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# Vegetation-Associated Enhancement of Azo Dye Removal in Constructed Wetlands Without External Carbon Addition

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**Abstract**

Constructed wetlands (CWs) are a low-energy alternative for treating dye-containing wastewater; however, the mechanisms enabling azo dye removal without external carbon supplementation remain unclear. This study demonstrates that azo dye reduction can proceed under oxic bulk conditions in CWs through vegetation-induced microscale redox heterogeneity. Lab-scale CWs planted with cattail and papyrus were evaluated for the removal of Reactive Orange 16 (RO16, monoazo) and Reactive Black 5 (RB5, diazo) at influent concentrations of 10–50 mg/L under varying ambient temperature (2–36 °C) and hydraulic retention time (1–15 days). Vegetated CWs consistently outperformed the unplanted system, achieving 60–95% removal for RO16 and up to 98% removal for RB5, whereas the unplanted CW showed substantially inferior performance, with removal efficiencies below 54% for RO16 and below 37% for RB5. Dye-decolorizing bacteria, including *Priestia megaterium* and *Clostridium* spp., were isolated exclusively under anaerobic conditions from vegetated CWs despite oxic bulk dissolved oxygen levels. The isolates did not decolorize dyes under aerobic conditions or when dyes were provided as sole carbon sources, indicating that azo dyes functioned as electron acceptors and required additional electron donors. These results suggest that vegetation promotes localized reductive microenvironments and supplies endogenous organic carbon, enabling anaerobic azo bond reduction within otherwise oxic systems. The findings indicate a mechanistic basis for plant–microbe interactions in CWs and support the design of sustainable treatment systems for dye-containing wastewater without external carbon input, particularly in warm regions. This study resolves a long-standing question of how azo dye reduction proceeds in CWs without external carbon input.

**Keywords:** constructed wetlands; azo dye decolorization; rhizosphere microenvironment; root exudates; anaerobic bacteria

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## 1. Introduction

The discharge of dye-containing wastewater from textile industries remains a significant environmental concern because of its intense coloration, chemical stability, and potential toxicity. Azo dyes account for more than 60% of synthetic dyes produced worldwide and are extensively used in textile manufacturing [1]. Due to their widespread production and discharge, they are frequently detected in industrial wastewater [2]. Their azo bonds (–N=N–) confer resistance to conventional biological treatment, and incomplete degradation

can generate aromatic amines with carcinogenic and mutagenic properties [3]. Therefore, the development of low-cost and effective treatment technologies for azo dye-containing wastewater is an urgent environmental management priority.

Physicochemical treatment methods such as adsorption [4], photocatalytic oxidation [5], and ozonation [6] have been investigated for dye removal. Although these approaches can achieve high decolorization efficiencies, they generally require high operational costs, chemical consumption, and energy input, limiting their feasibility for decentralized or resource-limited regions [7,8]. Constructed wetlands (CWs) represent a low-energy and nature-based alternative that integrates physical adsorption, plant uptake, and microbial transformation processes [9,10].

Previous studies have demonstrated effective removal of azo dyes, particularly Reactive Black 5 (RB5), in CWs [11–13]. In some cases, enhanced decolorization in CWs was achieved through bioaugmentation of dye-decolorizing bacteria [12,14] or supplementation with external carbon sources such as acetate [13], suggesting that electron donor availability plays a critical role in azo bond reduction. However, the mechanisms governing dye removal in CWs remain incompletely understood. It is unclear whether vegetation primarily enhances dye removal through evapotranspiration and phytoaccumulation [15] or whether plant–microbe interactions actively modify redox conditions and stimulate microbial reductive processes.

Reactive Orange 16 (RO16) and RB5 were selected as representative azo dyes due to their widespread use in textile industries in this study. These dyes differ in chemical structure, with RO16 classified as a monoazo dye and RB5 as a diazo dye, leading to differences in electron demand and degradation behavior during reductive processes. While RB5 has been extensively studied in CWs [12–14], RO16 has received comparatively limited attention. Moreover, comparative assessments of monoazo and diazo dye removal under conditions without external carbon supplementation remain scarce. Therefore, these dyes were used as model compounds to investigate how molecular structure influences azo dye removal mechanisms in CWs and to provide insights for the design of treatment systems without continuous chemical input.

We hypothesized that vegetation enhances azo dye decolorization in CWs by supplying endogenous organic compounds that may serve as electron donors for indigenous microorganisms, potentially promoting reductive cleavage of azo bonds even when bulk dissolved oxygen (DO) concentrations remain high. These endogenous organic compounds may include low-molecular-weight organic acids (e.g., acetate and lactate), sugars, and amino acids released from plant roots, which can serve as electron donors for microbial reductive processes [16]. Previous studies have shown that anaerobic bacteria such as *Clostridium* spp. [17,18] and *Bacillus* spp. [19–21] can enzymatically reduce azo dyes, and that electron shuttles may facilitate extracellular reduction [22]. However, the mechanisms underlying these processes remain unclear.

This study provides new insight into azo dye removal in CWs without external carbon addition, highlighting the role of vegetation in promoting reductive conditions. Previous studies have shown that operational parameters such as temperature, influent concentration, and hydraulic retention time (HRT) influence azo dye removal in CWs. In addition, dye-decolorizing bacteria have been reported to play important roles in azo dye removal processes. The objectives of this study were to (i) evaluate the effects of vegetation and operational parameters (HRT and dye concentration) on the removal of RO16 and RB5, (ii) isolate and characterize dye-decolorizing bacteria associated with vegetated CWs, and (iii) propose a mechanistic framework describing plant–microbe interactions that facilitate reductive decolorization.

