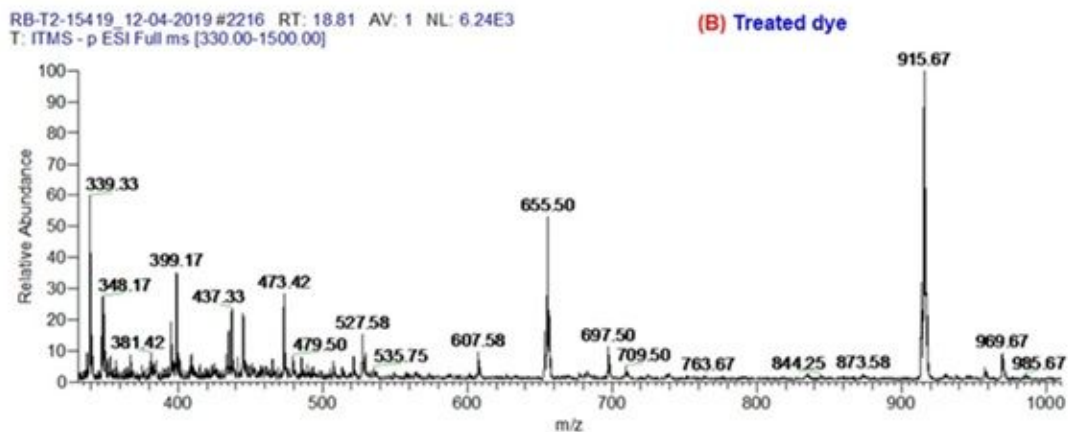
Figure 2

Effect of dye concentration on growth (length and weight of root and shoot) of P. australis inoculated with the bacterial consortium. Control (C3) (tap water with P. australis and without bacterial consortium), T1 = RB5 dye solution (200 mg L-1) with P. australis and bacterial consortium, and T1* = RB5 dye solution (1000 mg L-1) with P. australis and bacterial consortium. Values are the means of three

replicates, error bars represent the standard error and means followed by the same letter are not significantly different at a 5% level of significance.
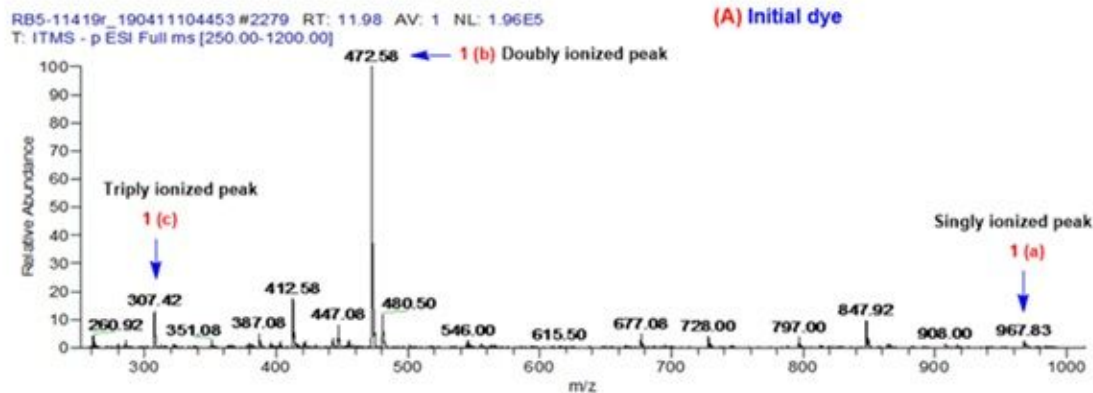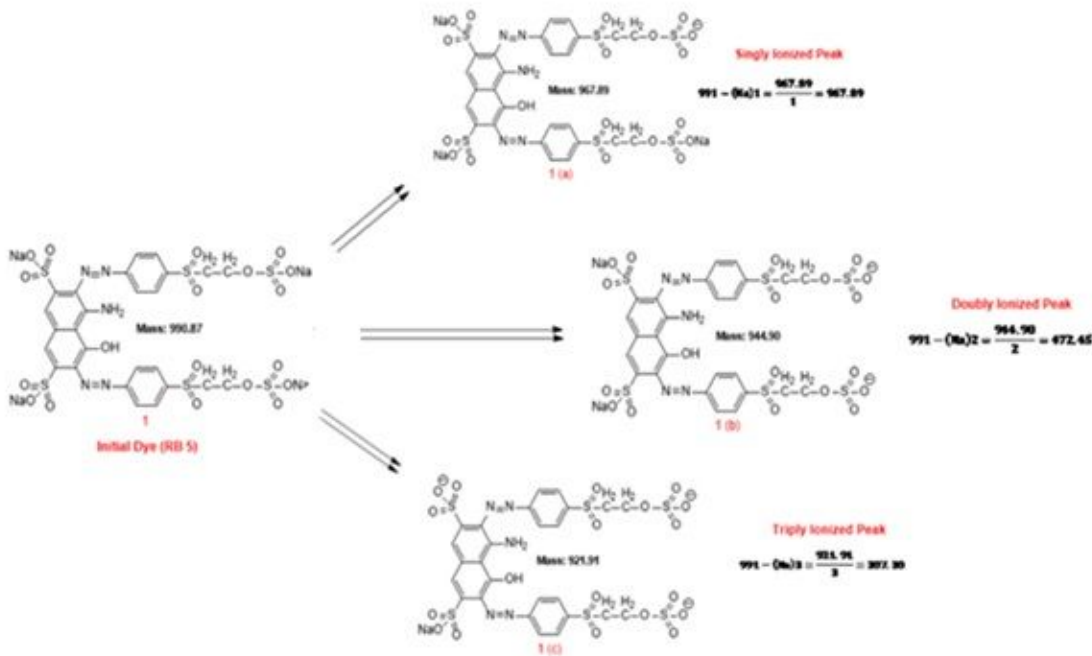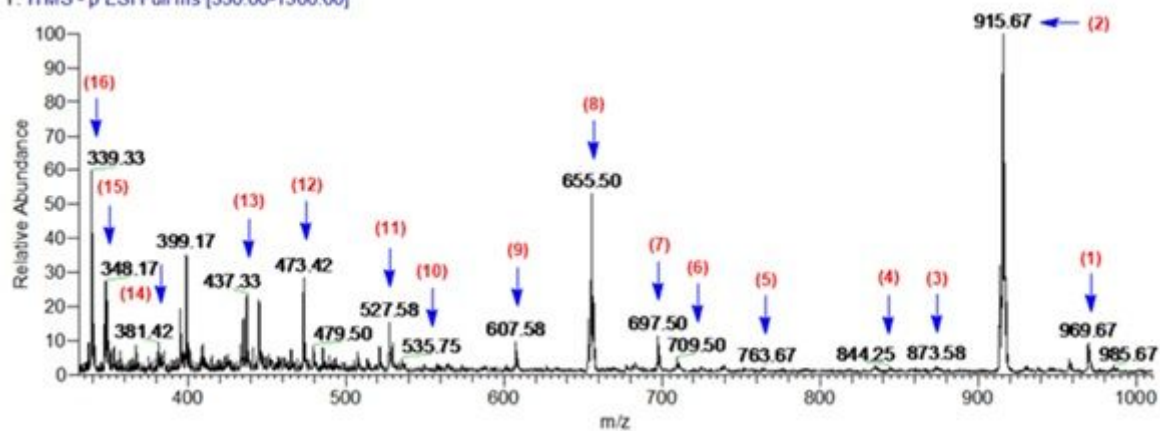

Figure 3

The concentration of color of Reactive Black 5 in water treated by FTWs vegetated with Phragmites australis at different time intervals. Control 1 (C1) (unvegetated and uninoculated): dye solution having 200 mg RB5 L-1 water, Control 2 (C2) (unvegetated and uninoculated): dye solution having1000 mg RB5 L-1 water, T1: FTWs having dye (200 mg RB5 L-1 water) with the bacterial consortium, T1*: FTWs having dye (1000 mg RB5 L-1 water) with the bacterial consortium. All values are the means of triplicates, whereas error bars represent the standard error, and means followed by the same letter are not significantly different at a 5% level of significance.