## 2. Materials and Methods

### *2.1. Synthetic Wastewater and Culture Media*

Synthetic wastewater consisted of a basal salt medium (BSM), whose inorganic composition was based on a previously reported formulation [23], supplemented with either RO16 ($C_{20}H_{17}N_3Na_2O_{11}S_3$, MW 617.54; Sigma-Aldrich, St. Louis, MO, USA) or RB5 ($C_{26}H_{21}N_5Na_4O_{19}S_6$, MW 991.82; Sigma-Aldrich) at concentrations of 10–50 mg/L.

The BSM (per liter of deionized water) contained $K_2HPO_4$ (21.8 mg), $KH_2PO_4$ (8.5 mg), $Na_2HPO_4{\cdot}12H_2O$ (44.6 mg), $NH_4Cl$ (1.7 mg), $MgSO_4{\cdot}7H_2O$ (22.5 mg), $CaCl_2$ (27.5 mg), and $FeCl_3{\cdot}6H_2O$ (0.25 mg). The pH of the BSM, containing multiple phosphate salts, was measured prior to use and was approximately 7.2 $\pm$ 0.2. No external readily biodegradable organic carbon source (e.g., glucose or acetate) was added to wastewater throughout the experiments, although plant-derived organic carbon may have been present in the vegetated CWs. The theoretical dissolved organic carbon (DOC) concentration of this synthetic wastewater was only 19.5 mg/L and 15.7 mg/L, even at a high dye concentration of 50 mg/L for RO16 and RB5, respectively.

R2A agar and potato dextrose agar plates (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) were used for microbial isolation. Dye-supplemented plates contained 50 mg/L RO16 or RB5. In addition, a dye-amended BSM solidified with 15 g/L agar and containing 50 mg/L RO16 or RB5 was prepared.

### *2.2. Lab-Scale CWs*

Laboratory-scale CWs were installed in a greenhouse at the Biwako Kusatsu Campus of Ritsumeikan University (Shiga, Japan). Each CW consisted of a plastic container with internal dimensions of 10.7 cm $\times$ 8.8 cm $\times$ 25.0 cm, filled with 1.3 kg of washed gravel (3–5 mm diameter; porosity 37.5%). The water level was initially set approximately 1 cm above the gravel surface; however, it fluctuated during operation due to evapotranspiration and occasionally dropped below the gravel surface.

Three configurations were prepared: (i) unplanted CW, (ii) cattail-planted CW (*Typha orientalis*; three plants; initial height 88 cm), and (iii) papyrus-planted CW (*Cyperus isocladus*; three plants; initial height 71 cm). Photographs of CW units are provided in the Supplementary Information (Figure S1). Plants were obtained from a commercial nursery (Tojaku Engei Co., Ltd., Kyoto, Japan) and acclimated in tap water for one week prior to wastewater treatment. Cattail and papyrus were selected based on their use in full-scale CWs treating batik wastewater in Indonesia [24]. These species differ in morphological characteristics, such as root structure and biomass production, allowing comparison of plant-dependent effects on azo dye removal. The relatively short acclimation period was selected to minimize changes in plant condition prior to the experimental phase and to ensure comparable initial conditions across treatments. An unplanted CW was included as a control to isolate the effect of vegetation on dye removal performance and to distinguish plant-mediated processes from those occurring in the substrate alone. All experimental conditions were conducted in duplicate.

### *2.3. Operation of CWs*

The CWs were operated without external carbon supplementation to evaluate the removal of RO16 and RB5 under varying environmental and operational conditions. Ambient temperature varied naturally during the experiment and was monitored but not controlled as an independent variable. Synthetic wastewater was treated in the CWs in a sequencing batch mode. The dye concentration, period, and batch count are shown in Supplementary Table S1. On the first day, wastewater of 500 mL (influent) was poured into the CWs. Each CW treated 500 mL of wastewater per batch cycle in a container with a surface area of

approximately 94 $cm^2$. This corresponds to a laboratory-scale hydraulic loading condition and was intended to enable controlled comparison of treatment performance rather than to replicate full-scale operation. After the defined HRT, the treated water (effluent) was drained from each CW. Then, fresh wastewater (500 mL) was again poured into the CWs. This operation was repeated for all CWs at every defined interval.

The operation consisted of two experimental phases for each dye: (i) a concentration–temperature phase at a fixed HRT of 5 days, and (ii) an HRT phase at a fixed influent concentration of 50 mg/L. For RO16, the influent concentration was gradually increased from 10 to 50 mg/L under natural ambient temperature conditions (August 2020–May 2021). Subsequently, the HRT was varied from 1 to 15 days at 50 mg/L RO16 (April–September 2021). For RB5, the influent concentration was gradually increased from 10 to 50 mg/L at a fixed HRT of 5 days (July–August 2021), followed by HRT variation from 1 to 15 days at 50 mg/L RB5 (August–September 2021). The same CW units were used continuously throughout the entire experimental period.

### *2.4. Isolation and Identification of Azo Dye Degrading Microorganisms*

Effluent samples from the CWs were serially diluted, and 0.1 mL aliquots were spread onto PDA and R2A agar plates supplemented with 50 mg/L RO16 or RB5. Plates were incubated at 28 °C for up to 14 days under both aerobic and anaerobic conditions. Anaerobic conditions were established using an AnaeroPack system (Gas Chemical Co., Inc., Tokyo, Japan). Colonies exhibiting clear decolorization zones were selected and purified through repeated streaking.

Genomic DNA was extracted from the isolates, and approximately 500 bp fragments of the V3–V4 region of the 16S rRNA gene were amplified using the universal primer pair 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′), and amplicon sequencing was performed by Seibutsu Giken Co., Ltd. (Sagamihara, Japan) following the protocol described previously [25]. Sequence similarity was determined by comparison with reference sequences. Accession numbers in the DDBJ database are provided in Tables 1 and 2.