Figure 4

ESI-MS full scan analysis of Reactive Black 5 at negative ion mode. (A) initial dye (without treatment), and (B) treated dye through plant assisted bacterial degradation in floating treatment wetlands (T1).
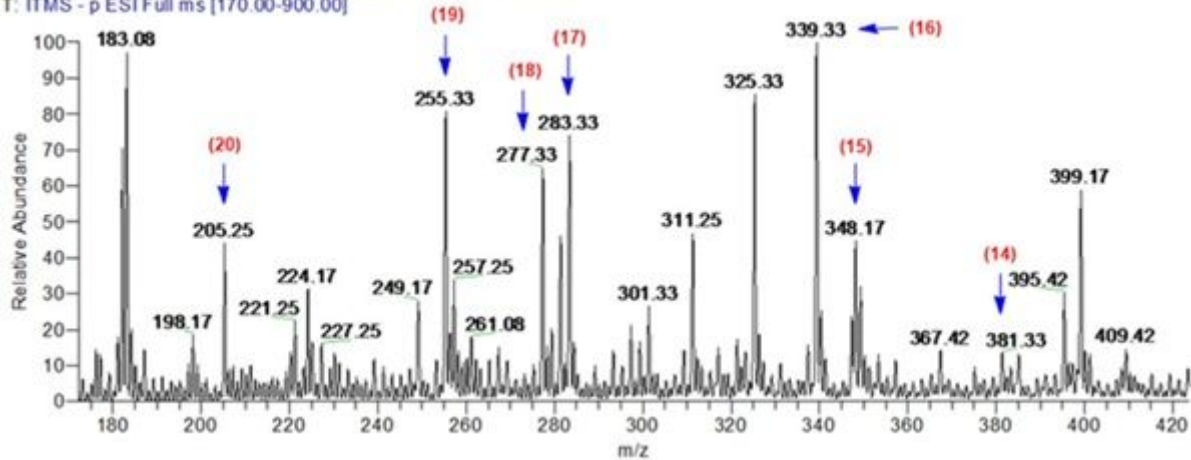
Figure 5

Identification of organic intermediates by ESI-MS analysis of RB5 at 200 mg L-1 concentration treated by FTWs vegetated with P. australis and augmented with the bacteria (T1).
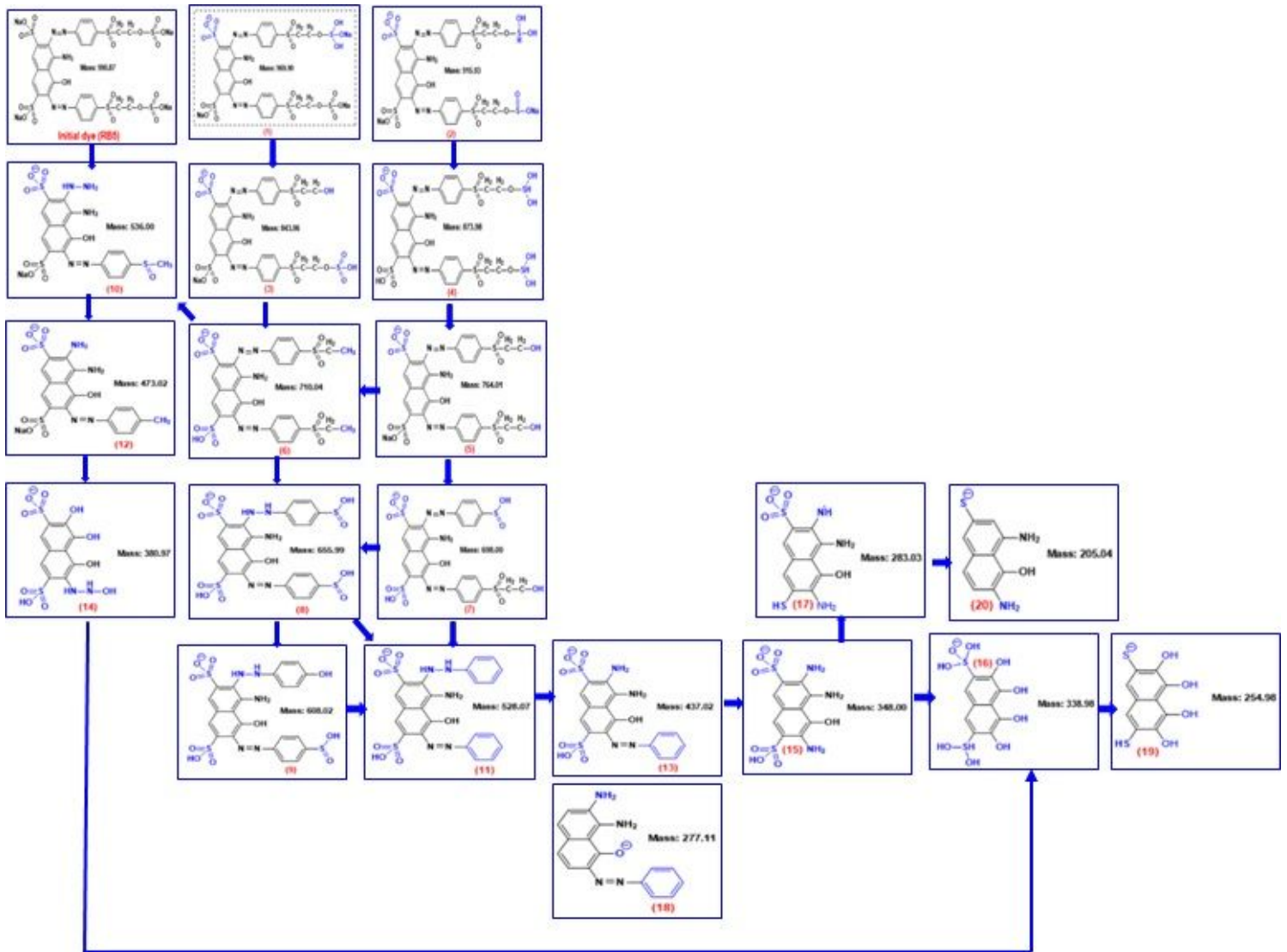
Figure 6