**Table 1.** Partial 16S rRNA gene sequence identities and accession numbers of RO16-decolorizing isolates obtained from vegetated CWs using R2A agar plates.

| Isolate | Source | DDBJ Accession No. (This Study) | Closest Relative | Accession No. (Reference Strain) | Sequence Similarity (%) | Oxygen Tolerance |
|---|---|---|---|---|---|---|
| T1 | Cattail-planted CW | DRA26315 | *Priestia megaterium* | CP049296.1 | 100 | Facultative anaerobe |
| T2 | Cattail-planted CW | DRA26316 | *Clostridium* sp. | MN334627.1 | 100 | Obligate anaerobe |
| P1 | Papyrus-planted CW | DRA26317 | *Clostridium Beijerinckii* | CP010086.2 | 100 | Obligate anaerobe |

**Table 2.** Partial 16S rRNA gene sequence identities and accession numbers of RB5-decolorizing isolates obtained from vegetated CWs using R2A agar plates.

| Isolate | Source | DDBJ Accession No. (This Study) | Closest Relative | Accession No. (Reference Strain) | Sequence Similarity (%) | Oxygen Tolerance |
|---|---|---|---|---|---|---|
| T3 | Cattail-planted CW | DRA26312 | Unclassified Alphaproteobacteria | LC106216.1 | 98.5 | Anaerobic |
| T4 | Cattail-planted CW | DRA21942 | Unclassified Porphyromonadaceae | KF504773.1 | 96.9 | Anaerobic |
| P3 | Papyrus-planted CW | DRA26313 | *Clostridium diolis* | CP043998.1 | 100 | Obligate anaerobe |
| P4 | Papyrus-planted CW | DRA26314 | Unclassified Clostridiaceae | KY124215.1 | 99.6 | Obligate anaerobe |

### *2.5. Analytical Procedures*

During each batch cycle, 15 mL of water was collected for water quality analysis. Dye concentrations were determined using a UV–Vis spectrophotometer by measuring absorbance at 494 nm for RO16 and 600 nm for RB5. Absorbance spectra were recorded

between 420 and 680 nm. The effluent from the CWs was centrifuged at 5000× *g* for 10 min, and the supernatant was collected for DOC measurement using a total organic carbon analyzer (TOC-V; Shimadzu Corp., Kyoto, Japan). Effluent DO was measured using a multiparameter meter (HQ30d) equipped with an LDO10101 probe (Hach, Loveland, CO, USA). Ambient air temperature in the greenhouse was continuously recorded using a TR-74Ui data logger (T&D Corporation, Matsumoto, Japan). Plant condition was assessed based on qualitative visual observations (e.g., leaf color, wilting, and overall vigor).

Mass removal efficiency (%) was calculated based on influent and effluent dye mass. Water loss due to evaporation and evapotranspiration was accounted for using a mass balance approach based on measured influent and effluent volumes, as previously described [24].

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by post hoc multiple comparison tests to evaluate differences among treatments. Differences were considered statistically significant at $p < 0.01$.

## 3. Results

### *3.1. Removal of RO16 in CWs*

#### 3.1.1. Effects of Influent Concentration and Temperature

Batch-wise variations in RO16 removal efficiency over an HRT of 5 days are presented in Figure 1, where removal efficiency is plotted against operational batch number. These data illustrate temporal trends under progressively changing influent concentrations and ambient temperatures.

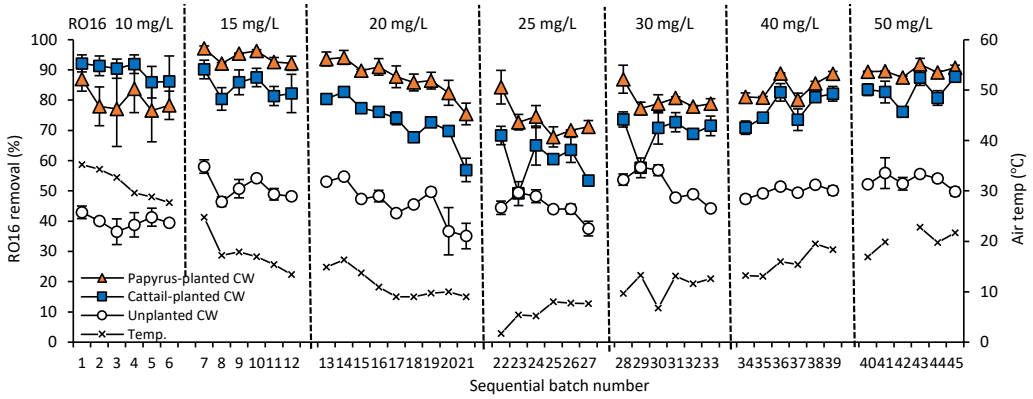



**Figure 1.** Batch-wise variation in RO16 removal efficiency in unplanted, cattail-planted, and papyrus-planted CWs in a sequencing batch mode overs a HRT of 5 days.

To provide an integrated overview of the relationship between influent concentration and RO16 removal, the same dataset is summarized in Figure 2. Ambient temperature varied naturally during the experimental period (Figure 1) and is included here as an environmental parameter. Across the tested concentration range (10–50 mg/L), the vegetated CWs consistently achieved higher removal efficiencies than the unplanted CW ($p < 0.01$). Except at 10 mg/L RO16, the papyrus-planted CW showed significantly higher removal efficiency than the cattail-planted CW ($p < 0.01$). Removal efficiency generally increased with ambient temperature. At temperatures above 15 °C, the vegetated CWs frequently achieved removal efficiencies exceeding 80%, whereas removal in the unplanted CWs remained below 54%. At an HRT of 5 days, the average removal rates at influent RO16 concentrations of 10–50 mg/L were 42–283, 95–440, and 85–476 mg/m$^2$/d in the unplanted, cattail-planted, and papyrus-planted CWs, respectively.

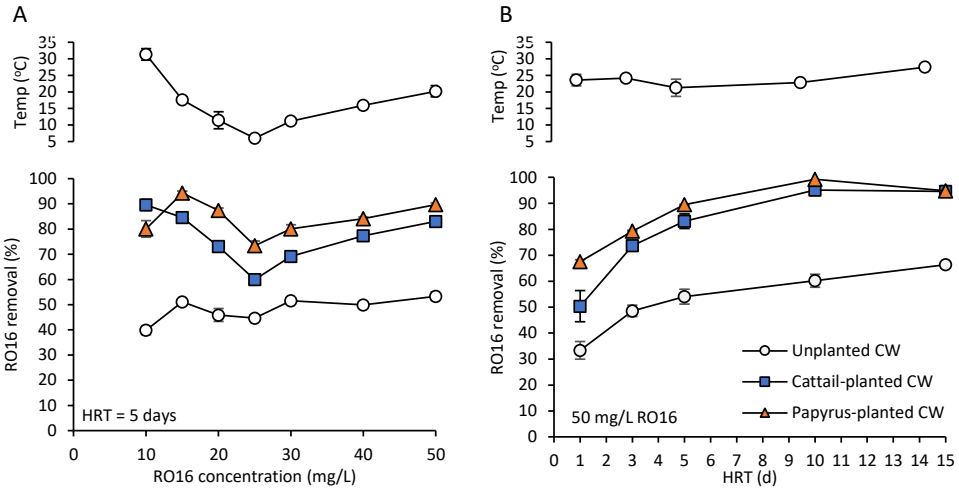



**Figure 2.** Effects of (**A**) influent RO16 concentration and (**B**) HRT on RO16 mass removal in CWs. Ambient temperature varied naturally and was not controlled as an independent variable.

Evaporation and evapotranspiration were consistently higher in vegetated CWs than in the unplanted system ($p < 0.01$), particularly under warm conditions (>27 °C), when water loss reached 15–28% in the unplanted CW, 22–50% in the cattail-planted CW, and 35–72% in the papyrus-planted CW. As temperatures declined to 2–8 °C, water loss decreased to <5% in the unplanted CW and <10% in the vegetated CWs. Effluent DO concentrations remained within oxic ranges throughout the study (7.2–11.5 mg/L in the unplanted CW, 4.5–8.3 mg/L in the cattail-planted CW, and 5.5–12.3 mg/L in the papyrus-planted CW). Even when the influent RO16 concentration was 50 mg/L, the effluent DOC remained low at approximately 10 mg/L, based on several measurements. Plant condition varied seasonally: both species remained physiologically active with green leaves at 13–25 °C, exhibited progressive senescence at 9–16 °C, and showed aboveground withering at 2–8 °C. With warming in spring (16–23 °C), new shoots emerged, indicating renewed growth.

Representative absorbance spectra for batches 1 and 41 showed a pronounced decrease in the characteristic RO16 peak at 494 nm in the vegetated CWs (Figure 3). In contrast, the unplanted CW exhibited incomplete attenuation of the main absorption band, and residual coloration remained in the effluent. When treating 10 mg/L RO16 (batch 1 at 35.2 °C), the effluent from the unplanted CW appeared more brownish than the influent, accompanied by a red shift in the maximum absorbance to 515 nm. By comparison, the effluents from the planted CWs were nearly transparent. Additionally, a new absorption band was observed below 420 nm in the CW effluents. For wastewater containing 50 mg/L RO16 (batch 41 at 19.9 °C), the absorbance at 494 nm decreased in all treated effluents, with a greater reduction observed in the planted CWs. Although the overall color intensity was reduced, a light orange coloration remained visible in the effluents from all CWs.

### 3.1.2. Effects of HRT

The influence of HRT on RO16 removal was evaluated at an influent concentration of 50 mg/L (Figure 2B). Increasing HRT from 1 to 15 days enhanced removal in all systems. The vegetated CWs consistently achieved higher removal efficiencies than the unplanted CW ($p < 0.01$). In the unplanted CW, removal increased from 33 to 66%. In the cattail-planted CW, removal increased from 50 to 95%, while in the papyrus-planted CW, removal increased from 67 to 99%.