Degradation pathways of Reactive Black 5 treated by FTWs vegetated with P. australis and augmented with the bacteria (T1) based on mass spectral data.
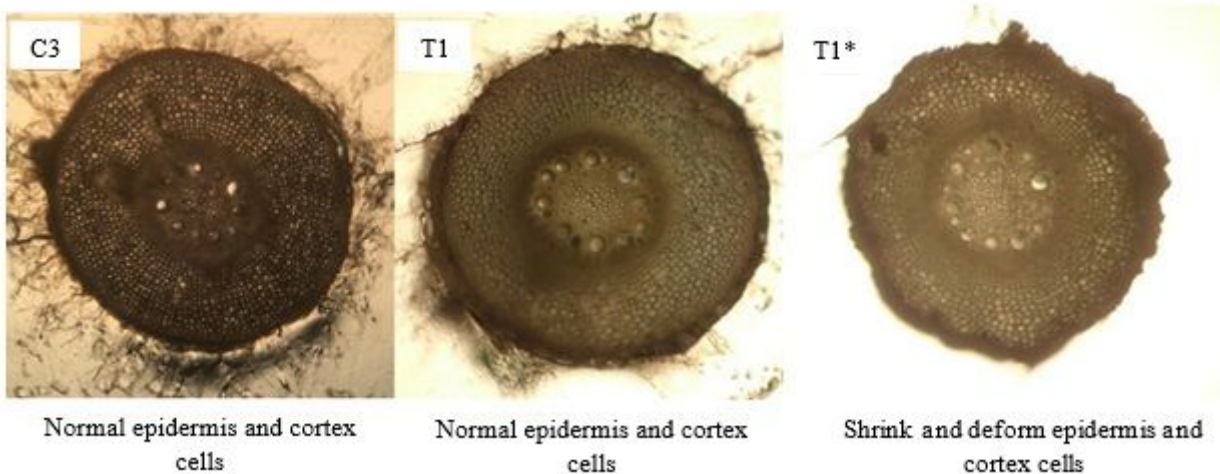

Figure 7

Anatomy of the root of Phragmites australis. Plants were grown in tap water (C3), plants exposed to low RB5 dye concentration (T1), and plants were exposed to high RB5 dye concentration (T1*).

Supplementary Files

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