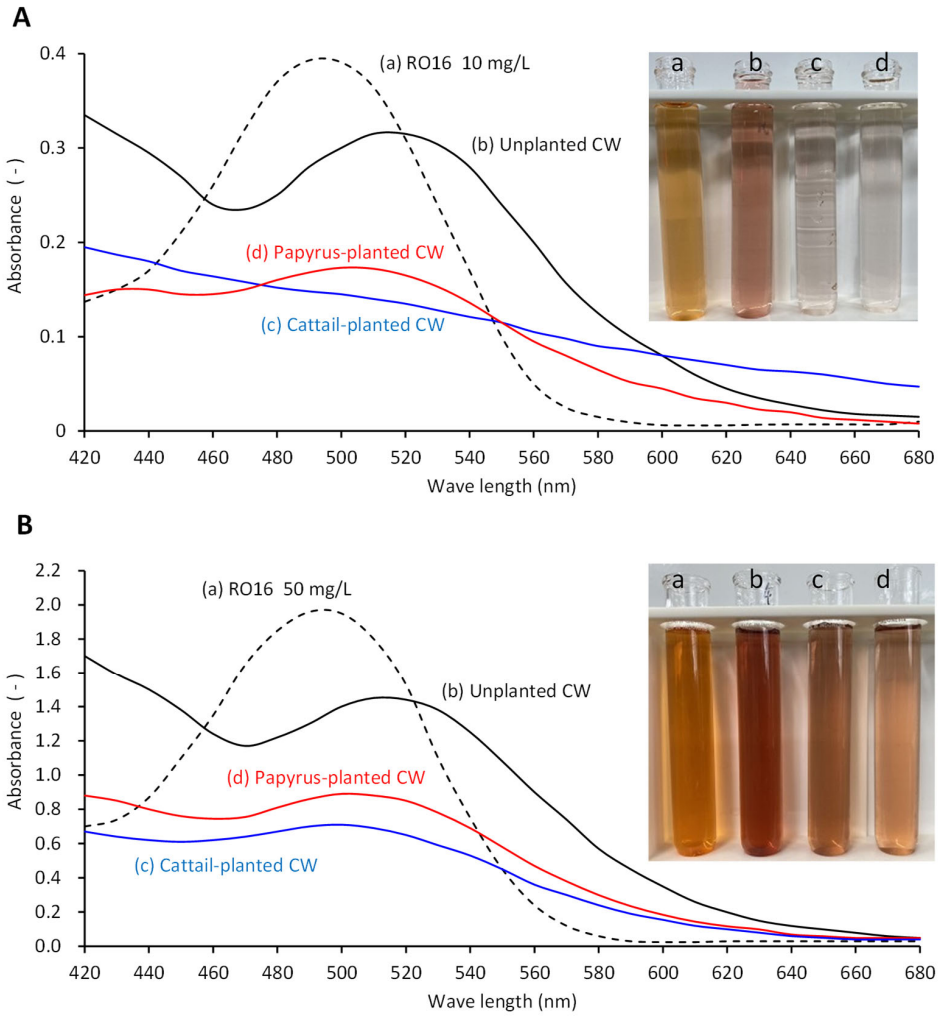



**Figure 3.** Absorbance spectra and corresponding visual appearance of influent and effluent from CWs operated over an HRT of 5 days, treating wastewater containing (**A**) 10 mg/L (batch 1 at 35.2 °C) and (**B**) 50 mg/L RO16 (batch 41 at 19.9 °C). The spectra and images illustrate the extent of decolorization.

### 3.1.3. Isolation of RO16-Decolorizing Bacteria

RO16-decolorizing bacteria were isolated exclusively under anaerobic cultivation conditions from effluents of vegetated CWs. Clear decolorization zones formed on RO16-supplemented R2A agar after 7–14 days of incubation. Decolorization extended beyond colony boundaries and spread across the agar surface, suggesting diffusion of soluble reducing compounds (Supplementary Figure S2). Halo size was not quantified due to the difficulty of frequent observation under anaerobic conditions and the rapid progression of decolorization. No decolorization occurred under aerobic conditions or when RO16 was supplied as the sole carbon source. These findings indicate that azo dyes functioned as electron acceptors during anaerobic decolorization by the isolated strains.

Strains T1 and T2 were isolated from the cattail-planted CW, whereas strain P1 was isolated from the papyrus-planted CW. Partial 16S rRNA gene sequencing revealed 100% sequence similarity to *Priestia megaterium* (formerly *Bacillus megaterium*) for strain T1, *Clostridium* sp. for strain T2, and *Clostridium beijerinckii* for strain P1. Detailed sequence information and accession numbers are provided in Table 1. The *Priestia* strain was facultatively anaerobic, whereas the *Clostridium* strains were obligately anaerobic; all isolates were Gram-positive and spore-forming.

### *3.2. Removal of RB5 in CWs*

#### 3.2.1. Effects of Influent Concentration and Temperature

Batch-wise variations in RB5 removal efficiency of an HRT of 5 days are presented in Figure 4. To provide an integrated overview of the relationship between influent concentration and RB5 removal, the same dataset is summarized in Figure 5. Ambient temperature varied naturally during the experimental period (Figure 4). Across the tested concentration range (10–50 mg/L), the vegetated CWs consistently achieved higher removal efficiencies than the unplanted CW ($p < 0.01$), and the cattail-planted CW further outperformed the papyrus-planted CW ($p < 0.01$).

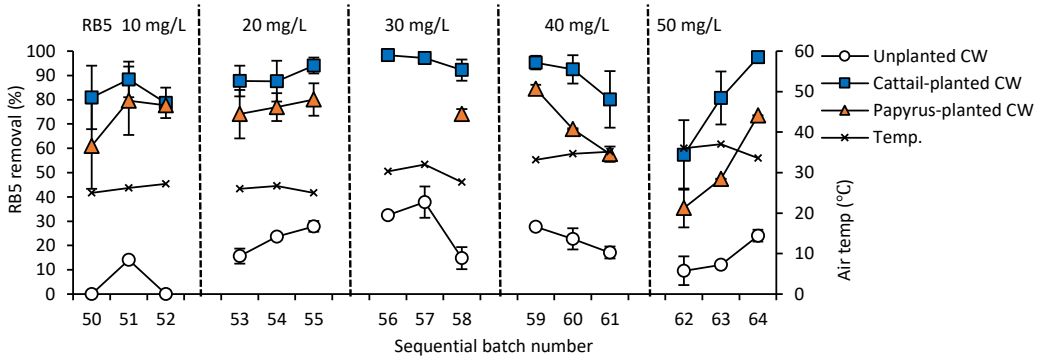



**Figure 4.** Batch-wise variation in RB5 removal efficiency in unplanted, cattail-planted, and papyrus-planted CWs in a sequencing batch mode of a HRT of 5 days.

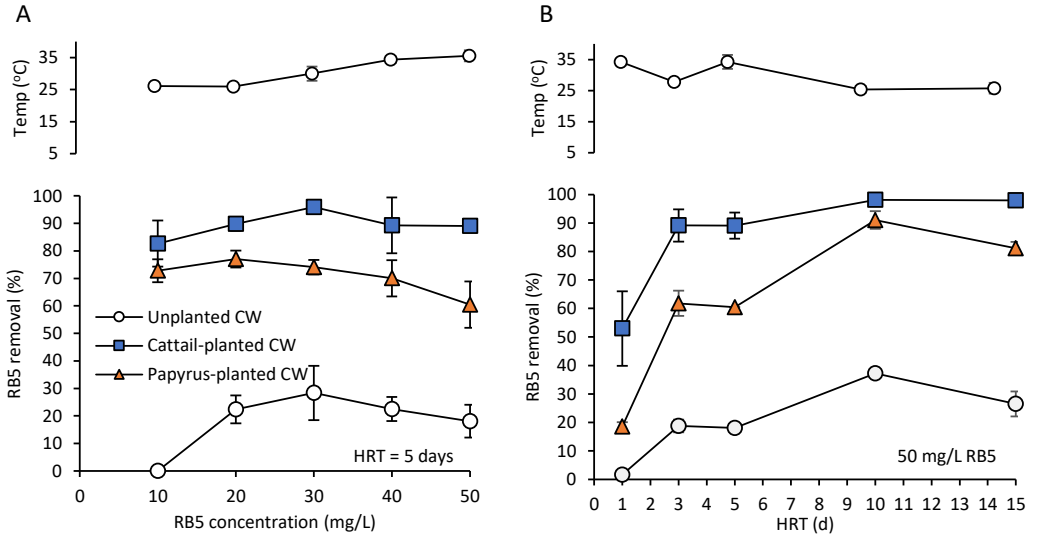



**Figure 5.** Effects of (**A**) influent RB5 concentration and (**B**) HRT on RB5 mass removal in CWs. Ambient temperature varied naturally and was not controlled as an independent variable.

Under warm conditions (approximately 25–36 °C), removal in the unplanted CW ranged from 0 to 28%, whereas the cattail-planted CW achieved 82–95% removal, and the papyrus-planted CW achieved 70–74% at 10–40 mg/L. At 50 mg/L, removal efficiency decreased in the papyrus-planted CW under warm conditions (25–37 °C), evaporation and evapotranspiration were consistently higher in vegetated CWs than in the unplanted CW ($p < 0.01$). With an HRT of 5 days, the average removal rates at influent RB5 concentrations of 10–50 mg/L were 0–96, 88–473, and 77–321 mg/m$^2$/d in the unplanted, cattail-planted, and papyrus-planted CWs, respectively.

Water loss ranged from 3 to 14% in the unplanted CW, 13–54% in the cattail-planted CW, and 22–73% in the papyrus-planted CW, generally increasing with HRT. Effluent DO concentrations remained within oxic ranges, measuring 6.1–9.3 mg/L in the unplanted

CW, 4.2–6.5 mg/L in the cattail-planted CW, and 6.2–8.2 mg/L in the papyrus-planted CW. Even when the influent RB5 concentration was 50 mg/L, the effluent DOC remained low at approximately 10 mg/L, based on several measurements.

Both cattail and papyrus remained healthy and exhibited active growth throughout the experiments. No visible withering or chlorosis was observed, and the plants maintained normal leaf coloration and structure despite exposure to RB5 concentrations up to 50 mg/L and varying HRT conditions. Plant vitality was sustained even under high dye loading and extended HRT.

For wastewater containing 20 mg/L RB5 (batch 53 at 26.0 °C), the absorbance at 600 nm decreased substantially in all treated effluents, with the greatest reduction observed in the cattail-planted CW, which produced the most transparent effluent among the CWs (Figure 6). For wastewater containing 50 mg/L RB5 (batch 62 at 36.0 °C), the absorbance at 600 nm likewise decreased in all treated effluents, again with a greater reduction in the cattail-planted CW. However, despite the decreased peak intensity, a discernible blue coloration remained in the effluents.

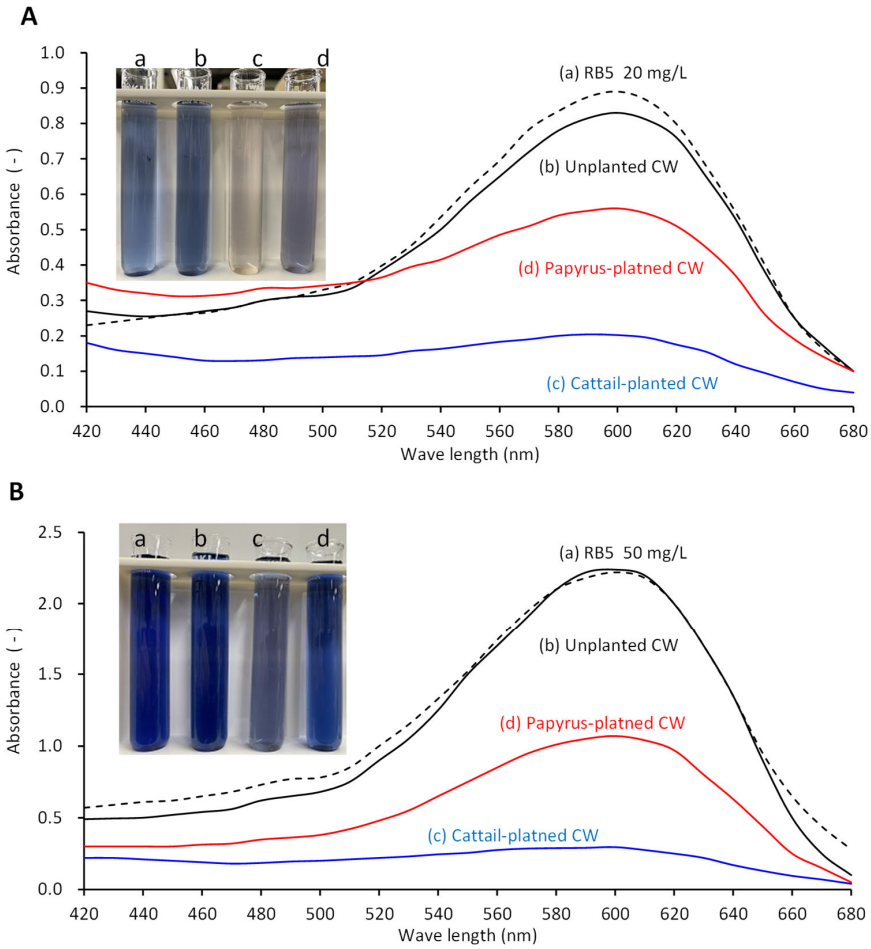



**Figure 6.** Absorbance spectra and corresponding visual appearance of influent and effluent from CWs operated at an HRT of 5 days, treating wastewater containing (**A**) 20 mg/L (batch 53 at 26.0 °C) and (**B**) 50 mg/L RB5 (batch 62 at 36.0 °C). The spectra and images illustrate the extent of decolorization.

### 3.2.2. Effects of HRT

The influence of HRT on RB5 removal was evaluated at an influent concentration of 50 mg/L (Figure 5B). Increasing HRT from 1 to 15 days enhanced removal in all CWs. Removal increased from 2 to 37% in the unplanted CW, from 53 to 98% in the cattail-planted

CW, and from 18 to 91% in the papyrus-planted CW, with significant differences observed among the three systems ($p < 0.01$).

#### 3.2.3. Isolation of RB5-Decolorizing Bacteria

RB5-decolorizing bacteria were isolated exclusively under anaerobic cultivation conditions from effluents of the vegetated CWs. Clear decolorization zones formed after 7–14 days of incubation. In some plates, decolorization extended beyond colony boundaries and spread across the entire agar surface (Supplementary Figure S3). Strains T3 and T4 were isolated from the cattail-planted CW, whereas strains P3 and P4 were isolated from the papyrus-planted CW. Partial 16S rRNA gene sequencing revealed sequence similarities of 98.5%, 96.9%, 100%, and 99.6% for strains T3, T4, P3, and P4, respectively, corresponding to an unclassified Alphaproteobacteria sequence, an unclassified Porphyromonadaceae sequence, *Clostridium diolis*, and an unclassified Clostridiaceae sequence. Detailed sequence information is provided in Table 2.

## 4. Discussion

### *4.1. Comparison with Previous CW Studies and Overall Performance*

RB5 has been widely studied in CWs, with reported removal efficiencies ranging from 45 to 95% depending on plant species, HRT, temperature, and external carbon addition [12,13]. For example, a cattail (*Typha angustifolia*)-planted CW achieved 91% removal of 50 mg/L RB5 with external carbon addition (1500 mg/L $CH_3COONa{\cdot}3H_2O$) over an HRT of 3 days in India [13]. In another study, a reed (*Phragmites australis*) system bioaugmented with *Acinetobacter junii*, *Pseudomonas indoloxydans*, and *Rhodococcus* sp. achieved 95.5% removal at 200 mg/L RB5 under ambient temperature conditions (20.5–36.5 °C) in Pakistan [12].

In contrast, the vegetated CWs in this study achieved comparable or higher removal efficiencies without external carbon addition, whereas unplanted CWs performed substantially worse. Higher removal efficiencies were observed at higher temperatures, suggesting enhanced microbial activity under warmer conditions. However, ambient temperature was not controlled as an independent variable in this study, and therefore, its effects on treatment performance should be interpreted with caution. Higher influent dye concentrations increased the loading to the system; however, no clear decrease in removal efficiency was observed. This suggests that sufficient electron donor availability was maintained even under higher dye concentrations, despite the absence of external carbon addition. Nevertheless, potential inhibitory effects of higher dye concentrations on microbial activity cannot be excluded. These findings highlight the critical role of vegetation in enhancing azo dye removal and demonstrate that high treatment performance can be achieved without external carbon addition.

To the best of our knowledge, RO16 has not previously been investigated in CWs. The present results, therefore, provide new insights into the behavior of monoazo dyes in such systems. Differences between monoazo (RO16) and diazo (RB5) dyes suggest that molecular structure influences treatment performance. RO16 contains a single azo bond, which likely requires fewer electrons for reductive cleavage and may influence removal performance. While the papyrus-planted CW achieved higher RO16 removal, the cattail-planted CW performed better with RB5; however, these results may be confounded by variations in plant growth conditions, precluding a definitive conclusion. Overall, these findings suggest that molecular structure plays an important role in treatment efficiency in CWs, although the underlying mechanisms remain inferential.

### *4.2. Vegetation-Induced Reductive Microenvironments and Carbon Supply*

Although aerobic degraders such as white-rot fungi have been reported for azo dye transformation [26], a wide range of bacteria capable of degrading azo dyes have also been identified, including *Aeromonas* [27], *Bacillus* [19–21], *Pseudomonas* [28], *Shewanella* [29] for RB5, as well as *Bacillus* [19,30] and *Nocardiopsis* for RO16 [31]. However, in the present study, dye-decolorizing bacteria were isolated exclusively under anaerobic conditions from vegetated CWs, including obligate anaerobic *Clostridium* spp., despite relatively high bulk DO concentrations. This contrast suggests that azo dye reduction in CWs is primarily governed by localized reductive microenvironments rather than bulk aerobic conditions.

The coexistence of obligate anaerobes and oxic bulk water provides strong indirect evidence for the formation of microscale anoxic niches within the substrate. Such spatial redox heterogeneity is likely driven by intense microbial respiration fueled by plant-derived organic carbon. Decolorization extending beyond colony boundaries further indicates that dye reduction was not limited to direct cell contact but involved diffusible reducing metabolites or extracellular redox-active compounds, consistent with the secretion capacity of Gram-positive bacteria and the reported involvement of redox mediators in azo reduction [22,32].

In addition, the isolates did not decolorize dyes when supplied as sole carbon sources, confirming the requirement for additional electron donors. Because no external carbon was added during CW operation, plant-derived organic matter—such as root exudates and decaying biomass—likely served as endogenous electron donors supporting anaerobic azo bond reduction. Taken together, these results demonstrate that vegetation plays a dual role by creating localized reductive microenvironments and supplying organic carbon, thereby enabling anaerobic azo dye reduction within otherwise oxic systems.

### *4.3. Proposed Sequential Anaerobic–Aerobic Transformation Pathway*

Based on these findings, a sequential redox mechanism is proposed. Plant-derived organic compounds stimulate rhizosphere microbial respiration, generating localized reductive zones within the substrate despite oxic bulk water conditions. Under these conditions, azo dyes function as alternative electron acceptors, and anaerobic bacteria reduce azo bonds to form aromatic amines [2]. It has been reported that the degradation of RB5 in constructed wetlands produces compounds such as 3,4,6-triamino-5-hydroxynaphthalene-2,7-disulfonate, 2-((4-aminophenyl)sulfonyl)ethyl sulfate, 2,7,8-triaminonaphthalen-1-ol, naphthalene-1,2,7,8-tetraol, and naphthalene-1,2,4-triol [13]. These intermediates may subsequently diffuse into more oxygenated regions, where aerobic microorganisms promote further oxidation and degradation [33]. The spatial coupling of anaerobic reduction and aerobic transformation enables integrated treatment within a single passive system. Therefore, redox heterogeneity represents a fundamental design principle of vegetated CWs.

### *4.4. Implications for Environmental Management*

Previous field investigations conducted by our research group in Pekalongan City, Indonesia, demonstrated the applicability of CWs for treating textile (batik) wastewater under real conditions, where cattail and papyrus were planted [24]. The laboratory-scale results provide mechanistic insights that help explain the dye removal performance observed in such field systems. Vegetated CWs achieved high azo dye removal without external carbon supplementation, supporting their application in decentralized textile wastewater treatment. In warm climates, CWs provide a low-energy alternative to conventional physicochemical processes. This study provides mechanistic evidence that vegetation may promote reductive microenvironments and endogenous carbon supply, thereby enhancing azo dye removal. Plant species also influenced dye-specific removal, indicating that appropriate vegetation selection and management can enhance system efficiency. These

findings bridge field observations and laboratory evidence, providing a scientific basis for optimizing vegetation-based CW design.

Limitations of this study should be acknowledged. Plant condition was evaluated based on qualitative visual observations rather than quantitative physiological measurements, and thus should be interpreted as indicative. Root exudates and redox conditions (e.g., Eh) were not directly measured. In particular, DOC concentrations in the CWs were low, making accurate evaluation difficult under the experimental conditions. Therefore, the proposed mechanisms regarding plant-derived carbon supply and localized reductive microenvironments remain hypothetical. In addition, the electron donors responsible for dye reduction were not identified. Furthermore, the chemical nature of the biodegradation products was not analyzed, and thus the potential formation of toxic intermediates (e.g., aromatic amines) remains uncertain. Moreover, toxicity reduction was not evaluated, and thus, the overall treatment performance cannot be fully assessed. Future studies incorporating chemical analysis and microscale redox measurements would be valuable to verify these mechanisms and assess the environmental implications of dye degradation.

## 5. Conclusions

Vegetated CWs achieved substantially higher removal of RO16 and RB5 than unplanted CWs across varying temperature and HRT conditions. High decolorization efficiencies were maintained without external carbon supplementation, indicating that biological transformation likely played a dominant role in dye removal.

Dye-decolorizing bacteria were isolated exclusively from the vegetated CWs under anaerobic conditions. The absence of decolorization under aerobic cultivation suggests that localized reductive microenvironments may have been established within the vegetated substrate despite oxic bulk conditions. The inability of isolates to utilize dyes as sole carbon sources further indicates the requirement of additional electron donors.

These findings support the hypothesis that vegetation enhances azo dye decolorization in CWs by promoting localized reductive conditions and supplying endogenous organic carbon. However, because direct measurements of redox conditions and organic carbon dynamics were not conducted, this hypothesis should be regarded as a plausible explanation rather than a confirmed mechanism.

Vegetated CWs therefore represent a low-energy and low-maintenance treatment option for dye-containing wastewater, particularly in warm regions where elevated temperatures can enhance microbial activity. The ability to achieve dye removal without external carbon addition further highlights the practical potential of this approach. CWs are also well-suited for decentralized wastewater management in areas with limited infrastructure, such as Indonesia. Future research should focus on validating this hypothesis under pilot- and full-scale conditions treating real wastewater, as well as conducting direct measurements of redox conditions, organic carbon dynamics, and transformation pathways to support mechanistic understanding and practical application.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/environments13050237/s1, Figure S1: Photographs of the CW units used in this study; Figure S2: Anaerobic decolorization of RO16 in R2A medium by microorganisms in CW effluent: (A) cattail-planted CW and (B) papyrus-planted CW; Figure S3: Anaerobic decolorization of RB5 in R2A medium by microorganisms in CW effluent: (A) cattail-planted CW and (B) papyrus-planted CW; Table S1: Operating conditions for dye wastewater using CWs in a sequencing batch mode.

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H.E.; writing—original draft preparation, S.G. and H.E.; writing—review and editing, S.S. and A.A.A.; visualization, S.G., H.E. and S.S.; supervision, S.S.; project administration, S.S.; funding acquisition, S.S. All authors have read and agreed to the published version of the manuscript.

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## Abbreviations

The following abbreviations are used in this manuscript:

| | |
|---|---|
| BSM | Basal salt medium |
| CW | Constructed wetland |
| DO | Dissolved oxygen |
| HRT | Hydraulic retention time |
| RB5 | Reactive Black 5 |
| RO16 | Reactive Orange 16 |

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